6th International Workshop on PLANT GROWTH PROMOTING RHIZOBACTERIA

Souvenir

Indian Institute of Spices Research

(Indian Council of Agricultural Research)

Calicut - 673 012, Kerala

In Harmony with Nature

AGRICULTURE SECTOR

BIOPESTICIDES

BIOLEP

Bacillus thuringiensis var. kurstaki for control of *Helicoverpa, Spodoptera*, Bollworms, *Plutella*, Fruit & Shoot borers, pests of agricultural crops & fruit trees.

BIOVIRUS-H

Nuclear Polyhedrosis Virus (NPV) for control of *Helicoverpa armigera*, pest of agricultural crops.

BIOVIRUS-S

Nuclear Polyhedrosis Virus (NPV) for control of *Spodoptera litura*, pest of agricultural crops.

BIODERMA

Trichoderma viride for control of fungal plant pathogens and nematodes.

BIORIN

Beauveria bassiana for control of lepidopteran caterpillar pests of vegetables and fruit plants, mites and red spidermites of vegetables and ornamental plants, whiteflies of vegetables, cotton, coffee berry borer and colorado beetles of potato and vegetables.

BIOMONAS

Pseudomonas flourescens for control of fungal and bacterial plant pathogens.

BIOLINE

Verticillium lecanii for control of whiteflies, aphids, mites, spidermites and thrips of various crops, particularly vegetables, oilseeds, cotton, tobacco & coccids of coffee.

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BIOGRAMMA

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MESSAGES

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President's Secretariat
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New Delhi - 110004

24th September 2003

MESSAGE

The President of India, Dr. A P J. Abdul Kalam, is happy to know that the Indian Council of Agricultural Research, New Delhi, Auburn University, Alabama, USA, the Indian Institute of Spices Research, Kozhikode and the Indian Society for Spices are jointly organising the 6th International Workshop on Plant Growth Promoting Rhizobacteria during October 5-10, 2003

The President extends his warm greetings and felicitations to the organisers and the participants from India and abroad and wishes the Workshop all success.

Pres Secretary to the President

A. K. ANTONY
CHIEF MINISTER KERALA

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MESSAGE

23.9.03

I am happy to learn that the sixth International workshop on Plant Growth Promoting Rhizobacteria (PGPR) is being held at Kozhikode from 5th to 10th October 2003. I congradulate the Indian Council of Agricultural Research, New Delhi; Aubum University; Alabama USA, Indian Institute of Spices Research, Kozhikode and Indian Society for Spices for organizing this workshop.

I send my good wishes for the success of the workshop and the souvenir published to highlight this event.

A.K.ANTONY

सत्यमेव जयते

Dr. MANGALA RAI

SECRETARY
AND
DIRECTOR- GENERAL

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भारतीय कृषि अनुसंधान परिषद
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MESSAGE

I am pleased to know that the 6th International Workshop on Plant Growth Promoting Rhizobacteria (PGPR) is being held at Kozhikode during 5-10th October, 2003 jointly by Indian Council of Agricultural Research, New Delhi, Auburn University, Alabama, USA: Indian Institute of Spices Research, Kozhikode and Indian Society for Spices.

I am sure the participants will deliberate on the application of plant growth promiting rhizobacteria to address emerging issues of sustaining agricultural productivity

I wish all success for the Workshop.

(Mangala Rai)

Dated the 19th September, 2003
New Delhi

MESSAGE

The Indian Institute of Spices Research, Calicut under Indian Council of Agricultural Research, over the years has contributed substantially to spices production in the country by generating various technologies for crop production. The eco-friendly crop protection technologies like biocontrol of soil borne diseases of spices are outstanding. At IISR, Calicut the PGPR research started a decade ago has established the potential in management of the soil borne diseases of spices like Phytophthora foot rot, slow decline and rhizome rot of ginger. I appreciate the International Organizing Committee of PGPR has chosen IISR, Calicut as venue for the conduct of 6th International Workshop that would ensure a greater interaction of both national and international scientific community to develop microbial technologies for sustainable crop production at global level. I wish the workshop deliberations a grand success.

(G. KALLOO)
Dy. Director General (Horticulture)
ICAR, New Delhi – 110 001.
24-9-2003

MESSAGE

I am indeed delighted to participate in the 6th International PGPR workshop to be held in Calicut, "the Spice Coast of the World", India - my mother country. Over the years, while working on PGPR abroad, and participating in previous PGPR workshops overseas, I have dreamt of organizing a PGPR workshop in India. Four years ago, while traveling with Dr. Joe Kloepper (Founder and Father of PGPR workshops) throughout India, visiting various government, academia and private research laboratories, I postulated the idea of organizing the 6th workshop in India. After seeing vast PGPR interest in various parts of India, Dr. Kloepper readily agreed with great enthusiasm. It is a pertinent topic at an equally relevant time, especially in that area of the world.

In preparing for this occasion, it has been a pleasure to work with Dr. Y. R. Sarma and his organizing team, Mr. K. P. Mayan, Silk n Spice Travel & Communications Co. (P) Ltd., and Kadavu Resort management. Dr. Sarma and team have accomplished a lot and should feel proud of their efforts. We wouldn't be where we are today without your passion, energy and commitment over the last three years. I thank you and hope we will continue to work together to make sure our participants have an enjoyable, informative, and relaxing time.

I am immensely pleased to welcome so many delegates from around the globe to an event that brings us all together and for the first time in India. The world has seen a great expansion at arm's-length of research in PGPR, especially in the last 10 years. This expansion has created an unprecedented opportunity for us to share the richly diverse cultural experiences of our respective countries and our many innovative approaches to the support of PGPR research. While all of us here are rooted culturally in our places of origin and residence, increasing global connections also shape our lives. This workshop on PGPR builds on for an international presence and signals a renewed interest in agriculture. This new global interconnection presents both extraordinary opportunities and formidable challenges, which can best be addressed through international cooperation. That cooperative spirit has already manifested itself in the input from the representatives of almost 20 countries that has helped shape the PGPR workshop agenda and in the eager interest delegates have expressed in this opportunity to work together. I am confident that this workshop will nourish the science of PGPR by exploring means of strengthening international cooperation.

The variety of topics planned for discussion in our working sessions already shows just how broad a mandate this PGPR workshop could have. The task you are beginning this week is both complex and tremendously rewarding. The impressive array of intellects and talents assembled here this week is a sure portent of productive and stimulating interchange.

To each and every one I extend my best wishes for stimulating and productive discussions.

M. S. Reddy
Associate Professor
Auburn University
Auburn, AL, USA

CONTENTS.....

Potential role of plant growth promoting bacteria in coastal sustainable agriculture

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Introduction

Increasing population and demands for food has pressured areas with water deficits to develop more water resources to meet their food and fiber production needs. By the year 2025, an estimated 1.4 billion people will be dependant on these water deficient regions for their food, feed and fiber (Seckler *et al* 1999, Intl. J. Water Res Develop. 4:34-45). This will represent a quarter of the world's population or one third of the population of developing countries. In this developmental paradigm soil salinity is one of the most serious stresses in agriculture. It has been estimated that about one billion ha of the worlds land is affected by salt, sixty percent of which is cultivated and approximately 8 to 12% of the irrigated land has lost productivity through accumulation of salts (Umali, 1993 in Key trends in feeding the World, World Bank Washington D.C).

The expansion of irrigation in India has been one of the key strategies in achieving self-sufficiency in food production. A large expansion in the irrigated area has been achieved through transported canal water. In most canal irrigated areas the problems of soil deterioration through accumulation of salts have reached serious dimensions. According to one estimate nearly 50% of canal-irrigated areas are affected by salt problems due to a lack of or inadequate artificial and/or restricted natural drainage, inefficient use of irrigation water and socio-political reasons. Salinization of soils has also increased where saline ground waters have been used for irrigation in the absence of good quality irrigation water which results in major crop losses particularly in semi-arid and irrigated agriculture (A.K. Tripathi *et al* 1998, J. of Biosciences 23. 463-471). Climate change arising from global warming and its impact on agriculture and vice versa have also emerged as new threats and challenges.

Salinization and Plant Growth Promoting Rhizo-bacteria

One of the major manifestations of this process of increasing salinization is increase in the concentration of soluble salts in the root zone of soils, which affects the rhizosphereic microorganisms including the plant growth promoting bacteria. This is more evident in the coastal agri-ecosystems.

With recent intensification of researches on natural resource and input management for sustainable practices importance of beneficial plant-microbe interaction to mitigate the problems of soil pollution/stress, compliment plant mineral nutrition, minimize soil diseases etc. can be hardly emphasized. To improve soil health and increase crop productivity the role of biological software's is critical as inputs especially in low input farming practices, which is the predominant way in most developing countries. To harness them optimally it is necessary first to thoroughly understand the individual growth

and survival characteristics of each particular beneficial microorganism, including their nutritional and environmental and ecological relationships. Since high variability of the soil environment and its microbial habitats has worked as major impediments in successful introduction many questions in this area remain to be answered.

The identification of bacterial strains and in some cases host cultivars that are tolerant to these stresses opens the way for alternate, lower cost solutions to these problems. Although we will not be able to eliminate many of the stresses currently limiting crop production under low-input conditions, we would be able to throw some light on identifying better host-strain combinations and identifying efficient ones as we use a polyphasic approach (phenotypic/ biochemical and genotypic) in characterizing the various groups of PGPR.

PGPR and the coastal niche

It is on the above-mentioned lines that the microbial group at MSSRF has worked in the last few years. Extensive studies have been carried out in relation to near normal and moderately saline soils to understand the diversity and functional efficiency of beneficial organisms like BNFs/PO_4 solubilizers and fluorescent Pseudomonas sps towards developing efficient biological software including biofertilizers and biocontrol agents for the coastal niche.

There are several reports on the sensitivity of nitrogenase to salt variation in different species of *Azospirillum* (Hartmann, 1991 Plant and Soil 137: 105-109, Riou and Le Rudulier 1990 J. of Gen. Microbiol 136: 1455-1461) and that excess salt in soil adversely affect survival, nodulation, N fixation and legume-*Rhizobium* symbiosis (Rai.R 1992 Biol. Fertil. Soil 14: 293-299, Rai.R 1999 Biol. Fertil. Soil 29:187-195). To establish reliable information on diversity, assessments in relation to the phenotypic and genotypic (RAPD, PCR-RFLP of 16SrDNA and nifD genes) characterization of *Bradyrhizobium*, *Rhizobium*, and *Azospirillum* and its functional efficiency have been carried out to facilitate a more extensive use of BNF's. It was observed that soil characteristics, especially salinity seem to play a predominant role in the selection of natural populations. Our studies have revealed that the adapted strains of *Azospirillum* perform well upto 500mM NaCl and performed better than the normal strains in growth promoting activities when tested with paddy under green house evaluations. There was definite pattern in the occurrence of genotypes of both Rhizobia and Bradyrhizobia when a comparison was done between their occurrences in saline and non-saline soils. Salt tolerant Rhizobia and Bradyrhizobia perform well in nodulation when tested under saline soil conditions in cowpea/ blackgram and groundnut (Saleena *et al* 2001 Microb Ecol 44: 271-272, Saleena *et al* 2001 Can J. of Microbiol 47: 118-122 and Saleena *et al* 2002 Biol. Fertil. Soils 34: 276-281).

Many investigators world wide are now interested in studying diazotrophic endophytes in non-legumes with a hope that a nitrogen fixing association can be established which could be of enormous economic value (Dobereiner. J. et al 1997 Soil. Biol. Biochem 29: 5/6, 911-922, and Nowak et al 2000 Critical Reviews in Plant Sciences 19 (1): 1-30). In our studies new hosts for BNF endophytic nitrogen fixing systems in non-legume crops like ragi and wild rice have been established for the first time. Two genotypically distinct forms of Gluconacetobacter diazotrophicus have been reported to be colonizing and fixing nitrogen in *E. corocana* (Loganathan et al 1999 J Applied. Microbiol 87: 167-172).

While screening endophytic populations from *Porteresia coarctata* (wild rice), salt-tolerant, N-fixing and phosphate-solubilizing bacteria was isolated from wild rice. Based on the overall analysis of the tests and comparison with the characteristics of *Acidomonas*, *Asaia*, *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia* these isolates have been proposed as *Swaminathania salitolerans* gen. nov., sp. nov. (Loganathan and Sudha Nair 2002 – communicated to IJSEM). The isolates tagged with *gusA* gene, colonized *Porteresia coarctata*

(wild rice) and Pokkali (salt-tolerant variety) more intensively when compared to Ponni (salt-sensitive variety), (Loganathan and Sudha Nair 2003, Biotech Lett. 25: 497-501).

With reference to Phosphate solubilizing bacteria we have observed that the count is normally very low in sandy saline soils. However we have been able to identify bacterial strains isolated from the root free soil, rhizosphere, and rhizoplane of crops cultivated along the coastal belt adapted to solubilization in salt concentrations up to 500 mM. Broth assay using tri-calcium phosphate showed that the isolates tested released 300–400 mg/ml from the initial 500mg/ml inorganic phosphate supplied. Green house and field level trails have given very positive results.

Pseudomonas spp have been studied mainly because of their widespread distribution in the soil, their ability to colonize the rhizosphere of host plants and ability to produce a wide range of compounds inhibitory to a number of serious plant pathogens (Thomashaw L.S and Weller, D.M. 1995 in Plant- Microbe Interactions, Stacey, G and Keen, N.T (eds) 187-235, Chapman and Hall, New York). In our attempts to understand their distribution pattern/diversity and functional efficiency in terms of their biocontrol activities more than 700 strains of Pseudomonas isolates were screened for their diversity. A highly selective PCR protocol for detecting 16S rRNA genes of the genus Pseudomonas in environmental samples was used to confirm their identity at the genus level. An interesting observation was that organic farming in the saline sites was found to be capable of mitigating the harmful effects of saline stress to a large extent and restoring the *Pseudomonas* diversity, thereby making it comparable with the diversity encountered in the non-saline sites. It was observed that increasing salinity caused a predominant selection of salt tolerant species in particular *P. alcaligenes* and *P. pseudoalcaligenes* irrespective of the host rhizosphere (Sunita *et al* 2001 J Applied Microbiol 91: 742-749, Sunita *et al* 2002 Micor Ecol 43: 280-289)

A few of the strains screened could suppress both bacterial leaf blight and sheath blight diseases under non-saline and saline conditions. These strains hold great potential for development as biological control agents most suited for the coastal environment and an important finding for the development of commercially viable biocontrol strains for this specific niche. (Sunita *et al* 2003 Plant and Soil 251: 73-82).

Such studies only goes to reiterate that the combination of molecular tools with conventional techniques have now opened up new pathways to understand the unraveled diversity especially with their functional efficiency in relation to the stresses to enable us to harness them more optimally.

Conclusion

The objective of agriculture in coming decades must be to optimize soil productivity while preserving its capacity to function as a healthy system. Increasing productivity in perpetuity without associated ecological or social harm - a phenomenon termed 'ever-green revolution' (*Swaminathan 1996 in Sustainable Agriculture*: Towards Food Security, Konark, New Delhi) is the need of the hour. In this evergreen revolution and with increasing interest being shown in organic farming biological inputs play a very vital role. There is a growing genuine interest in developing bacterial products that are reliable and that can act as complements to chemicals already on the market. Research and limited field trails of PGPB over the last decade have opened up new horizons for the inoculation industry. Agriculture in developed countries is definitely the major promoter of microbial inoculants that are 'environmentally friendly". Nevertheless, special attention should be paid to the needs and constraints of developing countries that need easy-to-use and inexpensive formulations

Developing countries practice mainly low-input agriculture in which fertilizers, pesticides and agrotechnical machinery are scarce. Naturally, this type of farming does not have the resources to invest in improved agricultural techniques. Artificial inoculation, in particular, requires an

infrastructure to store and transport biological products in large quantities into rural areas, and this infrastructure are not available.

The main reasons for the inadequate popularity of biological inputs is because of lack of strong promotion and extension work, insufficient publicity and lack of availability of quality products in time, especially in rural areas. Once the farmers are convinced that the biological inpts are an inexpensive but effective way of keeping their soil fertility, they will accept them thereby leading to higher crop production and higher economic returns

Therefore the first objective when considering inoculation with beneficial bacteria is to find the best bacteria available. Next, a study of the specific inoculants formulation is generally undertaken. Semiarid conditions make survival difficult for the introduced bacteria. Harsh conditions, including frequent droughts, lack of sufficient irrigation high salinity and soil erosion, may quickly diminish the population of any bacteria introduced into he soil unless precautions are taken to select the proper inoculants and provide irrigation concomitant with inoculation. Inoculation should be timed to coincide with sowing into moist soil or be delivered quickly with irrigation to assure rapid colonization of the target plants, as practiced in developed countries. However in this type of agriculture, beneficial microorganisms may make the greatest contribution, if inexpensive and easy-to-use formulations, which can be niche specific, can be developed by understanding them both in terms of diversity and functional efficiency as done by this group. Since microbial inoculants can be produced and marketed inexpensively, with some organized financial aid and better information transfer, these farmers are potential clients for microbial technology.

Overview of Microbial Biodiversity and Bio-resources in India and Role of National Bureau of Agriculturally Important Microorganisms (NBAIM)

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All life on earth is part of one great interdependent system. It interacts with and depends on the non-living components of the planet, atmosphere, oceans, freshwaters, rocks, and soils. Humanity depends totally on this biosphere of which we are an integral part. Biological diversity is the blanket term for the natural biological wealth that undergirds human life and well-being. The breadth of the concept reflects the interrelatedness of genes, species, and ecosystems. The concept of biodiversity has provoked considerable debate and misunderstanding among the general public, decision-makers, and even the scientific community. What is biodiversity, what threatens it, why is it important, and what are natural scientist doing to better understand it? Conserving biodiversity is not just a matter of protecting wildlife in nature reserves. It requires safeguarding natural systems that purify water, cycle oxygen and carbon, maintain soil fertility, yield food and medicine, and provide the genetic richness we tap in the ceaseless struggle to improve our crops and livestock.

Microbial diversity is an unseen resource that deserves greater attention. Too small to be seen no longer means too small to be studied or valued. Microbial diversity encompasses the spectrum of microscopic organisms including bacteria, fungi, algae, protozoa. These organisms populate the soil, water and air that surround us as well as live in more unusual environments such as the boiling water of hydrothermal vents, deep ocean trenches and alkali lakes. An understanding of microbial biodiversity is of utmost importance for a variety of reasons. It is well known that bacteria play an integral role in the cycling of carbon, nitrogen, sulphur and phosphate. Indeed, solely bacteria carry out key aspects of the carbon and nitrogen cycles, i.e. anaerobic fermentation of carbohydrates and fixation of atmospheric nitrogen. As such, it is important to determine the types of bacteria present in a particular ecosystem, the role they play in the functioning of that system, and to gauge the effects that anthropogenic forces (particularly pollution) are exerting on the diversity of microorganisms (Hill *et al.*, 1993).

The microbial diversity in marine environment also proving a valuable source of novel bioactive compounds with antibacterial, antiviral, and anticancer properties. The link between biotechnology and biodiversity is another key reason for cataloguing and conserving microbial biodiversity. Both free-living bacteria and bacteria that are symbionts of marine invertebrates are likely to be a good source of useful bioactive compounds. Seventy percent of the world's antibiotics originate from terrestrial actinomycetes. However, Russell Hill (Centre for Marine Biotechnology, USA) has shown that the diversity of marine actinomycetes is considerably

different to that of the terrestrial actinomycetes, and as such, is expected to yield novel antibacterial compounds that are urgently required in the battle against drug-resistant human pathogens.

India occupies a vast land area of 320 mha, of which over 140.3 mha are cultivable, covering diverse agro-ecological zones, rarely found in other countries, and the country is also endowed with enormous variability in agriculturally *important microorganisms (AIMs)*. The variability in fungi, bacteria, actinomycetes, viruses, cyanobacteria etc. is coevolved with their hosts vis-a-vis environment, and form invaluable gene pool resources. It is well recognized that once a variant is lost, it is lost forever. Therefore, it imperative to conserve and characterize the variability of AIMs for its optimum utilization by the coming generations. A better understanding of microbial diversity promises to provide an array of new products and processes as well as a better awareness of the microbial biosphere-the earth's life support system. The microorganisms present in the diversified agro-ecosystems of India will also provide a valuable source of novel bioactive compound

Although India is endowed with a rich microbial diversity, which unfortunately, has not been adequately enumerated and catalogued. Many laboratories in India over the years have been working on Indian microflora, and have described new genera and species or have exploded their biotechnological potentials. Data on Indian microbial resources has remained mostly with the investigators and in papers published by them. It is not known how many of them have been conserved in different Universities, Institutes and other nonorganized scientific centres. As a result we do not have systemic information about the microbial preservation and conservation of our country. However, a program was initiated to collect, collate and digitize data available on Indian microbial resources. The Department of Biotechnology (DBT), Govt. of India under the aegis of the National Bioresource Development Board (NBDB), supports this program. Data on microorganisms (bacteria, fungi, algae, viruses) will be collected in a uniform format (can be downloaded from http://imtech.res.in/mtcc/mdiv).

Microbes: the earth's engine

Microorganisms have been evolving for nearly 4 billion years and are capable of exploiting a vast range of energy sources and thriving in almost every habitat. For 2 billion years, microbes were the only form of life on earth. During this long history, all of the basic biochemistries of life evolved, and all life forms have developed from these microbial ancestors. It is estimated that 50% of the living protoplasm on this planet is microbial. Microorganisms represent by far the richest repertoire of molecular and chemical diversity in nature. They underlie basic ecosystem processes such as the biogeochemical cycles and food chains, as well as maintain vital and often elegant relationships between themselves and higher organisms. Microbes provide the fundamental underpinning of all ecosystems. Without microorganisms, all life on earth would cease.

Microbes: the biological frontier

Because microorganisms are small, they are least known, and this gap in knowledge is particularly apparent for bacteria and other small organisms. Current evidence suggests that perhaps 1.5 million species of fungi exist yet only 5% are described. For bacteria there may be 300,000 to 1 million species on earth yet only 3,100 bacteria are described in Bergey's Manual, the treatise of described bacteria. A gram of typical soil contains about 1 billion bacteria, but only 1% of those can be cultured. Similarly low fractions of microorganisms have been cultured from fresh water and ocean environments. Hence, most microbes remain to be discovered.

The value of microbial diversity

Diverse microorganisms are essential to a sustainable biosphere. They are able to recycle nutrients, produce and consume gases that affect global climate, destroy pollutants, treat

our wastes, and they can be used for biological control of plant and animal pests. The study of microbial diversity is also important to solve new and emerging disease problems and to advance biotechnology. New technologies, particularly in nucleic acid analysis, computer science, analytical chemistry, and habitat sampling and characterization place the study of microbial diversity on the cutting edge of science. Humans over the ages have been highly successful in applying processes carried out by microorganisms to solve problems in agriculture, food production, human health, environmental quality, and industry. Recently developed technologies in molecular biology and genetics offer great promise for new opportunities to develop the potential of microbial diversity.

Threats to the microbial biodiversity

The loss of microbial biodiversity is a significant issue for scientists and policy-makers and the topic is finding its way into living rooms and classrooms. Species are becoming extinct at the fastest rate known in geological history and most of these extinctions have been tied to human activity. Habitat loss and destruction, usually as a direct result of human activity and population growth, is a major force in the loss of microbial species.

The introduction of exotic (non-native) species can disrupt entire ecosystems and impact populations of native microbes. These invaders can adversely affect native species by eating, infecting, competing, or mating with them. The over-exploitation and human-generated pollution (pollution of soil, water, and atmosphere) and contamination can affect all levels of biodiversity. Microbial species and populations may be lost if they are unable to adapt to new conditions. Relatively undisturbed ecosystems have shrunk dramatically in area over past decades as human population and resource consumption have grown.

The value of microbial biodiversity research

- to expand the frontiers of knowledge about the strategies and limits of life, especially those thriving at extreme conditions and on unusual redox couples,
- microorganisms are of critical importance to the sustainability of life on our planet,
- the untapped diversity of microorganisms is a key resource for new genes and organisms of value to biotechnology,
- diversity patterns of microorganisms can be used for monitoring and predicting environmental change,
- microbes play a role in conservation and restoration biology of higher organisms and degraded landscapes,

Microbial diversity is the largest untapped resource for both understanding how biological systems function as well as for new biotechnologies. Advances in the molecular, chemical, optical, computer and information sciences has now made the exploration of this frontier practical. Microorganisms are the major sources of anti-microbial agents and produce a wide range of other important medicinal compounds including enzymes, enzyme inhibitors, anti-helminthics, anti-tumor agents, insecticides, vitamins, immunosuppressants, and immunomodulators. These agents have all been discovered during the past 50 years and represent only a small portion of what is likely present in nature. Pharmaceuticals of microbial origin have a market value (at the wholesale level in the developed world) of approximately $35-50 billion annually.

Workshop held at Michigan State University, USA, has recommended the following topics as high priority needs if we are to better understand, manage, and utilize the vast microbial resource

1. understand the origins and patterns of microbial biodiversity

a) Select key habitats for coordinated, focal, and long-term study. A few habitats should be intensively studied; others should be studied extensively.

b) Achieve a better understanding of spatial and temporal patterns of microbial diversity, and how environment determines those patterns.

c) Enhance our understanding about the rate and range of global dispersal, evolution, and extinction of microbial species.

2. Discover and characterize microbial diversity

a) Refine appropriate taxonomic units that define microbial diversity, and develop the appropriate methodology to measure those units.

b) Discover new microbial forms, biochemistries, evolutionary branches, and habitats.

c) Improve methodologies to characterize, isolate, and identify non-culturable and rare members of communities.

d) Develop advanced instrumentation and software that provides new and more rapid methods to characterize isolates and communities.

e) Foster research on polyphasic taxonomy, particularly the integration of phenotypic, genetic, and ecologic information.

3. Preserve microbial diversity

a) Conduct research leading to the preservation of mixed communities, e.g., consortia, natural communities.

b) Improve culture preservation strategies, such as robotic preservation, miniaturization, and optimized regimes for difficult to preserve microbial groups.

c) Maintain and coordinated network of culture collections that are accessible worldwide.

d) Conserve habitats with rare or threatened species.

4. Organizational and infrastructure needs

a) Coordinate microbial diversity research at an international level.

b) Develop integrated electronic databases that include habitat, geographic, phenotypic, genotypic, morphological, and accession information.

c) Involve researchers from other fields, especially computer science, optics, electronics, device engineering, chemistry, remote sensing, and microbial ecologists and systematists.

d) Centralized efforts are recommended where specialized facilities are needed, routine measurements are made, and for database and archival activities.

e) Expand the training of new scientists knowledgeable in modern microbial diversity, physiology, and taxonomy.

f) Enhance the public's awareness about the vital role microbial diversity plays in their lives.

Tools to study of microbial diversity

Historically, microbial biodiversity has been conducted by using a variety of physical and biochemical tests that allow the grouping of microbial isolates into genera and species. This approach requires laboratory cultivation of the microbes in order to separate the various isolates into monocultures. This approach (classical taxonomy) has been used to identify and characterize many of the culturable microorganisms. However, typically less than one percent of the bacteria can be cultivated in the laboratory. Indeed, only 3 000 - 4 000 species of bacteria have been described (Hawksworth & Colwell, 1992), even though it has been estimated that the number of bacterial species world-wide is close to three million. Because of this limitation, microbial biodiversity can only be accurately determined using molecular taxonomic tools that obviate the need for laboratory cultivation of isolates. Thus, the use of techniques such as polymerase chain reaction (PCR; Jansa et al., 2002; Latha et al., 2002) amplification and sequencing of 16S ribosomal RNA genes (Arora et al., 1996), randomly amplified polymorphic DNA (RAPD;

Mishra et al., 2000; Jiminez-Gasco et al., 2001; Young et al., 2001; Jana et al., 2003), microarray technology (Lockhart, 2000; Schena, 2002), enterobacterial repetitive intergenic consensus (ERIC) sequences (Stern et al., 1984; Sharples and Lloyd, 1990) ,whole community nucleic acid hybridization (Shi et al., 2003) etc. provide a more reliable approach to determining microbial biodiversity. Furthermore, hybridisation of DNA probes specific to genes coding for particular enzymes to either DNA (potential function) or messenger RNA (expressed function) isolated from seawater samples allows one to monitor microbial metabolic processes *in situ*.

PCR-based methods. Any DNA or RNA sequence that is specific for particular microorganism can be used for PCR detection of that microorganism. The sensitivity, speed, and versatility of PCR are primary factors in its wide acceptance in microbial diversity study. It is adaptable to many experimental objectives, and used with a wide range of starting material, including purified nucleic acids, intact cells or tissues, or complex environmental samples. As PCR methods for detection of micoorganism become available, more research were focused on using these tools to study antagonist populations, their biology, ecology, variability, and more prominently their interaction with other microbes or host. PCR-based methods offer many new tools that are directly applicable to systematic of microorganisms. These can be used to determine relationships among species, either by direct comparison or through phylogenetic analyses. Attempts have been made to increase the specificity of PCR reactions using other methods, including post-PCR hybridization, PCR-ELISA reactions, Molecula techniques like RFLP analysis of the PCR products (PCR-RFLP; Yamagishi *et al.*, 1999), denaturing gradient gel electrophoresis (DGGE; Cocolin et al., 2001), florescent capillary electrophoresis (Turenne et al., 1999), or nested PCR.

Random amplified polymorphic DNA (RAPD). One approach used for developing suitable species- or strains-specific probes for the detection of fungi is based on the random amplified polymorphic DNA technique (RAPD). RAPD-PCR assays have been used extensively to define fungal populations at different taxonomic levels. In general, most studies have concentrated on intra-specific grouping, although others have been directed at the species level.

Intergenic transcribed spacer (ITS) and *Intergenic spacers (IGS).* The ITS region has been most frequently used as target for species-specific detection of fungi. The ITS consists of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit, and the large subunit rRNA genes. The ITS region is a particularly useful area for molecular characterization studies in fungi (Sreenivasaprasad et al., 1996).

In contrast to ITS region, fewer studies have considered IGS region (Arora et al., 1996) to determine the variability within the species *Verticillium chlomydosporum* and other closely related species. They found that there was in general a low level of heterogeneity in this region within species, and that distinct IGS types could be associated with particular species

Hybridization based methods and microarray technology. The direct hybridization techniques used for the detection of antagonistic fungi include *in situ* hybridization and colony or dot blot hybridization methods (Sterflinger et al., 1998). Besides DNA probes, peptide nucleic acid (PNA) probes were also developed. PNA are pseudopeptides in which the sugar phosphate backbone of DNA is replaced by a polyamide backbone. A recent development of hybridization-based techniques is the microarray technology. DNA microarrays are glass slides containing an ordered mosaic of the entire genome as a collection of either oligonucleotides (oligonucleotide microarrays) or PCR products representing individual genes (cDNA microarrays; Shi et al., 2003).

Enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP) sequences. Families of short intergenic highly conserved palindromic inverted repeated sequences reported in enteric bacteria can be divided into two classes: repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences (Stern et al., 1984; Sharples and Lloyd, 1990). Repetitive DNA sequences have been used to amplify inter-repeat sequences in order to differentiate between fungal isolates (Arora et al., 1996).

Ribosomal DNA gene cluster. The DNA sequences that encode for RNAs have been extensively used to study the taxonomic relationships and genetic variations in fungi. The ribosomal RNA gene cluster is found both in nuclei and mitochondria, and consists of both highly conserved and variable regions (White et al., 1990). The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. The conserved sequences found in the large subunit (LSU) and small subunit (SSU) genes have been exploited to study the many relationships among distantly related fungi (Bridge et al., 1998).

Protein coding genes

There are numerous gene sequences that have been examined in the systematics and phylogeny of fungi. These include genes for the production of actin, tubulin, elongation factors, cytochromes, proteases, chitin synthatase and many others (Schoch et al., 2001). These genes are generally highly conserved between distant organisms, but can contain short introns that can be very variable in insertion position and number (Edelmann and Staben, 1994). This variation in introns can be useful as a molecular marker among closely related organisms.

Major culture collection center in India

India has a long history of studying microorganisms and research is carried out at a variety of institutes including the Centre for Cellular and Molecular Biology (CCMB), Institute of Microbial Technology (IMTECH) and the Indian Institute of Technology (IIT) and several institutes under the umbrella of CSIR and ICAR. Indian Agricultural Research Institute (IARI), New Delhi, has been began for the conservation and characterization of AIMs at National Fungal Culture in 1935, Rhizobial Collection in 1992 and Blue Green Algal Collection in 1988. Indian Type Culture Collection (ITCC) was established in the Division of Mycology and Plant Pathology, IARI. The main objectives of ITCC are to act as a storehouse, to supply authentic fungal cultures and identification of fungi as well as provide related services to farmers, technocrats, and scientists working in research Institutions, universities and industries. The collection consists of only fungal cultures of agriculturally, medical and industrial value. More than 4,200 strains/ isolate (VI-Edition; 2002) of all groups, specially Zygomycetes, Ascomycetes, Hyphomycetes, Biasidiomycetes including biocontrol, biotechnology and of academic value are maintained. It is well-established laboratory for identification of all kind of fungi.

Microbial Type Culture Collection and Gene Bank (MTCC) is also a well-equipped modern facility housed at the Institute of Microbial Technology (IMTECH), Chandigarh. Main objectives of this center are to act as a depository, to supply authentic microbial cultures and to provide related services to scientists working in research institutions, universities and industries. Relevant information about the strains held in MTCC is computerized for easy search, analysis and retrieval.

About 950 fungal strains and 600 yeast strains has been preserved in National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune 411008. Defence Material & Stores Research & Development Establishment Culture Collection, Defence R & D Organization, New Delhi, has also preserved more than 1,100 fungal strains.

Biodiversity Documentation Centre, Jawaharlal Nehru Centre for Advances Scientific Research

(JNCASR), Jakkur, Bangalore, a sister organization of Indian Institute of Science, focuses on selected areas of topical significance. One of these is biodiversity and the unit is engaged in laboratory studies on microbial biodiversity, and field research programmes, in the Western Ghats and the Himalaya.

There also some other microbial collection centers/universities, which are not mentioned above, have also been contributing remarkable roles in collection, identification and conservation of microorganisms.

India has decided to become a member of the Budapest Treaty on deposition of microorganisms. This will enable India to gain the advantage of depositing patentable microorganisms within the country for the purpose of patent protection involving invention of microorganisms. The instrument of Accession to the treaty would be signed and deposited with the Director General of World Intellectual Property Organization (WIPO) in Geneva. This will also make India eligible for depositing patentable of microorganisms in recognized International Depository Authority.

Role of National Bureau of Agriculturally Important Microorganisms (NBAIM)

Despite the well recognized importance of microorganisms, only less than 5% of the world's microorganisms are on record. Microbial systemic has received relatively little attention in the last fifty years. The very basis of molecular biology and genetics lies in microbiology, and given the present growing interest in the development of products from genetic engineering, introduction of genetically-modified microorganisms to the environment, and other such applications–the absence of available knowledge of the identification and taxonomy of microorganisms must be remedied. Thus, the lack of basic information is a major justification of special targeting of microbial systemic and diversity under the umbrella of NBAIM. As special target area of research, microbial diversity will contribute innovative methods and techniques to accelerate the discovery and characterization of new microbes. Also, a database to select and exchange information on the biological characteristics of microorganisms may be developed. The link between the biotechnology and biodiversity is another key reason for cataloguing and conserving AIMs biodiversity.

Conserving biological diversity is a common concern of all nations. The importance of biological diversity in India was realized, as a result of which, the Indian Council of Agricultural Research (ICAR) established National Bureau of Plant Genetic Resources for exploitation, evaluation and conservation of agro-diversity, National Bureau of Fish Genetic Resources for conservation of fresh and marine fish, and National Bureau of Animal Genetic Resources for conservation of liverstock genetic resources. In India a modest beginning has been made for the conservation and characterization of microorganisms at IARI, New Delhi.

NBAIM will pool all the available resources and upgrade the facilities to meet the current and future requirement for the conservation and characterization of AIMs in the country. The characterization of microbes is of paramount importance, not only from the point of view of protecting the important gene-pool resource, but also for supporting integrated pest and disease management programmes. The identification of indigenous species, strains, races and types of microorganisms would also help in identifying and developing suitable biocontrol agents, which in coming century, will be the main armory for the eco-friendly management of biotic stresses. The Bureau will provide good opportunities for isolating and utilizing gene for conventional and unforeseen products of high economic, environmental and agricultural values. The effort will greatly strengthen the national capability in quarantine and other regulatory matters The Bureau will also perform an important function of depository of AIMs, which will facilitate the process of registration and patenting.

Above all, the Bureau helps in understanding our national heritage of microorganisms, which have not been understood and conserved so far. Main aims of the Bureau is summarized as follow-

i. Exploration and collection of microorganisms from soil, plants, fresh water etc.- covering different agro-climatic regions.

ii. Collection of AIMs from existing culture collection centers, institutions and universities. Bureau will function as repository for all the agriculturally important microorganisms available in the country.

iii. Repatriation of cultures of Indian origin from different culture collections located in other countries including international centers.

iv. Characterization of microorganisms on the basis of morphological, physiological, biochemical and molecular characters.

v. Development of molecular markers and diagnostic tools for diversity analysis.

vi. Database of the entire collection on electronic format for easy access to information.

vii. Development of National Gene Bank for conserving the variability of agriculturally important fungi, bacteria, viruses and cyanobacteria. The evaluation and characterization of microbes could find genes suitable for agricultural and industrial use. The project will boost the Integrated Pest Management Programmes (IPM), and other microbial productivity-based research.

viii. Conservation (both short–term and long–term conservation) and utilization of microorganisms. Identification of AIMs for utilization as biofertiliser, biopesticvides, growth promotion, bioindicators, biodegradation, bioremediation, biocomposting, food processing etc.

ix. Surveillance of indigenous/exotic AIMs.

x. Human resource development (HRD).

Moreover, some others activities like supply of authenticated cultures to the users under material transfer agreement (MTA) and material transfers acquisition (MTA), preparedness for International Agreements/Mechanisms, development of safety standards, to facilitate IPR protection for novel microorganisms/ biomolecules etc. will be performed in future

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PGPR: application and monitoring methods in crop soils

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The new millennium brings with it, renewed hope for a better livelihood for the populations of this earth. Hence the themes often discussed at international fora on human welfare and agriculture range from sustainability, food security and safety to the provision of a productive and healthy environment to humankind and its future generations. Current scientific strategies to maintain and improve yields in support of high-input agriculture place great emphasis on 'fail-safe' techniques for each component of the production sequence with little consideration of the integration of these components in a holistic, systems approach. The fact remains that stable and lower human population is an integral component of sustainable agriculture and more so in the Indian context. Thanks due to the high yielding varieties we are still self-sufficient in rice and wheat, but for how long? High input agriculture is increasingly recognised as an environment degrading and not profitable. We now recognise that technical progress may have social and environmental costs we cannot pay. In any case considerable crop damage by insects and pathogens still prevails. In an Indian context, use of bioinoculants assumes special significance because the size of individual farm holdings is going down gradually due to growth in population. Farmers are struggling to earn enough income from farming to meet the minimum essential requirements of their families. The cost of inputs is going up while the price of output is going down. Indeed this is the very reason for much of the research into biological methods of using bioinoculants as possible alternative to intensive chemical fertiliser, fungicide and pesticide use. Under the present scenario, microbes will probably assume an increasingly prominent role as we look forward to new responsible technologies, if agriculture is to evolve towards both economic and environmental sustainability. The interaction between microbes and plants developed with the process of evolution in plants, and hence the use of bioinoculants are pre-adapted to fit into long-term sustainable agricultural systems. Thus, biological approaches through the use of bioinoculants are pre-adapted to fit into long-term sustainable agricultural systems.

Although the concept of using bioinoculants to increase plant yields goes back over 100 years, relatively very few reliable bioinoculant products are currently available in the market. More often the following reasons result into the failure of the optimal performance and wider use of the bioinoculants:

1. Soil may not be conducive for the propagation of the bioinoculants: The majority of the commonly used fungicides, insecticides, nematicides and herbicides are toxic to soil microflora. High fertility level or adverse physical condition of the soil due to extreme pH etc. or presence of acidic chemical fertilisers also cause the failure or diminish the performance of the inoculum.

2. Inoculum may not be able to compete with indigenous microflora: The biggest lacuna for the low key success of commercialised biologicals inclusive of bioinoculant is because the performance of products based on biologicals falls below that of chemicals. This is simply due to the fact that by virtue of being a product based on microorganisms, the performance of the product is based on how the living organisms reacts to the alien environment of its application, unlike that of chemicals. If the inoculum is not rhizosphere competent then it will fail to establish itself into the introduced environment. This is one of the major causes of the failure of the inoculum.

3. Lack of reproducible performance of the bioinoculants: The overwhelming problem in the area of bioinoculants is to get repeatable results (especially in field, rather than in the laboratory or greenhouse), which are consistent from year to year, and over different climatic and soil types. The variability has many causes including the sensitivity of many potential control agents to these environmental factors, especially when control depends upon the growth and spread of the antagonist, as it usually does. The soil, though very variable, is also remarkably stable: the organisms present are often assumed to be well adapted community in which there may be changes in individual species or populations but which overall remain quite constant. It is, therefore, difficult to introduce 'foreign' organisms into the environment where it does not already exist.

4. Poor quality bioinoculants: This problem arises because of the incongruous people handling the inoculum development work at the government & non-government level and because of some of the small time regional biotechnology companies who jumped into biotechnology bandwagon to make fast bucks. This unfortunately has resulted into the supply of inferior quality products, impairing the product reliability and subsequently adverse name to the inoculum industry. However, if the inoculant is prepared, marketed and applied correctly then significant increase in the yields can be obtained.

5. Treating bioinoculats in a chemical rather than a biological paradigm: The tendency to treat introduced microbes in a chemical rather than a biological paradigm is an unrealistic expectation. This epitome probably traces to product based on *Bacillus thuringiensis* (*Bt*). *Bt* is a marvelous success story for insect control, but probably is atypical of microbial biocontrol of the future. *Bt* works dead or alive. But it would be interesting to know whether this agent would be successful if, like other introduced biocontrol agents, in order to work it had to establish and function temporally and spatially within an ecosystem.

6. Lack of coordination among researchers and bioinoculant manufacturers: There is ample literature available on potentially useful bioinoculants but in reality a minuscule amount of these strains actually are put in use beyond few small-scale field trials conducted by the researchers. Even fewer are actually used for industrial scale production. It is very difficult to find a bioinoculant with ideally required 15 or 20 site years (number of sites x number of years) of performance data before considering the strain for scale up. There is need for the development of industrial scale production of bioinoculants. It is due to lack of interest among both researchers and bioinoculant manufacturers to coordinate efforts to look into the need for technology transfer from basic research to product development.

7. Lack of proper knowledge about bioinoculants in the farming community: Irrespective of their advantages, the use of bioinoculants in India has been low. This is partially due to lack of dissemination of proper knowledge in the farming community and lack of extension support. Farmers should be educated about the utility of bioinoculants by conducting field demonstrations. This will increase awareness about bioinoculant use and will help in a better adaptation of the technology.

The establishment of beneficial bacteria on root systems of plants via seed bacterisation has long been of a major interest to agricultural researchers. This is due to the secretions of nutrients into the surrounding environment. This environment, or the volume of soil that is influenced biologically and biochemically by the living root, is known as the rhizosphere. Root exudates and secretions create a "rhizosphere effect" which manifests itself in the intense microbial activity that is associated with the immediate vicinity of the root. Root-associated microorganisms must compete with other soil microbes for sources of carbon, nitrogen, and/or energy. The ability to utilise these nutritional resources present in the rhizosphere contributes to the survival and competitiveness (rhizosphere competence) of root-associated plant growth promoting rhizobacteria (PGPR). Processes in the rhizosphere influence plant disease and plant nutrition by affecting the dynamics of microbial populations and communities. The action of both plant-beneficial and plant-deleterious soil borne microbes depends on the ability to establish in the rhizosphere. Therefore the knowledge of bacterial growth conditions in the rhizosphere is important for understanding rhizosphere colonisation. In general, a proper characterisation of target soils and rhizosphere as habitats for introduced microbes, as well as adequate strategies is the key to success, to enhance the plant-microbial interactions. As climatic conditions, soils, plants, and microbes are all variable and/or diverse, there is no general rule for how introductions into soils can be optimised. However, it is clear that since soil generally represents a hostile environment to microbial introduction of an alien microbe and since the microbes in the soil are subjected to a range of adverse abiotic and biotic conditions, the rhizosphere competence of the introduced microbe depends to a large extent on how favorable to its survival and functioning the target environment is or can be made, in terms of either natural or induced ecological selectivity (through genetic engineering) or available protective niches. It is anticipated that knowledge of key ecological and molecular characteristics that determine bacterial survival and adaptation in soil may also lead to methods for genetic manipulations directed to improve the survival of bacteria introduced into soil. This in turn is critical for the application of beneficial microorganisms as inoculants to support plant growth. Therefore bacteria that can grow in the rhizosphere are ideal for use as bioinoculants.

The isolation and development of plant beneficial bacterial strains applicable to a variety of crops, soils and locations will depend upon the development of improved detection and screening procedures that more rapidly screen and identify beneficial strains. Few methods have been developed for selecting rhizosphere competent bacterial strains. Recently we have developed a raw soil assay for the large scale screening of native rhizosphere microflora of chickpea, which identifies and characterises naturally occurring rhizosphere competent bacteria with plant growth promotion potential, those which effectively colonise chickpea roots. In this assay system-screening time was reduced on two counts. First, spontaneous chromosomal Rifr strains were directly inoculated to seeds without any check for the stability of the mutation and secondly, no attempts were made to taxonomically identify all the strains being screened for chickpea rhizosphere competence. These findings indicated that the isogenic or equally rhizospheric competitive second non-isogenic strains should be compared for their survival and competition with that of the isogenic parent and with each other for specific ecological niche, before using mixture of isolates, for stable and consistent biological seed treatment to control soil borne pathogens or pests or to promote plant growth. To monitor the presence or absence of *Pseudomonas* on plant roots grown in sterile or non-sterilised soils, spontaneous rifampicin-resistant (Rif^r) strains were isolated on *Pseudomonas* isolation agar, containing 100 mg rifampicin. One spontaneous Rif^r strain

showing growth comparable to the wild type based on the size of colony, on agar plates containing 100 mg rifampicin, was selected, an amount sufficient to inhibit the growth of other native bacteria from the phytosphere. The rifampicin derivative of *P. fluorescens* NBRI1303 (NBRI1303), designated *P. fluorescens* NBRI1303R (NBRI1303R), was selected as a model for monitoring its survival and movement in the phytosphere of plants, its effect on indigenous microbial populations and to test the feasibility of its use as a seed treatment using a suitable carrier, for control of phytopathogenic fungi and growth of chickpea in fields. The rationale for the use of rifampicin resistance to mark bacteria in ecological studies is based on the unique advantages of recovery and enumeration, without interfering with the organism's fitness. Rif^r strains of bacteria have been used to assess the persistence of indigenous and nonindigenous microorganisms inoculated into natural systems. Using this method we have isolated a chickpea rhizosphere-competent bacteria, NBRI1303 for suppression of the chickpea pathogenic fungi *Fusarium oxysporum* f. sp. *ciceri*, *Rhizoctonia bataticola* and *Pythium* sp. In greenhouse test chickpea seed bacterization with NBRI1303 increased the germination of seedlings by 25%, reduced the number of diseased plants by 45% , compared to non-bacterised controls. Increases in seedling dry weight, shoot length and root length ranged from 16 to 18%. Significant growth increases in shoot length, dry weight and grain yield, averaging 11.59%, 17.58% and 22.61% respectively above untreated controls were attained in field trials in Agra and Jhansi. A rifampicin-resistant mutant NBRI1303R of the NBRI1303, used to monitor chickpea root colonisation confirmed the rapid and aggressive colonisation by the bacterium, making it a potential biocontrol agent against chickpea phytopathogenic fungi. The results demonstrate an increase in the efficiency of screening and detection of plant beneficial strains that will greatly benefit future studies. Ecological fitness of our strain was established by demonstrating that NBRI1303R is equally competitive compared to that of its parent NBRI1303. The continued presence of NBRI1303R for 60 days in non-sterilised soil shows that it has reached homeostasis after undergoing exchange with indigenous micro flora and is not affected by the active and passive processes restricting soil community. This makes NBRI2650R ideally suited as soil inoculant because of its potential for rapid and aggressive colonisation.

A key concern for the safety, efficacy, and commercial potential for any environmental application of bioinoculant is the ability of the microbe to survive in target habitats. However, the ecology of rhizosphere competent bacteria is not yet well enough understood to predict the behavior and efficacy of PGPRs in phytosphere (leaf, stem, rhizosphere, and endorhizosphere) colonisation and of the existence of crop specificity. Given their often high densities, intimate association with crop or other plants, physiological versatility, and ease of genetic transformation, it would be an advantage if they were to remain confined within the soil, but the evidence suggests otherwise. Therefore, bacterial movement is an important consideration in future field releases of both native and genetically modified rhizobacteria. It is anticipated that the study of how bacteria proliferate and function to improve plant growth will prove critical to ensure that the commercial products consist of safest as well as the most effective bioinoculants. An increasing number of studies indicate that some strains of common soil bacteria are capable of colonising internal plant tissues. Therefore, depending on the bacterial strain, it may be necessary to monitor colonisation of internal plant tissues in addition to exterior plant surfaces. Survival and movement of rifampicin derivative of *P. fluorescens* NBRI2650 (NBRI2650), designated *P. fluorescens* NBRI2650R (NBRI2650R) was evaluated in the phytosphere of chickpea, cotton, cucumber, and tomato. Unlike, chickpea and tomato, NBRI2650R did not remain confined to the rhizosphere of cotton

and cucumber, but after invading roots it moved into the stems and leaves. Frequent movement has also been detected with other rhizosphere inhabiting bacteria in several plants. Our study illustrates that rhizosphere competent NBRI2650R isolated from the rhizosphere of chickpea has the capability to invade cucumber, and tomato tissues through roots. When NBRI2650R was artificially introduced by injecting chickpea, cotton, cucumber, and tomato, it was recovered in the leaves, stems, and roots even in plants it does not normally colonise, such as chickpea and tomato. However the presence of NBRI2650R in chickpea and tomato could not be detected beyond 7 days when injected. The inability of NBRI2650R to invade and survive inside chickpea and tomato may be due to a hostile host environment. Our results clearly demonstrate host preference by NBRI2650R for its survival within plants. We could demonstrate the presence of chickpea rhizosphere-inhabiting NBRI2650R in the phytosphere of cucumber, and tomato. The variable ability of the NBRI2650R to invade and survive in the phytosphere of plants is in agreement with earlier reports that the ability of bacteria to enter and colonise internal plant tissues appears to be strain-specific. Thus depending upon the bacterial strain, it may be necessary to monitor colonisation of internal plant tissues in addition to exterior plant surfaces and and soil adjacent to roots. Therefore, when considering any large-scale release of phytosphere inhabitating bacteria, it would be desirable to elucidate the range of plant species and all possible colonisation niches the strain is able to colonies, before field release. No difference in the total viable bacteria, actinomycetes, and fungi counts was observed in the rhizosphere of control and bacterised 60-day old chickpea plants. Treatment of chickpea seeds with NBRI2650R resulted in increases in germination, 26%; survival, 20%; dry weight, 24%; shoot length, 20% and root length 27% compared to non-bacterised seeds, using nonsterile fungal disease-conducive field soil from Jhansi. Treatment of chickpea seeds with NBRI2650R using nonsterile fungal disease-conducive field soil from Kanpur resulted in increases in germination, 17%; survival, 35%; dry weight, 41%; shoot length, 26% and root length 39% compared to non-bacterised seeds. Chickpea is grown in low rain fed area and is not irrigated during the duration of the five months of crop growth. The recovery of NBRI2650R from field grown chickpea plant roots up to 5 Log10 CFU g/roots, and the fact that it persisted on the roots of the chickpea for the duration of the growing season is an indication of its aggressive rhizosphere colonisation capacity and potential as a rhizosphere competitor. This feature alone is suggested as a disease control mechanism, via the exclusion of microorganisms from the root surface. The positive chickpea root-colonisation ability of *P. fluorescens* and enhanced germination, survival and yield of chickpea reported here confirm that fluorescent *Pseudomonas* sp. are indeed a promising group of plant growth-promoting rhizobacteria (PGPR) involved in the biocontrol of plant diseases. Chickpea does not require any special care for its cultivation. Therefore, experimental field plot was not subjected to mechanical cultivation methods, like ploughing and harrowing, for the duration of five months. Under these conditions, the horizontal and vertical movement of NBRI2625R was restricted to 30 and 60 cm, respectively. The horizontal movement of the bioinoculant could be due to dispersion in surface water of rain and the vertical movement likewise could be due to migration and movement in water films along roots and the associated channels and cracks. The possibility of any inadvertent dispersion by humans or insects (on or in soil) also cannot be ruled out. NBRI2625R could not survive in field soil for seven months. Lack of survival of NBRI2625R in the soil could be due to either absence of a host plant, and/or a hostile soil

environment. In general, the microorganisms intended for use in biological control present no greater risk to the environment than presented by *Rhizobium*, or by mycorrhizal fungi; and in virtually all cases they present less risk to the environment and society than presented by the target diseases left uncontrolled. Besides a suitable rhizosphere environment, it is generally assumed that for the successful bioinoculant treatment root colonisation by introduced bacteria is required and that increasing the population of an introduced bacterium on the root should enhance disease control. Rhizosphere colonisation of NBRI2650R persisted throughout the growing season of five months at the time of harvesting. The ability of the rhizosphere competent NBRI2650R to sustain high level of population may explain its ability to control *Fusarium* wilt of chickpea, since *Fusarium* wilt infection occurs throughout the season. Our results indicate that NBRI2650R is a potentially useful biocontrol agent for improving yields in *Fusarium* wilt infested chickpea fields.

Engineering of the rhizosphere is another emerging field in which little information is available, both at ecological and molecular level. There is interest in engineering the rhizosphere for several reasons. Such plants might resist soil borne pathogens more effectively, be better hosts to beneficial microbes, remediate toxic waste, or attract communities of soil microbes that enhance plant health). The ecology of rhizosphere is a relatively new research area. Consequently, there is little understanding of how environmental factors will affect bacterial colonisation effects and persistence on roots and the resulting effect on plant. However, it is known that the bioinoculants, which are introduced to the rhizosphere, are involved in a complex of biological interactions with the host plant and with the surrounding rhizosphere microbes. The introduced bacteria are nourished by the root exudates and are thus dependent on the host plant. At the same time, the introduced bacteria may affect the host by inducing physiological changes in the plant. At an ecological level, it has been shown that ecological selectivity worked in the rhizosphere by using the concept of antibiosis. Competing or antagonistic indigenous microbes were temporarily suppressed by using streptomycin, in conjugation with an antibiotic resistant inoculant strain. Ecological selectivity can also be based on the use of a specific substrate, which is unavailable to a majority of other soil microbes. Central among the strategies to engineer the rhizosphere is the effort to create a "biased rhizosphere", which involves engineering plants to secrete nutrients that specifically enhance the growth of desirable microbes. Perhaps the most elegant example of the hypothesis that novel substrate utilization is a component of a microbe-host association is the relationship between *Agrobacterium tumefaciens* and the plants on which it induces crown gall tumors. These neoplasias result from expression by the plant genes transferred to them by the infecting bacteria. The tumors are characterized, in part, by the production of novel, low molecular weight compounds generically called opines. The DNA transferred from the bacterium to the plant during tumor initiation also codes this trait. In turn, the bacteria can catabolise those opine classes produced by the tumors that they induce. The observation that the families of opines produced by a given tumor are dependent upon the inducing bacterium, and that the bacterium can use only those opines that are produced by the tumor it induces led to the opine concept. In its general form, the opine concept states that a specific interaction between a microbe and a host may be driven in part by the capacity of the microbe to use a novel resource produced by the host. We have reported the capability to catabolise opines by the pseudomonads, as carbon and nitrogen sources. Later on, the impact of a novel substrate mannopine (MOP), by creating the nutritional biasing on

rhizobacterial colonisation was examined using mannopine utilisation (Mut) as the model system. The relative competitiveness of two *Pseudomonas* strains that differed only in Mut (one strain was a Tn*5* generated Mut- mutant of its parent wild type Mut+) was determined. Near isogenic tobacco lines differing in mannopine production (MOP+) were obtained by transformation with *A. rhizogenes*. Tobacco seeds were inoculated with a mixture of the two bacterial strains and the ratio between the two was determined (input ratio). Four weeks after planting, bacteria were recovered from seedling roots and ratio between the Mut+ and Mut- were again determined (output ratio). On MOP- plants (no mannopine in the environment), the input and output ratios were essentially the same, indicating that the two bacterial strains were equally rhizosphere competent in the absence of MOP. On MOP+ plants (mannopine in the environment), however, Mut+ bacteria increased relative to the Mut- bacteria, indicating that, in the presence of MOP, the ability to utilise MOP conferred a competitive advantage. We have used this method to engineer biased rhizosphere. It is anticipated that the discovery of a genetically controlled mechanism that would bring about a closer relationship between root and biocontrol agent, and that could be expressed in either the root, biocontrol agent, or both, would open the way for many advances in biological control of root pathogens, including, possibly, a means to lessen the influence of soil factors.

Conclusion

To conclude, the bioinoculants have a tremendous potential in India as a cheap source of plant nutrients, provided the quality of product is assured. The successful development of bioinoculants that meet farmers' expectation must deliver function and value. The subject is so challenging that one should approach it hopefully and with optimism, not with skepticism and doubts. As Samuel Johnson observed long ago, "Nothing will ever be attempted if all possible objections must first be overcome." Each successful application, no matter how limited, increases the level of familiarity with bioinoculants among user groups, governmental regulatory agencies, private investors, and the public. Looking ahead, it is relatively easy to visualise thousands of microbes in use worldwide for control of specific diseases on specific crops in specific cropping systems and produced by public or private enterprises as necessary. This requires development of new technologies on a constant basis and assimilates the same in their production set up. To succeed, those manufacturing the bioinoculants must deliver the knowledge. Indian farmer is conscious of the fact that chemical fertilisers adversely affect soil health. Therefore they prefer to use green manure and farm yard manure to preserve the soil health. It may probably be more important for us to convince our farmers about the ecofriendly and soil rejuvenating nature of bioinoculants along with limitations. For example, farmers must understand that bioinoculant application is prone to environment like any other living object, unlike chemical fertilisers. Once these limitations are understood, the benefits of bioinoculants such as efficacious season long control, flexible timing of application, crop, and soil health and environment safety may be established. Limitations and benefits may then be combined to arrive at consistently attainable expectations of value of the bioinoculants. Finally, the success of the wider use of bioinoculants will partly depend on the total commitment of companies for maintaining the quality of the bioinoculants and partly on our government regulators to implement regulations to ensure that companies are responsible for maintaining consistently high standards, as the bitterness of poor quality remains long after the sweetness of low price is forgotten. Bioinoculant products that meet and exceed farmers' expectations will drive productivity gains of benefit to all.

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BNF and Biofertilizers in Integrated Plant Nutrient Supply Systems

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Introduction

Biological nitrogen fixation (BNF) plays an important role in sustainable crop production and maintaining the fertility of the low-nitrogen (N) containing soils of the semi-arid tropics. The oil crisis of the early 1970's and the consequent rise in fertilizer N prices resulted in intensive researches on BNF. Subsequently, the momentum slowed but was sustained due to environmental concerns associated with the manufacture of nitrogenous fertilizers and their usage. There is a world-wide consensus now that sole dependence on chemical input based agriculture is not sustainable in the long run and only integrated plant nutrient systems (IPNS) involving a combination of fertilizers, organic/green manures and biofertilizers (bioinoculants) are essential to sustain crop production, preserve soil health and soil biodiversity in the long run. IPNS involves optimization of all possible organic, inorganic and biotic resources of plant nutrients required for plant nutrition and quality in an integrated manner appropriate to each cropping system and farming situation in its ecological, social and economic possibilities.

Sustainability of nutrient inputs would mean putting back into the soils what has been taken out. There is a huge gap of 12 million tonnes between crop removal of nutrients and additions each year through chemical fertilizers in Indian Agriculture. In the sate of Madhya Pradesh alone the gap is about 1.1 million tonnes each year. Recycling organic wastes and use of bio-inoculants have therefore a promising role to play in reducing this gap between demand and supply of nutrients. Secondly, as population is increasing, producing enough food will require us to increase N consumption by 2.5% per annum. The nitrogen fertilizer demand in Indian agriculture is expected to go up from the present level of 11.4 million tonnes (2001-02) to 13.9 mt by 2006-07 and 16.2 mt by 2011-2012 AD. The economic burden and environmental cost of applying such a huge quantity of additional fertilizer nitrogen is obvious. Even if a part of this increase in the need for nitrogen can be met from BNF, the likely savings will be enormous. The achievements in BNF in the country have recently been reviewed by the QRT of AICRP on BNF. The future course of action on biological nitrogen fixation and biofertilizers in the X five year plan has also been charted by several committees and working groups.

Integrating legumes in farming systems as grain legumes, green manure crops, forages, inter-crops, partial green manuring of vegetable legumes, live fences etc., are some of the most successful examples of BNF applications. Legumes can potentially fix about 80% of their own nitrogen and in addition can contribute to the yield of subsequent crops. However the

potential is seldom realized due to one or the other constraints, abiotic or biotic. Abiotic constraints include moisture stress, salinity, acidity, high pH, P deficiency due to fixation in calcareous and acid soils, iron deficiency in calcareous soils, molybdenum and cobalt deficiency in acid soils etc. Thus, the critical need is to enhance the efficiency of the externally applied nutrients and increase the bio-availability of the nutrients present in the soil. The latter becomes possible by the application of efficient strains of rhizosphere microorganisms that can influence the nutrient availability to plants in many ways including transformation of the unavailable forms of minerals to the available forms. The identification of competitive, efficiently nodulating, nitrogen fixing strains of rhizobia and their inoculation can solve the problem of ineffective nodulation by native rhizobia. Beneficial free-living soil bacteria are usually referred to as plant growth promoting rhizobacteria or PGPR. A number of different bacteria may be considered to be PGPR, including *Azotobacter* spp., *Azospirillum* spp., *Pseudomonas* spp., *Burkholderia* spp. and bacilli. They facilitate plant growth through fixation of atmospheric nitrogen, solubilization of fixed forms of phosphates, production of phytohormones like IAA and gibberellins, production of siderophores for chelating iron, and synthesis of low molecular weight compounds or enzymes that can modulate plant growth and development. PGPR are also reported to produce antibiotics that suppress deleterious rhizobacteria /plant pathogenic fungi or through some other unidentified mechanisms and thus provide a healthy environment for better root growth.

Past achievements in BNF and Biofertilizers

Research on BNF and biofertilizers in India is nearly eight decades old and was particularly intensified by ICAR during the last 25 years through the AICRP on Biological Nitrogen Fixation. Various bio-inoculants for all crops in various agro-ecological regions of India have become available for augumenting the supply of mainly N and P in cropping systems and for promoting plant growth. An enormous wealth of information on basic as well as applied aspects of biofertilizers based on field experiments and demonstration in farmers fields have become available which has recently been summarized. The inoculation technology has been successfully demonstrated through vigorous extension programme on front line demonstrations in farmers fields carried out for last 10 years to popularize the use of biofertilizers in four states namely Tamilnadu, Maharashtra, Madhya Pradesh and Uttar Pradesh. A rich germplasm collection of effective strains of agriculturally useful microorganisms are now available which are currently being commercially exploited. A range of stress (temp., salinity) tolerant microorganisms and strains efficient in fixing N in the presence of recommended dose of N fertilizers and herbicides have been developed. Largely, the local isolates are more effective than the best of the introduced isolates showing the importance of the ecological adaptation of the strains in a given habitat and the difficulty of introducing an organism in an alien environment. The methods for tracking inoculated strains using lac Z, gus A and gfp markers were used in nitrogen fixing associative and symbiotic bacteria. It has been shown that *Rhizobium* inoculation of legumes helps in increasing yields by 15-30% with an absolute yield increments of 0.5-2.0 q/ha and in some cases even upto 3 q/ha. In legumes additional plant N uptake of 10-15 kg/ha have been shown from field experiments conducted for three years. In terms of fertilizer nitrogen equivalence these amounted to residual benefits of about 30-40 kg N/ha. Application of micronutrients in acid soils boosted nodulation and BNF in pulses.

Inoculation with *Azotobacter* and *Azospirillum* in rice and wheat gave 8-10% yield increase, and 10-20% higher increases in millets, sunflower etc.. The benefits were equivalent to about 10-30 kg N/ha in the former and 10-20 kg/ha in the

latter group of crops with absolute yield benefits ranging from 1.0-3.0 q/ha. These beneficial effects were primarily through promotion of plant growth. In acid soils of Orissa additional rice yield of 1.1 q/ha was harvested by *Azotobacter* and *Azospirillum* application over and above application of 80 kg N/ha. Integrated use of 50% N + Biofertilizers showed higher recovery of NPK than sole fertilizer treatments at 50 and 100% level of N. Similarly in West Bengal for rice crop cultivated by application of 5 t ha^{-1} compost, inoculation of either *Azotobacter* or *Azospirillum* singly or in combination alongwith 50 kg N ha^{-1} was as effective as 100 kg N ha^{-1} thus saving 50 kg N /ha. The effects varied between 25-50% N substitution in rice depending on whether compost was applied or not. In Uttar Pradesh field trials on inoculation with *Azotobacter*, *Azospirillum* and Blue green algae in rice and wheat produced benefits equivalent to about 15-20 kg N/ha and gave yield increases ranging from 10-15 %. Azolla fixed nearly 40-50 kg N/ ha, producing yield increase of 10-20%.

Co-inoculation of *Rhizobium*, *Azospirillum*, VAM and PSB has been found to be significantly better than their single inoculation. Mixed biofertilizer formulations involving diazotrophs and phosphate solubilizers/PGPR's are developed. Methods for enhancing the shelf life of bacterial inoculants are also developed. Inoculation with PSB or VAM was shown to save upto 8-10 kg P_2O_5 /ha in rice, wheat, groundnut, soybean and other crops. In cereal-oilseeds rotation, biofertilizer application to both showed residual benefits of 40-60 kg N /ha in soybean-wheat and groundnut-rice system.

Increasing success is now being achieved with inoculation of 'Plant growth promoting rhizobacteria' (PGPR) including competitive antagonists, which stimulate plant growth and supress disease, particularly the soil borne ones. More than 50% of current production of biofertilizers in India is of PSBs, mainly *Bacillus* spp. and *Pseudomonas* spp. Much of their beneficial effects may be a PGP/biocontrol effect mediated by a variety of ways including P solubilization as one but not the exclusive cause.

Organics have been found to boost the proliferation of *Rhizobium* and enhance nodulation and nitrogen fixation in legumes and oilseeds. *Rhizobium* inoculation of groundnut increased the pod yield by 3.9q/ha while FYM alone @ 5t/ha increased it by 1.5 q/ha, combined application of FYM and *Rhizobium* increased it by 7.3 q/ha. Nodulation, N and P uptake, *Rhizobium* population in soil etc., were all boosted due to combined application of FYM and *Rhizobium*. Similarly in green gram, use of *Rhizobium* alongwith FYM gave 2.8 q/ha additional grain yield respectively over unmanured, uninoculated control. These and similar results in pigeonpea led to the recommendation from AICRP on BNF at Parbhani 'Apply *Rhizobium* inoculants alongwith FYM @ 5 t/ha'. In the presence of organics, non- and associative diazotrophs could substitute for 40 kg N/ha for rice in Eastern India.

As a result of concerted efforts' the biofertilizer consumption in the country has risen steadily, from 2005 t/yr in 1992-93 to nearly 10,000 t/ yr in 1998-99 and 13,000 t/yr in 2000-01. Biofertilizers are being currently produced in about 84 biofertilizers production units and it has been estimated that only about 18% of the potential demand is being met by current production. Yet I have seen conflicting figures of the actual demand from various agencies. We need to work out a realistic estimate of the demand for biofertilizers by the year 2010 and 2020, so that planning can be made to set up production units accordingly, region-wise. Reports of spurious inoculants or ones loaded with large number of contaminants are common, so we need to switch over to a completely sterile method of manufacturing. We should be prepared to pay a higher price for quality cultures. The role of research institutions and state agricultural universities in producing BNF packets on pilot scale or for the NBDC and state agricultural

departments in producing packets on a slightly larger scale has outlived its purpose and should be phased out. Subsidies on biofertilizers are already being phased out by many states and production must be entirely privatized and market forces should determine what is the actual need.

A Perspective on BNF and Biofertilizer Researches

The Research and Development status of biofertilizers is a matter of concern. The research agenda has fallen into a predictable pattern, based on limited isolations in 4-5 known media, testing on limited scale, field inoculation and measuring agronomic parameters like yield and fertility equivalents or at the most integration with organics in an IPNS mode. Screening of strains is not done in a stringent manner at most places, even in greenhouse trials. Even our methodology of glass-house screening is imperfect and bound to make us miss promising organisms. Assessment of actual benefits of nitrogen fixation by isotope dilution or simple methods like inclusion of non-nodulating hosts or cereal controls is more of an exception. Survival of introduced inoculum has been rarely assessed by biochemical or molecular markers. Our taxonomy not only of plants but also microbes is very poor. This is a dangerous neglect. Hardly 10 new genera of diazotrophs have been reported in last 20 years. Biodiversity of nitrogen fixers and other agriculturally useful microorganisms should be a top most research priority. The establishment of the National Bureau of Agriculturally Useful Microorganisms by the ICAR at New Delhi is a timely step in the right direction.

In view of the increasingly complex challenges the future researches must involve multi-disciplinary efforts involving microbiologist, soil scientist,agronomist and plant physiologist. Public perception of biofertilizers is moulded by the fact that the word 'fertilizer' is unjustifiably tagged with microbial preparations which are essentially 'ecological' inputs and not 'chemical' inputs and it is unrealistic to expect dramatic effects. The dirt cheap cost of biofertilizers, the perceived ease of their production often in a cottage industry style employing skilled labour with non-microbiologists supervision, has contributed not only to poor quality but also to a poor image for the product. Improper handling at the time of application by even scientists has further worsened the problem. No wonder that except in isolated pockets like Tamilnadu, the enthusiasm of the biofertilizer researcher is not shared by farmers or planners due to inconsistent response or exaggerated claims.

Some components of the problem that need to be tackled in the next ten years are discussed below:

1. Greater emphasis needs to be given to isolate newer strains from diverse habitats representing extreme environments as well as unexplored or under-explored areas in various agro-ecological zones of India. We have hardly made any attempts to categorize our indigenous diversity on a systematic basis. A beginning has been made by the AICRP on BNF for categorising the genetic diversity of soybean rhizobia in Madhya Pradesh during the last two years. We need to carry out more rigorous strain selection. By rigorous strain selection established ineffective native strains in some soils can be successfully replaced by introduced ones. We should place due emphasis on the crucial importance of soil moisture, soil salinity/alkalinity, toxicity of P, Fe, Al, Mn, heavy metals etc. in inhibiting the symbiotic process and develop strains resistant to such abiotic stresses. Our enormous area under rice fallow pulses is a potential area for isolating rhizobia with PGP action on cereals.

2. Newer formulations like mixing N fixers, P solubilizers and PGPR's in the same biofertilizer packet should be evaluated. There is also need to isolate and test widely organisms which can improve potassium, silicon, zinc, copper, manganese, molybdenum and cobalt nutrition of plants. Seed inoculation which is conventionally followed often fails to deliver the required number of rhizobia due to desiccation. Soil inoculation

in liquid or slurry form may be a more suitable method in many cases like groundnut and soybean and should be explored.

3. We need to exploit the host-variability of legumes and select for plants that can nodulate effectively with indigenous rhizobia. By screening in low N soils, high-nodulating varieties in groundnut, chickpea and soybean have been successfully developed that show early nodulation, higher yield, higher seed N and more proportion of nitrogen derived from fixation. Also a number of diazotrophs capable of epiphytic colonization on seeds (e.g., azorhizobia) are known that do not require inoculation as I have observed repeatedly on *Sesbania*. Further work may throw light on ensuring efficient rhizobial transfer through seeds itself.

4. The methodologies used by microbiologists need to be more sophisticated. The inoculated organisms must be tracked by molecular or other markers wherever feasible to ascertain survival of inoculated species. All screenings of strains must be done in undisturbed soil cores to minimize the effects of mineral N released on grinding soils, so that effective strains are not missed during the screening process. ^{15}N isotope dilution technique should be used to quantify fixation where possible, others must at least include non-nitrogen fixing isolines if available, failing which at least non-fixing cereal crops should be used as controls.

5. Culturing of VAM in synthetic media for biofertilizer supply to agricultural and plantation crops must be attempted. This will ensure easy propagation and distribution like bacterial cultures.

6. Since inoculants are manufactured as per demand and for use in the particular crop season the normal shelf life of those manufactured in India of about six months is usually enough. The shelf life of biofertilizers can be improved by addition of organic polymers. Indigenous waste-products/by-products of industry need to be screened for use as possible carriers. However the exclusive concentration of many workers only on improving shelf life in packets without simultaneous assessment of survival, tolerance to desiccation and high temperatures obtaining in the field is a simplistic approach with little merit in it. Merely improving shelf life is not an issue that will benefit farmers. Superior formulations are likely to result from exploiting the additive effects of physical protectants, physiological conditioning of rhizobia to promote rhizobial tolerance to rapid changes in water potential, inactivating toxic substance diffusing from seed and genetic improvement of rhizobia for survival. To help maintain quality, support for biofertilizer testing (Enzyme kits with marked strains) and certification standards should be strengthened. Sterile methods of production, industrial hygiene, double packing, better carriers can all contribute to high titre and quality. There is hence a need for introducing more sophisticated methods in bio-inoculants manufacture. We need to switch over to a completely sterile method of manufacturing inoculants. With the advent of liquid inoculants in the market there is a need to rigorously evaluate these vis-à-vis good quality conventional inoculants. Not only for VAM but also for other agricultural inocula we should intensify research on less bulky delivery systems based on lyophilized/pelletised formulations

7. The strides made in inoculant researches in all pulses, cereal and oilseeds crops in last 40 years is impressive. Now the need is for diversification. There is a good potential to increase production of leguminous vegetables by *Rhizobium* inoculation and of vegetable crops and floricultural crops by use of non-symbiotic diazotrophs with or without VAM co-inoculation. This ensures not only increased yields but improved quality of the product. For agroforestry industry involved in large scale plantation of commercial forests, supply of VAM propagules in solid substrate and *Rhizobium* inoculants which could be applied to the planting mixtures used for raising seedlings would be a profitable way of increasing agroforestry/forestry productivity.

8. Agriculture in the tribal and hilly areas has remained subsistence oriented. There is need to infuse new technology. Biofertilizers have a great promise because they work best under low fertility conditions. There is a need to focus on North-East, Chattisgarh region and Northern hilly tracts in view of their contribution to agriculture production.

Finally some thoughts on how to re-invigorate biofertilizer research and promote their wider use by farmers. Firstly Research- Biofertilizers research or researchers are not an island in themselves. Excellence can only be fostered by creating an all round enabling environment reducing the researchers to devote more time for research, more funding for longer duration, and greater autonomy. For promoting their under use by farmers we need to follow the lead shown in states like Tamilnadu, Maharashtra and Madhya Pradesh involving a close linkage by research, demonstration and industry involvement.

Thus, in addition to testing of the technologies already generated the areas of further concentration for BNF researches in X plan and beyond should include:

- ❒ Characterization of Soil Biodiversity
- ❒ More rigorous screening of strains
- ❒ Development of mixed consortia of biofertilizers
- ❒ Exploiting host variability in legumes
- ❒ Improved research methodology
- ❒ Development of technology for VAM multiplication
- ❒ Improvement of quality control methods for biofertilizers
- ❒ Development of new carriers for biofertilizers
- ❒ Expanding the BNF applications (vegetables, minor millets)

Extend BNF technologies to tribal, hill and mountain regions, and NEH regions.

Plant-associated bacteria for biological control of rice diseases

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I. Introduction

The early 1960s witnessed a dramatic increase in the global population and a mood of despair regarding the world's ability to cope with the food – population balance. Concerned at this impending crisis, several organizations sought to promote rice cultivation in the developing, rice growing regions of the world. High yielding, fertilizer responsive cultivars like IR-8, which could be grown throughout the year, were therefore introduced for cultivation in several parts of Asia, where 92% of the worlds rice is grown. Today, 23 countries contribute more than one million tons of rice in the global scenario.

This acceleration of growth in rice production has been possible largely due to the replacement of traditional agricultural practices with modern ones. While the deployment of high yielding cultivars contributed to a direct enhancement of grain yield, the development of effective strategies for pest and disease management minimized crop losses and consequently increased the net availability of rice.

Annual losses of up to 40% are said to be incurred due to biotic stresses like insects pests, pathogens and weeds and more than half the worlds rice crop is estimated to be lost hence. Among several diseases caused by bacterial, fungal and viral pathogens that devastate rice yields all over the world, *Magnaporthe grisea* (rice blast), *Rhizoctonia solani* (Sheath blight), *Sarocladium oryzae* (Sheath rot), *Xanthomonas oryzae* pv. *oryzae* (bacterial leaf blight) and the rice tungro virus (Tungro disease) are considered serious constraints.

Disease management strategies aimed at reducing losses and averting outbreak of epidemics have been developed in the past and have been in practice ever since. The use of chemicals, specially compounds of mercury and copper and diverse antibiotics were widely resorted to, in order to achieve high levels of disease suppression. However, the persistent, injudicious use of chemicals has been discouraged owing to their toxic effects on non-target organisms and due to the undesirable changes they inflict upon the environment. Many of these chemicals are also too expensive for the resource-poor farmers of Asia. Though the exploitation of host resistance and introgression of R-genes into local high yielding cultivars appear promising, the large-scale and long term use of resistant cultivars is bound to result in significant shifts in the virulence characteristics of pathogens, culminating in resistance breakdown.

Biological control therefore assumes special significance in being an eco-friendly and cost-effective strategy for disease management, which

can also be used in integration with other strategies to afford greater levels of protection and sustain rice yields. Though fungi, viruses, insects or any organism (other than the damaged host or the pathogen causing the disease) can be used as agents mediating biological control, bacterial antagonists to various plant pathogens have received enormous attention and are considered ideal candidates for biological control, due to obvious reasons like rapid growth, easy handling and aggressive colonization of the rhizosphere. These bacteria may mediate biocontrol by the production of antibiotics, iron-scavenging siderophores, lytic enzymes and microbial cyanides or by initiating a cascade of events resulting in the induction of systemic resistance in the host. Efficient bacterial antagonists to many rice pathogens affording appreciable levels of disease suppression have been identified and a few strains demonstrating potential for very high levels of suppression have been studied in detail. Also, the discovery that many of these antagonists may function as 'plant-growth promoting rhizobacteria' (PGPR), contributing directly to the enhancement of plant growth and health has enthused enormous interest in biological control and favors its deployment as a sound, ecology-conscious strategy for disease management. This paper aims at highlighting the usefulness of biological control agents in suppressing some major diseases of rice.

II. Candidate bacterial strains for biological control of rice pathogens

Diverse groups of microbes exist in nature. Obviously, biological control agents are not limited to any specific group – however, only very few groups of microbes have received attention and have been widely acclaimed as ideal candidates for biocontrol. Bacterial antagonists in general, *Pseudomonas* and *Bacillus* in particular, are thought to be the most appealing candidates for biological control. Bacilli are Gram-positive endospore producing bacteria that are tolerant to heat and desiccation – a feature that makes them very attractive for effective deployment. The pseudomonads are Gram negative rods and have simple nutritional requirements. They are known to be excellent colonizers and are widely prevalent in the rice rhizosphere. This paper describes for a larger part, the use of bacterial biocontrol agents for rice disease management.

Both fluorescent and non-fluorescent bacteria have been implicated in the suppression of rice diseases (Table 1). A number of antagonistic bacteria identified from the rice rhizosphere soils of upland and lowland fields, diseased and healthy plants and from rice field flood waters have been broadly categorized as fluorescent or non-fluorescent strains. Among them, 91% of the former and 33% of the latter inhibited mycelial growth of *R. solani in vitro*. When used for seed bacterization, these strains reduced rice sheath blight (ShB) severity in greenhouse and field tests.

In the Philippines, researchers have identified different groups of bacterial antagonists to seed-borne, foliar and sclerotium-forming rice pathogens. These antagonists belonged to the genera *Bacillus*, *Pseudomonas*, *Serratia* and *Erwinia*. All of 23 antagonists screened, inhibited mycelial growth of *R. solani*, while few of them could inhibit growth of other fungal pathogens like *Sclerotium oryzae*, *Helminthosporium oryzae*, *Pyricularia oryzae*, *Sarocladium oryzae* and *Fusarium monoliforme*.

Also, studies from our laboratory have revealed that a large number of bacterial strains possess the ability to protect rice plants from diseases such as blast, sheath blight, sheath rot and stem rot. About 40 bacterial isolates antagonistic to the rice bacterial blight pathogen were identified through dual plate assays (Figure 1).

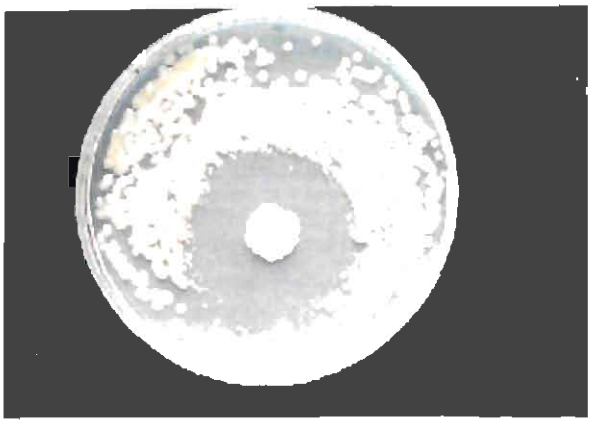


Fig. 1 Inhibition of *Xanthomonas oryzae* pv *oryzae*, the rice bacterial blight pathogen bacterial antagonists in dual plate assays.

Treatment of susceptible rice plants with these antagonists appears to effect statistically significant reductions in bacterial blight lesion lengths (Table 2) as evident from net-house and field assays. Also, there seems to exist a direct correlation between the endophytic survival of a biocontrol agent applied to rice plants through various treatments and the extent of bacterial blight suppression by *Pseudomonas putida* strain V14i. The exploitation of endophytes for biocontrol is therefore an exciting possibility, especially for the control of vascular pathogens.

Methods of bacteria application: The assessment of the potential of any organism to bring about disease suppression demands that it is applied on to the plant system in suitable form. This may be achieved either by direct inoculation (dipping seeds in a bacterial culture, aerial spraying or spreading the bacteria in sowing furrows by drip systems) or by the use of various solid-phase inoculants.

In our laboratory, bacterization is achieved by application of biocontrol agents to the seeds before sowing and/or as foliar sprays to the foliage of the plant. Surface sterilized seeds are dipped overnight in bacterial suspensions (OD at 600nm = 0.1) prepared in 0.5-1% carboxymethylcellulose for seed bacterization and are then air-dried before sowing. Besides, this suspension may also be used to bacterize plants as foliar sprays. Biocontrol agents thus applied have been effective in bringing about significant suppression of several serious foliar rice diseases. Greenhouse and field experiments for sheath rot suppression revealed that *P. fluorescens* treatments applied to the seeds and rice plants prior to inoculation with the pathogen could reduce disease severity by 20 to 42% in 5 rice cultivars tested. Bacterization of rice plants was also found to enhance plant height, number of tillers and grain yields from 3 to 160%. Seed treatments with bacterial antagonists to *Fusarium monoliforme* causing the rice bakane disease could bring about 72 to 96% disease reduction in seedbed experiments.

Similarly, net-house experiments for the biological control of the bacterial blight pathogen revealed that different species of *Bacillus* applied to rice plants as a seed treatment before sowing, a root dip prior to transplantation and 2 foliar sprays prior to inoculation could afford up to 59% suppression of the disease. These treatments could also bring about a two-fold increase in plant height and grain yield. Efforts are underway to characterize the mechanism(s) mediating the biological suppression of bacterial blight disease.

Also, recent work from our laboratory has demonstrated the insecticidal activity of *P. fluorescens* strains to the rice green leafhopper (GLH), *Nephotettix virescens*. The GLH is the insect vector of the rice tungro virus (RTV) causing the Tungro disease. This is one of the most devastating rice diseases, whose successful management has been a challenge to rice growers all over the world. Bacterial strains of *Pf* 7-14 and *Pp* V14i showed maximum toxicity to the insect vector, bringing about death of 90% of the insects that were fed on bacteria treated rice leaves for 7 days. Thin sections of the gut and

eyes of the dead insects, however, did not reveal the presence of the bacteria. The death of the insect vector therefore appears to be mediated by certain toxic substances produced by the bacteria. The possibility of using such bacteria to control vectors and thereby reduce incidence of the disease however needs to be explored and intensive studies with regard to the feasibility of such an approach has to be carefully assessed. The following paragraphs provide a detailed analysis of mechanisms involved in the biological control of a major fungal (blast) and bacterial (bacterial blight) diseases:

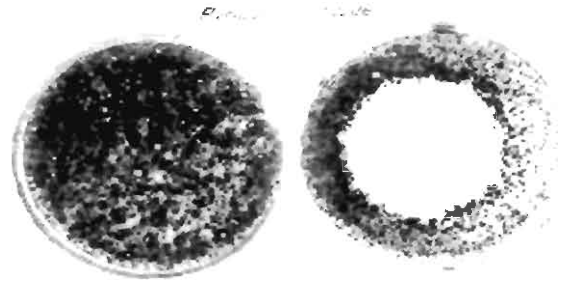
Mechanisms in blast suppression:

Rice blast is a serious production constraint in most rice growing regions of the world. Extensive screening of a large number of rice rhizosphere associated bacteria for antagonism against the rice blast pathogen has been carried out in our laboratory. Some of these strains have demonstrated up to 80% reduction of leaf blast in susceptible rice crop.

Strains of *Pseudomonas fluorescens* were probably the first agents of biocontrol identified against the blast pathogen. A strain designated *Pf* 7-14 isolated from the rice rhizosphere afforded significant levels of blast suppression in field experiments. A preliminary effort was also made to characterize the mechanism involved in disease suppression by this promising biocontrol agent. Siderophore production, one of the most important mechanisms known to mediate bacterial antagonism to fungi, could not have been involved here, as the rice soils in which these experiments were carried out were highly acidic (pH=4). Moreover, Fe amendments to the medium did not reverse the antagonism of this strain to the blast fungus. Antibiotics partially purified from the culture filtrate of the bacterium by sephadex column chromatography were implicated in blast suppression. These antifungal antibiotics (afa) afforded 70-100% inhibition of condial germination of *P. grisea* at 1.0 PPM concentration. The exact chemical nature of this antibiotic was not elucidated.

Pf 7-14 is known to produce several antifungal metabolites and it is likely that each of these metabolites require a different set of genes for their synthesis. The *Pf* 7-14 mutants, lacking either totally or partially, in their ability to produce antifungal metabolites generated by transposon mutagenesis (mini Tm5-Km) were tested in field experiments along with the wild strain in attempt to compare their efficiency in mediating blast suppression (Fig.2). While the wild type strain could afford 79 and 82% leaf and neck blast reductions respectively, the mutants afforded a mere 24 to 40% and 3 to 25% suppression of leaf and neck blast (Table 3). Also, the wild type and mutant strain of *Pf* 7-14 controlled ShB by 82% and 10% respectively. It was also observed that the reductions in blast and sheath blight mediated by *Pf* 7-14 in the field was better than those achieved by treatment with a commercial fungicide tricyclazole. Antifungal antibiotics (afa) produced by the bacterium were therefore thought to be responsible for biological control of blast by *Pf* 7-14.

Many fluorescent pseudomonads and other PGPRs have been reported to induce systemic resistance in different plant systems against their respective pathogens. A recent study from our laboratory suggests that induced systemic resistance (ISR) in rice triggered in response to treatments with *Pf* 7-14 and *Pp* V14i is an important mechanism in the biological suppression of blast. While an increase in the endogenous levels of salicylic acid was detected, the rice phytoalexin momilactone-A could not be detected in rice plants treated with either strain of bacteria. This increase in SA levels by bacteria-induced ISR was found to contribute to rice blast suppression by 25%.



(a)

(b)

Fig. 2 Blast control by *Pseudomonas fluorescens* strain *Pf*7-14.

[a] Laboratory assay shows inhibition of *Pyricularia grisea* (rice blast fungus) by *Pf*7-14.

[b] Rice panicles from a field experiments show suppression of neck blast due to treatment with *Pf*7-14 (plants on the right) and lack of protection against neck blast in plants treated with its afa⁻ mutant (plants on the left)

Mechanism(s) of bacterial blight suppression

Data presented in Table 2 show that 2,4-diacetylphloroglucinol (DAPG) production is also one of the major mechanism for bacterial blight control by plant-associated bacteria. In net-house and in field experiments, these bacteria afforded up to 64% bacterial blight control.

IV. Formulations of biocontrol agents for the control of rice pathogens

The success of a biocontrol program depends largely on the ability of the introduced agent to establish itself in the new environment and maintain a threshold population on the planting material or rhizosphere. Also, the commercial application of biological control and its implementation as a farm-level strategy demands that these agents are preserved in a viable state for long periods of time and are designed to tolerate desiccation and other physiological stresses associated with transport, storage and application. Therefore, the development of cost-effective formulations of biocontrol agents that are easy to handle and have no adverse effects on seed germination or plant growth is essential.

Studies from our laboratory have revealed that among formulations of *Pp*V141 and *Pf* 7-14 with 8 different combinations of methylcellulose (mc), talc and $CaCO_3$ in different proportions, the combination of mc:talc (1:4) emerged the most satisfactory. The bacteria could survive on this formulation for up to 10 months. Formulated *Pp*V141 applied as seed treatment, root dip and foliar sprays, could effect ShB suppression of up to 60%. Similarly, a formulation of *Pf* 7-14, applied as seed and multiple foliar sprays afforded 60 and 72% suppression of leaf and neck blast respectively.

Seed treatments with a formulation of the marked *Pf* 7-14 strain have also provided useful insights into its survival and migration in rice tissues. Their persistence on rice roots for up to 110 days (almost the entire cropping period) can be correlated with the high levels of disease suppression encountered upon treatment. The limited ability of *Pf* 7-14 to migrate to aerial parts of rice plants (until 7-9 days after emergence) suggests that a direct contact between the bicontrol agent and the pathogen may not exist. Disease suppression in such circumstances may be attributed to a systemic resistance induced by the agent in rice or to the production of potent afa(s) in the rhizosphere and their transport to aerial parts.

VII. Conclusions and future directions

Inconsistent performance of biocontrol agents

Table 1: The major rice diseases, their causal organisms, symptoms and biocontrol agents identified.

Disease	Causal organism	Symptom	Biocontrol agents
Blast	*Pyricularia oryzae* Teliomorph: *Magnaporthe grisea*	Small, water soaked, whitish-grey spots that enlarge forming elliptical lesion with pointed ends and brown margins. In the pannicle, the neck blackens and rots causing it to fall as the grain sets	*Pseudomonas fluorescens*
Brown spot	*Drechslera oryzae / Helminthosporium oryzae.* Teliomorph: *Cochliobolus miyabeanus*	Initiated as a brown, oval or round spot on leaf or sheath. Spots coalesce in severe cases. Black or brown spots seen on glumes as well.	*Pseudomonas P. aeruginosa* *Bacillus* sp. *B. subtilis*
Bacterial blight	*Xanthomonas oryzae* pv. *oryzae*	Dull green water soaked lesions on leaf tip or margin that enlarges forming characteristic yellow lesions with wavy margins that extend up to the sheath. Leaves later turn straw-like and blight.	*Bacillus spp, (B. lentus, B. substilis, B. coagulans and Bacillus sp) and DAPG-producing P. fluorescens*
Stem rot	*Sclerotium oryzae / Helminthosporium sigmoideum* Teliomorph: *Nakataea sigmoidea/ Magnaporthe salvinii*	Small black irregular lesions on the outer leaf sheath. Lesions advance to the inner leaf sheath and leaf rots. Inter nodes rot causing stem to lodge	*P. fluorescens* *P. aeruginosa* *B. subtilis* *B. pumilus*
Sheath blight	*Rhizoctonia solani* Teliomorph: *Thanatephorus sasakii*	Ellipsoid to ovoid lesions on leaf sheath with a greyish white center and brown margin. Lesions enlarge and extend to the leaves causing severe blight symptoms. Sclerotia formed during advanced stages.	*P. fluorescens P. putida, P. fluorescens, Bacillus sp. B. subtilis, B. laterosporus, B. polymyxa B. pumilus, Serratia marcescens, Pseudomonas, P. aeruginosa*
Sheath rot	*Sarocladium oryzae*	Rotting of the upper most leaf sheath enclosing young panicles. Lesions begin as oblong, irregular spots with brown margin and grey centre, which enlarge and coalesce later. Young panicles remain within sheath or only partially emerge	*P. fluorescens* *B. subtilis* *P. aeruginosa* *Pseudomonas*
Tungro disease	*RTV* *Vector: Nephotettix virescens*	Stunting, discolouration of leaves to yellow to orange. Young leaves mottled, old leaves have rusty specks.	*P. fluorescens* For green leaf hopper vector

Table 2. Plant-associated fluorescent pseudomonads which inhibited the growth of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and produced 2,4-diacetylphloroglucinol (DAPG).

S. No	Place of collection	Code number of strain	Diameter (in cm) of inhibition zone of *Xoo*	Production of 2,4-DAPG[1]	Percent (%) suppression of rice BB	
					Net-house[2]	Field[2]
1	Karnataka	KAD7	1.8	+	56.87	53.93
2	Karnataka	IMV14	2.8	+	58.68	56.74
3	Karnataka	IMV2	2.8	+	36.78	33.18
4	Karnataka	BGR19	0.5	+	27.48	42.71
5	Kerala	PTB9	2.1	+	58.78	64.46
6	Kerala	MON1	3.3	+	19.70	37.13
7	Tamil Nadu	TVM8	1.2	+	16.32	23.51
8	Tamil Nadu	VEL17	2.3	+	23.33	54.08
9	Tamil Nadu	VEL10	0.7	+	17.03	17.52
10	Tamil Nadu	GDY4	2.1	+	30.73	41.44
11	Tamil Nadu	GDY7	2.3	+	51.57	45.30
12	Tamil Nadu	TRP5	1.7	+	33.68	34.54
13	Tamil Nadu	TRP18	2.3	+	53.15	48.34
14	Tamil Nadu	MDR9	2.1	+	30.68	23.29
15	Tamil Nadu	MDR7	1.3	+	57.11	54.43
16	Tamil Nadu	STR7	1.8	+	19.51	36.04
17	Tamil Nadu	VGP13	2.5	+	55.39	51.62
18	Tamil Nadu	MDR16	1.4	+	37.60	46.12
19	Tamil Nadu	PDY5	1.8	+	58.83	56.88
20	Tamil Nadu	VLB7	1.7	+	33.64	9.08
21	Tamil Nadu	KVR5	2.5	+	55.92	49.75
22	Tamil Nadu	TNI13	2.2	+	26.05	28.19
23	Tamil Nadu	KOV8	3.0	+	38.69	9.71
24	Tamil Nadu	RJP31	2.4	+	10.31	0.50
25	Tamil Nadu	KOV3	1.8	+	21.18	49.21
26	Tamil Nadu	PDU1	2.9	+	8.16	11.80
27	Tamil Nadu	PDU9	2.7	+	16.94	2.04
28	Control	-	-	-	0.00	0.00

[1]Production of DAPG was identified through a PCR-based screening procedure which amplified a 745 bp DNA fragment in these strains

[2]Results of a replicated field experiment (RBD) conducted at the Regional Agricultural Research Station, Pattambi, Kerala. Each figure is a mean of 3 replications.

in the field thus far has plagued researchers and their efforts to exploit them for commercial application. Therefore, there is a compelling need to identify efficient and dependable biocontrol agents to be used singly or as mixtures, so as to ensure consistent performance in the farmer's field.

In a recent evaluation, we have observed that biocontrol agents complimented the role of major genes for blast resistance (Table 4). This appears to be a worthwhile direction for the future as the combination the two major strategies afford higher levels disease control.

Choice of right microbial candidates is indeed one of the most important factors governing the success of biocontrol programs on a commercial

Table 3. Evaluation of *Pseudomonas fluorescens Pf*7-14 and its mini-Tn5 mutants in the field for the suppression of leaf blast in IR50 rice.

Bacterial strain	Leaf blast		Neck blast	
	Percent disease incidence	Percent disease control	Percent incidence	Percent control
Pf 7-14	21.30	78.70	12	82.4
Afa⁻ mutant AC2003	75.30	24.70	51	25
Afa^{leaky} mutant AC2007	52.90	47.10	44	35.3
Fungicide (Tricyclazole)	31.00	69.00	21	69.9
LSD		*(P=0.02)* = 54.7 *(P=0.01)* = 74.6	*(P=0.02)* = 11.02 *(P=0.01)* = 15.05	

Table 4. Levels of rice blast control observed due to the combined use of blast-resistance genes and bacterial biocontrol agents. Field experiment, RARS, Pattambi, Kerala.

Rice cultivar	Mean percent blast suppression due to bacterial strains[1,2]				
	B. pumilus (IM3)	*B. polymyxa (VLB 16)*	*B. polymyxa (KRU 22)*	*B. coagulans (PD 7)*	*Enterobacter agglomerans (UPM18)*
IR50	47.03	55.63	52.76	47.82	54.48
CO39	45.24	29.51	37.65	46.82	55.61
Pi-1	71.40	66.87	67 63	53.68	96.05
Pi-2	65.50	62.70	59.68	54.10	69.01
Pi-1+Pi-2	65.18	76.03	71.06	68.70	77.93

[1] Mean percent disease suppression from three replications
[2] Means followed by common letters are not significantly different at 5% level by DMRT

basis. It is therefore important to consider biocontrol agents with ability to reduce the severity of more than one pathogen, as this will make their application cost-effective. Once such agents are selected after stringent testing, it is imperative to have them formulated for high levels of consistent performance. For all these, *Bacillus* strains are attractive candidate agents, as they withstand desiccation and storage conditions better than fluorescent pseudomonads or other bacteria.

It needs to be remembered nevertheless, that most of the world's rice farmers who live in Asia are resource-poor. Therefore, cost-effective formulations of biocontrol agents that perform consistently in their fields when made available, either by themselves or, as part of an integrated disease management (IDM) package will benefit these small income group rice farmers with increased grain harvests. In this, lies the key to the ultimate success of biocontrol research for rice disease management.

Progress and Perspectives of Using Plant Growth Promotoing Rhizobacteria for Management of Aflatoxin Contamination and Late Leaf Spot of Groundnut

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Introduction

India is the largest grower of groundnut and the second largest producer after China. Andhra Pradesh, Gujarat, Karnataka, Maharashtra and Tamil Nadu account for about 89% of the total groundnut area and contribute nearly 88% of the total groundnut production of our country. About 81% of the Indian groundnut crop is rain-fed where productivity fluctuates between 500 and 1500 kg/ha with a national average at about 1000 kg/ha. This low productivity is partly due to diseases. Biocontrol agents that also possess plant growth promoting activity could help in minimizing losses due to diseases.

Use of *Bacillus* and *Pseudomonas* for management of two main constraints i.e. aflatoxin contamination and late leafspot are discussed here. The plant growth promoting activity has been demonstrated for *Bacillus subtilis* (AF-1) on cotton, cucumber, pigeon pea, tomato and egg plant. Groundnut seeds bacterized with *B. subtilis* reduced incidence of collar rot (Podile and Prakash, 1996). In greenhouse tests, 99% of groundnut plants were protected from *S. rolfsii* infection when inoculated with *P. fluorescens* @ 10^8 cfu/ml (Ganesan and Gnanamanickam, 1987). Groundnuts responded most favourably to the bacterial seed treatment exceeding 10^4 cfu/g of root tissue esp. when subjected to stresses, such as limited water availability, poor rotational practices, or cool soils, caused by early plantings (Turner and Backman, 1991). Populations of *Bacilus cereus* 304, applied along with chitin, increased considerably and reduced late leafspot significantly (Kokalis-Burelle *et al.*, 1992). Groundnut yield could be enhanced by 24.69% and 22.53% by the seed coating with the non-cyanogenic fluorescent *Pseudomonas*, and non-fluorescent *Pseudomonas*, respectively (Dey *et al.*, 2000). Based on fatty acid methyl-esters of isolates, higher bacterial diversity was noticed in geocarposphere and rhizosphere compared to soil, suggesting the association of these bacteria to host and its immediate ecological niche (Kloepper *et al.*, (1992).

Materials and Methods

Aspergillus flavus management

Out of 102 strains of bacteria, isolated through a double-layer agar plate technique from geocarposphere and rhizosphere of 'healthy plants' of groundnut, 12 strains were identified as effective in inhibiting the growth of *A. flavus* in dual-culture. These strains were also inoculated on groundnut plants to test their adverse effects on plant growth, biomass and pod yield.

Selected bacterial antagonistic strains were characterized for their morphological and physiological traits (Anjaiah *et al.*, 1998). For pseudomonads, a multiplex PCR amplification with lipoprotein gene (*oprI*, *oprL*) primers (De Vos *et al.*, 1993), for biochemical analysis of bacterial siderophores by isoelectric focusing (Koedam *et al.*, 1994) and, whenever possible a Biolog® system identification were performed. Also, the possibility of *P. aeruginosa* was ruled out by *Opr* analysis as *P. aeruginosa* is a known human pathogen.

Antifungal compounds from the culture supernatants were extracted into ethyl acetate from the 48 h grown cultures. Concentrated fractions were dissolved in 1 ml of 50% methanol and tested for the growth inhibition of *A. flavus* on GCY agar plates. Active fractions were indicated by HPLC chromatograms.

Pot culture studies were conducted using clay soil artificially amended with *A. flavus* multiplied on pearlmillet seeds. *A. flavus* population in pots before and after mixing the inoculum was recorded. Simultaneously, cell mass of BCB-135, AF-52, *Pseudomonas fluorescens* -2 and *Bacillus sp.*, identified as antagonistic to *A. flavus*, multiplied on nutrient broth for 48 h on a shaker, was collected and used for seed bacterization. One hundred healthy seeds of groundnut cv. GG20 were bacterized with slurry prepared by mixing one g each of CMC and kaolin and 10 ml of bacterial culture. The overnight dried groundnut seeds were sown in earthen pots in eight replications. The soil population of *A. flavus* was monitored.

Late leafspot management

One strain of *Bacillus* sp. isolated from phyllosphere of groundnut was effective in reducing leafspot disease. For mass multiplication, three broths viz. King's B broth, molasses yeast broth and nutrient broth were tried on shake culture. Subsequently, molasses-yeast extract broth was used for liquid fermentation using a 10 L fermenter.

In the field trials from 2000 to 2002, two sprays of fermenter biomass (oil-based spray fluid) were administered where as cell-free filtrate was sprayed after dilution. Late leafspot intensity was recorded at regular intervals. Pod yield and fodder yield were recorded.

Results and Discussion

Aspergillus flavus management

Out of 102 bacterial cultures, 12 were effective in inhibiting the growth of *A. flavus*. The log population of total bacteria in the rhizosphere soil of groundnut ranged from 4.72 to 7.53. Under pot culture conditions, BAF3 was more effective as compared to other strains (Figure 1). None of the strains produced either disease symptoms or adverse effects on plant growth.

Morphological characterization of bacterial strains showed that they all were non-sporulating and gram-negative rods. Physiological characterization indicated that most of the strains (more than 70%) are fluorescent *Pseudomonas*, which were further confirmed by molecular characterization. Molecular analysis for multiplex PCR amplification of outer membrane lipoprotein genes of *Pseudomonas* showed the amplification of *oprI* gene, which is specific to all the members

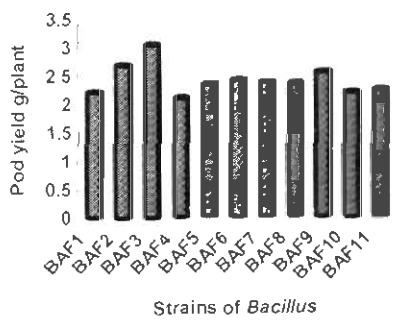

Fig. 1. Effect of bacterial antagonists on pod yield of groundnut plants (glasshouse conditions)

of rRNA group I fluorescent pseudomonads. There was no amplification of *oprL* gene in these bacterial antagonists suggesting that these were not *P. aeruginosa* strains. Further sequence analysis of *oprI* gene and iso-electric focusing (IEF) of bacterial siderophores confirmed that the majority of strains were from the rRNA group I fluorescent pseudomonads other than *P. aeruginosa*.

Biolog® strain identification system was used for further identification of the strains at species level. Among five effective strains, three strains were identified as *P. fluorescens*, *P. fluorescens* biotype F and *P. aurantiaca* on the basis of biochemical and molecular analysis. These strains are further evaluated for their effectiveness in suppressing *A. flavus* population in greenhouse experiments. The antagonists produced antifungal metabolites that inhibited growth of *A. flavus*. All these fluorescent *Pseudomonas* strains were compatible to *Trichoderma* species *in vitro* in a dual-culture and they could therefore be used together in dual inoculations.

The bacterial inoculation at sowing (as seed dressing) and *Trichoderma* inoculation at pegging was found to be more effective in reducing *A. flavus* population. In single strain inoculation, both the bacterial strains and four of the *Trichoderma* isolates significantly reduced *A. flavus* population both in rhizosphere and geocarposphere, and reduced kernel and peg infection by *A. flavus*. However, combined application of bacteria and *Trichoderma* proved to be more effective than single strain application. Further, *Bacillus* sp. treated plants showed significant increase in fresh biomass (31.49%); fresh root mass (30.30%); fresh shoot mass (23.64%); dry shoot mass (35.48%); shoot height (37.96%); and root volume (24%) –as compared with other strains.

In pot culture studies, application of all four PGPR strains reduced soil population of *Aspergillus* considerably. However, the reduction in soil population was maximum with the application of AF 52 (Table 1).

Dry pod mass was also maximum in *P. fluorescens* (39.94%) followed by Af-52 (25.15%), *Bacillus* sp. (18.07%) and BCB-135 (6.32%). Both *P. fluorescens* and AF-52 treated plants showed maximum number of mature pods as compared to control.

Late leafspot management

King's B broth was found supported maximum growth of *Bacillus* sp. antagonistic to late leafspot pathogen (111.8×10^8 colonies per ml) followed by Molasses Yeast Broth (95.5×10^8 colonies per ml) and Nutrient Broth (80.5×10^8 colonies per ml). Molasses @30g.L^{-1} with soy-flour @ 10g.L^{-1} also supported optimum growth of the bacterium and economical. The optimum fermentation conditions were 28 to 30°C for 96h

Table: 1 *Aspergillus flavus* population in the pots as influenced by the application of antagonistic strains of *Bacillus* and *Pseudomonas*

Treatment	Soil population after inoculation* x10^3cfu/g soil	Population after 4 days of inoculation and before sowing x10^3 cfu/g soil	Soil population after 45 days x10^3 cfu/g soil	Reduction (%)
Bacillus	37.71	53.29	28.00	47.45
BCB-135	54.88	71.71	33.46	53.33
AF-52	53.46	70.29	31.33	55.43
PF-2	51.08	64.46	27.46	57.40
Control	48.70	61.63	52.75	14.41

*After neutralizing with the native population

with a pH 0f 7.5, 40% DO and a stirring rate of 200 rpm for first 48h and 350 rpm for rest of the period.

In station trials during the rainy seasons of 2000 and 2001, spray of cell suspension of *Bacillus* sp. reduced the mean late leaf spot intensity by 50.21% increased mean pod yield by 20.85% and mean fodder yield by 16.4% in GG2 cultivar. Similarly, sprays of cell-free filtrate of *Bacillus* sp. reduced the mean late leaf spot intensity by 50.44% and increased mean pod yield by 24.64% and mean fodder yield by 26.55% over control (water spray).

Conclusions

Strains of *Bacillus* and *Pseudomonas* were effective against A. flavus and P. personata, the major constraints in groundnut production systems. They also possessed plant growth promoting activities. Thus, they could be further exploited for commercial scale up.

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Plant growth promoting rhizobacteria for beneficial agriculture - Current concepts and future outlook

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Introduction

To keep pace with the ever-increasing demand for crop yield by the exploding population, use of chemical fertilizers and pesticides in agricultural production has become indispensable. Production cost of agriculture has skyrocketed owing to the abandoning of green, organic farmyard manure and use of chemicals. However, the problems associated with chemical pesticides are abundant. The excessive application of chemical fertilizers cause soil sealing, fertility diminishing, and residual problems. The leftover of chemical fertilizers and pesticides have seriously affected the quality of agricultural products, people's health and caused environmental pollution. The over use of fertilizers has also damaged the soil's original micro-ecological balance and deteriorated the diseases spread by soil. While some pesticides must be abandoned because of their unacceptable non-target effect, there will always be a need in agriculture for safe and selective chemicals to limit the effects of pests. More significantly, it is becoming increasingly more difficult and expensive to find new kinds of synthetic chemical pesticides. Implications of chemical pesticides in ecological, environmental and human health problems have increased public awareness and concern regarding the continued use of agrichemicals that are damaging to human or environmental health thereby obviating the search for effective alternative approaches which have minimal deleterious effects, more environmentally friendly, and will contribute to the goal of sustainability in agriculture. In this line Plant growth-promoting rhizobacteria (PGPR) present immense potential and promise as effective substitutes for chemicals. PGPR enhance plant growth and survival, control soil-borne fungi, and induce systemic resistance to phytopathogens and are used as inoculants for biofertilization, phytostimulation and biocontrol.

Plant growth promoting rhizobacteria

Rhizosphere refers to the area surrounding the root whose physiological and physical features are affected by the root. Rhizosphere constitutes: internal rhizosphere, surface and external rhizosphere. Internal rhizosphere refers to the cells of the root components. Surface refers to the root surface; the external rhizosphere refers to the area around the root. The number of microbes in rhizosphere soil is many times higher than other areas.

Rhizobacteria refer to the bacteria living in plant rhizosphere soil forming a bacterial cover around the root. These bacteria are abundant in

number, quick in reproduction and strong in activity. They select and regulate the root's function in secreting and absorbing nutrients. PGPR can be divided into three categories according to their functions: beneficial, harmful and neutral. Those bacteria that promote plant growth are called plant growth promoting rhizobacteria. PGPR mainly constitute species of *Azotobacter*, *Azospirillum*, *Pseudomonads*, *Acetobacter*, *Burkholderia*, *Enterobacter* and *Bacilli*. The beneficial effect of these bacteria has been variously attributed to their ability to produce various compounds including phytohormones, organic acids and siderophores, fixation of atmospheric nitrogen, phosphate solubilization, antibiotics that suppress deleterious rhizobacteria or to some other unidentified mechanisms. PGPR are also implicated in plant disease control. PGPR multiply rapidly, occupy all available niches, absorb nutrients and oxygen, restrain the secretion of active substances, form a biological protective screen around the root, preventing the breeding and invasion of harmful bacteria groups as well as preventing the growth of rhizosphere pathogenic microbes. As a result, soil-borne diseases will be reduced and the disease-resistant ability of the crop will be strengthened. At the same time, these beneficial bacteria group will produce various plant growth substances in the process of breeding and metabolism. This will promote the growth of root, absorb sufficient nutriments and foster luxuriantly. Besides, through symbiosis among the, microbes, the product can increase the number and activity of beneficial bacteria such as Nitrogen-fixing bacteria, phosphorus decomposition bacteria and potassium decomposition bacteria, quicken the decomposition of organic materials and transfer of effective nutriments, improve rhizosphere nutriment environment, heighten soil nutrient supply level and utilization rate of fertilizer.

Role of PGPR in growth promotion

There are several reports that PGPR have promoted the growth and reproductive parameters of plants ranging from cereals, pulses, ornamentals, vegetable crops, plantation crops and even tree species. Treatment with PGPR has increased the germination percentage, seedling vigor, emergence, plant stand, root growth, shoot growth, total biomass of the plants, seed weight, early flowering, increased grain, fodder, fruit yields etc., (reviewed by Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001). The exact mechanisms involved in growth promotion are still to be understood. Several methods have been suggested to explain the phenomenon of plant growth-promotion when agronomic crops are inoculated with rhizobacteria. These include increases in the nitrogen fixation, the production of auxin, gibberellin, cytokinin, ethylene, the solubilizaion of phosphorous and oxidation of sulfur, increases in nitrate availability, the extracellular production of antibiotics, lytic enzymes, hydrocyanic acid, increases in root permeability, strict competition for the available nutrients and root sites (Enebak and Carey, 2000). ACC deaminase activity, siderophore production, phosphate solubilization, IAA production, enhancing biological nitrogen fixation and enhancement in the uptake of essential plant nutrients could be the best possible explanations. Furthermore, the plants grow faster and greener with longer roots and shoot that the untreated plants. It has been established that fluorescent *Pseudomonads* enhance plant growth in several ways viz., producing plant growth regulators, such as gibberellins, cytokinins and indole acetic acid, which can either directly or indirectly modulate the plant growth and development (Dubeikovsky *et al.*, 1993; Glick, 1994). Possible mechanisms of growth promotion by PGPR also include symbiotic N2 fixation, mobilization of insoluble nutrients (e.g. phosphate solubilization) (Subba Rao, 1982), suppression of deleterious rhizobacteria, and production of plant growth substances (Gaskins *et al.*, 1985; Kloepper and Schroth, 1981).

Role of PGPR biological control

PGPR are known to control a wide range of phytopathogens like fungi, bacteria, viruses, nematodes etc., they are known to control these pathogens by biocontrol mechanisms which may be by competition or antagonisms, however, the most studied phenomenon is the induction of systemic resistance by these bacteria in the host plant thereby containing the invading pathogens (Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001). Plant growth-promoting bacteria control the damage to plants from phytopathogens by a number of different mechanisms including: out-competing the phytopathogen, by physical displacement of the phytopathogen, secretion of siderophores to prevent pathogens in the immediate vicinity form proliferating, synthesis of antibiotics, synthesis of variety of small molecules that can inhibit phytopathogen growth, production of enzymes that inhibit the phytopathogen and stimulation of the systemic resistance of the plants. Several studies have indicated that PGPR may stimulate the production of biochemical compounds associated with host defense, massive accumulation of phytoalexins, phenolic compounds, increases in the activities of PR-proteins, defense enzymes and transcripts, and enhanced lignification.

Structural mechanisms: PGPR treatment is known to induce structural changes in the host like the formation of appositions and papillae, deposition of callose, lignin, phenolic compounds, and development of hypersensitive response. These changes are characterized by a considerable enlargement of the callose-enriched wall appositions deposited onto the inner cell wall surface in the epidermis and the outer cortex. In tomato PGPR treatment significantly reduced germination of sporangia and zoospores of *P. infestans*, triggered the hypersensitive reaction (HR), H_2O_2 increased significantly with all treatments compared to the non-induced control. *Serratia plymuthica* strain R1GC4 sensitizes susceptible cucumber plants to react more rapidly and efficiently to *Pythium ultimum* attack through the formation of physical and chemical barriers at sites of fungal entry. A *P. fluorescens* strain functioned as an activator of plant disease resistance by inducing callose synthesis in tomato (M'Piga *et al.*, 1997). *P. fluorescens* induced accumulation of lignin in pea root tissues (Benhamou *et al.*, 1996b). Roots of bean plants which were bacterized with a saprophytic fluorescent pseudomonad, had higher lignin content than control plants (Anderson and Guerra, 1985).

Biochemical mechanisms: PGPR are known to produce antibiotics, antifungal metabolites, enzymes, phenolics, signal compounds and other determinants of defense in response to pathogen attack. Various antibiotics can be produced by rhizobacteria like *Bacillus* and *Pseudomonas*, like bacilysin, iturin-like lipopeptides, diacetylphloroglucinol and pyrrolnitrin, HCN, phenazine-1-carboxylate (Thomshow *et al.*, 1990). De Meyer *et al.* (1999) reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated Phenlyalanine ammonia lyase (PAL) in bean roots and increased the salicylic acid levels in leaves. Increased activity of PAL was observed in *P. fluorescens* treated tomato and pepper plants in response to infection by *F. oxysporum* f. sp. *lycopersici* and *C. capsici* (Ramamoorthy and Samiyappan, 2001). In bean, rhizosphere colonization of various bacteria induced Peroxidase (PO) activity (Zdor and Anderson, 1992). The higher PO activity was noticed in cucumber roots treated with *P. corrugata* challenged with *Pythium aphanidermatum* (Chen *et al.*, 2000). Foliar application of P. fluorescens caused increases in the chitinase and glucanase activities in rice (Meena *et al.*, 1999). Groundnut plants, when sprayed with *P. fluorescens* strain Pf1, showed increase in activity of PAL, phenolic content, chitinase and glucanase activities increased significantly 23-kDa thaumatin-like protein (TLP) and a 30-kDa glucanase (Meena *et al.*, 2000). Earlier and increased activities of

phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) were observed in *P. fluorescens* Pf1 pretreated tomato and hot pepper plants challenged with Pythium aphanidermatum. Moreover, higher accumulation of phenolics was noticed in plants pretreated with *P. fluorescens* isolate Pf1 challenged with *Pythium aphanidermatum*. Phenolic compounds are toxic to pathogens in nature and may increase the mechanical strength of the host cell wall. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f. sp. pisi (Benhamou *et al.*, 1996a). Benhamou *et al.* (2000) reported that an endophytic bacterium *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots following infection by *P. ultimum*. *P. fluorescens* Pf1 isolate also induced the accumulation of phenolic substances and PR-proteins in response to infection by *F. oxysporum* f. sp. *lycopersici* in tomato (Ramamoorthy *et al.*, 2001) and *C. capsici* in pepper (Ramamoorthy and Samiyappan, 2001). Hynes and Lazarovits, (1989) reported that the levels of a PR-protein increased in bean leaves following seed treatment with PGPR strains. Maurhofer *et al.* (1994) reported that PR-proteins viz., PR-1a, 1b, 1c, endochitinase and b-1,3-glucanases were induced in the intercellular fluid of tobacco leaves of plants grown in the presence of *P. fluorescens* strain CHA0. Increase in lignin content, peroxidase activity and , 4-coumarate CoA ligase activity were observed after challenge inoculation with *X. oryzae* pv. *oryzae* in rice leaves pre-treated with *P. fluorescens* (Vidhyasekaran *et al.*, 2001).

Molecular mechanisms: The molecular mechanisms underlying rhizobacteria-mediated ISR are to a large extent unknown. Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulated the expression of the jasmonate-inducible gene *Atvsp* upon challenge. Gene expression studies were performed with Arabidopsis gene-speicifc probes for the defense-related genes *PR-1.*, *PR-2.*, *PR-5.*, *Hel.*, *ChiB*, *Pdf*1.2, *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2*. Responsiveness of the genes to the defense signaling molecules SA, ethylene, and jasmonate was verified by analyzing their expression in leaves treated with SA, the ethylene precursor 1aminocyclopropane-1-carboxylate (ACC), or methyl jasmonate(MeJA). Although variation in the expression of most genes was apparent, roots and leaves of WCS417r-treated plants never showed an enhanced expression of any of the genes, at any of the time points tested. (van Wees *et al.*, 1997).

PPO transcript levels increased in young leaves of tomato when mature leaflets were injured (Thipyapong and Steffens, 1997). Increase in mRNAs encoding PAL and chalcone synthase were recorded in the early stages of the interaction between bean roots and various rhizobacteria (Zdor and Anderson, 1992).

Integrated use of PGPR

It is likely that most case of naturally occurring biological control result from mixtures of antagonists, rather that from high populations of a single antagonist. Consequently, application of a mixture of introduced biocontrol agents would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of control (Duffy and Weller, 1995), and allow the combination of various mechanisms (Janisiewiez, 1988). Plant growth-promoting rhizobacteria tested singly and in combinations for biological control against multiple cucumber pathogens recorded a general trend across all experiments toward greater suppression and enhanced consistency against multiple cucumber pathogens in combinations compared to individual strains (Raupach and Kloepper, 1998). Previous studies on combinations of biological control agents for plant diseases have included mixtures of fungi

and bacteria and mixtures of bacteria. Most of these reports on mixtures of biocontrol agents showed that combining antagonists resulted in improved biocontrol. Different mechanisms of action for different PGPR strains may explain why combinations of strains provided more consistency in disease suppression. These results are in agreement with studies by Pierson and Weller, 1994; and Duffy and Weller, 1995; both of which demonstrated that certain mixtures of fluorescent pseudomonads were significantly more suppressive of take-all than either treatment used alone. Sung and Chung, 1997, demonstrated that chitinase-producing *Streptomyces* spp. and *Bacillus cereus* isolates used in conjunction with antibiotic-producing *P. fluorescens* and *Burkholderia cepacia* isolates had a synergistic effect on the suppression of rice sheath blight.

Commercialization

The development of biological products based on beneficial micro-organisms can extend the range options for maintaining the health and yield of crops. As early as 1897 a "bacteriological fertilizer for the inoculation of cereals" was marketed under the proprietary name Alinit by Farbenfabriken vorm. Friedrich Bayer & Co." of Elberfeld, Germany, Today's Bayer AG. The product was based on a Bacillus species now known by the taxonomic name *Bacillus subtilis* (Kilian *et al.*, 2000). In the mid-1990s in the USA, *Bacillus subtilis* started to be used as seed dressing, with registrations in more than seven crops and application to more than 2 million ha (Backmann *et al.*, 1994). This was the first major commercial success in the use of an antagonist. In Germany, FZB 24 *Bacillus subtilis* has been on the market since 1999 and is used mainly as a seed dressing for potatoes (Kilian *et al.*, 2000). The application of five commercial chitosan-based formulations of carefully chosen plant growth promoting rhizobacteria developed at Auburn University, USA has previously shown demonstrable increase in the growth of nursery-raised plants such as cucumber, pepper and tomato among others.

Advantages and disadvantages

Bio-pesticides are cheaper than synthetic pesticides by 50 per cent. They are eco-friendly, have a high cost-benefit ratio and do not pose risk of the pathogen developing resistance. They are easy to apply and are compatible with bio-fertilizers. The advantages of a seed treatment with rhizobacteria in a biological control system are: 1) their saprophytic nutritional status makes large scale production feasible, 2) only small amounts of inoculum are required, 3) application is simple, 4) independence from energy sources for survival, 5) systemic spread along the surface of the developing root system, and 6) antagonistic activity on the root surface during the economically important phase of early root infection by the pathogens. Their versatile metabolism, fast growth, active movement, and ability to readily colonize the root surface make these rhizobacteria especially suitable for seed bacterization. Further, seed treatments provide targeted application of PGPR, allowing earlier protection than could be provided with foliar sprays. In addition, the additional plant growth-promotion provided by some PGPR treatments in comparison to chemical adds another advantage of PGPR over chemical inducers. However, microorganisms as biological control agents typically have a relatively narrow spectrum of activity compared with synthetic pesticides (Janisiewicz, 1988) and often exhibit inconsistent performance in practical agriculture, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (Backman *et al.*, 1997). One reason for its growing popularity is its record of safety during the past 100 years considered as the era of modem biological control. No microorganism or beneficial insect deliberately introduced or manipulated for biological control purposes has, itself, become a pest so far as can be determined, and there is no evidence so far of measurable or even negligible negative effects of biocontrol agents on the environment.

Effective biological control demands thorough knowledge of biological interactions at the ecosystem, organismal, cellular, and molecular levers. Biological control is also likely to be less spectacular than most physical or chemical controls but are usually also more stable and longer lasting. In spite of biological controls having been used in agriculture for centuries, as an industry biological control is still in its infancy.

Future outlook

Diseases are very common in plants and are responsible for the loss of approximately one third of the crop yield (Lugtenberg *et al.*, 1994). Chemical pesticides that control plant diseases have become a threat to health and to the environment and hence being banned worldwide. This has increased the interest in microbiological control of plant diseases by the use of conventional and safe agricultural practices. PGPR mediated agriculture is now gaining worldwide importance and acceptance for an increasing number of crops and managed ecosystems as the safe method of pest control. Another reason for considering biological control over other methods is untapped potential; biological control is underused, under exploited, underestimated and often untried and therefore unproven. The new tools of recombinant DNA technology, mathematical modeling, and computer technology combined with a continuation of the more classical approaches such as importation and release of natural enemies and improved germplasm, breeding, and field testing should quickly move biocontrol research and technology into a new era. Although activity and effects have been reported for a number of antagonists, the underlying mechanisms are not fully understood. This deficiency in our knowledge often still hinders attempts to optimize the biological activity by employing tailored application strategies. One can envision a number of different ways in which biocontrol PGPB might be improved by genetic engineering. Biocontrol PGPB may be improved by genetically engineering them to overexpress one or more of these traits so that strains with several different anti-phytopathogen traits, which can act synergistically. More detailed studies are needed on the composition of the rhizosphere population, the effect of cultivar on bacterial population dynamics, the influence of inoculum density on antagonistic activity, the survival of the inoculum under adverse conditions, and the role that environmental conditions play in altering the activity of rhizobacteria. An attempt to overcome problems of varying efficacy may be attained by strain mixing, improved inoculation techniques, or gene transfer of the active genetic source of antagonism to the host plant Ooestendorp and Sikora, 1986). The basis of agriculture is soil microbes are active elements for soil development. Form the standpoint of sustained agricultural development, and good eco-environment establishment, we propose a scientific fertilizer addressing measure, that is to apply organic, inorganic and microbial fertilizers in a balance and rational way to keep high and stable yield.

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Plant Growth Promoting Rhizobacteria for Sustainable Management of major Pests and Diseases in Crop Plants

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Introduction

Due to frequent use of the chemical pesticides in the recent past for the management of plant diseases and insect pests, several new problems developed mainly due to the development of pesticide resistant insects/strains, environmental pollution and human health hazards. Thus, the use of pesticides for the management of pests will not be a permanent solution. Biological control using microbial inoculants is an alternative method to chemical control and it is safe, cost effective and eco-friendly approach for the management of pests and diseases. Microbial inoculants including bacteria, actinomycetes and fungi are part of the natural ecosystem of the healthy plants and they occupy in the habitats of rhizosphere, leaf surface and as endophytes in the plant system. Among the various microbes, some of the fluorescent pseudomonads and the *Bacillus* spp. are commercially being exploited for the management of major soil-borne and foliar diseases of major crop plants in India. The role of fluorescent pseudomonads in the biological control of soil-borne diseases was reported as far back as 1976 by Cook and Rovira (1976). Moreover, certain isolates of fluorescent pseudomonads are found to be effective for the management of foliar diseases as these isolates induce plant defense mechanisms systemically throughout the plant system (Zehnder *et al.*, 2001; Van Loon *et al.*, 1998). Fluorescent pseudomonads also increase the plant growth and yield by production of various plant growth promoting substances (Dubeikovsky *et al.*, 1993) thus they are called as Plant Growth Promoting Rhizobacteria (PGPR). Hence, fluorescent pseudomonads are more beneficial biocontrol agents when compared to other antagonistic microbes. The saprophytic pseudomonads associated with plants include *P. fluorescens, P. putida, P. aeruginosa* and *P. aureofaciens.*

Major mechanisms in PGPR mediated suppression of plant pathogens and insect pests include a) production of antibiotics and lytic enzymes which are related to direct inhibition of the pathogens or pests b) induction of systemic resistance (ISR) by activating the PGPR treated plants to express genes encoding Pathogenesis-related (PR) proteins and genes involved in phenyl propanoid metabolic pathway. Many of the PGPR strains are isolated from disease suppressive soils or rhizosphere of healthy plants worldwide. Suppressive soils of microbial origins generally contain variety of antagonistic microorganisms responsible for the suppression of soil-borne pathogens (Weller, 1988). Thus, the natural suppressiveness of soil to pathogenic organism indicates that concerted action of several microorganisms and their various modes of action is implicated in consistent suppression

of the disease incidence. Natural suppressiveness of soil indicates the association of various antagonistic microflora. Introduction of isolated rhizobacterial strain at sufficient population level suppresses the pathogen propagules. Several studies indicate that inoculation of single strain of a biological control agent rarely leads to a level of suppression as observed in naturally disease suppressive soils and positive effect with single inoculants are often inconsistent (Weller, 1988). Thus, application of a mixture of PGPR is likely to more closely mimic the natural situation and represent an effective method of biological control.

Improvement of efficacy of formulation by Chitin amendment

While developing formulations, several molecules have been reported to be added to enhance the survival and efficacy of the PGPR. Chitin, as a carbon source / substrate for the growth of chitinolytic bacteria, increased the chitinase production when bacteria were grown in chitin amended medium (Gooday, 1990). Chitosan, a nontoxic polymer obtained from the chitin of crustacean shell wastes is not only the inhibitor of fungal growth but also activates genes encoding defense-related proteins in plants (Hadwiger *et al.*, 1986; Lafontaine and Benhamou, 1996). In addition, chitin oligomers which are released during degradation of chitin substrate by chitinolytic bacteria are also found to elicit plant defense reactions (Benhamou and Theriaut, 1992). Incorporation of chitin in King's medium B (KMB) supported the multiplication of *Pseudomonas fluorescens* when compared to the medium without incorporation of chitin and enhanced chitinase activity was observed in chitin amended medium (Viswanathan and Samiyappan, 2001).

Chitin and chitosan are reported to reduce diseases caused by soil borne pathogens (Benhamou *et al.*, 1998). Tomato plants treated with chitosan showed enhanced protection against crown and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Lafontaine and Benhamou, 1996). In groundnut, treatment of leaves with chitosan before challenge inoculation with *Puccinia* pathogen reduced the rust disease (Sathiyabama and Balasubramanian, 1998). *Bacillus pumilus* strain SE 34 in combitnation with chitosan showed enhanced biocontrol efficacy (Benhamou *et al.*,1998). Recently, Radjacommare *et al.*(2002) demonstrated that chitin amended PGPR formulation significantly controlled the sheath blight disease and leaf folder insect pest in rice besides enhancing the grain yield and attracting more number of natural enemies of the leaf folder insect in rice field.

In chitin amended treatments, biocontrol efficacy of Rhizobacteria (as mixture of *P. fluorescens* Pf1+FP7strains) was enhanced due to induction of defense-related enzymes *viz.*, chitinase, â-1,3-glucanase, peroxidase (PO), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) etc. Expression of defense related genes in chitin amended treatments contributed more towards durable ISR against fungal pathogens and insect pests simultaneously in rice, tomato and cotton plants under field conditions.

Combining microbial strains for increasing the biocontrol activity

Generally, application of PGPR singly leads to inconsistent performance, because a single PGPR is not likely to be active in all kinds of soil environment and agricultural ecosystems (Raupach and Kloepper, 1998). Several approaches including strain improvement through mutation, protoplast fusion and genetic engineering yielded successful results to overcome these problems. Concern about the ecological safety of releasing large numbers of these genetically modified microorganisms has been raised. These genetically modified microbes might evoke an impact on the soil ecosystem and thereby affect soil ecosystem. Moreover, use of

genetic engineering techniques for strain improvement is a laborious process. Another approach to improve disease suppression by biocontrol bacteria is the use of combinations of strains which have different modes of actions. Combination of strains with different biocontrol mechanisms is essential in this approach (Raupach and Kloepper, 1998; Schisler *et al.*, 1997). It has been well demonstrated in wheat in which application of several fluorescent pseudomonads has greater biocontrol activity against take-all disease (Pierson and Weller, 1994). Mixture of microbial inoculants include mixture of fungi (Paulitz *et al.*, 1990; Budge *et al.*, 1995), mixture of bacteria (Raupach and Kloepper, 1998; Schisler *et al.*, 1997; Pierson and Weller, 1994; Singh *et al.*, 1999), mixture of yeasts (Janisiewicz, 1996), mixture of bacteria and fungi (Duffy *et al.*, 1996; Janisiewicz, 1988; Leibinger *et al.*, 1997) and bacteria and yeast (Janisiewicz and Bors, 1995). In addition to disease control, strain mixtures enhanced the plant growth in terms of increased seedling emergence (Dunne *et al.*, 1998), plant height (Raupach and Kloepper, 1998) and yield (Duffy *et al.*, 1996; Cook *et al.*, 1988; Pierson and Weller, 1994). Use of formulation containing different antagonistic microbes may broaden the spectrum of activity; enhance the efficacy and reliability of the biocontrol and more importantly it may allow the combination of various traits without employment of genetic engineering (Janisiewicz, 1996)

The biocontrol efficacy and plant growth promotion activity of fluorescent pseudomonads were increased either by mixing two or more strains of *Pseudomonas* spp. (Raupach and Kloepper, 1998; Singh *et al.*, 1999) or mixing with other bacterial or fungal antagonists (Duffy *et al.*, 1996; Janisiewicz, 1996) or mixed with chitin or other substances (Benhamou *et al.*, 1998). Incorporation of chitin in King's medium B (KMB) supported the multiplication of *P. fluorescens* when compared to the medium without incorporation of chitin and enhanced activity of chitinase was observed in chitin amended growth medium (Viswanathan and Samiyappan, 2001). Application of *P. fluorescens* grown in chitin amended KMB medium showed the maximum suppression of red rot disease incidence caused by *Colletotrichum falcatum* (Viswanathan and Samiyappan, 2001). Chitin is a carbon source/substrate for the growth and multiplication of chitinolytic bacteria and increased chitinase production was observed when bacteria were grown in chitin amended medium (Gooday, 1990). Chitosan, a non-toxic polymer obtained from the chitin of crustacean shell wastes is not only the inhibitor of fungal growth but also activates genes of defense responses in plants (Hadwiger *et al.*, 1986; Benhamou and Theriaut, 1992; Lafontaine and Benhamou, 1996). Chitin oligomers which are released during degradation of chitin substrate by chitinolytic bacteria are also found to elicit plant defense reactions (Benhamou and Theriaut, 1992). Yuen *et al.* (2001) also found that incorporation of chitin in the medium increased bacterial population when compared to the non-amended medium.

Microbial mixtures for suppression of major pests and diseases

Combination of PGPR antagonistic organisms increases the efficacy and consistency of biocontrol agents (Weller, 1988). *P. fluorescens* isolate Pf1 is applied as seed/soil/foliar application in rice for the control of major diseases such as blast caused by *Pyricularia oryzae*, brown spot caused by *Helminthosporium oryzae*, sheath blight caused by *R. solani* and sheath rot caused by *Sarocladium oryzae* (Samiyappan *et al.*, 1999). It is also effective for the management of rice leaffolder incidence (Radja commare *et al.*, 2002). However, the mixture consisting of Pf1 plus FP7 was the most effective in reducing sheath blight incidence and promoting plant growth and grain yield as compared to application of their individual strains under field conditions. Similarly,

combination of chitinase- producing *Streptomyces* spp and *B. cereus* with antibiotic-producing *P. fluorescens* and *Burkholderia cepacia* showed a synergistic effect on the suppression of rice sheath blight (Sung and Chung, 1997). In cucumber biological control has been more beneficial when mixtures of antagonists are used rather than a single antagonist. Seed treatment with mixture of three PGPR strains *viz., B. pumilus* strain INR 7, *B. subtilis* strain GBO3 and *Curtobacterium flaccumfaciens* strain ME1 enhanced growth promotion and disease reduction when compared with the strains tested singly (Raupach and Kloepper, 1998). In tomato, combination of three strains *viz., P. fluorescens* CHAO, COP and COT was found to be more effective for the suppression of tomato spotted wilt virus when compared to individual strain application or all possible two strains combination (Kandan, 2000). Combination of chitinolytic bacterial strains was also found to be more effective for the management of fusarium wilt of cucumber (Singh *et al.*, 1999). *Erwinia herbicola* is antagonist for *E. amylovora* causing fire blight disease in pome fruits. Spraying a mixture of *P. fluorescens* A 506 and *E. herbicola* C9-1S was found to be effective for the suppression of fire blight of pear (Nuclo *et al.*, 1998).

Combination of fungal and bacterial antagonist is also successful and increases the biocontrol efficacy against diseases. These microbes should be compatible. Certain *Trichoderma* spp and fluorescent pseudomonads are compatible and application of these antagonists together results in greater suppression of the disease incidence. In wheat, take all disease was suppressed greatly due to combined application of *T. koningii* and bacterial antagonists (*P. fluorescens/P. achlororaphis*) than the fungal and bacterial antagonists which were applied separately (Duffy *et al.*, 1996). Similarly, combined application of *P. fluorescens* and *Glomus mosseae* brought about higher reduction in galling caused by *Meloidogyne javanica* than their individual application. The combined application also showed the significant reduction in nematode reproduction (Siddiqui and Mahmood, 1998). Concomitant inoculation of bacterial antagonists (fluorescent pseudomonads) and ectomycorrhiza in conifer seeds resulted in higher seedling survival and lower root rot incidence caused by *F. moniliforme* and *R . solani* (Pedersen *et al.*, 1999).

All these experiments indicate that combined application of different strains of antagonistic microbes will increase the biocontrol efficacy and consistency in suppression of the diseases.

Conversely, certain mixtures of biocontrol agents have no synergistic effect and sometime they have negative effect on biological control. Moreover, a mixture which is effective in certain environmental conditions may not be effective in other environmental conditions (Schisler *et al.*, 1997). So, emphasis should be given during development of microbial mixture that has synergistic, broadspectrum effect and effective under different environmental conditions.

Formulation development

Development of PGPR formulation for biocontrol purpose with suitable carrier is the last step in the commercialization of biocontrol agents which is practically applicable on a large scale field level. The carrier should be easily available in the particular location, cheap and support the maximum population of microbes during the longer period of storage. Suslow (1980) first developed the formulation of *P. fluorescens* using talc as a carrier. Seed treatment with bacterial cell suspension was found effective in controlling several diseases. However, for commercial and field application, this methodology would be impractical due to difficulties in handling, transport and storage. Vidhyasekaran and Muthamilan (1995) found that the colonies of *P. fluorescens* in the suspension culture drastically declined when stored for a period of 10 days whereas the population was maintained when the culture

was mixed in the carrier. The talc-based formulation of two *P. fluorescens* strains (Pf1 and FP7) and its mixture (with and without chitin) were tested against sheath blight and leaffolder incidence in rice. In this, combination of two strains performed better when compared with individual strain application. In leaffolder bioassay with *Pseudomonas* treated leaves, it was showed that the feeding behaviour of larvae was altered resulting in reduction in the larval and pupal weight and increase in larval mortality and adult malformation were observed under *in vitro*. In addition, the increased population of natural enemies of leaffolder *viz.*, hymenopterans and spiders were noticed in *Pseudomonas* treated plots under field conditions which accounted for suppression of the pest incidence (Radjacommare *et al.*, 2002). Earlier Nandakumar *et al* (2001) tested the efficacy of three PGPR strains *viz.*, Pf1, FP7 and PB2 individually and also in combinations for suppression of rice sheath blight disease and found that mixture consisting of Pf1 + FP7 was most effective in reducing the disease in addition to promoting plant growth and grain yield. Combined application of talc-based PGPR formulation as seed, root, soil and foliage treatment was the most effective method for reducing the disease (Nandakumar *et al.*, 2001). Rhizosphere and phyllosphere population of *P. fluorescens* suppressed the development of the disease (Vidhyasekaran and Muthamilan, 1999). The addition of chitin in the talc-based *Pseudomonas* formulation containing Pf1+FP7 strains further reduced leaffolder and sheath blight incidence (Radjacommare *et al.*, 2002). Application of chitin and chitosan are found to reduce soil-borne disease incidence (Lafontaine and Benhamou, 1996; Benhamou *et al.*, 1998). Tomato plants treated with chitosan enhanced protection against crown and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici* (Benhamou and Theriaut, 1992; Lafontaine and Benhamou, 1996). In tomato, application of endophytic rhizobacterium restricts the entry of *Fusarium* pathogen in the cortex region of the roots and application of chitosan alone also showed less effect in restricting the mycelial growth of the pathogen whereas, combined application of chitosan with the endophytic rhizobacterium resulted in more inhibition of fungal growth by restricting the mycelium in the intercellular space itself. Thus, incorporation of chitosan with the bacterial formulation would certainly enhance the biocontrol efficacy synergistically (Benhamou *et al.*, 1998). Treatment of groundnut leaves with chitosan before challenge inoculation with the pathogen reduced the rust disease (Sathiyabama and Balasubramanian, 1998) indicating chitin products are activating the resistance mechanism of plant. The commercial formulation LS213, containing spores of *B. subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a and chitin as a carrier was found to be effective for increasing the growth of tomato, cucumber, tobacco and pepper transplant and also effective against bacterial spot and late blight of tomato, angular leaf spot of cucumber and blue mold of tobacco (Reddy *et al.*, 1999). The formulation (LS213) also exhibited significant protection against nematode damage and anthracnose in cucumber under field conditions (Kenney *et al.*, 1999). The formulation has also enhanced pine seedling root and shoot growth in the production of containerized seedlings (Enebak and Reddy, 1999). Thus, the development of suitable formulation of biocontrol strains mixture as that of chemical pesticide would certainly be beneficial for field application.

Conclusion

In India, development of biological control technologies involving PGPR for pest and disease management is a newly emerging field. The major criteria for consideration of successful biocontrol technology involving PGPR are identification of effective agents, formulation, mass production technology and delivery systems. In addition, keeping in view of the current and future restrictive legislation against chemical pesticides, the process of commercial

formulation with good delivery system should be implemented with compatibility to industrial and commercial development methods and field applications.

Most of the research activities involving biocontrol bacteria have been carried out in the laboratory and green house and very rarely field tested for only few crops against only less number of pathogens. In the last 2-3 years, the scenario is fast changing as more scientists are actively involved in field studies related to bacterial biocontrol agents (funded by ICAR, DBT, DST and some private agencies in India).

Although biocontrol of plant pathogens with PGPR has not dramatically solved the diseases so far, we are at a turning point in this technology to make significant advances. More attention is needed to orient our future research on some of the areas *viz.*, PGPR survivability, identification of crop specific strains, mixture formulation, biochemical/molecular markers for identification of effective PGPR and genes activated in octadecanoid pathway and phenyl propanoid pathway due to bacterial treatment in order to enhance the performance of biocontrol bacterial inoculants for managing plant pests and diseases in sustainable manner. Further, greater cooperation between industrial and non-industrial scientists is needed for transferring basic scientific information into research and development activities and this will eventually further improve the efficacy and commercial viability of the bio-products.

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Dual inoculation of rhizobia and PGPR inoculants increase the productivity of legume crops

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1. Excess use of chemical fertilizers led to decrease in fertilizer responsiveness

Modern green revolution targeted at increased food grain production was largely dependent on chemical fertilizers, and high yielding crop varieties. There was a steady increase in the global use of chemical fertilizers in different agricultural systems, and worldwide use of NPK fertilizers in 1999/00 was >14 million tons (Fig. 1). Continuous and large scale use of chemical fertilizers, and the resulting shift in cropping patterns led to yield stagnation and decrease in fertilizer responsiveness of the crops. In addition, there were adverse effects on the rural population, the environment and food safety as well. These ill effects of chemical fertilizers emphasize a need for changes in agricultural production technologies, with an objective of sustainability, economic production, conservation of natural resources and minimal use of synthetic chemicals. In this context, the large-scale application of bioinoculants would be attractive as it can substantially reduce the use of chemical fertilizers. These bioinoculants can effectively increase the crop yield, and contribute to feed the growing world population.

2. Beneficial microorganisms are present in the root zone

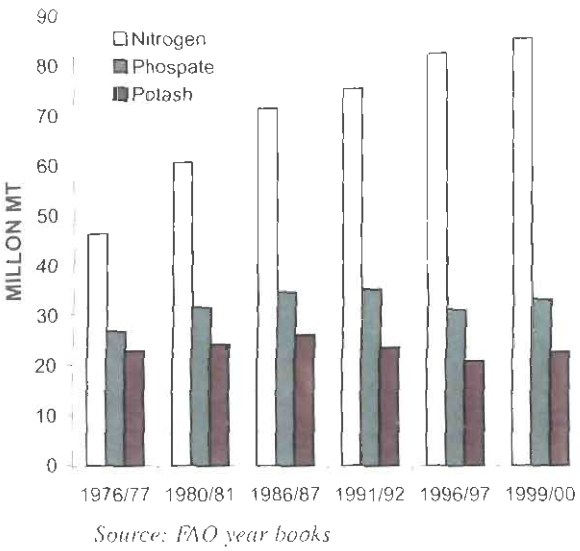
The plant rhizosphere, colonized by diverse microorganisms, is a dynamic ecological environment of plant-microbe interactions. The colonizing microorganisms form symbiotic, associative, neutralistic or parasitic relations with the plant, which in turn is dependent on the type of microorganism, available nutrients, plant defense responses and environment. The symbiotic association between legumes and *Rhizobium* is a well known phenomenon which contributes to biological nitrogen fixation (BNF) and soil fertility. Some of the free living rhizobacteria utilize nutrients secreted by the plant roots and influence the plant in a direct or indirect way resulting in growth stimulation. This set of beneficial bacteria termed as plant growth promoting rhizobacteria (PGPR), comprises of a broad-spectrum of bacterial genera. Additionally, PGPR also protect the plant from infection by phytopathogenic organisms within the vicinity of the root system.

3. Nitrogen fixing bacteria convert atmospheric nitrogen to ammonia

Nitrogen is an essential macronutrient and deficient in most of the agricultural lands resulting in reduced yields. Molecular nitrogen (N_2) makes up >70% of the atmosphere but is metabolically unavailable directly to higher plants or animals. BNF that occurs in the root nodules of legumes, converts the atmospheric nitrogen into ammonia (>200 kg N/ha/year) using the enzyme nitrogenase. BNF in legumes had a significant contribution in the total nitrogen fixed globally (90 million tons/year) but still is

needed for the most vulnerable cropping systems in developing countries. The most efficient symbiotic nitrogen fixers are bacteria belonging to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium*. Free living rhizobacteria such as *Azospirillum*, *Herbaspirillum*, *Acetobacter*, *Azotobacter* and *Azoarcus* are also able to fix atmospheric nitrogen.

Rhizobia form a host-specific symbiosis with the roots of leguminous plants and fix the atmospheric nitrogen (Fig. 2). The first step in the establishment of the symbiotic system is signal exchange between plant roots and rhizobia. The plant-to-bacteria signals are isoflavonoids, which induce rhizobial *nod* gene expression. In turn, rhizobia secrete *lipooligosaccharide* signal molecules, the Nod factors, which play a pivotal role in root nodule formation. The bacteria penetrate the cortex, induce root nodules, multiply and subsequently differentiate into bacteroids. The root nodules maintain a low oxygen concentration, which allows bacterial nitrogenase to convert atmospheric nitrogen into ammonia. In return, the plant supplies the bacteria with a carbon source. Rhizobia can also promote the growth of non-legumes such as gramineae and crucifers following root colonization without nodule formation.



Source: FAO year books

Fig. 1. Global utilization of chemical fertilizers - nitrogen, phosphorous and potash in different agricultural systems

4. Soil fertility can be enhanced by legumes

Legume crops by virtue of their ability to fix nitrogen with the help of rhizobia are commonly used as green manures. Crop rotation with legumes increases the soil fertility and sustainability of other cropping systems dependent on organic farming. Cereal or vegetable crops grown after legumes will have an increased biomass because of the improved nitrogen availability. Crop rotations are essential in farming systems where the subsistence agricultural practices resulted in excessive removal of nutrients from the ecosystem, causing a negative balance of plant nutrients in the soil. Legume crops also alter the rhizobacterial community of the soils and increase the proportion of beneficial bacteria.

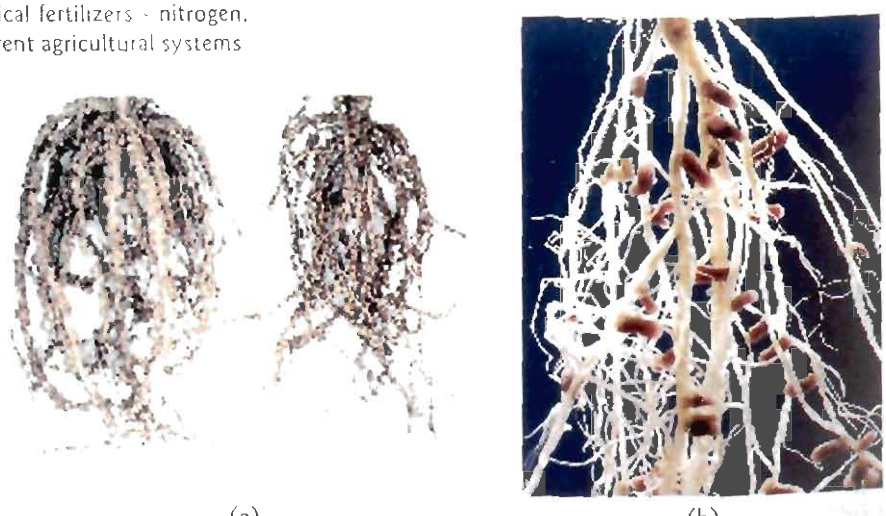


Fig. 2. *Rhizobium* nodules on the roots of (A) groundnut, and (B) Pea

5. PGPR enhance plant growth and yield in different crops

PGPR naturally exist in different cropping systems. Enrichment of their populations in the rhizosphere profoundly interferes with the plant growth (Fig. 3 and 4). The beneficial bacteria belong to several genera, of which *Bacillus* and *Pseudomonas* spp. were found appealing in a wide range of crops, including legumes (Table 1). In addition to nitrogen-fixing ability, *Azospirillum* spp. secrete phytohormones such as auxins, cytokinins and gibberellins and favor the plant growth.

Growth promoting activity of different groups of bacteria involves both direct and indirect mechanisms. Direct growth promotion is due to enhanced availability of nutrients like phosphorous and production of growth promoting hormones by PGPR. Next to nitrogen, phosphorous is the vital nutrient for plant growth and most of the phosphorous in the soil is in insoluble form. Phosphate solubilizing bacteria (PSB) solubilize the inorganic phosphorous (Fig. 5) and makes it available for plant nutrition. Indirect growth promotion is by suppression of deleterious or plant pathogenic microorganisms through competition for space and nutrients, production of antibiotics, siderophores or HCN and/or induction of systemic resistance in the host plant.

Fig. 3. Plant growth promoting activity of *Bacillus subtilis* AF 1 applied as seed treatment in pigeonpea under field conditions

Fig. 4. Plant growth promoting efficiency of *Bacillus firmis* GRS 123 applied as seed treatment in groundnut under field conditions.

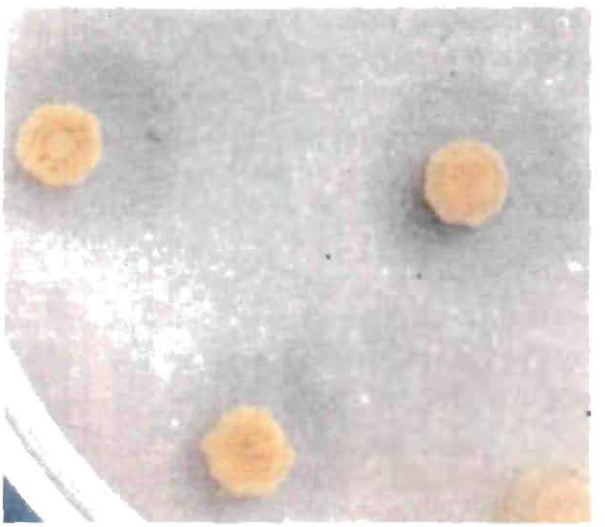


Fig. 5. Solubilization of tricalcium phosphate by phosphate solubilizing rhizobacteria

6. PGPR can control root diseases in legumes

Plant growth promoting rhizobacteria, in addition to growth promotion, also protect the legume crops from the attack of soil-borne pathogens among which *Fusarium* spp., *Rhizoctonia solani*, *R. bataticola* and *Sclerotium rolfsii* are highly destructive (Table 2). Competition for the available nutrients and space, antibiosis, and induced systemic resistance were the major mechanisms responsible for the observed disease control. Pyrrolnitrin, phenazine-1-carboxylic acid, and 2,4-

Table 1. Plant growth promoting rhizobacteria (PGPR) in enhancing the growth of legume crops.

Crop	Bacterial isolate	Observed growth promotion
chickpea	*Pseudomonas fluorescens*	
	Pseudomonas spp.	enhanced seed germination, root and shoot dry weight, and yield
	Azospirillum brasilense	increased nodulation, root and shoot development
	Azotobacter	increase in shoot length, number of shoots/plant, yield and seed protein content
pigeonpea	*Bacillus subtilis* AF 1	increase in shoot and root length, and biomass
groundnut	*Bacillus subtilis*	increase in root and shoot length, and biomass
	Pseudomonas aeruginosa	
	Pseudomonas fluorescens	
	Bacillus amyloliquefaciens	increase in root and shoot length, biomass and yield
faba bean	*Azospirillum brasilense*	increase in growth of root and shoot, and nodulation
mungbean	*Azotobacter*	increase in yield
pea	*Azotobacter*	increase in yield
soy bean	*Bacillus cereus* UW 85	increase in root growth, nodulation and yield

diacetylphloroglucinol produced by *Pseudomonas* spp. were the major antibiotics involved. The production of these antifungal metabolites by Pseudomonads is subjected to a complex regulation and influenced by both global regulation and quorum sensing. Seed bacterization or foliar application of PGPR was effective in control of foliar diseases of legumes, for example powdery mildew of pea and late leaf spot of groundnut.

7. Co-inoculation of PGPR and rhizobia can be combined for legumes

Plant growth promoting rhizobacteria are capable of promoting the yield of agriculturally important legume crops in different soils and climatic regions. Different PGPR and rhizobia applied as seed treatment enhanced the growth of legume crops as evidenced by an increase in root and shoot length, plant biomass and grain yield. Some of the introduced PGPR were antagonistic to soil-borne pathogens and also increased the crop survival both in native and pathogen infested soils.

Combined inoculation of PGPR and rhizobia was observed to exert positive effects on the growth of legumes, and the interaction between these two groups of microorganisms can be exploited for economic gains. Co-inoculation of PGPR and *Rhizobium* increases the root nodulation. The beneficial effects of co-inoculation are strain dependent. The basic mechanisms involved in this synergistic activity were not completely known and remains a challenge. One possibility is that PGPR, by altering the host secondary metabolism and/or creating antibiosis in the rhizosphere, out-compete the pathogens and eliminate competition of *Rhizobium* with deleterious microorganisms for colonization of the plant parts. Alteration of the plant flavonoid metabolism was proposed as another mechanism of synergistic activity of PGPR and rhizobia. Co-inoculation of PGPR and *Rhizobium* was also effective in other legumes including pea, clover, common bean and cowpea. The applied rhizobacteria stimulate formation of additional infection sites that may later be occupied by rhizobia, thus increasing the nodulation. Isolates

Table 2. Plant growth promoting rhizobacteria (PGPR) in control of root diseases of legume crops.

Crop	Disease	Pathogen	PGPR used
bean	root rot	*Sclerotium rolfsii* *Rhizoctonia solani* *Pythium aphanidermatum* *Fusarium solani*	*Pseudomonas cepacia*
chickpea	wilt	*Fusarium oxysporum* f. sp. *ciceri*	*Bacillus subtilis* *Bacillus* spp. *Pseudomonas aeruginosa* *Pseudomonas fluorescens*
	dry root rot	*Rhizoctonia bataticola*	*Pseudomonas fluorescens*
cowpea	stem and root rot	*Phytophthora vignae*	*Brevibacterium linens*
	root rot	*Rhizoctonia bataticola* Rhizoctonia solani *Fusarium solani*	*Pseudomonas fluorescens*
groundnut	crown rot	*Aspergillus niger*	*Bacillus subtilis* AF I
lentil	wilt	*Fusarium oxysporum* f. sp. *lini*	*Pseudomonas fluorescens*
pea	root rot	*Fusarium solani* *Rhizoctonia solani* *Sclerotium rolfsii*	*Azotobacter chroococcum* *Azospirillum lipoferum* *Pseudomonas fluorescens*
	damping-off	*Pythium ultimum* *Pythium sylvatium*	*Pseudomonas cepacia* *Pseudomonas fluorescens* *Pseudomonas putida*
pigeonpea	wilt	*Fusarium udum*	*Bacillus brevis*, *Bacillus subtilis* AF I *Pseudomonas fluorescens*
soy bean	damping-off	*Rhizoctonia solani*	*Bacillus megaterium*
urdbean	root rot	*Rhizoctonia bataticola* Fusarium solani Rhizoctonia solani	*Pseudomonas aeruginosa* *Pseudomonas fluorescens*

of *Azospirillium* spp. co-inoculated with *Rhizobium* increased the nodulation in several legume crops. *Azospirillum* sp. produce large quantities of auxins and stimulate the formation of epidermal cells that become root hair cells or additional infection sites for rhizobial colonization. Certain isolates of PGPR enhance legume nodulation by affecting the signal exchange between plants and rhizobia. These isolates produce signal molecule analogues and/ or stimulate the plant to produce more signal molecules.

8. Rhizosphere competence is a key factor for establishment of PGPR

The introduced PGPR should be able to establish themselves in the rhizosphere at population densities sufficient to produce a beneficial effect. In many of the bioassays poor root colonization was mainly responsible for the inconsistence performance of PGPR. The complex phenomenon of root colonization is dependent on the root exudates and competition from other microorganisms. Other abiotic factors that influence the root colonization are soil temperature and soil type (pH, proportions of

organic matter, clay contents and the quantity of polyvalent cations). Therefore, efficient bioinoculants should survive in the rhizosphere, make use of nutrients exuded by the plant root to proliferate, be able to efficiently colonize the entire root system, and compete with native microorganisms. Hence, determination of the survival of the PGPR in their introduced environments is very important.

9. Formulation of the bioinoculants is essential for large-scale application

Development of effective formulations that support long shelf-life of PGPR and *Rhizobium* strains, and also their establishment in the introduced environments, is critical for the large scale adoption and commercialization of these strains. Effectiveness of the formulation of *Rhizobium* and PGPR depends on the nature of the carrier, physiological state and viability of microbes in the final product, microbiological purity of the stored product, use of adjuvant and final application method. Peat-based formulations of *Rhizobium* were suitable for the commercial inoculation process, as *Rhizobium* has a good adaptation to the peat carrier under different storage conditions. Gum arabic was an effective protector of *Rhizobium* inoculated on to seeds. Different carrier materials - peat, vermiculite, spent compost and alginate supported the formulation of PGPR isolates.

10. Dual inoculation of PGPR and rhizobia helps in increasing legume crop yields in a more safer route

In a global scenario of reaching the nitrogen and phosphorous resource plateau, rising concerns over possible environmental effects of chemical fertilizers, as well as their cost for small-scale farmers in developing countries, it is essential to expand the use of BNF and PGPR technologies that offer the greatest environmental and economic benefits for specific agroecosystems. Unlike chemical fertilizers, PGPR exert a beneficial effect on rhizobial nodulation of legumes and should be exploited for the economical benefit of subsistence farming systems. A complete understanding of the mechanisms of the synergistic activity between PGPR and *Rhizobium* facilitates the better utilization and improvement of bioinoculants in legume-based cropping systems.

Acknowledgements

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PGPRs and their potential for the sustainability of plantation crops and spices

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***Central Plantation Crops Research Institute, Kasargod

Plantation crops and spices form major components of agrarian economy in view of their commercial potential and also the employment they provide to millions. Their production and productivity thus becomes the major focus for sustainability. Decreasing the cost of production through low inputs and increasing productivity have become imperative. Maintaining optimum health of any crop is the key for productivity and these perennial crops are amenable for microbial intervention that would ensure optimum root health and consequent increase in productivity. In order to increase the production and productivity per unit area, per unit time, with maximum utilization of soil energy, CO2 and water, ingenious farming community has practiced various cropping systems (mixed / inter cropping systems) over years, which are still in vogue as practical models. Agriculture scientists have given scientific explanation behind this approach by fine tuning, taking into consideration the root patterns, their distribution and optimization of resources with in the cropping system.

It is in this context Plant Growth Promoting Rhizobacteria (PGPRs) have become relevant *specially to ensure the productivity and longevity,* of these perennials, since loss of a single plant would result in cumulative loss over years. Coconut, arecanut, coffee and tea based mixed and high density multiple (HDMSCS) cropping systems have become relevant. These systems would partly act as an insurance against price fluctuations of the commodities so that farmer's income from these crops is sustained.

Biotic and abiotic stresses have become major production constraints thus affecting the sustainability of the cropping systems. It is here the role of Plant Growth Promoting Rhizobacteria (PGPR) has become an important focus.

The following are some of the successful cropping systems.

Coconut	Cocoa	Black pepper	
Arecanut	Cocoa	Black pepper	
Coconut	Ginger	Turmeric	Tuber crops
Arecanut	Cardamom	Black pepper	
Coffee	Black pepper	Orange	
Tea	Black pepper		
Coffee	Tree spices		

Nutrient Management

There are several instances where some of these crops continue to remain productive in spite of any specific inputs. This would indicate the role of microbes in ensuring crop growth and

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productivity. In majority of plantation crops, organic recycling through biomass addition to the soil resulted in organic status of the soil that improved the structure and nutrient sources. The mineralisation of the organic matter through increased microbiological activity in the rhizosphere of coconut based cropping systems has been studied intensively. Majority of these plantations are in acidic soils. Specially the availability of P becomes a limitation because of fixation.

In coconut – cocoa mixed cropping system the increased yield of coconut by 95 % in the double hedge of cocoa and 65% in single hedge cocoa was attributed to the increased rhizosphere activity in coconut particularly IAA producing bacteria like *Eschericia sp.* and also *gibberillins* producing *Aspergillus flavus* and *A.fumigatus*. The population of *Bacillus*, *Pseudomonas* and *Micrococcus sp.* increased considerably in many of the coconut and arecanut based cropping system, which are efficient P.solubiliseres.

Recent studies at Central Plantation Crops Research Institute, Kasaragod clearly established the role of asymbiotic nitrogen fixers like *Beijerinckia indica*, *Azospirillium*, *Herbaspirillium*, *Azoacrus*, *Burkholderia*, *Arthrobabacter*, in the rhizosphere of coconut in the coconut based mixed cropping system. The increased rhizosphere (microbial) activity in these systems resulted in increased crop growth. Increased microbial activity was noticed when lower fertilizer does like one third or all the fourth was applied, indicating the role of these rhizosphere microflora in ensuring the nutrient availability, thereby reducing fertilizer inputs.

High density multiple cropping adopted in root wilt affected coconut belts as a disease management strategy decreased the root wilt intensity based on the foliar index and consequently increased yields ranging from 29-68%. This increase was attributed to increased microbial activity specially PGPRs in the rhizosphere and nutrient build up. Many of these microbial intervension clearly indicated the importance of the PGPRs in the integrated nutrient management in cropping systems. However the potential of the PGPRs as an ecofriendly technology through bioinoculants in the perennial crop ecosystem remain largely untrapped and needs to be exploited systematically. In view of the huge biomass of these crops, their discernable effects on increased yields would be gradual and steady.

Biotic stress management

Disease suppressive activity of *Bacillus amyloliquifacieus* in suppressing bud rot of coconut caused by *Phytophthora palmivora* recorded at CPCRI, Kasaragod is of considerable interest and need to be field tested to establish its disease suppressive potential. Similarly some of the native fluorescent pseudomonad isolate *(P.fluorescens) from the coconut leaves in root* wilt affected areas were found effective in reducing the leaf rot caused by *Colletotrichum gloeoosporoides* and *Exerohilum rostration*. This is yet another important funding which needs field testing.

Role of PGPRs in plant disease suppression is well known. Their role through antibiotic production, induced systemic resistance (ISR) and through nutrient availability and growth mediated defense received considerable attention in spice crop disease management in recent years.

Both basic and applied research of PGPRs and their effectiveness in disease management of spice crops particularly in foot rot and slow decline of black pepper and rhizome rot of ginger and clump rot of cardamom have been intensified in recent years at Indian Institute of Spices Research. Strains of *Pseudomonas fluorescens* and *Bacillus* sp. were found to increase the growth and vigor of these crops apart from suppressing the soil borne diseases. A repository of rhizobacteria consisting of about 1000 isolates has been set up at IISR, Calicut.

Mechanism

Efficient strains of fluorescent pseudomonads and *Bacillus* sp. have been short listed based on disease suppression, growth promotion and induction of systemic resistance in black pepper. The short listed strains of fluorescent pseudomonads in black pepper were found to produce various volatile and non-volatile metabolites including HCN against the *P.capsici*. Antibiotics viz. pyoluteorin, and pyrrolnitrin were detected in these strains by TLC. Siderophore mediated antagonism is implicated in *P. capsici* - *P. fluorescens* antagonistic system. The culture filtrate of the bacteria not only inhibited the mycelial growth of *P. capsici*, but also the explosive asexual phase, sporangial production, release of zoospores and germination of zoospores. These isolates produced mycolytic enzymes viz. b-1,3 glucanases, b-1,4 glucanases and lipases. Strains of *Pseudomonas fluorescens* caused cytoplasmic coagulation in the mycelium of *P. capsici* when they were cultured together.

The growth promoting strains of Fluorescent pseudomonads were found to synthesize phytohormones viz. IAA and GA as detected in TLC. The other determinants for growth promotion in black pepper were enhanced production of feeder roots in the plant and also the increased absorptive surface area of the roots.

Increased levels of Peroxidase (PO), Catalase, Phenylalanine Ammonia Lyase (PAL) and Polyphenol Oxidase (PPO) were induced in leaves apart from the roots of treated black pepper plants. There also found a relatively higher quantity of lignification (30 – 100% over control) in the bacterized roots. This was correlated with the lesser root rot in the bacterized plants upon challenge inoculation with the pathogen.

It is suggested that a consortium approach for disease management in plantation and spice crops would be rewarding. The bioefficacy of *Trichoderma harzianum* in disease management of soil borne disease of spices has been well established. *Trichoderma harzianum* & *Pseudomonas fluorescens* were found compatible and consortium was found effective for the management of foot rot of black pepper caused by *P. capsici*.

The biocontrol consortium was found successful not only for black pepper but also for ginger and cardamom disease management. The maximum disease suppression (63%) obtained by the treatment combination of *T.harzianum* isolate, IISR-1369 and *P.fluorescens* strain- IISR-6 in black pepper and in cardamom, it was 36% over control. The same treatment could impart 66.2% survival of ginger tillers after challenge inoculation with *P.aphanidermatum*. The efficient isolate from black pepper can be used in a cropping system involving black pepper, ginger and cardamom.

The nematode pathogens of black pepper viz. *Meloidogynes incognita*and *Radopholus similis* also were inhibited by these strains of *P.fluorescens*. An economical and ecofriendly multiplication medium was developed for the large scale production of the bacteria to log 14 cfu/ml in 32h. The Indian Institute of Spices Research has recommended this *P.fluorescens* strain IISR-6 for release to the benefit of farmers.

The role of PGPR s in vanilla disease management is of great relevance since efficient isolate that could suppress diseases caused by *Phytophthora meadii* and *Fusarium oxysporum*, have been identified. Isolate IISR 859 was found growth promotive and *Phytophthora* suppressive. Where as IISR 147 and IISR 148 were suppressive to both the pathogens.

A greater thrust is given for development of biological consortia with multiple modes of action to suppress both fungal and nematodal pathogens since there is no spatial segregation of these pathogens in many of the cropping systems practiced. The biocontrol technology with PGPRs and consortia are of immense

importance specially for export oriented spice crops since organic spice production is the present trend and is being popularized.

PGPRs and planting material production

Being primary colonisers with high rhizosphere competence, PGPRs can be effectively utilized in production of healthy robust planting materials. Since many of the soil borne pathogens like *P.capsici*, *P.aphanidermatum*, *Fusarium* and *R.similis* infect the roots right at the nursery stage but go unnoticed. PGPRs act as good root protectants, and also can further offer protection in the field. In a recent study carried out at IISR, Calicut in black pepper, rooted cuttings treated with *P.fluorescens* inoculum, exhibited better growth optimum root health and good field establishments. Vegetative planting materials like cardamom, ginger, turmeric and vanilla are highly ameable for such treatment. Root dip of coconut, arecanut and cashew and also other plantation crops like coffee, tea and rubber with PGPRs and their effect on the field establishment and subsequent growth deserve a careful study in the nursery management.

Future Strategies

Basic research on the molecular mechanism of biological control, ISR and growth promotion need to be intensified in order to identify the genes controlling these, since strainal improvement for bioefficacy is important to reduce the cost of the products. Molecular techniques such as DNA hybridization and PCR techniques such as RAPD and AFLP provide a sensitive means of detecting pathogens and biocontrol agents without culturing. PCR technique can be used for targeting functional genes involved in biocontrol such as genes for antibiotic production.

Besides the plant microbe interaction, the stability of the released PGPR strains in the environment through antibiotic markers and the factors governing this ecological fitness need indepth study for the success of the biological control. Besides product development, the quality parameters particularly shelf life should receive high priority in future programmes to realize the benefits of this eco-friendly technology for sustainability in these perennial crop ecosystem.

Best wishes from.....

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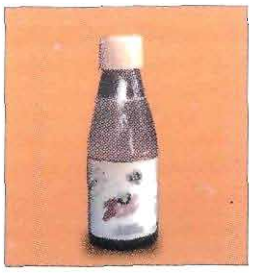

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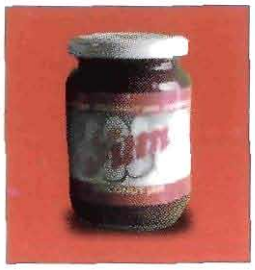

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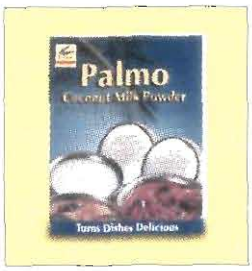

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