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FAMILIAR MICROBIAL ORGANISMS AS BIO-FERTILIZERS

Mohan Agarwal, Biswa Mohan Purohit, Bhirab Chandra Patra, Durga Prasad Pattanaik, Bodroth Rabi Prasad & Thadepalli Venu Gopala Rao*

Department of Biotechnology, Gandhi Institute of Engineering and Technology, Gunupur-765022, Rayagada-District, Odisha, India.
*Corresponding Author

ABSTRACT

The general term for the 'bio-fertilizers that are microbial inoculants' is used for the mixture of microorganisms. In this short communication it is focused on the standardized results of laboratory to land supplementation source of "nitrite and nitrate" converting bacteria from atmospheric nitrogen, by using a mixture of bacterial organisms (include around 21 different strains of prokaryotic bacterial cells except common nitrogen converting bacteria). In addition, the results of another group of microorganisms e.g., PSB (Phosphate Soluble Bacteria, in a separate mixture include around 11 different strains) as a source of phosphorus supplementation to different crops and vegetable plants, which are growing on different types of lands. The soil which is selected here for the crop growth and production experiments is "crude red".

Key words: Bio-fertilizers, bacteria, nitrite, nitrate, phosphate.

1. INTRODUCTION

The bio-fertilizers which acquired here are for the extension work of constant and consistent observatory results of a small biotechnology industry (Sneha Biotech, Vijayawada-520010 in India). This laboratory is one of the stable bio-fertilizers producing industries in south part of India. The farmers buy these bio-fertilizers for the growth and production of crops such as legumes, paddy, turmeric, zinger, and vegetables etc. Because of industrial competition, the secrets of composition of these bio-fertilizers have not been disclosed to us. It is generally recognized that the microbial cultures accelerate the decomposition of organics residues and agricultural by-products through various metabolic processes, and gives healthy harvest of crops (Hargitai, 1993). It is also often perceived to be more expensive than the chemical fertilizers due to the lack of skills and modern technology to produce bio-fertilizer products from abundant wastes. Besides, the effect on the crops is slow, compared to chemical fertilizers. However, the two groups of bio-fertilizers which used in this communication are nothing but the mixtures of prokaryotic bacteria where the main organisms included examples like *Bacillus* and *Pseudomonas* genera (Das *et al.*, 2003). They may have the plant growth promoting rhizobacteria (PGPR) properties of *Bacillus subtilis* and *Pseudomonas aeruginosa* as representatives of their two genera, which have been conformed as per the simple tests of identification, by staining and by simple enzyme activities with the help of biochemical tests (Aneja, 2001). Generally, there are different bacteria that have been reported as PGPR belong to the following genera *Pseudomonas, Bacillus*, *Azospirillum, Agrobacterium, Azotobacter*, *Arthrobacter, Alcaligenes, Serratia, Rhizobium, Enterobacter, Burkholderia, Beijerinckia, Klebsiella, Clostridium, Vario-vovax, Xanthomonas,* and *Phyllobacterium* (Vessey, 2003). The microbial organisms that are expected in mixtures were obtained by Sneha Biotech from MTCC, Chandigarh, India and/or from DSZM, Germany. All the "general bio-fertilizers" which used here are for large number of crops like paddy, turmeric and legumes, and "P.S.B" which has been producing different vegetable crops is generally worth promising in the growth and production (Gaind & Gaur, 1991). One of the phases of work in this communication used is the "general bio-fertilizer" for legume plants such as green grams (*Vigna radiata*). The second phase of work used is "P.S.B." against vegetable plants (Adesemoye *et al*., 2008) that are beans (*Phaseolus vulgaris L* of family Fabaceae) for growth and production.

2. MATERIALS AND METHODS

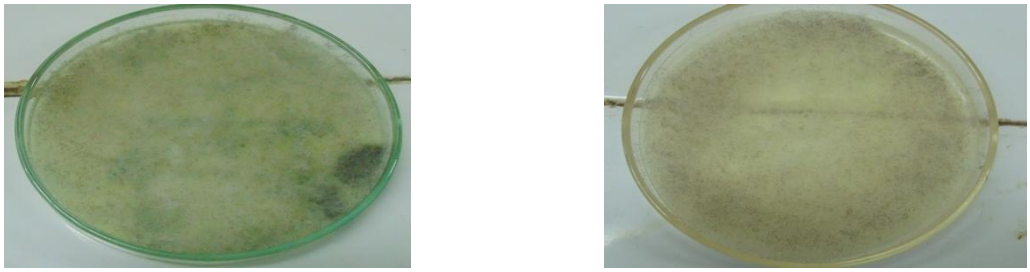
Cells (named as 'general bio-fertilizer' bacteria in this communication) from Petri plates (mother cultures grown on nutrient agar which is purchased from Hi-media, Mumbai, India) were inoculated into nutrient broth (containing Peptic digest of animal tissue, Sodium chloride, Beef extract, and Yeast extract which was purchased from Hi-media, Mumbai, India) culture media and supplemented by two different methods to the piece of land (1 square meter) after germinating the legume green grams (*V. radiata*) seeds. The methods selected for bio-fertilizer supplementation to land are (i) the stationary phase cultures of control (normal culture from broth grown) and experimental {random *EcoR1* cut DNA (method of DNA isolation is standard by using lysozyme incubation to remove the exiting cell wall of prokaryotic microbes and by adding chloroform and phenol followed by ethanol precipitation) fragments transformed cells in the nutrient broth} have mixed separately with the moderate wet soil in a plastic basin were supplemented to the 3 serial bits of one square meter land leaving 1 bit as control, and allowed the plants to grow up to 6 days after germination. After 10 days (ii) the second phase of culture, which is the *EcoRI* digested (*EcoRI* obtained from Helini Biomolecules, Chennai, India) genomes with the isolated as total DNA from the total number of mixed strains were transformed randomly (by simple 0.5% calcium chloride incubation method of linear transformation/uptake without a vector) to mid-log bacterial set of cultures and were grown up to stationary phase (Daniel, 2014). This stationary phase mixed CaCl2 transformed

culture again inoculated into fresh broth medium, as 'second generation culture' in large scale, and directly supplemented to the crude red land soil's third bit of 1 square meter, and allowed to grow the green grams (*V. radiata*) plants up to production. In the transformation method that is by the uptake of exogenous DNA by cells that alters the phenotype or genetic trait of a cell, the cells to uptake exogenous DNA they must first be made permeable so the DNA cut fragments (with higher and lower base pairs) can enter the cells. This state is referred to as competency (Daniel, 2014). In nature, some bacteria become competent due to environmental stresses. We can purposely cause the cells to be competent by treatment with chloride salts of cationic metals such as calcium, rubidium or magnesium and cold treatment (Chen & Dybnau, 2004). The changes in presence of cationic metals affect the structure and permeability of the cell wall and membrane so that restriction cut DNA (gene fragments) can pass through. However, this renders the cells very fragile and they must be treated carefully while in this state. The amount of cells transformed per 1 µg of DNA is called the transformation efficiency (Meddeb *et al.,* 2012). Too little DNA can result in low transformation efficiencies, but too much high concentrations of DNA also inhibit the transformation process. It is here maintained transformation efficiencies generally are in the range between 1 x 10^4 and 1 x 10^7 cells per µg of added DNA in the cases of experimental samples that is approximately of mid-log cells.

2.1 ***Phosphate Solubilizings Bacteria culture growth methods***

P.S.B used in these experimental studies is a mixture of microbes and is in the form of dried powder and is made as mother culture by inoculating the dried powder. It is accepted as around 11 strains are there in the mixture (uncharacterized different colonies with varied morphologies were carefully observed on agar plates) and it is also an industrial secret for the analysis and for its applications towards agriculture purpose for sake of farmers. Initially the mother culture was inoculated for the regular use in presence of tri calcium phosphate (0.5% w/v). They were also conformed as phosphate solubilizing bacteria by adding drops of sodium phosphate and potassium phosphate on the agar lawn of P.S.B as phosphorus sources for the conversion of inorganic into utilizable form of phosphorus, and for the growth of liquid culture. Since the results of tri calcium phosphate for the 'zone' is worth promising for the culture growth and conversion of inorganic phosphate to utilizable form of phosphate by microbes, we selected tri calcium phosphate as phosphorous source for further experimentation on growth of plants (Rodriguez & Fraga, 1999). The media which we used are nutrient broth (Hi-media, Mumbai, India) and nutrient agar (Hi-media, Mumbai, India) as the same composition of above mentioned. Slants were maintained for the regular sub-culture of P.S.B.

In another set of experiment, phosphate source (tri calcium phosphate) with iron chloride has been supplemented for the constant source of iron phosphate. In all experiments the concentration of tri calcium phosphate (0.5% w/v) and iron chloride (1% w/v, which was obtained from Merck, India) were made constant. In addition, the interesting experimentation has been performed with the crude iron (1% w/v iron dust granules obtained from a local welder). Iron dust of 1 gram is added with the tri calcium phosphate in the medium as phosphorous source for an occurred chemical reaction during the growth of microbes, as calcium chloride and iron phosphate (Simon *et al.,* 2013). It was also observed that the air exposed grown culture was developing iron absorbing micro-organism in crude iron as well as iron chloride. P.S.B. which was grown in iron chloride and crude iron separately with the supplementation of tri calcium phosphate was exposed to air which caused the further infection of environmental mycelia. Unexpectedly these mycelia are accumulating iron and change in their color to reddish brown which is interesting for the production of large scale iron for the human application (Figure.1 A and B). The P.S.B grown in iron chloride + tri calcium phosphate and with crude iron + tri calcium phosphate was also supplemented to growth of beans plants where there was stimulation of growth (Figure. 2 A-G).. P.S.B. lawn was made on nutrient agar and 0.5% of sodium/potassium/tri calcium phosphate was added for the zone of solubilizing bacterial growth (Keleli, *et al.,* 2014). The tri calcium phosphate grown cultures were also made as slants for further use.



(A) **(B)**

Figure.1. P.S.B. grown cells in Iron chloride and crude iron after exposing to air have developed the mycelia which accumulate iron observed in the form of reddish brown color.

(A).Iron chloride grown and (B).Crude iron grown

Figure 2.(A). Manual culture media were made with rice cake (2% w/v) + Hi-media nutrient broth mixture (1% w/v) + tri calcium phosphate (0.5% w/v) in one tub as normal and in the second tub addition of tri calcium phosphate (0.5% w/v) /iron chloride (0.5% w/v) and/or iron dust (crude iron (1%w/v) for the chemical conversion as calcium chloride and iron phosphate which is used by P.S.B for the conversion into utilizable form. (B). Inoculation of P.S.B into the tubs (C). The growth of P.S.B (microbial culture) between 72 and 96 hours that is ready for land supplementation. (D). Three areas for supplementation as normal, with iron chloride, and with iron dust. (E). Growth of bean plant in presence of tri calcium phosphate + iron chloride, (F).Growth of bean plant in presence of tri calcium phosphate + iron dust. (G) Normal in absence of iron chloride and crude iron dust.

2.2 *Preparation of large scale culture*

The large scale culture of 6 liters fresh filtered tap water in plastic tubs in where the extra carbon source is supplemented by cake sugar (2% w/v) and Hi-media nutrient broth (1% w/v), and for the experimental set (a) the phosphorous source used was tri calcium phosphate (0.5% w/v), (b) iron chloride (1% w/v) + tri calcium phosphate (0.5% w/v) and (c) crude iron (1% w/v) + tri calcium phosphate (0.5%). It is ready for supplementation to the land after inoculation and 72-96 hr grown in the form of large scale culture. All sets in a nut shell are: 1. Normal or control. 2. Tri calcium phosphate added (0.5%) 3. Tri calcium phosphate+ iron chloride added (0.5% w/v +1% w/v) 4. Crude iron+ tri calcium phosphate (1% w/v + 0.5% w/v)

3. RESULTS AND DISCUSSION

'General bio-fertilizer' used in this paper contains approximately 21 prokaryotic bacterial strains of the Sneha Biotechnology, have a tremendous effect on the growth and production of the green gram (*V. radiata*) crop. The interesting results which have been observed here are, in the first phase, the green gram plants picked up the higher growth (Figure. 3 A, B, C, D, E, and F) which may be concluded that there was an instant massive stimulation of metabolites and plant hormones for growth and production (Akiyoshi, *et al.*, 1987). However, there are recent reports that bio-fertilizers are nothing but supplementary component to soil and crop management traditions that are crop rotation, organic adjustments, tillage maintenance, recycling of crop residue, soil fertility renovation and the bio-control of pathogens and insect pests, where the operation can significantly be useful in maintaining the sustainability of various

crop productions (Sahoo, *et al.*, 2013b). The production parameter which we have taken here is seed wet weight, dry weight and size which was more and was about 12 to 14% (data not shown).

Figure.3. Effect of 'general fertilizer' the growth of green grams (*Vigna radiata*) (A). 1 square meter crude red soil area for green grams growth after germination at 6th day. (B). Rectangular bit is with supplementation of the culture which has contained microbes of randomly transformed with EcoR1 fragments. (C). Normal culture that is in the absence of transformation of EcoR1 fragments. (D). Rectangular sector is with microbial cells of 'second generation' grown after the random transformation of EcoR1 fragments. (E). Green grams crop grown in this sector are without the bio-fertilizer. (F). Total grown plants after 40 days of the 1 square meter with noticed difference in the growth in presence and absence of bio-fertilizer.

Growth promotion and disease control by *Pseudomonas* and *Bacillus* are complex interrelated processes involving direct and indirect mechanisms that include synthesis of some metabolites (auxin, cytokinin and gibberellins), induction of 1-aminocyclopropane-1-carbocylate (ACC) deaminase, production of siderophore, antibiotics, hydrogen cyanide (HCN), and volatile compounds (Glick *et al.*, 1994; Joo *et al.*, 2004). Others include mineral solubilization (e.g., phosphorus), competition, and induced systemic resistance (Pablozalba *et al.*, 2007). It is also observed that the major percentage of crops which were growing in presence of 'general bio-fertilizer' is disease resistant. Of course, it may be also concluded that these bio-fertilizers are also containing the disease resistant genes obtained from these selected bacterial strains. In a nutshell, it may be concluded that the mixture of bacterial strains with their self genomes would be performing two kinds of functions that are growth and production of crops, and disease resistance *(*Ansari *et al.*, 2013). The same "general bio-fertilizer" supplemented to leafy vegetable plants (*Trigonella foenum-graecum* and *Amaranthus tricolor*) similar kind of disease resistance with good production up to 40 days have been noticed (data not shown). In a separate small scale observation of legume plant roots, it is also evident that the root nodules formation is quicker and higher in the zone of 'general bio-fertilizer' supplemented plants of green gram (*V. radiata*), than the control where in the plants zone not supplemented with the bio-fertilizer (data not shown) that is lacking nitrogen fixing bacteria.

3.1 *General growth and production versus disease resistance by bio-fertilizers*

The results of microbial strains showed that are used as 'general bio-fertilizers' have both the functions that are growth and production, and disease resistance. If we look back a bit about recent observations in 'scientific world' of the Bt-cotton (*Bt-Gossypium arboreum*), Bt-brinjal (Bt-*Solanum melongena*) the sources of the disease resistant seeds are for the production and disease-resistance of crops (Wu *et al.*., 2008; Doganlar *et al.*, 2002). However, we may expect similar kind of biotechnical rearrangements in genomes of different microbial strains which can be useful for both the production and disease resistance functions. The simple observation made by several groups for competent hosts by techniques that the transformation/uptake of genes with or without plasmids, where the different genes may be having a chance of integrating into the genomes (Chen & Dubnau, 2004). Our observations here are of random microbial cells of 'general bio-fertilizers' proved that a kind of gene rearrangements of within these prokaryotic bio-fertilizers, which may help in dual functions that are the growth and production, and disease resistance. This may be further concluded that the G+C percentage changes with the CaCl2 transformation of *EcoRI* digested DNA of total genomes of microbial organisms of 'general bio-fertilizer' mixture (Ussery *et al.*,2004).

3.2 *Phosphate Solubilizing Bacteria and their effect on beans plants* (*Phaseolus vulgaris L of family Fabaceae) growth*

Phosphorus (where there is no substitute for phosphorus for crop growth) is supplied through phosphoric fertilizers, animal manures etc., The P.S.B that are mainly capable of converting non-available inorganic phosphorous in the soil, which has to be supplemented in the growth medium of phosphorus solubilizing bacterial culture, for the conversion into

the form of utilizable phosphate. The main examples again are the *Bacillus* and *Pseudomonas* genera (Prasad, 2014). Phosphorus is very important and essential nutrient of plants which is required in larger quantities. The inorganic forms of this nutrient are as the compounds of Ca, Fe, and Al with larger amounts of phosphorus applied to various soils to get fixed which is unavailable to the plants. Several soil bacterial, particularly those belonging *'Pseudomonas'* and *'Bacillus'* and 'fungi' belonging to '*Penicillium* and *Aspergillums'* possess the ability to convert insoluble phosphates in soil into soluble forms by secreting organic acids such as acetic , formic , propionic lactic , glycolic , famaric and succinic acids (Beever & Burns, 1980 ; Cunnigham & Kuiack, 1992). These acids lower the pH and bring about dissolution of bound form of phosphate. Mineral fertilizers like nitrogen and phosphorus may generally lead to a substantial increase in agriculture plants growth like as shown in Figure. 2. D, E, and F where supplementation of phosphate solubilising bacteria on beans (*Phaseolus vulgaris L)* and their yields (Glick, 1995).

The perspective of bio-fertilizer came into existence through discovery of many organisms capable of nitrogen fixation, p-solubilization, p-mobilization, potash solubilization and micronutrient transformation in the soil. The bio-fertilizers assume special significance, in these years, due to increased cost of chemical fertilizer and their ill effects on soil health. The term bio-fertilizers for nitrogen and phosphorus accumulation refer to preparation containing live microbes which helps in enhancing the soil fertility either by fixating atmospheric nitrogen, solubilization of phosphorous or decomposing organic wastes or by augmenting plant growth by producing growth hormones with their biological activities. Plants have a number of relationships with fungi, bacteria, algae, and the most common of which are with mycorrhiza, rhizobium. These are known to deliver a number of ways for tolerance to adverse soil and climatic conditions. The techniques that have been proved to be successful bio-fertilizer form a healthy relationship with the number of benefits including plant nutrient, disease resistance. Thus, the supplementation of P.S.B. bio-fertilizers techniques have proved to be successful that forming a healthy relationship with the roots.

4. CONCLUSIONS AND BENEFITS FOR FARMERS

Keeping the set up for microbial fertilizers for the production of different crops by following different steps like (a). Selection of good strain that means for fertilizer production, the first priority must be given for the characteristics and sources of suitable strain which is specified in any one of the bio-fertilizer laboratories. (b). Isolation of the micro-organisms from their sources. (c). Then grow it in suitable culture media by keeping it in an incubators (manual or industrially manufactured). (d). Observation of colony formation that occurred to confirm our isolates of micro-organisms by which one can go for different tests by approaching bio-service centers. (e). Further, one may get it done for the pure culture by using different microbial techniques. (f) Finally we go for the pot experiment.

The main benefits to the farmers are of increase in crop yield by 20 – 30%. The replacement of chemical nitrogen and phosphorus by 25%. The stimulated plant growth was observed in all most considerable limits. The observable restoration of natural soil fertility, cost effective, eco-friendly, reduces the cost towards fertilizers of usages especially regarding nitrogen and phosphorus, and better germination.

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