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SOIL MICROBIAL DIVERSITY AND ITS USE IN CROP PRODUCTION

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ABSTRACT

Soil is the outermost layer of the Earth's surface that provides anchorage to plants and most of their nutrients. The microorganisms in soil represent a very small fraction of this ecosystem. Nevertheless they perform a vital function in the maintenance of soil health and providing nutrients to plants in a sustainable manner. This paper reviews the species diversity of microorganisms and their distribution in soil, the general methods employed to study them and the roles they play in this ecosystem. It also briefly describes the research studies conducted by the authors and their teams to utilize beneficial microbes for environmentally benign crop production. In conclusion the paper suggests that the widespread use of the technologies developed utilizing natural microbial biodiversity could revolutionize agriculture to a low cost, eco-friendly activity with long term sustainability. Having noted the dearth of knowledge on soil biodiversity of Sri Lanka, it is recommended to establish a task force to prepare a comprehensive document of the diverse organisms present in Sri Lankan soils and to draw up an action plan for their conservation and sustainable utilization.

Key words: *soil microbes, Rhizobiology, biofertilizers, biofilm biofertilizers, eco-friendly agriculture.*

INTRODUCTION

Soil is the outermost layer of Earth's crust formed largely by the weathering of the underlying bedrock together with deposition and transfer through water and wind erosion. It is composed of the mineral fraction, water, air and the organic fraction (living and non-living) and functions mainly as a substrate for the anchorage of plants and provides most of their nutrients. The organic fraction of a normal soil is around 5% and of this less than 1% consists of the living component which represents soil biodiversity. However, this small component ensures soil health and plays a critical role in the maintenance of soil fertility that is essential for sustainable crop production. Within this living component the diversity extends from microscopic organisms through semi-macro species like earthworms, millipedes, centipedes, termites and other arthropods to macro types such as snails, amphibians, reptiles, rodents etc. This paper reviews the diversity among microorganisms, the roles they play in this ecosystem and how they can be used to improve and sustain soil fertility in an environmentally benign manner.

PRESENT STATUS

Compared to documentations on terrestrial above ground biodiversity, reports on soil biodiversity are sparse. There is no comprehensive document on the overall diversity of soil organisms of Sri Lanka. This National Symposium should therefore be regarded as the gateway to initiate a project to bring together different research groups working on soil biodiversity and appoint a task force to prepare a proposal and a plan. That document should not only review the present knowledge on the diversity of soil organisms in Sri Lanka but also prepare a strategy for their conservation and utilization for the improvement of soil fertility and develop technologies for their application for low cost, eco-friendly and sustainable agriculture.

SOIL MICROBIAL DIVERSITY

Soil microbial diversity refers to the different microorganisms in soil and their study comes under the discipline of Soil Microbiology. Soil microorganisms fall into two major categories: the *micro-fauna* that includes amoebae, protozoa, ciliates and microscopic nematodes and the *micro-flora* under which bacteria, fungi, actinobacteria, cyanobacteria and certain micro-algae are included. Species diversity among the micro-flora falls into both prokaryotic and eukaryotic groups.

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The prokaryotes are largely represented by bacteria that include archeobacteria, eubacteria and actinobacteria with a smaller representation of cyanobacteria. Among them the eubacteria are largest and the most diversely distributed group, but occasionally, as in the wetland rice fields, the cynaobacteria could also become a significant population.

The cyanobacteria are entirely aerobic and photo-autotrophic (photosynthetic) and hence are confined largely to the photic and sub-photic layers of the soil. Certain cyanobacteria fix atmospheric nitrogen and such species are therefore completely independent in their carbon and nitrogen nutrition. A few species live symbiotically with all the other major groups of plants. They exhibit a wide range of morphological diversity (Figure 1). For unicellular forms this diversity extends from single cells such as *Anacystis* and *Synechococcus*, loose colonies like *Microcystis*, compact colonies like *Gloeocapsa*, to colonies of definite shape such as *Merismopeida (Agmenellum)* and *Eucapsis*. The filamentous cyanobacteria are represented by unbranched, undifferentiated types like *Oscillatoria* and *Lyngbya*, differentiated filaments like *Nostoc* and *Ananbaena*, tapering filaments like *Calothrix* and *Gloeotrichia*, filaments with false branching like *Tolypothrix* and *Scytonema* and filaments with true branching like *Mastigocladus* (both erect and prostrate systems being uni-seriate), *Fristcherella* (prostrate system multi-seriate and erect system uni-seriate) and *Stigonema* (both systems multi-seriate).

In comparison the morphological diversity among the bacteria is very narrow, being confined to three basic shapes of cells *viz:* cocci (spherical), bacilli (oval or rod shaped) and spirillum (spiral). They exist mostly as unicells *e.g. Pseudomonas* and *Azotobacter*, but occasionally form colonies that are either filamentous *e.g. Streptococcus* and *Streptobacillus* or clumps of irregular shape *e.g. Staphylococcus* or with cells arranged in a cuboidal manner *e.g. Sarcina*. On the other hand bacteria exhibit a wider range of nutritional types (Figure 2) which range from photo-autotrophy, chemo-autotrophy and heterotrophy, associative, symbiotic to parasitic (facultative and obligate) forms. They also exist in soil and other environments as aerobes, micro-aerobes, facultative aerobes/anaerobes and obligate anaerobes.

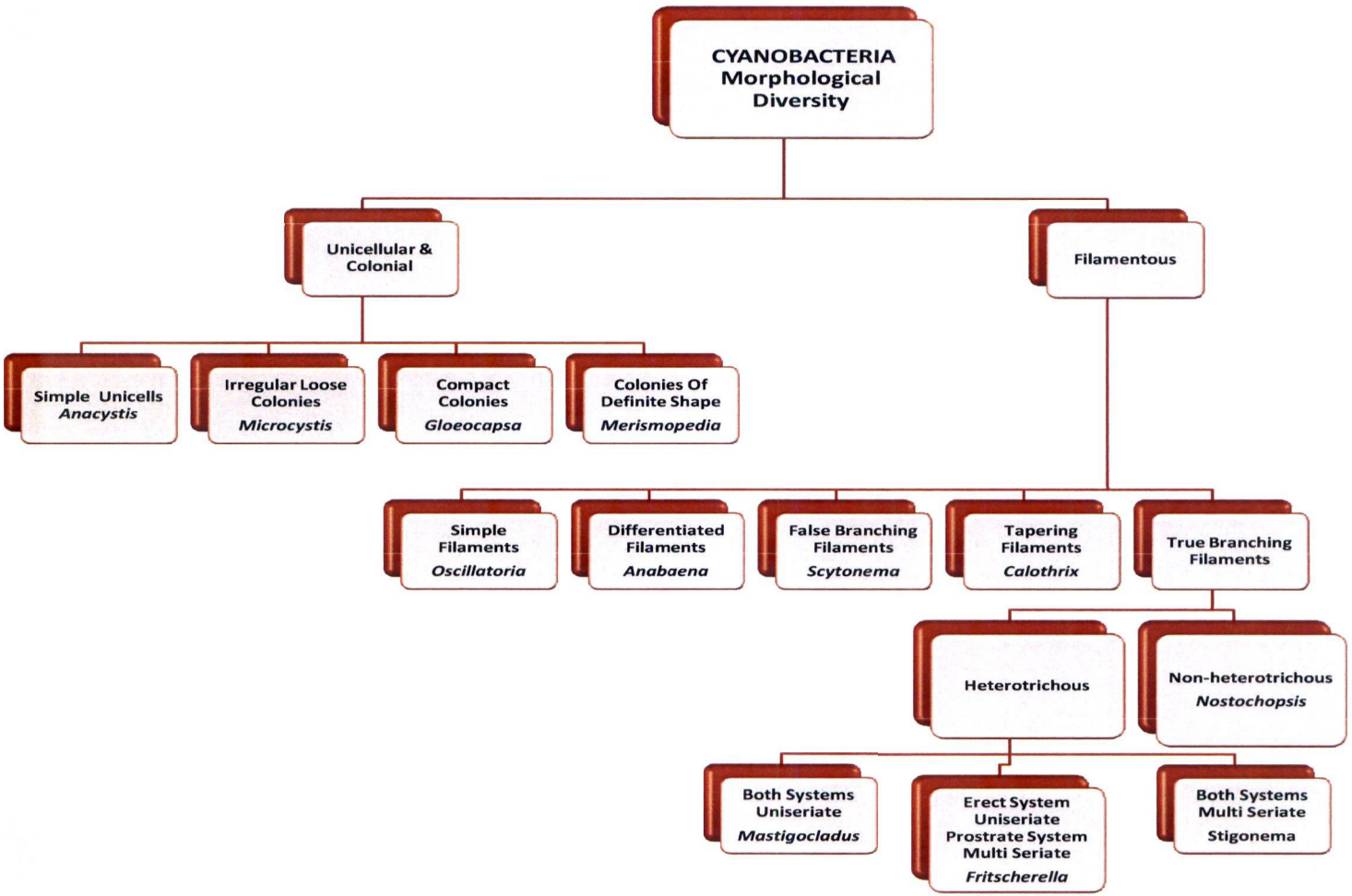

Fig. 1: Morphological diversity among the Cyanobacteria

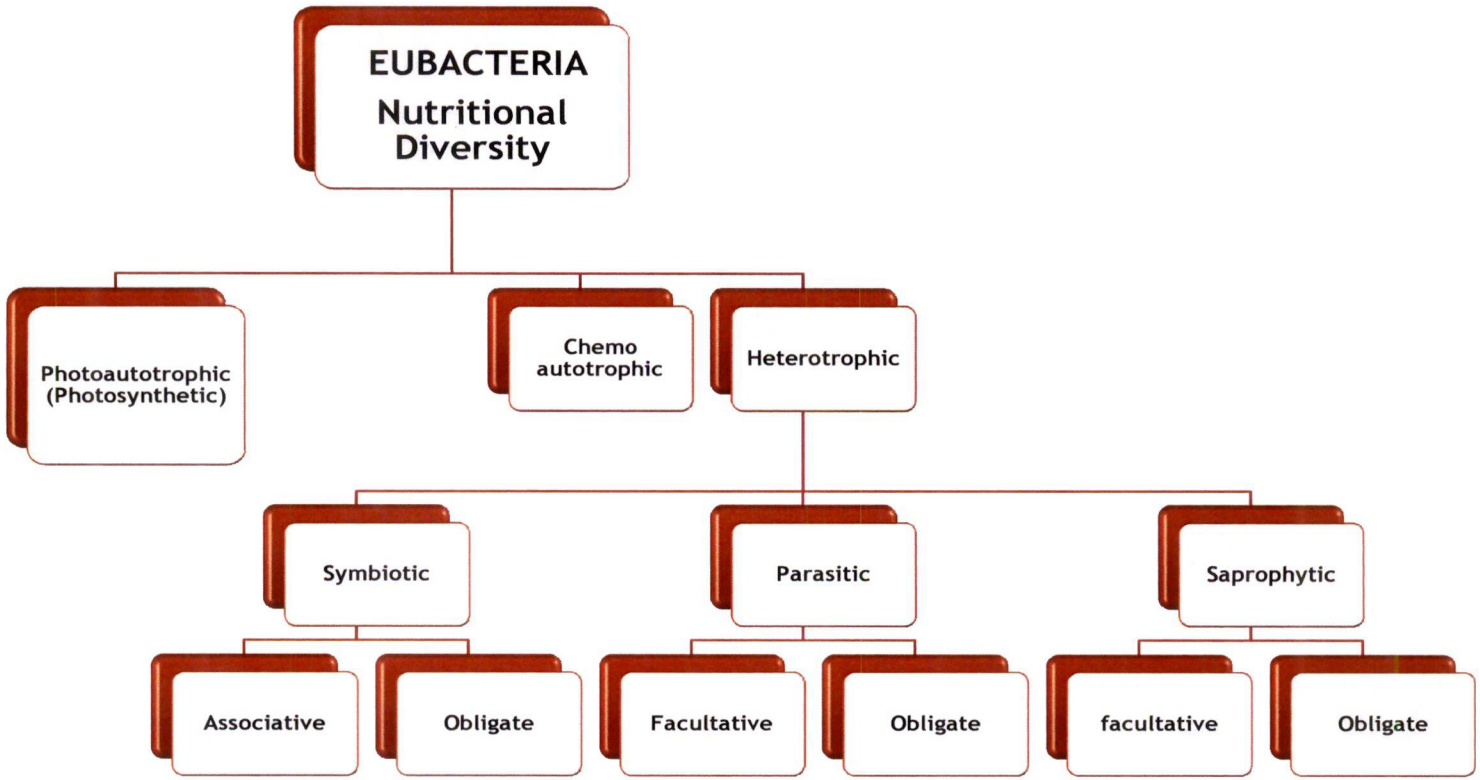

Fig. 2: Nutritional diversity among the Eubacteria

Therefore the distribution of bacteria in soil is far more diverse than all the other groups. While most of them occur in the surface and sub-surface layers of soil into which plant roots grow, they have been found even in deeper layers, rarely down to 10 m depths where they perennate as resistant spores and other reproductive units. However, being almost entirely heterotrophic bacterial distribution in any eco-system will depend upon the distribution of suitable substrates. Therefore bacterial species are never or rarely distributed in soil homogenously. Where there are suitable substrates like a rotting part of a dead organism or their excretory products the bacterial population could be billions of cells, but even a few centimeters away where there are no substrates it could be almost zero. Such a distribution (called a log normal distribution) has an implication in sampling for soil bacteria. In taking soil samples for estimating microbial populations it is necessary to take a large number of small samples (at least 20 or more) from a site, pool and mix them thoroughly and then take replicates from the pooled samples for evaluation, rather than take random replicates from the site itself.

The other major prokaryotic group the actinobacteria (formerly known as actinomycetes) is not as well represented in soil as the bacteria. They are however noteworthy inhabitants of certain niches like thermophilic compost heaps, decomposing recalcitrant substrates and certain species form symbiotic, nitrogen fixing, root nodules with non-leguminous plants like *Casuarina*. A number of their species also produce antibiotics.

Eukaryotic microorganisms in soil are fungi and allied organisms. While a few of them such as the slime molds are acellular and certain species of the Chytridiales and yeasts are unicellular, the vast majority of soil fungi are multicellular and filamentous and these filaments are called hyphae. Colonies of these hyphae exist as multi-filamentous mycelia. Though not so numerous as the bacteria fungal mycelia extends to great lengths and in extreme cases certain specialized rhizomorphs could grow to lengths of six meters or more. They are by far the highest biomass producers among all soil microorganisms. Among the soil fungi are found members of the Phycomycota, Ascomycota and Basidiomycota. Most of them live as free living heterotrophs decomposing organic matter mostly of plant origin. Some of them are closely associated with plant roots and a few are symbiotic forming mycorrhizae which could be either ecto (outside) or endo (within the root cells).

METHODS OF STUDY

Basic microbiological techniques can be applied for the study of soil microorganisms sometimes with certain modifications. As already mentioned it is necessary to pool a large number of sub-samples from a site to overcome the natural log normal distribution of substrate dependent heterotrophic bacteria. For the isolation and characterization of soil bacteria the common techniques of soil plating and dilution plating are often applied. However, the culture media used depend upon the aims of the study. If it is for the determination of total microbial populations, wide spectrum nutritional media like Nutrient Agar (NA) and Meat Extract Agar (MEA) are commonly used. If it is the study of a specialized group selective media are used. For example, to isolate nitrogen fixing bacteria nitrogen free culture media are used. For rhizobia Yeast Mannitol Agar (YMA) with Congo red is often used initially to distinguish *Rhizobia* from contaminants like *Agrobacterium*. The latter will absorb the red pigment while rhizobia will remain white and gelatinous. If it is necessary to detect coli form bacteria then specialized differential media such as Eosin-Methylene-Blue Agar (EMBA) may be employed. While *Escherichia coli* will appear as purple colonies with a greenish sheen on such media, *Aerobacter aerogenes* will grow as pink colonies with brown spots in the center. It is therefore necessary to employ several different media if a comprehensive picture of the diversity of soil bacteria is to be obtained. For the study of root associated bacteria direct plating of cut root pieces of a targeted plant is used. Such associative bacteria are often categorized as rhizosphere (the zone of root influence), rhizoplane (on the root surface) and histosphere (inside the root) organisms. Basic microbial techniques are slightly modified for such studies. After washing off the loose particles of soil and other debris attached to a collected sample, cut root pieces are bead beaten by shaking them with sterile glass beads contained inside a sterile container. This is expected to dislodge the bacterial cells growing in close proximity to the roots. The suspension so obtained is plated either directly or after dilution on suitable media to obtain rhizosphere organisms. The root pieces are then washed in several changes of sterile water and cut pieces are directly plated on to solidified media to culture rhizoplane organisms. Finally these root pieces are thoroughly surface sterilized using suitable sterilant solutions and macerated. Samples from the macerated material are

plated with or without dilution to obtain histosphere organisms. Initial isolations applying such different techniques usually result in the growth of several different colonies on a culture plate. These are separated by successive sub-culturing until pure cultures of different bacteria are obtained. Such axenic isolates are then subjected to a series of morphological, physiological and biochemical tests to enable their characterization and eventual identification.

For the study of photosynthetic cyanobacteria purely mineral media without any combined source of carbon, are used. In the case of nitrogen fixing cyanobacteria the media used are devoid of both combined carbon and nitrogen and the cultures are kept exposed to light either natural or artificial.

Several different methods can be adopted for the isolation of soil fungi depending upon the group under study. For water molds baiting with suitable substrate seeds is commonly employed. A few small seeds like bird seeds or lotus seeds are boiled in distilled water in a conical flask until the seeds just split and allowed to cool to room temperature. A few specks of the soil sample is added to this and left in the dark. The flask is observed daily and if the baiting has been successful fluffy colonies of water molds could be observed growing out of the split seeds. Sub-culturing from such colonies which are often highly contaminated with bacteria is a tedious process. The entire seed with its associated microbial growth is observed under either a dissecting microscope or the low power of a compound microscope on a sterile slide. Fungal hyphae from such a culture can be cut and either transferred to suitable liquid media or plated onto agar media for subsequent growth and isolation. If the fungus has produced spores they can be used for the sub-culture. The other common method is to do direct soil plating or dilution plating of soil suspensions. Very often antibiotics are incorporated into the culture media used for the initial isolation of fungi in order to minimize bacterial growth. Subsequent sub-culturing is then continued in antibiotic free media. Dilution plating is associated with the drawback that it selectively promotes the growth of spore forming and conidia producing fungi because these tiny propagules get easily dispersed in the suspension solution and eventually overgrow all the other mycelial types. Such methods therefore predominantly result in the isolation of *Aspergillus, Penicilium, Trichoderma, Ascogonium* and similar species. Among the different groups of fungi, Basidomycetes are the most difficult to isolate and culture and requires special media and techniques. To get an accurate view of soil fungi sterile slides are either buried in targeted locations or soil samples are sprinkled on to sterile slides and kept under a moist chamber to encourage the initial growth of mycelia. Certain soil substrates such as animal dung, leaf litter, cut pieces of decomposing plant parts, fruits and seeds are often incubated inside moist chambers and different fungi are observed and isolated as they appear in a retrogressive succession. In such a succession which passes through the stages of colonization, exploitation and exhaustion of the substrate the initial colonizers are the simple sugar fermenting fungi, followed by conidia producing Ascomycetes ending up with certain Basidiomycetes that are able to digest lignified tissues.

What is described above is a generalized, brief outline of the methods used in soil microbiology and the readers are advised to refer specific research papers or specialized text books for details of the methods used for the isolation and characterization specific soil microorganisms.

ROLE OF MICROBIAL BIODIVERSITY IN SOIL FERTILITY

In the broadest sense soil fertility is the overall ability of a soil to support and sustain healthy growth of plants and this is far more than simply providing nutrients for plant growth. While the micro-fauna is important primarily in relation to the natural ecological balance of a soil, it is the micro-flora that plays a crucial role in the sustenance of soil fertility. They are the organisms capable of decomposing organic matter and releasing its energy and nutrients to the soil. This ensures the cycling of nutrients and continuation of life in nature. Among the soil microbes are the nitrogen fixing forms and this is a process confined to prokaryotic microorganisms like certain eubacteria, cyanobacteria and actinobacteria. A number of them form symbiotic associations with roots of higher plants and in this manner converts a significant amount of inert atmospheric nitrogen to combined forms (Kulasooriya 2008). These ecosystem services of microbial decomposition, nitrogen fixation *etc.* and their contributions to nutrient cycling have been estimated to be equivalent to 33 billion US$ annually (Constanza *et al.*, 1997). The role of microorganisms in organic matter decomposition and nutrient availability in natural forests and cultivated lands in Sri Lanka was reported by

Ratnayake *et al.,* (2013). While organic matter production and its further breakdown is largely due to microorganisms, the semi-macro fauna like certain insects contribute by way of maceration of material such as plant leaf litter and twigs and earth worms contribute by burrowing through the organic matrix improving soil mixing and aeration and adding secretions that helps in soil aggregation. The combined effects of this biota converts the top layers of the soil into a moisture retaining, nutrient rich, healthy mass of spongy substrate through which roots can grow well, breathe and function actively. This is the status in a natural, undisturbed forest soil that exists in dynamic equilibrium and supports the long term sustenance of its vegetation.

The moment man interferes with this naturally balanced ecosystem to produce crops, its equilibrium breaks down and the system becomes unstable and unsustainable. With the clearance of the natural vegetation the soil gets exposed to both wet and dry erosion resulting in the loss of the top soil together with all its nutrients. To replenish such losses particularly for the cultivation of high yielding, annual crops chemical fertilizer applications become mandatory. Today chemical fertilizer application has become a routine practice in conventional crop production that most of our farmers identify soil fertility with fertilizer additions. Addition of fertilizers and other agro-chemicals to control weeds and pests destroys most the soil's natural micro-flora (Seneviratne 2009). To sustain crop productivity on such deteriorated soils application of chemical fertilizers is essential. However, such unhealthy soils cannot retain most of the chemical nutrients added to them and crop uptake is seldom beyond 30% of the applied amount. A large part of the balance 70% is lost and washed down leading to pollution that causes several environmentally related ailments like chronic kidney diseases (Jayatilleke *et al.,* 2013), blue baby syndrome and certain cancers (Jeyakumaran 2013).

While most of the soil organisms are either beneficial or harmless to plants and animals that occupy these lands, a few are pathogens and pests and under certain circumstances could cause devastating damages to crops, livestock and even humans. Preventive and destructive methods are necessary to control such infestations reaching epidemic proportions. A major thrust by all breeders of crops and domestic animals is to develop cultivars and breeds resistant to pests and pathogens. As the pathogenic organisms themselves undergo genetic changes, breeding programs have to continue but with the advent of modern techniques of molecular biology and genetic engineering the breeder's tasks have been made less cumbersome. On the other hand in undisturbed natural ecosystems disease causing organisms are under the bio-control of other organisms and predators. This is why epidemic diseases are seldom observed in natural vegetations. The other reason is the mixed vegetations in natural ecosystems compared to mono culture crop cultivations that allow the rapid spread of a disease to become an epidemic.

UTILIZATION OF SOIL MICROORGANISMS IN CROP PRODUCTION

One of the earliest beneficial effects of microorganisms to be recognized by man is biological nitrogen fixation. This process by which inert dinitrogen gas which constitutes 78% by volume of air is converted to combined nitrogen is reported to make a 60% contribution to the global nitrogen cycle (Kulasooriya 2008). The eubacteria, cyanobacteria and actinobacteria which are exclusively capable of this function both under free living and symbiotic existence are widely distributed in most soils. Rhizobia which are free living, gram negative, non-spore producing soil bacteria form nitrogen fixing nodules in symbiosis with roots of certain leguminous plants.

RHIZOBIAL INOCULANTS

Research studies on this symbiosis over several years led to the technology of isolating, screening and artificial culturing of selected rhizobia and producing rhizobial inoculants that could be used with targeted legume crops

A number of legumes commonly known as pulse crops such as *Glycine max* (soybean), *Vigna unguiculata* (cowpea), *Vigna radiata* (mung bean), *Vigna mungo* (black gram), *Arachys hypogea* (groundnut) and *Phaseolus vulgaris* (vegetable beans) represent an important component of our food that provide nutrition particularly to the poor sectors of our society. All these plant species are capable of nitrogen fixation. Rhizobial inoculants for legume food and pasture crops have been used quite successfully in many countries for several decades but in Sri Lanka this technology is not yet applied on a large scale and addition of

nitrogen fertilizers like urea is recommended for these crops. Having developed a low cost locally available carrier material (Seneviratne *et al.*, 1999) an inoculant for soybean (*Glycine max*) was field tested (Kulasooriya *et al.*, 2007) and found to be capable of replacing the entire requirement of urea fertilizer for this crop (Fig. 3) and produced a luxuriant crop in a farmer's field (Fig. 4). It is also evident from this photo that there is hardly any weed growth among the crop plants. This is partly because of the effective shading of weeds by the soybean plants and partly due to the nitrogen being available to the host plants through their root nodules unlike urea fertilizer which will provide nitrogen indiscriminately to all plants. The farmer will therefore not only save on fertilizer but could reduce the application of weedicides saving cost and labor and also minimize environmental pollution.

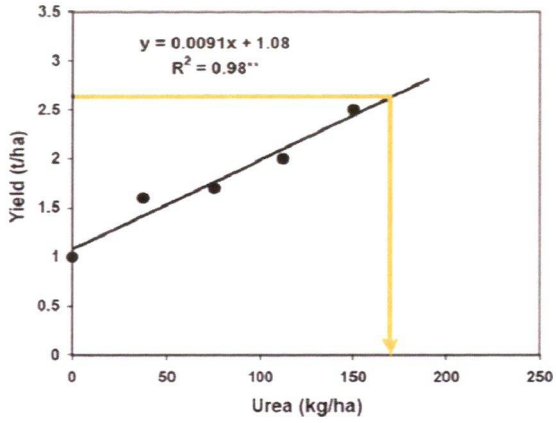

Fig. 3 : Yield response to urea fertilizer, yellow line shows response to the inoculants

This technology was successfully transferred to soybean cultivators from the year 2006 (Kulasooriya and Seneviratne 2012) and during the *Yala* season of 2013 nearly 10,000 acres of soybean was cultivated using this inoculant without the application of any urea fertilizer.

Fig. 4: An inoculated soybean field at *Madatugama*. Note the complete absence of weeds.

Table 1: Response of Mung bean (MI 5) to inoculation with rhizobia

Rhizobial strain	Seed yield kg/ha*	% yield increase over the control
M2	3726[a]	25
M11	3128[b]	16
M14	4370[a]	36
Tal420	4278[a]	34
Control	2806[b]	
CV% - 9.85 MSD – 1070		

* Values superscripted with the same letters are not significantly different at 5% probability level.

Following similar procedures rhizobial inoculants for mung bean, cowpea, groundnut and vegetable beans have been produced. These are being field evaluated in farmers' fields in different parts of the country. The results of a field trial done with mung bean (*Vigna radiata*) which shows that inoculation with different rhizobial strains has significantly increased grain yields over the control without inoculation are presented in Table 1.

Very encouraging results have also been obtained with inoculation of vegetable beans (*Phaseolus vulgaris*) in the *Hanguranketha* area (Fig. 5) and it is expected to release such inoculants for farmers' use in a few months.

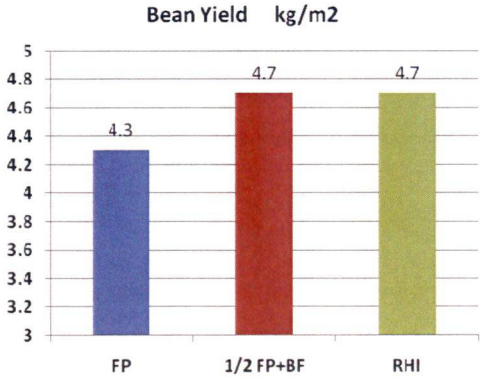

Fig. 5: Effect of inoculation on beans (*Phaseolus vulgaris*) FP: Farmers' practice, BF: Biofilm inoculant, RHI: Rhizobium inoculant

BIOFILMED-BIOFERTILIZER

Rhizobial inoculants are exclusive for legume crops and they provide only nitrogen to the host plants. There is still a need for a novel biofertilizer that could supply all the major NPK nutrients and also cater to a wider range of plants including cereals and plantation crops. Years of laboratory studies that investigated the preparation of inoculants using several different microorganisms including rhizobia, other nitrogen fixing bacteria, fungi and cyanobacteria resulted in the development of a novel group of biofertilizer called biofilmed-biofertilizer (BFBF) introduced to science for the first time by Seneviratne *et al.*, (2008a & b). In biofilm formation the component microorganisms not only associate themselves intimately and get embedded in a common polysaccharide matrix but the biofilms also develop properties unique to them.

BFBFs for tea were field tested during the past eight years with the participation of the Tea Research Institute of Sri Lanka. The results have clearly shown that N, P & K fertilizers applied during nursery, young and mature stages of tea can be reduced by 50% when combined with BFBFs (Fig. 6, Seneviratne *et al.*, 2011).

Fig. 6: A tea nursery and an estate applied with biofilmed biofertilizers.

Similarly BFBF inoculants for rice, maize and certain vegetable crops have confirmed the possibility of reducing chemical fertilizer additions by 50% or more, sometimes with increases in yields (Figures 7a, b, c and d).

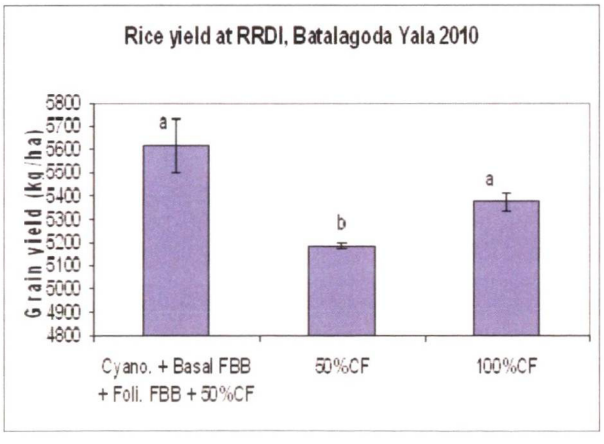

Fig. 7a: Effect on Rice Cyano: Cyanobacteria biofilm, FBB: Fungal-bacterial biofilm CF: Chemical Fertilizer NPK

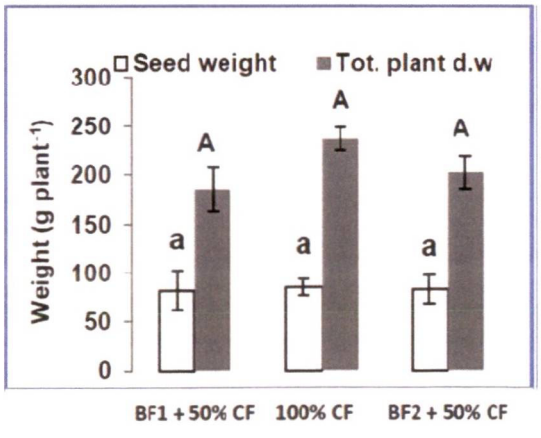

Fig. 7b: Effect on Maize BF1 & BF2: fungal-bacterial biofilms, CF: Chemical fertilizers NPK.

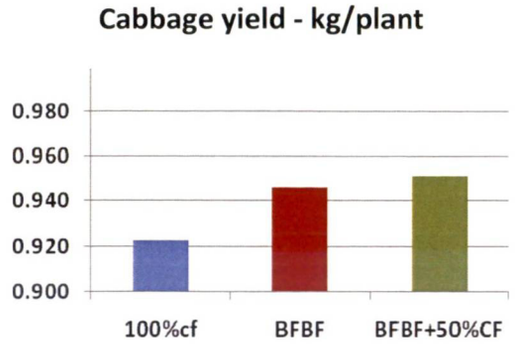

Fig .7c: Effect on Cabbage (BFBF: Biofilm Biofertilizer, CF: Chemical Fertilizer)

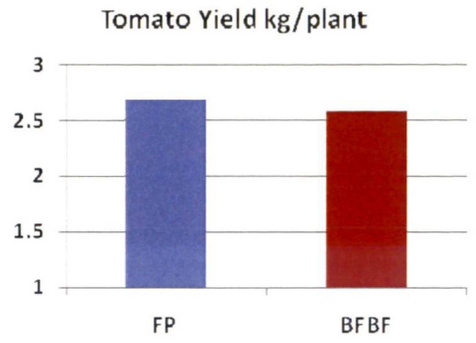

Fig .7d: Effect on Tomato (FP: Farmer's Practice, BFBF: Biofilm Biofertilizer)

These field trials have been conducted in several locations in Sri Lanka (Fig 8) in collaboration with the Rice Research and Development Institute of the Department of Agriculture for rice, Plenty Foods (Private) Limited at *Mahiyangana* for maize and Community Based Organizations at *Hanguranketha* for vegetables.

It has also been demonstrated that the soil application of biofertilizers in biofilm mode increases microbial diversity in agroecosystems through the breaking of dormancy of microbial seed banks (Seneviratne and Kulasooriya 2013). This contributes to strengthen the biodiversity–ecosystem functioning relationship, which leads to agro ecosystem sustainability.

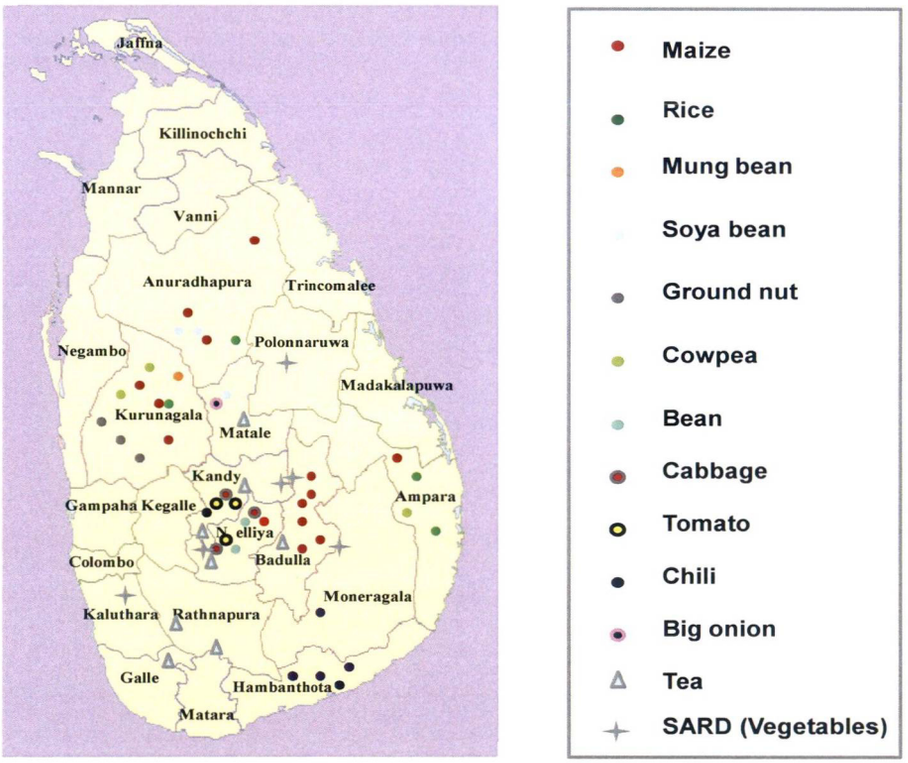

Fig. 8: Locations where field trials have been conducted on biofertilizers

In the preparation of BFBFs a large number of diverse bacteria, fungi and cyanobactria are initially isolated from the soil in which the targeted crop plants are grown. They are then subjected to a thorough scrutiny and the beneficial isolates are used to prepare biofilms. These biofilms are then screened for nitrogen fixation, production of growth promoting substances, secretion of beneficial products *etc.* and the most promising combinations are utilized to prepare BFBFs. After a series of laboratory and greenhouse testing the best selections are subjected to field trials in locations where the targeted crops are widely grown.

It is evident from these results that soil microbial biodiversity can be utilized for low cost, environmentally benign crop production. Adoption of these biofertilizer technologies can not only revolutionize agriculture in Sri Lanka as a non polluting, eco-friendly sustainable activity but also minimize environmentally related human and animal ailments significantly reducing the economic burden on the country's health services.

A WORD OF CAUTION ON BIOFERTILIZER PRODUCTS

At present there are a few private enterprises engaged in the preparation and marketing of microbial inoculants for use in Sri Lanka working in partnership with foreign countries. However there is hardly any regulatory mechanism to effectively monitor the quality of these products and the effects of their applications to our crops and soils. It is of paramount importance that Sri Lanka should have a firm control over the types of microorganisms that are being introduced to our soils and the effects of those introductions on our indigenous soil biodiversity. It is proposed to take immediate steps either to establish a regulatory body or to strengthen and expand the scope and powers of relevant authorities such as the Quarantine Department and/or the Sri Lanka Standards Institute to supervise the activities of these enterprises. It is also necessary to strengthen the capacity of these regulatory bodies with respect to qualified and competent personnel, equipment and other facilities for them to function in an effective manner.

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