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Methods for studying microbial diversity from soyabean rhizosphere by phenotypic and molecular approach

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ABSTRACT

Microbial diversity is considered as one of the most useful resources for bioprospecting. Genetic diversity of bacteria is being analyzed increasingly by PCR-based genomic fingerprinting methods. As more knowledge is acquired and isolates from unexplored environment such as soyabean rhizosphere are studied, new species are discovered and former species are split. Since then, this technique has been utilized extensively by various workers and several novel species have been reported during the last decade. The present study has been planed keeping in mind the importance of rhizobacterial isolates in plant growth promotion of soybean. The study of rhizobacteria from important plant of human consumption, i.e., soybean by using phenotypic and molecular tools would be useful for identification of potent functional rhizobacterial isolates. These rhizobacterial isolates would be use as a biocontrol of most important pathogen of host plant. By evaluation of plant growth promoting activity and biocontrolling properties and these efforts will make for search of potent bioagent to exploit it as biofertilizer and biocontrolling agent.

Key words: *Soyabean, Rhizosphere, Rhizobacteria, Microbial Diversity, phenotypic approach, molecular approach.*

INTRODUCTION

Biodiversity

Biodiversity or biological diversity is defined as variability among living organisms from all sources including terrestrial, marine and aquatic ecosystems. This includes diversity within species, between species and within ecosystems. In addition, biological diversity reflects on the large number of biological species on earth which are key to ensuring the continuance of life on earth. Biodiversity also encompasses expressions of the relative abundances of different

ecosystems, species, and genes. The conservation of all biological diversity is a common concern of human kind and it is vital to anticipate, prevent and tackle the cause of reduction of biological resources [1].

Microbial biodiversity describes complexity and variability among microorganisms at different level of biological organization. It includes genes, species, ecosystems and evolutionary and functional processes that link them (www.for.gov.bc.ca/pab.publctns/ glossary/b.htm).

The current estimates of the number of species of prokaryotes (3,000,000), fungi (2,700,000), algae (1,000,000) and nematodes (1,000,00) are a fair reflection of the relative numbers vis-à-vis the efforts required to enumerate this diversity [2].

Number of named district living species and estimated total number of species

Group	Number of named species (in thousands)	Estimated total number of species (in thousands)
Viruse	4	1000
Prokaryotes	4	3,000
Fungi	72	2,700
Protozoa	40	200
Algae	40	1,000
Plants	270	500
Animals		
Vertebrates	45	55
Nematods	25	1000
Molluses	70	200
Arthropods	855	4,650
Crustacean	40	200
Archnids	75	1000
Insects	950	100,000
Other animals	15	800

Mcinnerney *et al.*, 2003

While there is considerable information with respect to the origin, maintenance and distribution of biodiversity of plant and animal kingdoms only a beginning has been made investigate patterns and the forces that govern bacterial diversity scenario[3,4] understanding pattern of bacterial biodiversity is of particular importance since these small creatures comprise the majority of earth's species diversity. Bacterial diversity is a resource with great applied importance in bioremediation and bioprospecting. Patterns and principles of distribution of free-living bacterial diversity and compared these with plants and animals. Bacterial diversity exhibits regular patterns; in some cases these pattern are however qualitatively similar to those observed for plants and animals.

Modern molecular approaches have revealed an extraordinary diversity of microorganisms, most of which are as yet uncharacterized because of its non-culturable nature. This poses major challenge to microbial ecologists. How can one compare the microbial diversity of different environments when vast majority of microbial taxa are usually unknown? . Three statistical approaches have reported to analyze microbial diversity parameteric estimation, non-parametric estimation and community phylogenetics which are proving to be promising tools to meet this

challenge [5, 6]. Parametric and non-parametric estimation approaches are used to compare operational taxonomic unit (OUT) richness among environments while phylogenetic approach compares evolution any diversity among environments.

Soil-plant -microbe interaction

Soil plant microbe is highly complex and dynamic ecosystem. Among these, soil ecosystem represents a heterogeneous environment, since it comprises of several habitats each with own trophic characteristics, thus contributing towards specific microbial population structure. In soil, a compartment of major interest is rhizosphere defined as a part of soil under the direct influence of plant roots. Indigenous microbial population, specifically rhizobacteria exhibit positive interaction with plant roots (Plant growth promoting rhizobacteria, PGPR) and plays a key role in establishing microbial communities with beneficial properties [7] .

Rhizosphere is rich reservoirs of microbial gene pool on account of available nutrient resources derived from secretions, sloughed-root cells, mucilage and dead biomass. Both are important habitats wherein competitive interactions of the highest order prevail and niche diversification is a way of life. Many bioactive molecule secreting forms have been recovered from these hot spots of diversity including bacteria and fungi. Among the heterotrophic bacterial forms, bacilli and pseudomonads have attracted special attention since they are not only dominant in these ecosystems but are capable of helping plants withstand abiotic and biotic stress through direct and indirect mechanisms that lead to improved plant fitness and better soil health[8,9]. While the direct mechanisms revolve around release of growth promotory substances and action of ACC deaminase, indirect promotion is known to occur through release of siderophores that chelate iron (deprive phytopathogens), antifungals that help suppress the colonization capacity of pathogenic root/ shoot and soil borne fungi, and building up plant defense machinery through the induction of systemic resistance [10].

Why Soybean?

Among the crop ecosystems, soybean occupies a special place due to human consumption and rich source of protein. However, it is also prone to various diseases, major being collar rot, charcoal rot, root rot, bacterial pustule, anthracnose, powdery mildew, *Myrothecium* leaf spot, *Alternaria* leaf spot, Fusarial seedling rot, phyllody, rust and yellow mosaic and soybean mosaic [11]. Damages well over 10 to 30% yield losses are not uncommon in the Indian context. Among these, root and collar rots appear 1-2 week after planting but can attack the plants, all the way upto mid to late flowering stage. Fusarium seedling and collar rot is amenable to attack throughout the growing season of the crop. Anthracnose occurs throughout the season; whereas other leaf infecting pathogens have more even distribution. However, last years have seen several problems of which the root disease complex, caused by species of *Fusarium*, *Rhizoctonia* and *Sclerotium* has taken heavy toll and many a farmers are no longer enamored by the virtues of soybean as a cash crop. Besides the variability that one finds in the performance of the mutualistic partners, rhizobia under varied soil type and management practices, disease incidence is also plant and soil dependent [12].

Methods of studying microbial diversity

Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species [13]. Methods to measure microbial diversity in general

and compost can be categorized into two groups. Phenotypic based approaches and molecular based approaches. It is difficult with the current techniques to study true diversity since we do not know what is present and have no way of determining the accuracy of the extraction or detection methods.

Phenotypic based approaches

1. Plate count

Traditionally, diversity was assessed using selective plate and viable counts. Direct counting by fluorescent microscopy is reported to give 100-1000 times more than the number obtained by plate counting [14]. These methods provide information on the active, heterotrophic component of the population.

2. Carbon source utilization profile/ communities level physiological profile (CLPP)/ BIOLOG

Community level physiological profile is culture dependent methods of analyzing microbial communities. This technique takes advantage of traditional methods of bacterial taxonomy in which bacterial species are identified based on their utilization of different carbon source. CLPP have been fascinated by the use of BIOLOG which is now widely available to assess functional diversity of microorganisms in compost ecosystem [15].

3. Fatty acid methyl ester (FAME) analysis

Phospholipid fatty acid (PLFA) analysis has been used as a culture independent method of assessing the structure of microbial community. This method provides information on the microbial community composition based on grouping of fatty acids .Fatty acids are used as chemotaxonomic marker. This is a signature molecule which is present in all living cells. In microorganisms, phospholipids found in cell membrane are the key determinant for the purpose. Fatty acids make up a relatively constant proportion of the cell biomass and can differentiate major taxonomic groups with in the community. Therefore, change in the fatty acid profile can represent change in the microbial population in an environmental sample [16].

Molecular-based approaches

To understand rhizobacterial diversity and community composition of soybean plant, different approaches have been developed by taxonomists, these include DNA reassociation, DNA-DNA and mRNA-DNA hybridization, DNA cloning and sequencing and other PCR based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand-conformation polymorphisms (SSCP), ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA)

PCR-independent approaches

1. Guanine plus cytosine (G+C) content

Difference in the guanine plus cytosins (G+C) content of DNA be used to measure bacterial diversity of soil. Microorganisms have reported differ in their G+C content and that taxonomically related groups only differ by 3% and 5%. It provides a coarse level of resolution as different taxonomic groups may share same G+C range. G+C analysis is not influenced by PCR biases and since it includes all extracted DNA, it can uncover rare members in microbial population [17].

2. Nucleic acid reassociation and hybridization

DNA reassociation is used to measure genetic complexity of the microbial community and has been applied to evaluate environmental diversity. Total DNA is extracted from the environmental samples, purified, denatured and allowed to reanneal. The rate of hybridization or reassociation will depend on the similarity of DNA sequences. Various workers have reported that complexity of diversity of DNA sequences increases, at rates at which DNA reassociates decrease. Similarity between communities of two different samples can be studies by measuring the degree of similarity of DNA through hybridization kinetics [18].

Nucleic acid hybridization by using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology [19]. These approaches can be applied on extracted DNA, or RNA, or *in situ* conditions. Oligonucleotide or polynucleotide probes designed from known sequences ranging in specificity from domain to species can be tagged with fluorescent markers at the 5' end. However, dot blot hybridization is used to measure the relative abundance of a certain group of microorganisms. It provides valuable spatial distribution information on microbial community or with either environmental samples to which microbial community similarity is compared.

3. DNA microarrays

The latest development includes application of DNA microarray to detect and identify bacterial species or to asses microbial diversity. This rapidly characterizes the composition and functions of microbial communities because a single array contains thousands of DNA sequences with the possibility of very broad hybridization with wide identification capacity. The microarray can contain specific target genes such as nitrate reductase, nitrogenase or naphthalene dioxygenase to provide functional diversity information or can contain 'standard' of environmental samples (DNA fragments with less than 70% hybridization) representing different species found in the environmental sample [20].

4. Reverse sample genome probing (RSGP)

RSGP is used to analyze microbial community composition of the most dominant culturable species. RSGP has four steps (1) isolation of genomic DNA from pure cultures (ii) cross hybridization testing to obtain DNA fragments wit less than 70% cross hybridization, (iii) preparation of genome arrays on solid support, (iv) random labeling of a defined mixture of total community DNA and internal standard. RSGP is a useful approach when diversity is low, but several molecular biologists have bad difficulty when assessing community composition of diverse habitats [21].

PCR-based approaches

PCR based 16S rDNA profile provides information about prokaryote diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships [22,23,24]. Initially, molecular-based approaches for ecological studies relied on cloning of target genes isolated from environmental samples. Therefore, 16S rDNA based-PCR techniques such as, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation-polymorphisms (SSCP), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphisms (T-RFLP), ribosomal intergenic spacer analysis (RISA) and others can provide detailed information about

community structure of an ecosystem in terms of richness, evenness and composition and can be used to compare species present in a sample.

1. Restriction fragment length polymorphisms (RFLP)

RFLP, known as amplified ribosomal DNA restriction analysis (ARDRA) is used to study microbial diversity that relies on DNA polymorphism. PCR amplified 16S rDNA is have reported by digested with a 4-base pair cutting restriction enzyme [25]. Banding patterns can be used to screen clones and used to measure bacterial community structure. Various workers have reported that this is useful for detecting structural changes in microbial communities but not as a measure of diversity or detection of specific phylogenetic groups [26].

2. Ribosomal intergenic spacer analysis (RISA)/ automated intergenic spacer analysis (ARISA)

RISA and ARISA provide riobosomal-based fingerprinting of the microbial community. In RISA and ARISA, the intergenic spacer (IGS) region between the 16S and 23S ribosomal subunits in amplified by PCR, denatured and separated on polyacrylamide gel under denaturing conditions. IGS region may encode tRNAs and is useful for differentiating b/w bacterial strains and closely related species because of heterogeneity of the IGS length and sequence [27]. In RISA, the sequence polymorphisms is detected by using silver stain while in ARISA the forward primer is fluorescently labeled and is automatically detected.

3. Repititive (REP)-PCR

REP-PCR has been used for identification of bacteria since it provides genomic fingerprint of chromosome structure which is considered variable between strains **[28]**. Both prokaryotic and eukaryotic genomes contain dispersed repetitive sequences that range between 15 to several hundred base pair in length. These elements are non-coding but are present in high copy number relative to the longer repeated elements, which contain coding sequence. The interspersed repetitive sequences described in bacteria are Box element (154 bp), Repsequence (386 bP) and ERIC sequences (124 bp). These sequences may be diagnostic and allow differentiation down to the species or strain level [29].

4. Denaturing gradient gel electrophoresis (DGGE)/ Temperature gradient gel electrophoresis (TGGE)

DGGE and TGGE were first developed to detect point mutation in DNA sequences. DGGE and TGGE have used to study microbial genetic diversity. DNA is extracted from compost samples and amplified by using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The 5'-end of forward primer contains a 35-40 base pair GC clamp to ensure that at least part of the DNA remains double stranded. Seperation on a polyacrylamide gel with gradient of increasing concentration of denaturants (Formamide and urea) will occur based on melting behaviour of double stranded DNA. On denaturation, DNA melts in domains, which are sequence specific and will migrate differentially through polyacrylamide gel[30].

5. Single strand conformation polymorphisms (SSCP)

SSCP separates single strand DNA fragments differing in strand length and conformation by gel electrophoresis. SSCP-PCR was developed to detect novel polymorphisms or point mutations in DNA [30]. Single stranded DNA is separated on a polyacrylamide gel based on differences in

mobility caused by their folded secondary structure. The diversity of bacterial and fungal communities in the compost was analyzed by single-strand-conformation polymorphisms (SSCP) of approx 400 bp PCR products, which were amplified with universal primer for 16S rRNA (bacteria) and 18S rRNA for fungi, with compost DNA as a template.

Phylogenetic approaches

Taxonomy based on comparative analysis of 16S rRNA genes, first introduced by Carlwoose, presents a radical departure from classical taxonomic. Life could be divided into three primary phylogenetic 'Domains, Archara, Bacteria and Eucarya . use of rDNA and rRNA in phylogenetic analysis have reported [28]. For microorganisms, molecular data often provides greatest information because microorganisms such as bacteria simply do not have the diversity of form to make morphological characteristics useful in establishing phylogeny.Aside from derivation of taxonomies, phylogenetic analyses are important in identifying similarity between organisms, leading to the ability to understand physiology and ecology of non culturable species**.**

CONCLUSION

Association of microorganisms and plant system is considered not only intimate but one where positive and negative influences can be found within a group with ease and exploitative potential considerable. Besides a dominant constituent of the bulk soil, microbial populations resident in rhizosphere are known to be not only large but also much diverse. On account of resource availability and competition. However, from the point of view of resource availability and competition. However, from the point of view of beneficial influence on plant health and fitness, functionality of the effective microbial populations is essential. Since Indian soils are deficient in nitrogen and phosphorous, considerable research effort has been directed towards assessment of diversity that could lead to recovery of potentially exploitable forms. In doing so both, non-symbiotic and symbiotic nitrogen fixers have been surveyed across the country and evaluated for field performance utilizing the extension machinery of the agricultural system. While a great deal has been achieved here, variability, stability and effective root colonization has been difficult to maintain. This scenario has slowly changed with emphasis on selection protocols applied on large indigenous pools of bacterial diversity, multistep screening procedures, coupled to community dynamics data that permits closer monitoring of perturbations as a consequence introduction.

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