UPM Inaugural Lecture Series

SMART PARTNERSHIP: Plant-Rhizobacteria Associations

Prof. Dr. Zulkifli Hj. Shamsuddin

Diterbitkan di Malaysia oleh
Bahagian Komunikasi Korporat
Universiti Putra Malaysia
43400 UPM Serdang
Selangor, Malaysia

Tel : 603-8946 6003
Fax : 603-8948 7273
e-mail: cco@admin.upm.edu.my

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ZULKIFLI HJ. SHAMSUDDIN

Professor Zulkifli Hj. Shamsuddin was born in Georgetown, Penang in 1947. He started schooling at Sekolah Melayu Bandar Penggaram and High School, Batu Pahat, Johor, followed by his early secondary education at Sultan Ismail College, Kota Bharu, Kelantan and Ibrahim School, Sg. Petani, Kedah. These changes in school locations were necessary due to frequent transfers of his agriculturally-trained father, Tuan Hj Shamsuddin Bin Mohd. Arabee, K.M.N., a pioneering graduate of School of Agriculture Malaya (1933), the foundation institution for Universiti Putra Malaysia.

In 1963, he completed his secondary education at the Federation Military College (renamed Royal Military College) which had moulded his formative years with its motto "Serve to Lead". He was active in athletics and extra-curricular activities having won a gold medal in the Selangor School Sports Council and served as the school cricket captain. He had also represented the state of Kelantan in a National Scout Jamboree.

The first professional qualification he received was the Diploma of Agriculture (Malaya) from College of Agriculture Malaya (1970). It was where its motto "*Bertani Berbakti*" and later "*Berilmu Berbakti*" became the guiding philosophy in his professional career. This was followed by a Bachelor of Science (Soil Science) and a Master of Science (Soil Microbiology) from Louisiana State University (LSU), USA in 1973. He represented LSU in the inter-state soils competition. His education was funded by MARA (Majlis Amanah Rakyat) and a graduate research assistantship from LSU. He also received the Phi Kappa Phi and Gamma Sigma Delta award for academic excellence while at LSU. He successfully pursued for a Ph.D. at Murdoch University, Western Australia on an Australian-Asian Universities Vice-Chancellors' Scholarship (AAUCS).

Professor Zulkifli began his professional academic career as a lecturer at Universiti Pertanian Malaysia (renamed Universiti Putra Malaysia) since 1973 to date. He was promoted to an Associate Professor in 1982 and subsequently appointed as a Professor of Soil Microbiology in the Faculty of Agriculture.

Research is his passion being one of the earliest recipients of the MPKSN research fund, a national research granting scheme prior to the inception of IRPA in 1983. Since then, he has received more than 25 national and international research grants from IRPA Experimental Applied (EA) and Prioritised Research (PR) Grants, US-AID, JSPS (Japanese Society for the Promotion of Science), ACIAR (Australian Centre for International Agricultural Research), and UNESCO. In 1994, he was awarded the Asian Development Bank Research Fellowship for sabbatical research at Melbourne University where he managed to demonstrate the ability of a locally isolated rhizobacteria to substantially fix N_2 with oil palm plantlets *in vitro* using ^{15}N technique. Along with these scientific contributions, he accumulated a collection of local bradyrhizobia and plant-growth promoting rhizobacteria strains for various legumes and economic crops which are shared

with other researchers, locally and overseas. These cultures are listed in the UNEP/ UNESCO/RIKEN World Directory of Collection of Cultures of Microorganisms.

Professor Zulkifli is highly committed towards academic excellence and has been actively involved in the academic development and restructuring of the Faculty of Agriculture and Faculty of Food Science and Biotechnology at UPM. He was also instrumental in the establishment of the Bachelor's program in Biotechnology, the Bachelor of Science Bioindustry program and the initiative for a Master of Science program in Land Resource Management. This academic program, conducted since 1997 in collaboration with Cambridge University, is unprecedented in Southeast Asia. He has coordinated five study visits for these Masters students to United Kingdom, Holland, Belgium and France. Professor Zulkifli has also supervised or co-supervised more than 70 students at undergraduate and postgraduate levels.

The major administrative positions he has held were the Head of Department of Soil Science and Deputy Dean of Research, Faculty of Agriculture which enabled him to contribute effectively and successfully as the Founding Director of the Research Management Centre, UPM in 2001. He has also organized several agricultural conferences including the Faculty of Agriculture Conference on "Food and Agriculture Malaysia 2000", in conjunction with UPM's 1st Convocation (1977), the Malaysian Soil Science Conference on "Nitrogen in Malaysian Agriculture" (1982), "Biotechnology of Nitrogen Fixation in the Tropics" Symposium (1986), "Agricultural Exposition and Convention" (1999) and "Agriculture Congress 2004".

Professor Zulkifli has been the President of UPM Academic Staff Association (1985-1986) and Malaysian Society of Soil Science (1998-2000); Executive Secretary (1987-1991) and National Point of Contact Representative (1982-1992) of UNESCO Regional Network for Microbiology in Southeast Asia; and Founding Secretary (1976-1977) and Secretary (1983-1984) of Malaysian Society for Microbiology. Professor Zulkifli is also a Board Member of Felda Herbal Corporation Sdn. Bhd. and a Fellow of the Malaysian Society of Soil Science.

"SMART PARTNERSHIP: PLANT-RHIZOBACTERIA ASSOCIATIONS"

ABSTRACT

Malaysia is one of the twelve mega-biodiversity countries blessed with diverse forms of microflora and fauna together with their complex microbial associations. The unique smart partnership between root-associated and colonizing bacteria (rhizobacteria) and plants is vital for the sustainability of the global ecosystem namely the contribution towards nutrient cycling and plant growth. Various symbiotic and associative rhizobacterial associations are discussed in this paper with focus on the contribution of rhizobia and plant growth promoting rhizobacteria (PGPR) in legumes and non-leguminous economic crops under acidic, humid tropical environment. Different plant-rhizobacterial adaptations are exemplified to demonstrate the unique abilities of these associations to overcome the adverse effects of soil environment. Locally isolated *Bradyrhizobium* (UPMR29, 48), *Azorhizobium* (UPMR42, 43, 44), and PGPR (*Bacillus sphaericus* UPMB10, UPMB12, 13, 14) strains show varying competitiveness and compatibility in their symbiotic and non-symbiotic associations with several leguminous covers, pasture and food legumes (groundnut, winged bean, vegetable soybean) and non-leguminous food (sweet potato, banana) and economic crops (oil palm). The ability of these bacteria and their plant associations to overcome abiotic stresses, increase plant nutrient uptake, growth and yield are highlighted. The competitive PGPR strains namely *Bacillus sphaericus* UPMB10 was characterized and the optimum growth requirement, inoculum carrier (ground oil palm frond) and inoculum production technique identified. A new cost-effective and high quality inoculant production technology for these rhizobacteria with high commercial potential is featured. In Malaysia, there is greater possibility for more smart partnerships involving many plant-rhizobacterial associations to be developed for economic benefits to the nation.

INTRODUCTION

The humid tropics are blessed with plentiful rainfall (> 2500 mm/year) and sunshine essential for luxurious plant growth. This has resulted in lush and dense vegetation which encouraged the perpetuation of diverse species of flora and fauna, and the survival of many higher animal species. Twenty (20) biological diverse countries have been identified in the world; Malaysia is one of the 20 mega-biodiversity countries (Table 1).

Table 1. World ranking mega-biodiversity countries (Paine, 1997)

Country	Mammals	Birds	Flowering Plants
Mexico	450	1,026	25,000
Indonesia	436	1,531	27,500
Zaire	415	1,096	11,000
Brazil	394	1,635	55,000
China	394	1,244	30,000
Colombia	359	1,695	50,000
Peru	344	1,678	17,121
India	316	1,219	15,000
Venezuela	305	1,296	20,000
Ecuador	302	1,559	18,250
Cameroon	297	874	8,000
Malaysia	286	736	15,000
Australia	252	751	15,000
South Africa	247	790	23,000
Panama	218	926	9,000
Papua New Guinea	214	708	10,000
Vietnam	213	761	7,000
Costa Rica	205	850	11,000
Philippines	153	556	8,000
Madagascar	105	253	9,000

Sustainability of a biological species in a specific environment depends largely on its ability to survive and outcompete the other species – "survival of the fittest". It also hinges on the ability of that species to associate facultatively or symbiotically in an obligate fashion with other organisms for mutual benefit. This lecture focuses on the unique smart partnership developed between the root-associated and colonizing bacteria (rhizobacteria) and the plant systems (legumes and non-legumes). The mutual association is vital not only for the respective organisms but also for the sustainability of the global ecosystem. Besides the gaseous exchange (CO_2, O_2) during photosynthesis and the maintenance of the ratio of these gases in the atmosphere, another phenomenon which is less commonly appreciated occurs during this plant-rhizobacteria association; the contribution towards nutrient cycling. Nitrogen is generally considered the most demanded plant nutrient. Thus an

effective nitrogen cycling is vital especially because biological nitrogen (N_2) fixation by N_2-fixing bacteria, independently or in association with legumes or non-legumes, is the sole biological mechanism whereby gaseous nitrogen is recycled into the biological system again. Otherwise it would remain in the inert gaseous form not metabolized by any living organism.

The tropical ecosystem established is fragile and will collapse rapidly when physically disturbed and exposed for example, during indiscriminate logging and deforestation. The greatest loss will be the organic matter status which requires a very long period to accumulate; 1-2 cm organic matter layer formed in 100 years. Effective plant-rhizobacteria associations, however, can contribute towards improvement in the organic matter status and consequently increase soil fertility.

Soils of the humid tropics which characteristically receive abundant moisture and optimum temperature conditions for active microbial activities continuously undergo the weathering process throughout their profiles. This has led to the highly problematic low pH (< 4.5) and highly acidic conditions of these soils which cover an extensive proportion of the humid tropics (Figure 1).

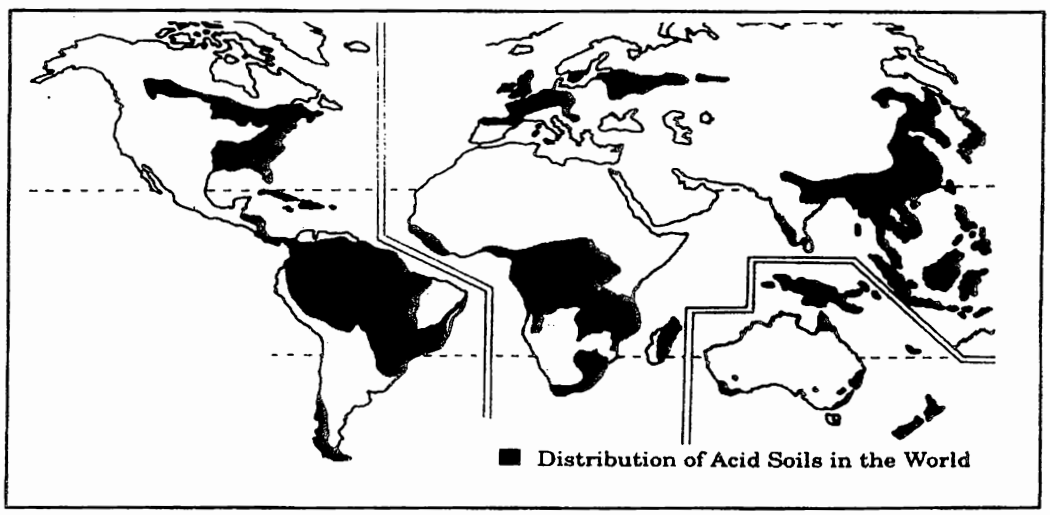

Figure 1. Distribution of Acid Soils in the World

An understanding of the plant-rhizobacteria associations can help alleviate the detrimental effects of soil acidity on plant growth. Such knowledge are highly required in the management and reclamation of problem soils (peat, acid sulphate soils, ex-mining land) for agriculture through green manuring and other organic inputs.

BENEFITS OF PLANT-RHIZOBACTERIA ASSOCIATIONS

Plant-rhizobacteria associations can occur through several mechanisms namely as endophytes or on the root surface. Most rhizobacteria remain confined to the root surface (rhizoplane), but some enter the root interior and behave as endophytes (Sturz *et al,*. 2000). Endophytes are microorganisms that spend most of their life cycle inside plants (Quispel, 1992). They are saprophytes, parasites or symbionts. Various endophytic N_2-fixing bacteria, named endophytic diazotrophs, have been identified to be associated with cereals and grasses. These endophytes do not cause damage to the host organism. Instead, they promote plant growth by one or more of three factors: the production and secretion of plant growth regulators or phytohormone (bioenhancer), antagonistic activity against phytopathogens (biopesticide) and the supply of biologically fixed nitrogen (biofertilizer) (Bashan and Holguin, 1997; Van Buren *et al.*, 1993). The rhizobacteria can develop an obligatory symbiotic relationship with the plant e.g. N_2-fixing rhizobia-legume, N_2-fixing actinomycete or cyanobacteria-non-legume association. Alternatively, a non-obligatory (facultative) associative relationship can develop with the rhizobacteria growing in the intercellular region of the roots or on the root surface.

Biofertilizer is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). This definition separates biofertilizer from organic fertilizer. The latter contains organic compounds which directly, or by their decay, increase soil fertility. Likewise, the term biofertilizer should not be used interchangeably with the terms, green manure, manure, intercrop, or organic-supplemented chemical fertilizer. Not all plant growth promoting rhizobacteria (PGPR) can be considered biofertilizers. Bacteria that promote plant growth by control of deleterious organisms are biopesticides, but not biofertilizers. Similarly bacteria can enhance plant growth by producing phytohormones and are regarded as bioenhancers, not biofertilizers. Interestingly, some PGPR can promote growth by acting as both biofertilizer and biopesticide or bioenhancer.

Symbiotic Association

Symbiotic associations of significance to agriculture occur predominantly between rhizobia and legumes with a relatively lower impact between cyanobacteria and non-legume (paddy) or actinomycetes and non-legume trees. These involve biological N_2 fixation (BNF) which is a vital component of the Nitrogen Cycle (Figure 2).

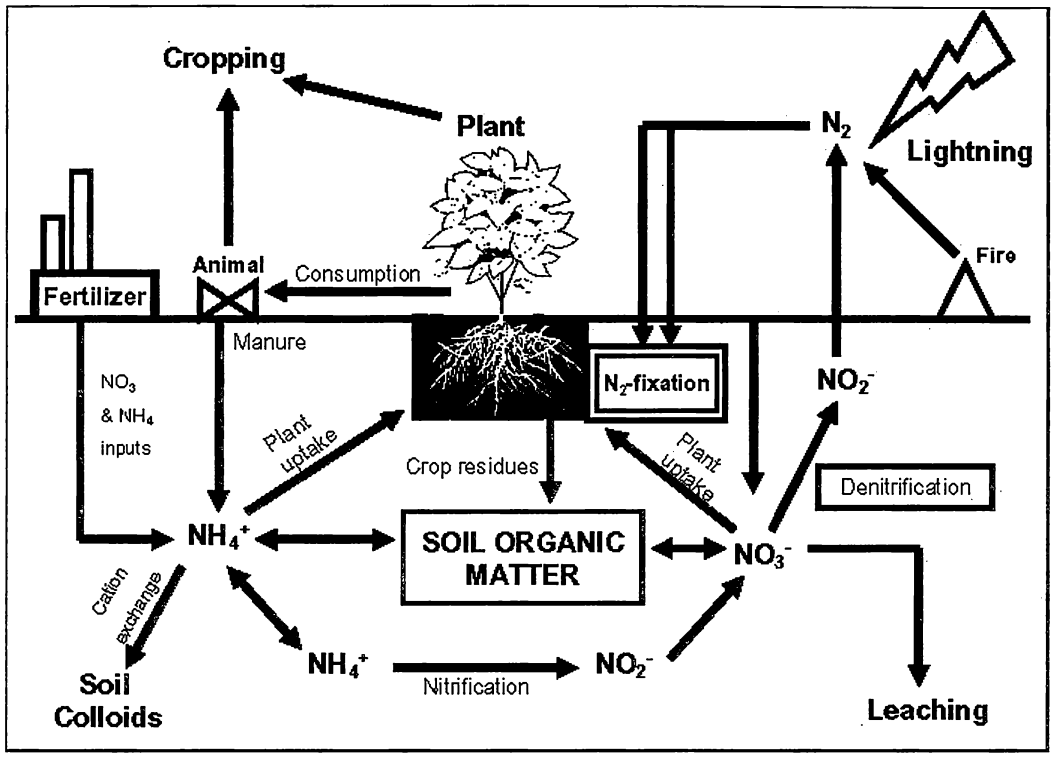

Figure 2. Nitrogen Cycle

Soil bacteria play a critical role in providing nitrogen for plant growth via the ***nitrogen cycle,*** a recycling of nitrogen through the environment by plants, animals and microbes. The nitrogen cycle involves the fixation of atmospheric nitrogen (N_2) into organic N, which is mineralised into ammonia N and nitrate N and finally returned to atmospheric N through denitrification.

Most organisms (including humans) cannot use nitrogen in the gaseous form (N_2) for their nutrition, so they are dependent on other organisms to convert nitrogen gas to nitrate, ammonia, or amino acids. "Fixation" is the conversion of gaseous nitrogen to ammonia or nitrate. The most common kind of fixation is "biological fixation" which is carried out by a variety of organisms, including cyanobacteria, the *Azobacter*, and the association of legume plants with the nodulating bacteria *Rhizobium (Table 2)*. Additionally, nitrogen can be fixed by some inorganic processes. For example, "high-energy fixation" occurs in the atmosphere as a result of lightning, cosmic radiation, and meteorite trails. Atmospheric nitrogen and oxygen combine to form nitrous oxides (NO_2^-) which fall to the earth as nitrate.

Table 2. N_2-fixing organisms and their cultural requirements

Cultural requirements	N_2-fixing organisms
STRICT ANAEROBES	*Clostridium spp.* *Desulfovibro* *Desulfotomaculum* *Methanogens e.g.Methanococcus*
PHOTOTROPHIC ANAEROBES	*Chromatium* *Chlorobium* *Thiopedia* *Ectothiospira*
FACULTATIVE (aerobic when not fixing N_2)	*Klebsiella* *Bacillus* *Enterobacter* *Citrobacter* *Escherichia* *Propionibacterium*
FACULTATIVE PHOTOTROPHS	*Rhodospirillum* *Rhodopseudomonas*
MICROAEROPHILES (normal aerobes when not fixing N_2)	*Mycobacterium* *Thiobacillus* *Spirillum* *Aquaspirillum* *Methanosinus* *Rhizobium*
MICROAEROPHILIC PHOTOTROPHS	*Plectonema* *Lyngbya* *Oscillatoria* *Spirulina*
AEROBES	*Azotobacter* *Azotococcus* *Azomonas* *Beijerinckia* *Derxia*
AEROBIC PHOTOTROPHS	*Anabaena* *Nostoc* *Calothrix* *Gloeocapsa* 7 other genera of blue-green algae

When plants and animals die, proteins (which contain organic nitrogen) are degraded by bacteria to form ammonia (NH_3). This process is called "ammonification". Ammonia is then degraded by other bacteria (*Nitrosomonas*) to form nitrite (NO_2^-) which is further degraded by another type of bacteria (*Nitrobacter*) to form nitrate (NO_3^-). This conversion of ammonia to nitrite and nitrate is called "nitrification" Nitrates can then be used by plants to grow.

Completing the nitrogen cycle, nitrates are reduced to gaseous nitrogen by the process of "denitrification". This process is performed by organisms such as the bacteria *Pseudomonas*, and *Thiobacillus denitrificans*. These organisms break down nitrates to obtain oxygen.

Estimates of the natural fixation of atmospheric-N gas, the N released and recycled annually are presented in Table 3. Terrestrial ecosystems gain an estimated 130-170 million metric tons of nitrogen (N) annually from biological nitrogen fixation (BNF) (Galloway *et al.*, 1995), with about 40 million metric tons, or 23-31 percent of the total, attributed to forested ecosystems (Burns and Hardy, 1975). The global fertilizer consumption clearly highlights the need for BNF (Figure 3).

Table 3. Global Estimates of N_2 fixation, N Release and Recycled Annually

N_2 fixation System	x 10^6 metric tons N yr^{-1}	Reference
Natural		
Marine ecosystems	40 – 200 (Fixation)	Galloway, 1998
Terestrial ecosystems	130 – 170 (Fixation)	Galloway *et al.*, 1995
Lightning	9.4 (Fixation)	Galloway, 1998
Anthropogenic		
Chemical-N fertilizer	80 (Fixation)	Galloway *et al.*, 1995
Legumes	25 (Fixation)	Smil, 1999
Wastes	25 (Recycled)	Smil, 1999
Combustion	20 (Release)	Galloway, 1998

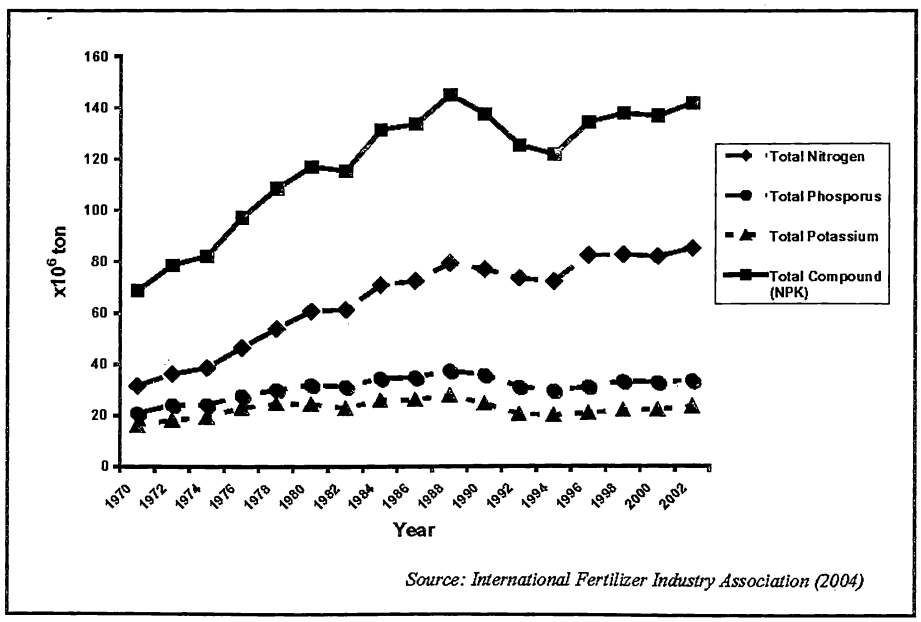

Figure 3. World fertilizer consumption (1970-2002)

The lecture will focus on the rhizobia–legume association. The rhizobia/bradyrhizobia ('slow-growing') associations with various temperate and tropical legumes can be classified into 10 cross-inoculation groups (Table 4). These are groups of rhizobial species (or biovars) that specifically nodulate the same legume host.

Table 4. Cross-inoculation groups of leguminous plants

***Rhizobium* spp.**	**Cross-inoculation grouping**	**Legume types**
R. leguminosarum biovar *viciae*[a,b]	Pea	*Pisum, Vicia, Lens*
R. phaseoli biovar *phaseoli*[a,b], *R. tropici*[b], *R. etli*[b]	Bean	*Phaseolus*
R. leguminosarum biovar *trifolii*[a,b]	Clover	*Trifolium*
R. meliloti	Alfalfa	*Melilotus, Medicago, Trigonella*
R. lupini	Lupini	*Lupinus, Ornithopus*
Bradyrhizobium japonicum[a,b,c], *R. elkanii*[b], *R. fredii*[b], *Sinorhizobium*[a]	Soybean, Siratro	*Glycine, Macroptilium*
Bradyrhizobium sp.[a]	Cowpea	*Vigna, Arachis*
Azorhizobium caulinodans[b]	Sesbania	*Sesbania rostrata*
R. galegae[c]	Galega	*G. orientalis, G. officinalis*

[a] (Rao, 1999); [b] (Madigan *et al.*, 1997); [c] (Holt *et al.*, 1994)

In the rhizobia-legume symbiosis, root nodules are formed and the bacteroids effectively fix atmospheric nitrogen into organic-N for the plants which provide photosynthate as energy source to the bacteroids. Among the N_2-fixing bacteria, the biological nitrogen fixation reaction has been exemplified into an equation illustrated below:

$$N_2 + 10\,H^+ + 8\,e^- \text{ ----------> } 2\,NH_4^+ + H_2$$

The reaction requires the input energy of 16 molecules of ATP and the enzyme nitrogenase. This nitrogenase enzyme is coded by *nif* (nitrogen fixation) genes. The nitrogenase is a metalloenzyme composed of two protein components: Fe protein and MoFe protein. The Fe protein domain serves as the ATP binding site, and the MoFe protein serves as the substrate binding site. The two protein domains are both oxygen sensitive (inactivated by oxygen) and require an anaerobic environment for nitrogenase catalysis to work. In the symbiotic diazotrophs with legume hosts, root nodules provide the oxygen-free site for nitrogen fixation. These nodules are elicited by bacterial Nod factor encoded by the bacterial *nod* (nodulation) genes (Truchet *et al.*, 1991). In the free-living diazotrophs such as cyanobacteria, nitrogenase fixes nitrogen within the nodule-like structure called heterocyst.

Molecular communication between the rhizobial and legume symbionts is mediated by signal molecules that activate expression of genes required for the symbiotic pathway (Figure 4).

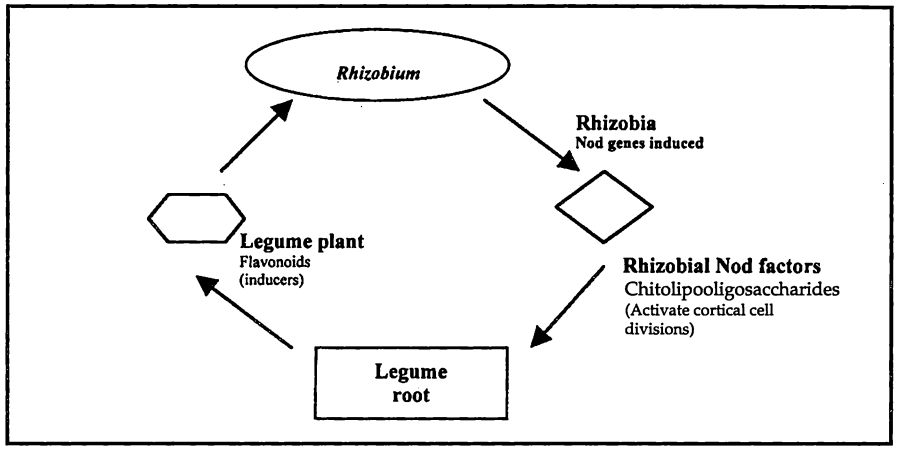

Figure 4. Molecular communication between rhizobia-legume symbionts

The host legume root secretes phenolic compounds called flavonoids. These are taken up by the rhizobial symbiont, where they activate expression of various symbiotic plasmid-encoded *nod* (nodulation) genes. These signal molecules trigger root hair deformations and cortical cell divisions within the root leading to nodule formation.

Within the root nodule, the bacteria are released from infection threads into the host cell while still enclosed within a host-derived membrane called the peribacteroid membrane. They then divide and transform into enlarged pleomorphic bacteroids and make the enzymatic machinery which carry out N_2-fixation. The entire endosymbiotic structure is called a symbiosome.

Associative Plant-Rhizobacteria Association

Plant Growth-Promoting Rhizobacteria (PGPR) generally include all root-associated bacteria that promote or enhance plant growth. In this lecture this refers namely to rhizobacteria with non-obligatory association but of benefit to the plant.

Plant associated N_2-fixing bacteria have been considered as one of the possible alternatives for inorganic nitrogen fertilizer to promote plant growth and yield in non-legumes (Ladha and Reddy, 2000). Though a variety of N_2 fixing bacteria like *Acetobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Derxia, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas* and *Zoogloea* have been isolated from the rhizosphere of various crops (James *et al.*, 2000), interest in the beneficial N_2-fixing growth-promoting rhizobacterial - plant association has increased recently due to their potential use as biofertilizers (Vessey,

2003). Their beneficial effects on plants have also been attributed to the production of phytohormones (Tien *et al.*, 1979; Haahtela *et al.*, 1990) and competitive suppression of pathogens (Glick, 1995).

SYMBIOTIC INTERACTIONS IN TROPICAL LEGUMES

Many legumes in Malaysia can form root nodules naturally but certain legumes such as soybean *(Glycine max)* will not form nodules unless inoculated. The indigenous rhizobia are not compatible with these introduced legumes. Selection of effective *Bradyrhizobium* is imperative to increase nodulation, N_2 fixation and legume yield. Local research in this area is relatively limited and no conclusive results have been established.

Rhizobial growth and symbiotic associations in tropical legumes are restricted by soil acidity stresses (high Al content, low pH (≤ 4.5), low levels of P, Ca, Mg). The inherent acidity is attributed mainly to the highly weathered condition and the high Al and Fe contents (>10% $Al(OH)_3$ and /or Fe_2O_3) in the soil.

Pasture Legumes

Tree legumes such as leucaena (*Leucaena leucocephala*) are suitable components in mixed signal grass (*Brachiaria decumbens*) pastures and have a great potential as a protein source for animal feed. In a field study with signal grass-based pasture, leucaena has been estimated to fix 240 kg N ha^{-1} $year^{-1}$ while stylo (*Stylosanthes guianensis* cv. Schofield) fixed 37 kg N ha^{-1} $year^{-1}$. When both legumes were grown together in association with signal grass, the total amount of N_2 fixed was 298 kg N ha^{-1} $year^{-1}$. Unlike leucaena, the dry matter productivity of the stylo component in mixtures was low and declined over time (Aminah *et al.*, 1992a,b).

Green Manure

Sesbania rostrata is a unique legume capable of root and stem nodulation, thus enabling it to fix large amounts of nitrogen (450 kg N ha^{-1} $year^{-1}$) (Figure 5). Stem nodulation also means that the N_2-fixation process is not subjected to any constraints associated with stresses in the soil.

Figure 5. Stem nodules of *Sesbania rostrata*

Three *Azorhizobium* strains (UPMR 42, 43, 44) have been isolated from stems and nodules of locally grown *Sesbania rostrata* from paddy and sandy soils. Characterization studies showed that *Azorhizobium* wild-type strains UPMR 43 and UPMR 44 may be the same, based on isoenzymes and plasmid profiles. However, antibiotic mutants of UPMR 43 (wild-type) and UPMR 44 are capable of increasing nodulation and plant growth of *Sesbania rostrata.*

A study on the use of legumes showed that *Sesbania rostrata* has been shown to be more effective than guar *(Cymopsis tetragonoloba)* as a source of green manure for growth of maize. However, the amount of nutrients released during mineralization, such as N and Ca, can be increased with a higher rate of green manure applied (> 5 t ha^{-1}).

On sandy soil, increased nodulation was observed for guar inoculated with *Bradyrhizobium* NC 92, and *Sesbania rostrata,* inoculated with three local strains of *Azorhizobium.* Inoculated *Sesbania rostrata* can nodulate and grow effectively on sandy and clayey soils (Radziah and Shamsuddin, 1990) making it a potential green manure crop in paddy soils and the reclamation of ex-mining land.

Food Legumes

Leguminous crops are important sources of protein and improve soil fertility from their N_2 fixing activities. Accurate estimations of the amount of N_2 fixed by different legume crops in a particular agro-ecosystem are necessary for reliable assessment on the contribution of biological nitrogen in a given cropping system. In grain legumes, nitrogen accumulation is closely linked to biomass accumulated and seed growth. Nitrogen accumulation in the grain is important in terms of both the physiology of pod filling and the nutritive value of the grain. Leaf N is the dominant source of mobilised N in many grain legumes.

Groundnut

Groundnut (*Arachis hypogea*) is a food legume processed largely as snacks or food ingredients. It is, however, not extensively cultivated locally and grown mainly as cash crops or intercrops.

Al toxicity is an important factor that limits plant growth in acidic soils. Its major effect is in limiting nutrient supply for legume growth, nodulation and nodule function. In a study on soil acidity effects under aluminium (Al) stress conditions (30μM Σa_{Almono}) using solution culture, the optimum amounts of calcium (Ca) and magnesium (Mg) required for nodulation and maximum symbiotic growth of groundnut were 2500 and 400μM, respectively (Figure 6). In a 21-day solution culture study it was shown that 2500μM external Ca concentration was required to alleviate Al toxicity at 24 μM Σa_{Almono} on non-symbiotic growth of groundnut cv. Matjam at pH 4.3 (Figure 7) (Kasran *et al.*, 1995).

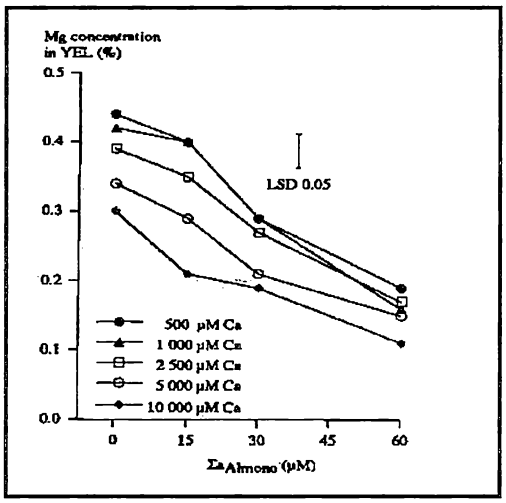

Figure 6. Mg concentration in YEL of groundnut grown in mineral N at different concentrations of Σa_{Almono} and Ca

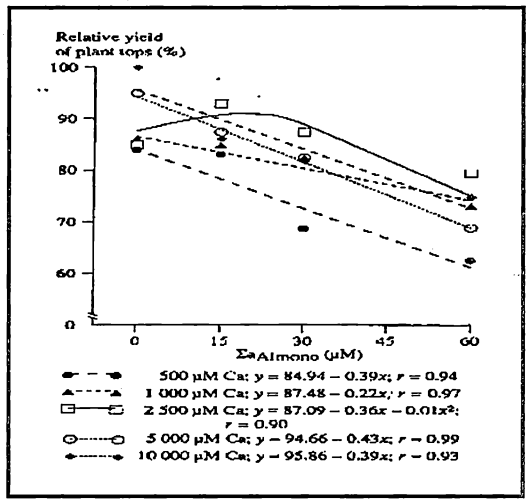

Figure 7. Relative yield of plant tops of groundnut grown in mineral N at different concentrations of Σa_{Almono} and Ca

This critical Ca concentration is equivalent to the soil solution Ca concentration when 2 t ha^{-1} ground magnesium limestone was applied to an Ultisol (Shamshuddin *et al.*, 1989). The result demonstrates the beneficial effect of Ca in alleviating Al toxicity for non-symbiotic growth of groundnut plants. However, the interaction between Ca and Al on nodulation and N_2-fixation of legumes is little understood. A 28 d solution culture experiment using groundnut cv. Matjam showed that the $\Sigma a_{Almono} \geq 30$ *μM* in solution delayed nodule development, N_2-fixation and plant growth (Figures 8; 9) (Shamsuddin *et al.*, 1992). The results also showed that an increase in Ca and Al solution concentration would decrease the Mg concentration in the youngest expanded leaf (YEL), indicating an interference in the Mg nutrition of the host legume. In summary the increasing order of tolerance of components in groundnut-bradyrhizobia symbiosis to Al toxicity are as follows:

infection process (nodulation) < nodule development < plant growth

A 35 d solution culture experiment using groundnut cv. Matjam showed that *Bradyrhizobium* strain NC92 was more effective than strain UPMR29 in nodulating of groundnut under Al stress, based on the greater number of nodules formed at 15 μM Σa_{Almono} and higher N concentration in YEL (4.7% vs. 3.0%) (Table 5) (Kasran and Shamsuddin, 1992). In a field study, *Bradyrhizobium* NC 92 and CB 756 enhanced nodulation of groundnut cv. Matjam grown on Ultisol (Bungor and Rengam series) and Oxisols (Munchong and Katong series).

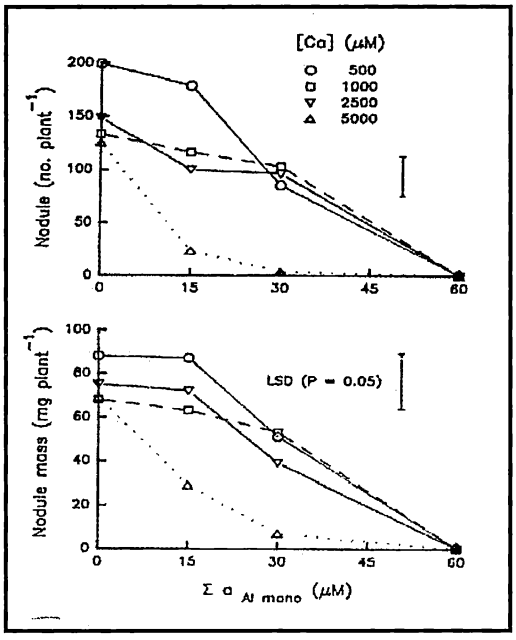

Figure 8. Effects of sum of activities of monomeric Al species (Σa_{Almono}) and Ca concentration on (a) nodule number and (b) nodule dry mass per plant in groundnut cv. Matjam inoculated with *Bradyrhizobium* spp. strain NC92

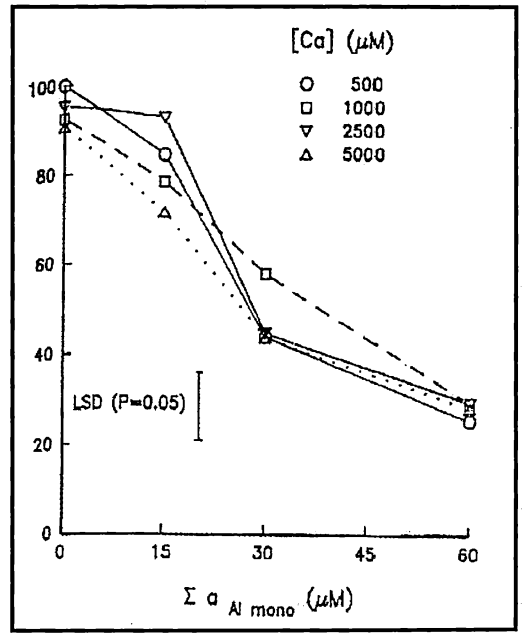

Figure 9. Effects of sum of activities of monomeric Al species (Σa_{Almono}) on relative yield of plant tops of groundnut cv. Matjam inoculated with *Bradyrhizobium* spp. strain NC92

Table 5. N, Ca and Mg concentrations in youngest expanded leaf (YEL) of groundnut grown at different concentrations of Σa_{Almono} and inoculated with *Bradyrhizobium* strains NC92 and UPM29

Σa_{Almono} (μM)	*Bradyrhizobium* strain	Nutrient concentrations in YEL (%)		
		N	Ca	Mg
0	Uninoculated	1.61	2.20	0.23
	NC92	4.91	1.52	0.31
	UPM29	4.21	1.56	0.30
15	Uninoculated	1.45	2.00	0.18
	NC92	4.74	1.70	0.31
	UPM29	3.04	1.58	0.31
60	Uninoculated	1.44	1.55	0.17
	NC92	1.38	1.54	0.19
	UPM29	1.31	1.54	0.17
$LSD_{0.05}$		0.94	0.50	0.07

Aluminium toxicity is also known to inhibit root elongation by reducing the mitotic activity (Roy *et al.*, 1988). The damaged root systems appear thickened and stubby, and the development of secondary roots is prevented (Ohki, 1986). It has also been reported that Al toxicity results in reduced tolerance to water deficit (Roy *et al.*, 1988), a condition which could result in a lowering of nitrogenase activity (Edmeades *et al.*, 1991). Al toxicity can cause changes in various physiological and biochemical functions of plants including a reduction in the rate of transport of photosynthates in legumes (Foy, 1988), an inhibition in nodulation (Shamsuddin *et al.*, 1992), and an increase in the concentration of phenols in legumes (Balsberg Pahlsson, 1990). In a solution culture experiment, Al at 10-30 μM Σa_{Almono} inhibited root elongation, suppressed nodulation and increased the activity of polyphenol oxidase (PPO) in groundnut (Marziah *et al.*, 1995)(Table 6; 7; Figure 10). Al toxicity affected nodulation by reducing the nodule number. This could be related to the shortage of photosynthate supply to serve as C skeletons in the nodules, since Al is known to reduce photosynthesis (Foy, 1988). PPO activity, which is an indicator of plant stress, was observed to increase as early as 10 days after planting (DAP), suggesting that PPO could be an effective indicator enzyme for Al toxicity in groundnut. The results indicate that Al interferes with the phenolic metabolism, the mechanism of which, however, is not clear.

Table 6. Effects of A1 on root length of groundnut at 10, 20, 30 and 40 days after planting (DAP)

Treatment Σa_{Almono} (μM)	**Root length (cm) at DAP:**			
	10	**20**	**30**	**40**
0	34.0def*	50.4c	66.9b	80.6a
10	32.8ef	47.4c	54.3bc	58.3bc
20	30.5f	47.2cd	52.2c	55.7bc
30	27.8f	45.6cde	50.1c	54.1bc

* Values followed by different letters within a column indicate significant differences at P=0.05 by Duncan's multiple range test.

Table 6. Effects of A1 on nodulation (nodule number) of groundnut at 10, 20 and 30 days after planting (DAP)

Treatment Σa_{Almono} (μM)	Nodule number at DAP:		
	10	20	30
0	0	250ab*	302a
10	0	204b	303a
20	0	225b	246ab
30	0	174b	228b

* Values followed by different letters within a column indicate significant differences at P=0.05 by Duncan's multiple range test.

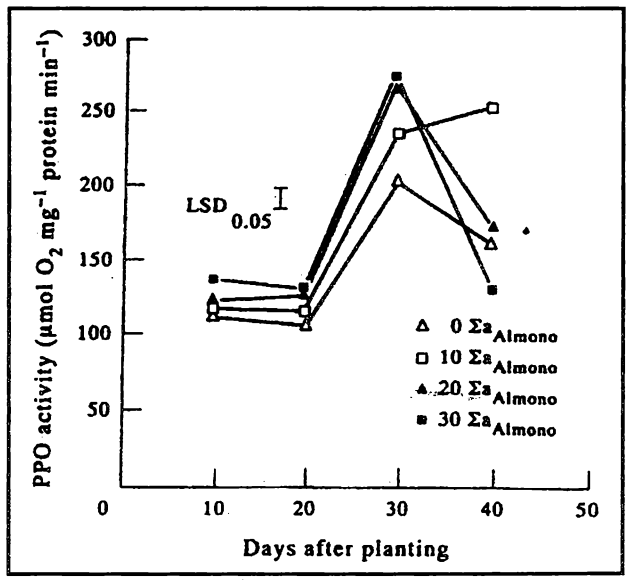

Figure 10. Polyphenol oxidase (PPO) activity in YEL of groundnut at different concentrations of Σa_{Almono} and 10, 20, 30 and 40 days after planting (DAP)

Means with the same letters are not statistically significant at 5% level

Winged bean

Winged bean (*Psophocarphus tetragonolobus* (L.) DC), an indeterminate, climbing perennial legume requires a support system to achieve high yields. Studies on winged bean yield have mainly been done on dry matter and nitrogen accumulation with single staked supports but no comparison has been made between plants grown on a support system and those without support. There is also little experimental evidence on nitrogen partitioning and its significance to seed yield of winged bean.

A field experiment on winged bean was conducted on Serdang sandy loam (pH 6.0-6.5) using three support systems: i) control plants grown on the ground surface i.e. unsupported, ii) plants grown with support height of one metre (wire trellis) and iii) plants grown with support height of two metres. Plants grown with 2-m supports produced substantial nodule mass, the highest rate of N_2 fixation, increased nitrogen accumulation of the plant, and seed yield compared to those grown with 1-m supports and unsupported plants. Nitrogenase activities increased and reached a peak at the onset of flowering i.e. 70 days of growth (D_{70}) but declined during the pod formation stage in plants grown with a support system. The descending order of total plant nitrogen accumulation at D_{140} was: plants with 2-m supports (6.30 g N plant^{-1}) > those with 1-m supports (4.06 g N plant^{-1}) > control plants (2.10 g N plant^{-1}) (Figure 11) (Motior *et al.*, 1998). The data clearly demonstrated that supported plants contributed significantly to higher N_2 fixation and leaf N at the vegetative stage; seed N was also significantly higher than in unsupported plants.

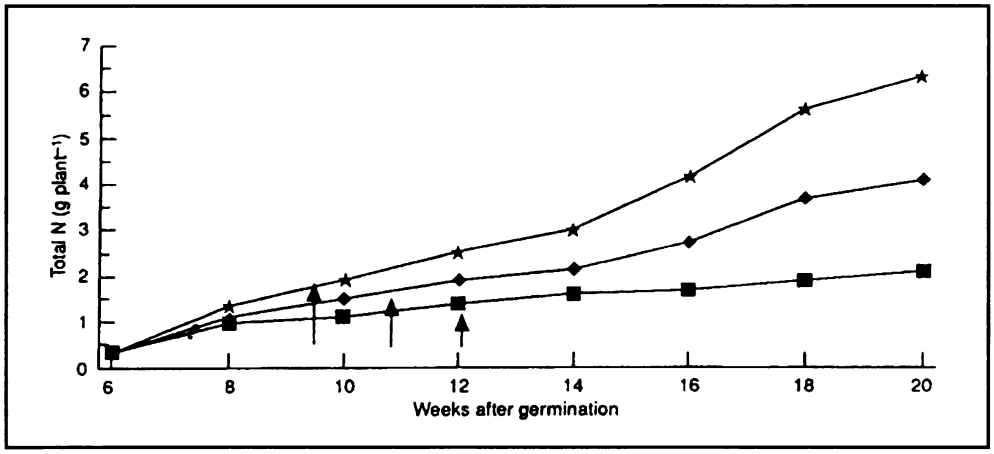

Figure 11. Total nitrogen (N) accumulation of winged bean grown on support systems of varying heights (——■—— unsupported control; ——◆—— support system height of 1 m; ——★—— support system height of 2 m). Arrow indicate time of flowering; s.e. at 6 weeks after germination (WAG) = 0.01; s.e. at 8 WAG = 0.11; s.e. at 10 WAG = 0.05; s.e. at 12 WAG = 0.09; s.e. at 14 WAG =0.14; s.e. at 16 WAG = 0.07; s.e. at 18 WAG = 0.34; s.e. at 20 WAG = 0.20

Soybean

Vegetable soybean is a highly nutritious and highly priced economic crop in Malaysia providing a possible net earning of RM11,960 ha^{-1} in 70 days. Local agronomic studies on vegetable soybean is minimal but reports on grain soybean using saturated soil culture technique have shown that it was possible to raise the grain yield of inoculated soybean from 1.5 to 3.0 t ha^{-1} (Leong *et al.*, 1991). In vegetable soybean, the short vegetative growth phase to harvest (70 days) makes it an ideal intercrop between the two rain-fed and irrigated paddy seasons. Exploiting its N_2-fixing potential through *Bradyrhizobium* inoculation would not only reduce the input cost but also raise its value as a cash crop.

Inoculation studies showed that *Bradyrhizobium* TAL 102 increased nodulation, N_2 fixation and seed yield of soybean cv. Palmetto grown on Bungor series soil.

Physical factors such as water stress can reduce nodulation and effectiveness of N_2 fixation. Saturated soil culture (SSC) system, which maintains moisture at saturation point 25-30 cm from the soil surface, is a planting system suitable for soybean. Under SSC, *Photorhizobium* MKAa2 can form nodules on soybean, consequently increasing the supply of carbohydrate required for increased nitrogen fixation while conserving the plant photosynthate for increased grain yield.

Bradyrhizobium inoculation increased nodulation, growth and pod yield of vegetable soybean grown in MADA and KADA padi soils (Figure 12) (Shamsuddin *et al.*, 2003b). Nodule dry weight were significantly higher in plants treated with .UPMR 48 than TAL 102; UPMR 48 formed more lateral nodules while TAL 102 encouraged more crown nodules (Figure 13). The latter phenomenon demonstrates the competitive ability of the local strain (UPMR48) to move further into the soil environment and colonize the lateral roots than the introduced TAL 102 strain which could colonize only the main roots around the geminated seeds.

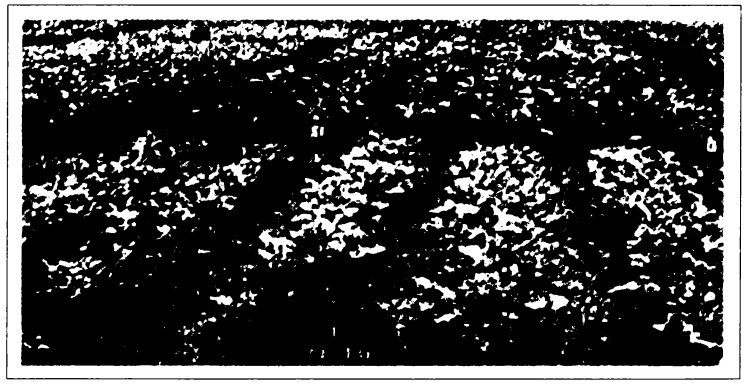

Figure 12. Field experiment in MADA. Fully grown pod-filling vegetable soybean inoculated with UPMR 48 and TAL 102 (background), uninoculated control (foreground)

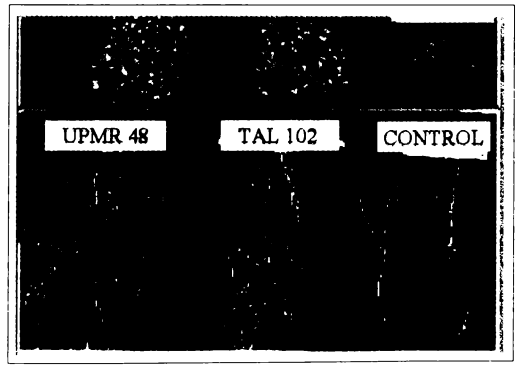

Figure 13. Nodulation of vegetable soybean inoculated with *Bradyrhizobium* UPMR 48, TAL 102 and uninoculated control

ASSOCIATIVE PARTNERSHIPS BETWEEN RHIZOBACTERIA AND NON-LEGUMES

Sweetpotato

Sweetpotato [*Ipomoea batatas* (L.) Lam.], the second most important root crop in Malaysia after cassava, is grown mainly on marginal soils such as peat and sandy tin-tailing. The sandy tin-tailing soil, with more than 90% sand, requires more fertilizer input. Thus, the cost of crop production on such soil can be very expensive due to the additional fertilizer cost. The use of diazotrophs as bio-fertilizer can substantially reduce fertilizer application and consequently the production cost.

A field study using sweetpotato variety UPMSS5 on sandy tin tailing soil was successfully undertaken using *Azospirillum brasilense* Sp7 and two locally isolated strains UPMB12 and UPMB14. The local strain UPMB14 was more compatible with the variety UPMSS5. Plants treated with this strain together with one-third (1/3) of the recommended total inorganic nitrogen at one week after planting produced 27.2 t ha^{-1} of root yield or about 74% more than the control (15.6 t ha^{-1}) (Figure 14) (Saad *et al.*, 1999). This study evidently showed that *Azospirillum* and the locally isolated PGPR strain are potential bio-fertilizers for sweetpotato grown on sandy soils. However, more compatible PGPR strains need to be selected for specific sweetpotato varieties. Furthermore, the initial low rate of inorganic-N supplied is adequate to provide the N requirement at the early stage of crop growth before the plant can derive the beneficial effect of the plant-rhizobacteria association.

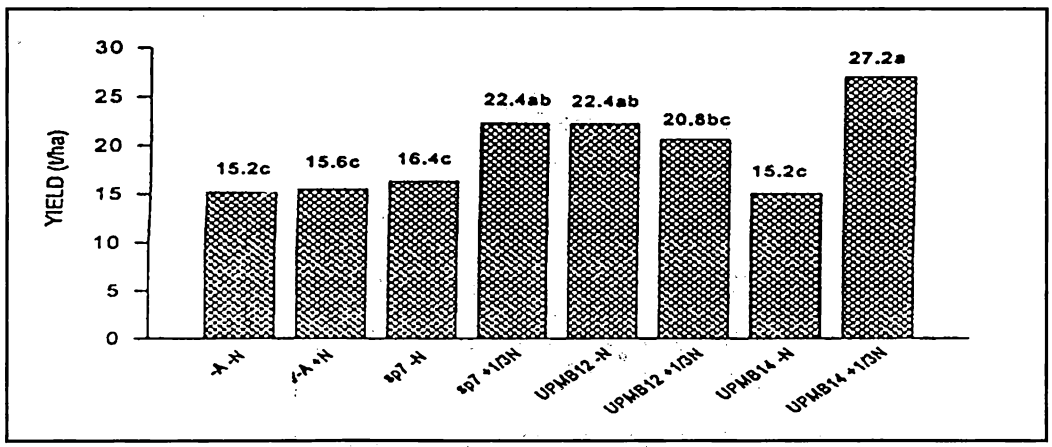

Figure 14. Fresh root yield (t ha-1) of sweetpotato variety UPMSS5 grown on sandy tin-tailing soil treated with various combinations of *Azospirillum* strains and N fertilizer. The symbols (-N)=no N fertilizer applied, (+N)= 100 kg N ha-1 applied in split application at 1 and 8 weeks after planting, and (1/3 N)= 33 kg N ha-1 (or 1/3 of 100 kg N ha-1) given at one week after planting.

Means with the same letter are not significantly different at P=0.05 according to Duncan's Multiple Range Test

Banana

Plant growth promoting rhizobacteria (PGPR) have a great impact on plant growth and development through direct or indirect mechanisms. Direct promotion of plant growth by PGPR is generally based on the ability of the bacteria to synthesize and facilitate plant nutrient uptake from the soil environment and the indirect mechanism involves the antagonistic activity of PGPR against phytopathogens.

Bacillus sphaericus UPMB10 is a locally isolated PGPR reported to have beneficial effects in stimulating growth and yield of many agricultural crops. *Azosprillium* inoculation benefits the host plant by promoting root development and nutrient uptake (Okon and Kapulnik, 1986). It has been suggested that these bacteria promote growth by producing growth promoting substances (Tien *et al.*, 1979) and consequently mineral and ionic uptake (NO_3^-, K^+, $H_2PO_4^-$) (Lin *et al.*, 1983) by the roots. Inoculation with a variety of *Azosprillum* strains and PGPR has increased root surface area and grain yield in gramineous crops such as maize, wheat, rice and sorghum (Murty and Ladha, 1988; Kapulnik *et al.*, 1985).

The findings from a series of hydroponics experiments demonstrated that inoculation of PGPR strains (Sp7 and UPMB 10) with minimal fertilizer-N supply are effective as a bioenhancer and biofertilizer to fix N_2 and increase plant growth, nutrient uptake, yield and fruit qualities of bananas (Mia *et al.*, 2001).

In a preliminary study, the effects of inoculation with two PGPR strains, Sp7 (*Azospirillum brasilense*) and UPMB10 (*Bacillus sphaericus* UPMB10), on plant growth and N accumulation of banana plantlets were observed under N-free hydroponics condition for 45 days. A marked increase in root growth namely length (33-44%), volume (76-168%) and mass (137-141%) were recorded due to the PGPR inoculation besides a higher shoot growth (123-202%) and N yield (94-144%) (Mia *et al.*, 2001).

An *in vitro* electron microscopy study was conducted to observe the pattern of colonization of PGPR strains Sp7 and UPMB10 on roots of banana plantlets (Figure 15) (Mia *et al.*, 2000; 2001). This study demonstrated that both strains could effectively colonize the banana roots and more bacterial cells were present in the root hair proliferation zone.

Figure 15. Colonization of banana roots by *B. sphaericus* UPMB 10

Two experiments with small (4L) and larger (1000 L) plastic containers showed that inoculation together with 33% of the total N requirement improved the bioenhancing activity by increasing root and shoot growth, and photosynthetic rate (25%) (Mia *et al.*, 2001). The PGPR inoculation with 33% fertilizer-N increased the N concentration in the pseudostem, and Ca concentration in root, corm and pulp. The total accumulation of nutrients was heavily influenced by PGPR inoculation due to enhanced root proliferation. PGPR inoculation also greatly increased the bunch yield (35-51%) and physical fruit attributes namely finger weight (62-65%), finger length (22-24%), grade and pulp/peel ratio. Plants also flowered three weeks earlier in PGPR-inoculated plants.

The N_2-fixing capacity studies with PGPR strains Sp7 and UPMB10 in association with banana roots by acetylene reduction assay (ARA) and ^{15}N isotopic dilution technique conclusively showed that roots of PGPR-inoculated plants produced higher ARA activities (129 nmole $plant^{-1}$ $hour^{-1}$). Inoculated plants together with the least fertilizer-N supply (3.2 ppm, 2.13% of the total plant N requirement) produced the highest amount of nitrogen derived from atmosphere (Ndfa; 37-39%) while those with higher inorganic-N fertilizer (50 ppm, 33% of the total N requirement) showed the lowest Ndfa (5%) (Mia *et al.*, 2001).

The PGPR has been known to decrease or prevent some of the deleterious effects of phytopathogenic organisms (usually fungus) by one or several mechanisms such as production of antibiotics and antifungal metabolites or enzymes that could lyse the fungal cell wall (Bashan and Holguin, 1997). Anti-fungal abilities of these beneficial microbes that were discovered in the 1930s, have been used extensively for plant disease control. However, they are only now beginning to be used commercially. It is believed that these advantages will lead to its potential as a biocontrol agent for *Fusarium* wilt of banana which has become one of the major banana diseases in Malaysia and other banana producing countries in Southeast Asia. This is largely due to the new aggressive Race 4 of the fungus (FOCR4) which is insensitive to most fungicide. *Bacillus sphaericus* UPMB10 has been observed to inhibit the growth of *Fusarium oxysporum* f.sp *cubense* (Foc) mycelium by 65-70% in culture medium using a dual culture test (Shamsuddin *et al.*, 2003a). Malformation of fungal hyphae by vacuole formation, swelling and thickening of the hyphal strands occurred with the presence of *B. sphaericus* UPMB10. Normal hyphal walls were smooth with no swellings or vacuolation (Figure 16).

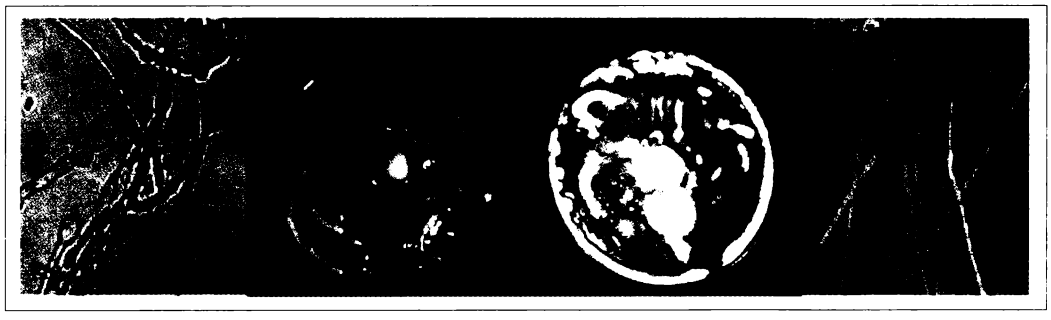

Figure 16. Malformed fungal hyphae cultured with *B. sphaericus* UPMB10 (left) and normal hyphae (right) of *Fusarium oxysporum* f.sp. *cubense*

Meanwhile, less germination of conidia occurred in the presence of UPMB10. A glasshouse study showed that inoculation with *B. sphaericus* UPMB10 increased plant growth and nutrient uptake and also reduced the disease severity as observed through foliar symptoms and internal pseudostem infection of Foc (Figure 17) (Illani *et al.*, 2004). These results suggest that *Bacillus sphaericus* UPMB10 has the potential to be used as a biocontrol agent against Foc.

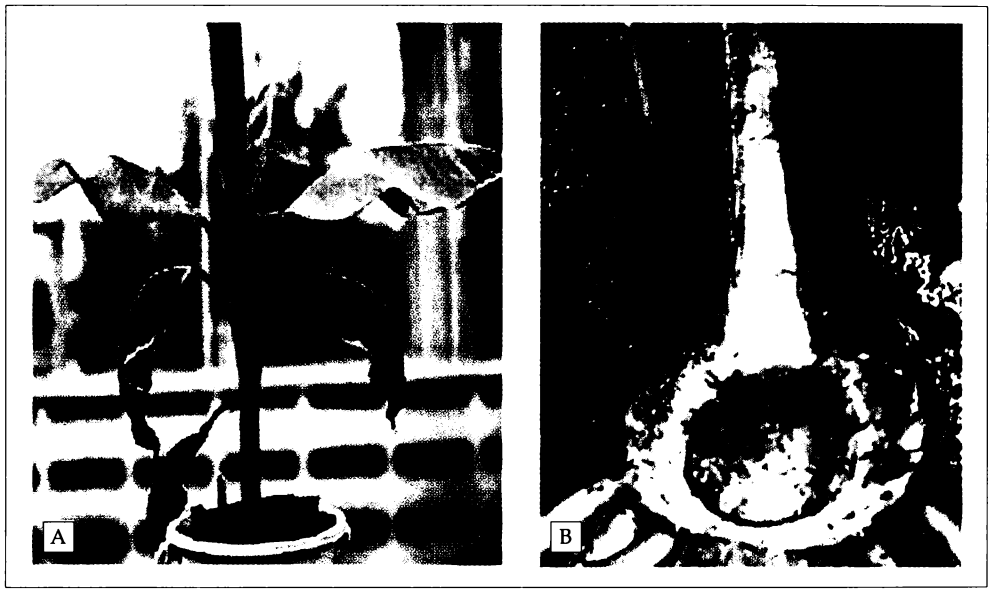

Figure 17. Foliar symptoms (A) and internal pseudostem (B) infection of *Fusarium oxysporum* f.sp *cubense*

Oil Palm

Nitrogen fertilizer is the most expensive management input in the oil palm industry. At a recommended rate of 0.5 to 1.0 kg N palm/year (148 palms/ha) and with the urea price at RM 760/tonne, total nitrogen fertilizer cost to the industry is estimated to be RM 611 million/year. Positive association of the N_2-fixing PGPR with various non-leguminous crops has reinforced the importance of biological nitrogen fixation on plant growth.

The use of N_2-fixing PGPR (e.g. *Azospirillum* spp. and *Bacillus* spp.) as a bio-fertilizer can reduce the application of nitrogenous fertilizer and consequently lower the production cost of this crop. These were recently established through our laboratory and glasshouse studies using ^{15}N isotope dilution technique as a tool to estimate N_2-fixation in oil palm seedlings. Laboratory experiment indicates that *Azospirillum* spp. (Sp7) could contribute up to 66% of the host plant N requirement (% Ndfa), while locally isolated *Bacillus* spp. (UPMB 13) recorded up to 55% Ndfa after 56 days of inoculation (D_{56}) (Table 8). The inoculation (especially Sp 7 and UPMB 13) also caused a significant increase in total N and

higher leaf chlorophyll content of the host plant (Table 9). The PGPR tested had also enhanced primary root numbers and length (Figure 18) (Amir *et al.*, 2002). A subsequent glasshouse experiment also demonstrated that inoculation of the rhizobacteria could contribute up to 20-50% of the total nitrogen requirement of the host plant through N_2 fixation process, further reaffirming the earlier laboratory finding. In addition, the inoculation process had also stimulated accumulation of nutrient and plant growth (tops and roots) comparable to the control with full inorganic nitrogen (N_i) fertilization after 390 days of growth (Table 10) (Amir *et al.*, 2003). However, this improvement in plant growth was less than the control with complete N_i fertilization after an intentionally prolonged nursery phase, D_{390}. A duplicated inoculation experiment (without application of ^{15}N isotope dilution technique) as above undertaken in the field nursery station, FELDA Bukit Mendi, Pahang showed positive response in enhancing higher concentration and accumulation of N, P and K for the host plants especially at D_{130}, enhanced root growth (root dry weight, volume, primary root number, and R/S ratio) until end of the growth stages (D_{390}) and top growth (total dry matter, top dry weight and chlorophyll content)(until D_{260}) of the host plants.

The above findings provided evidence that *Azospirillum* and locally isolated *Bacillus* spp. are potentially effective biofertilizers and bioenhancers for sustainable oil palm seedling production. However, more frequent inoculation and higher inoculum size will be required to maintain the inoculum population in the soil especially during a prolonged growth phase of the host plants (D_{390}) under an extended nursery condition. The study warrant further field research to categorically demonstrate the biofertilizer effect of PGPR on oil palm yields.

Table 8. N concentration and content, percentage of ^{15}N atom excess (a.e.), N_2 derived from atmosphere (% Ndfa) and N_2 fixed (mg N plant^{-1}) of inoculated oil palm plantlets at D_{56}

Treatments	N (%)	N content (g plant^{-1})	%^{15}N.a.e.	% Ndfa**	N_2 fixed** (mg N plant^{-1})
Sp 7 k (control)	3.19b	5.49c	3.59a		
+N_i (control)	4.81a	10.92a	0.319d		
Sp 7	3.38b	6.62b	1.218c	66.07a	4.38a
CCM 3863	2.98b	5.69bc	2.094b	41.67b	2.37b
UPMB 10	3.40b	5.98bc	1.918bc	46.57b	2.79ab
UPMB 13	3.36b	6.45b	1.631bc	54.56ab	3.52ab

Means with the same letters are not statistically significant at 5% level
** Estimate based on Sp 7 k reference plants

Table 9. Effects of rhizobacteria inoculation on plant growth and development of oil palm plantlets at D_{56}

Treatments	Chlorophyll content	Primary root no.	Secondary root no.	Primary root length	Top dry wt. (g)
Sp 7 k (control)	0.36b	2b	45b	12.8b	172a
$+N_i$ (control)	0.45a	3ab	86a	14.7b	227a
Sp 7	0.45a	3ab	76a	13.7b	196a
CCM 3863	0.45a	4a	75a	20.8a	191a
UPMB 10	0.46a	3ab	79a	14.3b	176a
UPMB 13	0.46a	3ab	50b	13.8b	192a

Means with the same letters are not statistically significant at 5% level

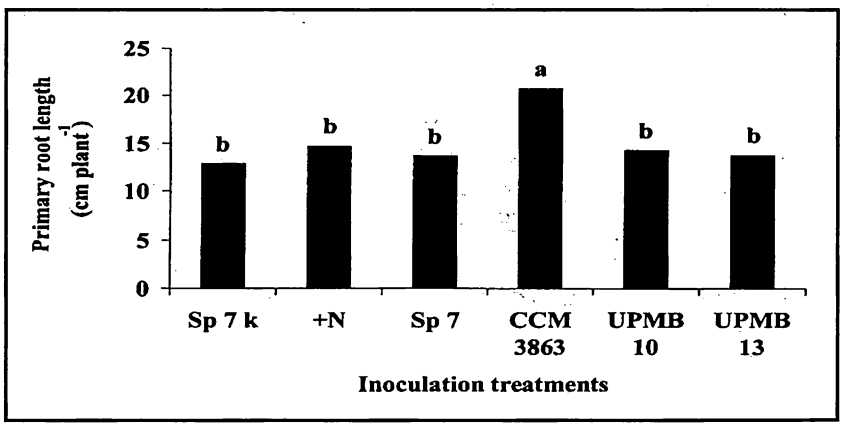

Figure 18. Primary root length (cm plant^{-1}) of inoculated oil palm plantlets after 56 days of inoculation

Means with the same letters are not statistically significant at 5% level

Table 10. Effects of rhizobacteria inoculation on plant growth and development of oil palm seedlings at D_{390} in Selangor series soil.

Treatments	Root dry wt. (g)	Root vol. (cm^3)	Top dry wt. (g)	Chlorophyll content
Sp 7 k (nss)	68.6bc	325b	112b	0.34c
Sp 7 k $+N_i$	55.1c	335b	163a	0.57a
Sp 7	89.1a	436a	117b	0.44b
CCM 3863	80.3ab	388ab	118b	0.44b
UPMB 10	93.7a	460a	114b	0.41b
UPMB 13	65.2bc	380ab	118b	0.40b

Means with the same letters are not statistically significant at 5% level

INOCULUM PRODUCTION

The commercial production of any legume and non-legume inoculant involves the integration of physical, chemical and biological parameters leading to both high target bacterial population and longer shelf life. To develop a high quality product, the two main components of a bacterial inoculant, namely processing of the raw material and bacteriological requirements, must be addressed.

Carrier Component

In our research on inoculum production, ground oil palm frond (GOPF) is used as an inoculum carrier and has yielded promising results. The GOPF carrier supported growth of the locally isolated *Bacillus sphaericus* UPMB 10 at 10^9 cfu/g for a minimum of six months (Table 11) but it only lasted for two months for *Azospirillum* Sp 7. In the case of rhizobia, coir-dust seemed to be a more suitable carrier than GOPF. Oil palm fronds (OPF) are one of the most abundant waste materials (approximately 24 million tonnes/year) from oil palm plantations (Islam, 1999). Pruned OPF are commonly arranged along oil palm inter-rows to be naturally decomposed. The nutrients released would be recycled to the growing palms. OPF with their high vitamin E content has previously been tested as animal feed (Ishida and Abu Hassan, 1997). Based on its abundance, ready availability and inherent beneficial characteristics, GOPF is most suitable to be used as an inoculant carrier. Furthermore, GOPF display other desirable characteristics of an inoculum carrier, which are: high water holding capacity, chemical and physical uniformity, acceptable pH, non-toxic to bacteria, promotes bacterial cell growth and is environmentally sustainable.

Bacterial Inoculum Component

The local isolates in our collection, namely several bradyrhizobia and *Bacillus sphaericus* UPMB10 (from oil palm roots) have been presented to have N_2-fixing and plant growth-promoting abilities on several legumes (groundnut, winged bean and soybean) and non-legumes (sweetpotato, banana and oil palm), respectively and are potentially encouraging as PGPR inoculum for biofertilizer production.

Table 11. Survival of *Bacillus sphaericus* UPMB10 in different carriers, moisture and storage temperature conditions over six months.

Treatments	Growth (Log cfu/mL)						
	D_1	D_7	D_{14}	D_{21}	D_{28}	D_{56}	D_{161}
Ground oil palm fronds (GOPF)							
T1:20°C, 2.54	7.758	8.268	8.616	9.306	8.546	9.132	9.226
T2:20°C, 2.19	7.388	8.134	8.992	9.346	8.820	8.864	8.844
T3:30°C, 2.54	6.690	8.530	9.198	9.272	9.118	8.222	9.252
T4:30°C, 2.19	6.564	8.956	9.186	9.138	9.084	8.290	9.194
T5:40°C, 2.54	8.358	9.020	8.668	8.850	8.308	7.676	8.256
T6:40°C, 2.19	8.320	8.200	8.798	8.452	8.092	7.336	7.566
Coir-dust (CD)							
T7:20°C, 2.54	7.362	7.024	7.094	6.936	7.364	6.932	7.426
T8:20°C, 2.19	7.444	7.096	7.018	7.180	7.328	7.038	7.388
T9:30°C, 2.54	7.894	8.100	8.142	8.106	8.212	8.102	7.984
T10:30°C,2 .19	7.06	7.592	7.856	8.244	7.996	7.718	7.582
T11:40°C, 2.54	8.416	9.058	8.236	8.172	7.236	6.736	7.106
T12:40°C, 2.19	8.294	8.906	8.190	8.414	6.796	6.762	6.970
GOPF + 50% Compost							
T13:20°C, 2.54	7.556	8.580	9.210	9.238	9.276	9.284	8.536
T14:20°C, 2.19	7.356	8.393	9.106	9.330	9.322	9.374	9.558
T15:30°C, 2.54	7.178	8.292	9.382	9.342	9.422	9.374	9.008
T16:30°C, 2.19	7.530	8.370	9.478	9.416	9.434	9.436	9.304
T17:40°C, 2.54	7.796	8.320	9.460	9.502	9.478	9.530	9.434
T18:40°C, 2.19	7.586	8.442	9.414	9.372	9.494	9.408	8.978
GOPF + 25% Compost							
T19:20°C, 2.54	8.424	9.184	9.206	9.194	9.122	9.218	8.860
T20:20°C, 2.19	9.002	9.152	9.216	9.232	9.136	9.362	8.430
T21:30°C, 2.54	9.346	9.394	9.740	9.482	9.222	9.358	9.114
T22:30°C, 2.19	9.400	9.306	9.614	9.490	9.318	9.410	9.330
T23:40°C, 2.54	9.512	9.176	9.478	9.458	9.292	9.466	9.338
T24:40°C, 2.19	9.312	9.302	9.578	9.458	9.278	9.428	8.796

The UPMB10 isolate was studied extensively, and systematically as it showed promising results as an associative N_2-fixing diazotroph with the potential of exploiting it in commercial biofertilizer production. It was identified by 16S rRNA analysis to be *Bacillus sphaericus* (Rene Bally, Universite Claude Bernard Lyon 1. UMR-CNRS 5557, Laboratoire d'Ecologie Microbienne, Villeurnebanne Cedex, France). It was also characterized in our laboratory and another overseas laboratory for various novel characteristics for enhanced plant growth promoting properties (Table 12a and 12b).

Table 12a. Characterization of *Bacillus sphaericus* UPMB10 by Hartmann, A. and Kirchhof, G. GSF-Research Centre, Institute of Soil Ecology, P.O. Box-1129, Oberschleissheim, Germany.

Test/isolates	**UPMB 10**	***B. subtilis* UPMB 13**[a]
Growth in *Azospirillum* medium	+	+
Growth in NFB semisolid	Pellicle	Pellicle
Growth in LGI semisolid		
Growth on Congo Red medium	Light red, small colonies	Very light red, small colonies
BIOLOG GN (from *Azospirillum* medium)		M4 (0.174) & Gsf5 (0.177)
Hybridization: Alpha-proteobacteria (alf 1b, 16S: 20% FA)	+	+
Hybridization: Azospirilla (AZO 23S; 0% FA)		
Hybridization: Beta-proteobacteria (bet23S, 35% FA		
Hybridization: *Herbaspirillum seropedicae* (HS 23S: 0% FA)		
Hybridization: Gamma proteobacteria (gam 23S: 35% FA)		+
Hybridization: *Xanthomonas spp.* (183 93 16S; 40% FA)		
Interline-PCR fingerprint	Identical banding pattern with UPMB 11[a]	Different banding pattern
Box-PCR fingerprinting	Not defined	Different banding pattern
PCR-product with nif-specific primers	+	N. A.[b]

[a] UPMB11 and 13 are other locally isolated rhizobacteria from oil palm.
[b] N. A. = not available

Table 12b. Characteristics of *Bacillus sphaericus* UPMB10 tested at Soil Microbiology Laboratory, Faculty of Agriculture, UPM.

	UPMB10	***B. sphaericus* type strain**[c]
Rod (Figure 17a)		
Width, μm	0.2-0.4	0.6-1.0
Length, μm	2.0-5.0	1.5-5.0
Gram reaction (Figure 17b and c)	Gram positive or variable	Gram positive or variable
Easily stainable body attached to one side of spore (Figure 17d)		
Mobility	+	+
Acid from		
L-arabinose		
D-xylose		
D-mannitol		
D-glucose		
Anaerobic growth		+/-
Growth in 7% NaCl	d[d]	
Production of alkaline in V-P broth	+	+
Deamination of phenylalanine	d	
Citrate utilization		
Acid from starch		
Capable of N_2 fixation (ARA)	+ (20 nmol C_2H_2 hr^{-1} mL^{-1})	

[c] Adapted from Priest (1989) and Holt (1994)
[d] d = Reaction differ

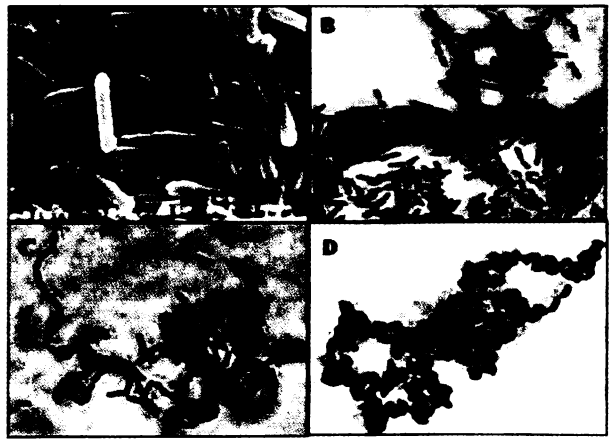

Figure 19. (a) UPMB10 morphology viewed under scanning electron microscopy (SEM); (b) UPMB10 cells stained Gram-positive (<18h); (c) Older UPMB10 cells stained Gram-negative (>24h); (D) UPMB10 endospores (stained with Malachite Green)

PCR-based DNA fingerprinting with random commercial primers of arbitrary sequence was used as a potential tool to distinguish and identify UPMB 10 (Figure 20) when applied as biofertilizer in the soil (Shamsuddin *et al.*, 2001). This method was effective in providing DNA polymorphic fingerprints unique to UPMB10 (Figure 21) among other *Bacillus* sp. However, the mechanism of colonization and the amount of inoculum needed for successful colonization and, consequently its beneficial effects on plant growth could not be elucidated. Thus, we embarked on a research project to biologically tag the UPMB10 strain with a unique genetic marker or reporter gene not present in soil microorganisms to detect *in vivo*, spatial and numerical colonization of the introduced inoculum. In our current research, the reporter gene, green fluorescent protein (*gfp*) was used to tag the UPMB10 strain. A chimaeric shuttle plasmid pSV101GFP was constructed and transformed into UPMB10. The UPMB10-GFP transformants yielded positive expression of green fluorescence under fluorescence microscopy. However, the signal detected was low and efforts are underway to improve the system.

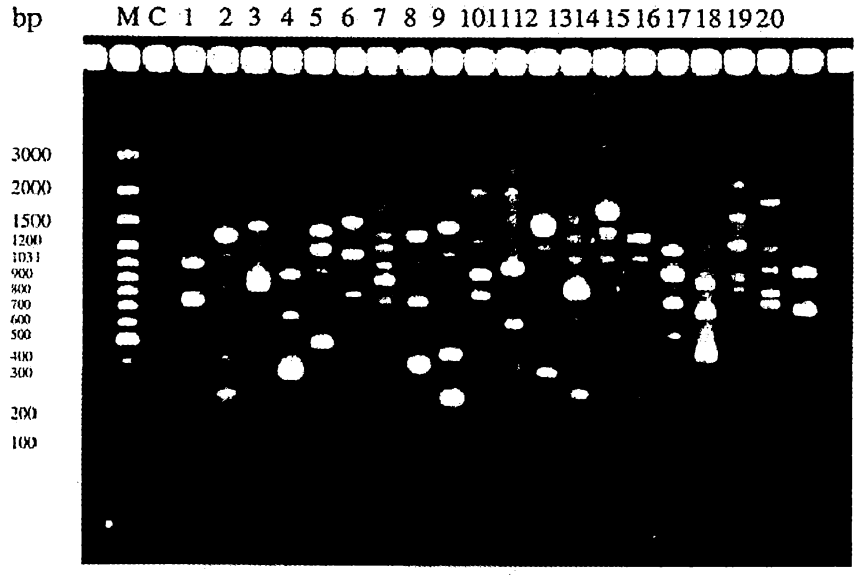

Figure 20. Twenty PCR primers (OPA-01 to 20) (Operon Technology, USA) were used to amplify DNA fingerprinting patterns from *B. sphaericus* UPMB10. M: 100bp ladder plus, C: Negative control

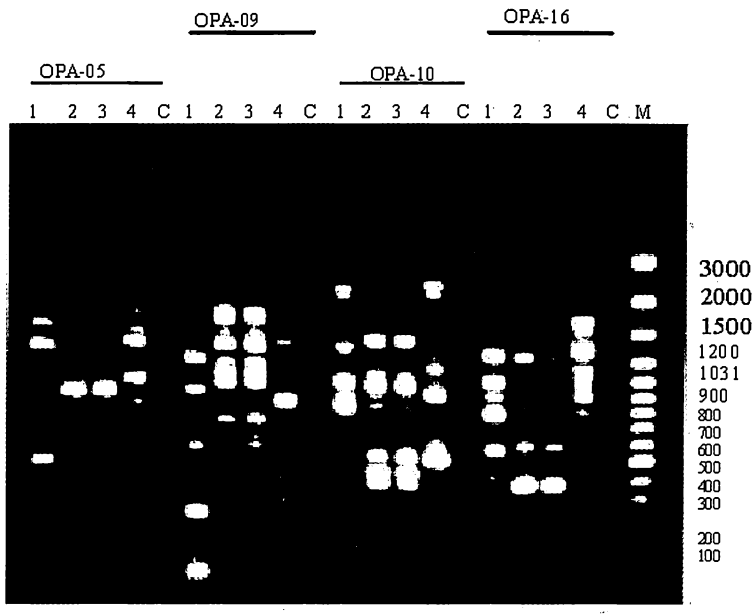

Figure 21. Four primers (OPA-05, 09, 10 and 16) produced unique banding patterns for UPMB10 when compared to other *B. sphaericus* strains. 1: UPMB10, 2: *B. sphaericus* ATCC 33203, 3: *B. sphaericus* ATCC 14577, 4: *B. sphaericus* ATCC 2362, M: 100bp ladder plus, C: negative control

Inoculant Preparation and Packaging

The preparation of a high quality inoculant is a process that requires optimization of relevant parameters at every step to ensure high and long survival rate of the bacterial inoculum. These parameters should be optimized for different genus of bacteria. Utilizing UPMB10 as the test strain, every possible parameter was tested, that is the inoculum growth (carbon and nitrogen source, pH, growth kinetics) in fermenter system, carriers (GOPF, coir-dust, compost), storage temperature (20-40°C), moisture potential (pF 2.19, 2.54) and storage time to yield maximum survival rates of the inoculum (Table 11).

Optimization of medium for cultivation of UPMB10 was achieved using 1.4 g L^{-1} of glycerol and 2.0 g L^{-1} of yeast extract with a cost of RM11.38 kg^{-1} cell and RM65.72 kg^{-1}, cell respectively. Optimal pH for growth was 6.0 to 8.0 while optimal temperature was 30°C at agitation speed of 600rpm and airflow rate of 0.5 vvm. Up to 10^{10} cfu mL^{-1} viable cells can be obtained with fed-batch fermentation. Fed-batch fermentation (specific growth rate, μ=0.4) produced four times more viable cells, three-fold more cell density and productivity compared to batch fermentation (Table 13).

Table 13. Growth performance of *B. sphaericus* UPMB10 using batch and fed-batch cultivation techniques

Kinetic values	Batch	Fed-batch (μ= 0.4)
Viable cell counts, X (cfu mL^{-1})	3.26×10^9	1.14×10^{10}
Cell density, X (g L^{-1})	1.62	5.27
Productivity, P (g L^{-1} h^{-1})	0.11	0.29

Various carrier materials were surveyed and a suitable carrier selected, prepared and tested (Table 11). The carrier material GOPF was air-dried and ground (<2mm) to increase the total surface area for bacterial cells. Then the GOPF was moistened prior to gamma irradiation at 50kGy for uniform moisture distribution. Sterile GOPF generally supports higher inoculum population and display much longer shelf life, a minimum of 6 months. Furthermore, the use of a sterile GOPF extensively reduces threats from possible contaminants. Sterile GOPF also lowers production cost through minimal use of broth culture to achieve the recommended population inoculum of 10^9 cfu g^{-1}. Irradiated two-layer polyethylene bags are used to prevent contamination during the transfer of broth culture to the GOPF inoculum substrate. Inoculated bags are incubated at room temperature for a minimum of two weeks and kneaded weekly to ensure a uniform distribution of the bacterial culture in the substrate. The bags are ready for field application or stored for later use within six months. For quality assurance the inoculum bags are randomly sampled to estimate the number of viable bacterial cells by plate counts using tryptic soy agar.

This study has demonstrated a new cost-effective and high quality GOPF-based inoculant production technique for rhizobacteria (Shamsuddin *et al.*, 2005) which has been tested to be effective in rhizobacteria-legume and non-legume associations.

CONCLUSION

In Malaysia, the rich pool of microflora and fauna provide enormous opportunities for the discovery, isolation and identification of more local rhizobacteria which can be beneficially exploited for sustainable agriculture.These rhizobacteria form smart and effective partnerships with legumes and non-legumes as biofertilizer, bioenhancer and/or biocontrol agents which contribute to cost saving and environmental-friendly and sustainable crop production.

Several of the plant-rhizobacterial associations presented in this lecture have unique abilities to overcome abiotic and biotic stresses to be competitive and productive under the adverse, acidic and humid tropical environment. In root nodulating legumes such as groundnut the acid sensitive root infection and nodule formation phase is easily overcome by a minimum application of 2 tonnes ha^{-1} Ground Magnesium Limestone (GML); the subsequent nodule development and function (N_2 fixation) processes are less sensitive and thus protected from soil acidity.

In the symbiotic N_2 fixing system of legumes, *Leucaena leucocephala*, a high N_2 fixing tree legume can effectively fix 240 kg N ha^{-1} $year^{-1}$ when incorporated with stylo and bracharia as a pasture legume. *Sesbania rostrata*, with its unique stem and root nodulating abilities, can fix 450 kg N/ha/yr and be used as a green manure. Vegetable soybean inoculated with a competitive local strain (*Bradyrhizobium japonicum* UPMR48) is highly suitable as an intercrop due to its short 70-days crop cycle. Winged bean cultivation with modified 2-m supports produced higher N_2 fixation and leaf-N accumulation. These are a few examples of smart partnerships in legume-rhizobacteria associations.

Smart partnerships involving non-legumes include the local isolate *Bacillus sphaericus* UPMB10, which could increase root development, plant nutrient uptake and yield of sweetpotato, banana and oil palm plantlets while saving 70% of total fertilizer-N cost. *B. sphaericus* UPMB10 is also a potential biocontrol rhizobacteria which can increase tolerance of banana towards *Fusarium oxysporum* f.sp.*cubense* (FOC) infection. These unique and versatile plant-rhizobacteria associations clearly demonstrate the smart partnerships potentially available in Malaysia's abundant natural resources.

The smart partnerships presented are further exploited for wider application and commercial production through the development of *B. sphaericus* UPMB10 as a cost effective and high quality inoculum utilizing local agricultural waste products as the carrier.

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