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Original Article

Synergistic Efficiency of Phosphate solubilizer associated with Nitrogen fixer on the Growth of Soybean (*Glycine max*)

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Abstract

Phosphorus is one of the major nutrients besides nitrogen and potassium, adequate amounts in available forms for the growth and reproduction of plants. Huge amounts of phosphorus end up as sedimentary rock in the form insoluble land inorganic phosphorus. This insoluble phosphorus cannot be used up by the plants directly. Chemical fertilizers have come up today posing several health hazards caused through pollution. Depleting fertilizers source and the residues left off by the pesticides has compelled man to divert his attention to another alternative, the so called biofertilizers. This investigation is going to identify the synergistic efficiency of phosphate solubilizer (*Pseudomona*s sp) associated with Nitrogen fixer (*Rhizobium* sp) on the growth of Soybean. In this research project, it was identified that the bio-inoculant of the Phosphobacteria, and *Rhizobium* sp showed the higher plant growth than the control. The co-inoculant of the Phosphobacteria and Rhizobium sp showed the symbiotic associated growth on the plant of "Soybean"
***Key words*:** *Rhizobium*; *Pseudomona*s , Soybean, Phosphorus

Introduction

Phosphorus is one of the essential major nutrients besides nitrogen and potassium and is needed inadequate amounts in the available form for the growth and reproduction of plants. As Pierre, (1938) put forth, phosphorus is also known as the "Master key" element in crop production which is associated with several vital functions and is responsible for many characteristics of plant growth such as utilization of sugar, starch and photosynthesis, nodule formation, cell division and organization, fat formation and transfer of heredity. (Arnon, 1956). Phosphorus cycle that is termed as sedimentary cycle generally end up as sedimentary rock in the form of imperfect insoluble and inorganic phosphorus. This insoluble phosphorus cannot be used by the plants directly, effect this, fertilizer factories one overworked in converting this insoluble phosphorus to a soluble one.

Many soil micro organisms have the capacity to solubilize insoluble inorganic phosphates. These insoluble phosphates include bone meal, rock phosphates, tricalcium phosphates, hydroxyl apatite, fluorapatite, aluminium phosphate, iron phosphates. (Kapoor *et al*., 1989). A large fraction of the autochthonous microbial population can dissolve insoluble inorganic phosphates that occur in soil itself. In some soils this ability has been found to be present in as high as 85 % of the total population (Hayman,1975). In addition to bacteria, actinomycetes belonging to the genera micromonospora , Nocardia and Streptomyces have also been reported to solubilize insoluble phosphates (Ahmad, 1968).

Solubilization of phosphorus by these phosphate solubilizing micro organisms is attributed to the excretion of organic acids like glutamic, succinic, lactic, oxalic, glyoxalic, maleic, fumaric, tartaric, alpha – ketobutyric, propionic acid and formic acid. Some of these acids (hydroxy acids) may form chelation with cations such as Ca^{2+} and Fe^{2+}, which result in effective solubilization of phosphates (Subbarao, 1986). The action of organic acids has been attributed Al^{2+}, Ca^{2+} and Mg^{2+}. In addition to phosphate solubilization these microbes can also mineralize organic phosphorus into soluble form.

The decomposition and mineralization of organic compounds of phosphorus occur under the influence of specific enzymatic complexes of some heterotrophic bacteria, actinomycetes, and fungi. It has been reported that many terrestrial bacteria produce phosphatase (Skujins,1967).

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These reactions take place in the rhizosphere and because the micro organisms render more phosphorus into the soil solution that is required for their own growth and metabolism, the surplus now becomes available for plants to absorb (Dubey and Gupta, 1996).

India, basically is an agricultural country. Population explosion is posing serious pressure on food supply. Various modern techniques are adopted in the agricultural industry to fulfill our food requirements. The cost of chemical fertilizers is increasing over the year and the low marginal farmers are find to be difficult to meet the increase in the price of chemical fertilizer. To overcome this defect of escalating energy costs, energy will be a key limiting factor for increasing agricultural production in future. It is impertinent to adopt a strategy of integrated nutrient supply by using a judicious combination of chemical fertilizers, organic manure along with biofertilizers.

Bacterial biofertilizers are classified into two types. They are nitrogen fixers and phosphate solubilizers. Both symbiotic and asymbiotic nitrogen fixers are used as fertilizers. More often *Rhizobium* sp is used as symbiotic nitrogen fixing bioferilizers in selective plants. These *Rhizobium* sp are host specific. Phosphate solubilizing micro organisms used as biofertilizers.

Nitrogen fixing micro organisms increases the amount of nitrogen in soil. These micro organisms enrich the soil with nitrogen there by increase the nutrient supply of the plant and enhance the crop yield. Phosphate solubilizing micro organisms solubilize the soluble, inorganic phosphates and making them available to the plant. Abundance of the microbes in plant root surfaces would greatly influence the plant growth and productivity and stability of the soil complex.

Rhizobia are considered as symbiotic nitrogen fixers. They elicit on their leguminous hosts, the formation of specialized organs “nodules”. In these root or stem structures the nitrogen fixing bacteria are able to convert atmospheric nitrogen into ammonia which the plant is used as a nitrogen source. The Rhizobia symbiosis is species specific.

The essential plant nutrient phosphorus usually present as insoluble inorganic and organic compounds in the soil. The phosphate solubilizing micro organisms solubilize the insoluble phosphate mainly by production of organic acids like acetic, lactic, formic and gluconic acid etc. (Rekha et al., 1991).

Combined inoculation of *Rhizobium* sp and phosphobacteria enhanced the yield in soybean. Kundu and Gaur (1980), also observed an increase in the production of groundnut using *Rhizobium* sp and phosphobacteria as bio-inoculants.

Soybean (Glycine max) is a potential crop (35-40 q/ha) and it is the cheapest source of high quality protein 42 % and oil 20 %. It is grown on a large scale in many countries. In recent years it is cultivated around 2.2 million hectare in India. (Singh, Mehar singh, and V.P.Singh, 1993).

It can produce the highest yield of protein per weight than any other plant or animal food source while at the same time producing calories. With continuing increase in the cost of living, Soybeans have gained importance as a source of protein. However long been used in Asia and are heavily consumed in affluent developed countries. Soybean is also valuable source for oil extraction which is used in numerous food stuff and in commercial products such as detergents, fertilizers, glue, lecithin, papers, plastics, soaps, paints and varnishes (Agboola, 1996).

Materials and Methods

Sampling Area

The sampling area selected for the isolation of Phosphobacteria and nitrogen fixer (Rhizobium species) for the present investigation were the fertile Rhizosphere soil in our college campus. The main cultivable crops in these areas are paddy, Brinjal, banana, groundnut, chilly, ladies finger etc. The beneficial micro organisms such as nitrogen fixers and phosphate solubilizers were added with seeds, during seed preparation. In addition BGA are also used in water lagging conditions to fix atmospheric nitrogen and reliable aminoacids, proteins, and other growth

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promoting substances which can be utilized by the plants.

Samples

In the present study, Rhizobium and Phosphobacteria species were isolated and identified from the following plants, such as:

1. *Arachis hypogea* - Groundnut
2. *Clitoria lablab* - Sanku pushbam
3. *Sesbania sesbania* - Sesbania
4. *Sesbania rostrata* - Akathi
5. *Glycine max* - Soybean.

Sampling

The rhizosphere soil from the agriculture field was collected in a sterile polythene pack and was immediately snapped up with a rubber band. The pack with the sample was placed in an ice box and transferred to the laboratory. Aseptic sampling and transfer was taken care of at each step.

Enumeration of Phospho bacteria

For the analysis of total bacterial counts in the soil samples, standard procedures as given in "Standard method for the examination of water and waste water (APHA, 1980) was followed. The media used for agricultural samples was nutrient agar medium.

To enumerate and isolate the PSB, the above procedure as adapted with spread plate technique. Here Pikovyskaya's agar media was employed for the enumeration of phosphate solubilizing micro organisms. The inoculated plates were incubated at 28^0C for the period of 3 days. The results for the enumeration of TVC counts were recorded after the incubation period.

Phosphate solubilizing populations were identified by clear solubilization zone around their colonies. The population was expressed in colony forming units (CFU).

Isolation of Phosphobacteria

Well isolated PSB colonies were picked up from the Petri plates and restreaked on agar plates, atleast for two to three times. This procedure was followed to get pure colonies. The pure colonies were restreaked onto agar slants and stored at 4^0C for further experimental work.

Identification of Phosphobacteria

The isolated pure cultures were identified up to generic level following the scheme of Simudo and Asio, (1962) Shewan et al, (1960) and the Bergey's manual of Determinative Bacteriology. (Buchanan and Gibbons, 1984).

Enumeration, Isolation and Identification of Nitrogen fixer (Rhizobium sp)

Spread plate technique was employed for the enumeration and isolation of *Rhizobium* sp of the leguminous nodules. 1 gram of nodules were surface sterilized using 0.1 % mercuric chloride solution and 70 % ethanol and washed with distilled water. The surface sterilized nodules were homogenized using sterile mortar and pestle. One ml of homogenized sample was serially diluted up to 10^8 using 9 ml of sterile distilled water blanks. From these dilutions, 0.1 ml of the sample was pipetted out and spread plated on Yeast Extract Mannitol Agar medium (YEMA). The inoculated plates were incubated at room temperature, 28^0C ± 2^0C for 3-10 days depending on the nature of Rhizobium.

The colonies of *Rhizobium* sp appeared as white, translucent, glistening and elevated with entire margin. The well isolated *Rhizobium* colonies were purified by streaking on the nutrient agar plates and pure strains were again streaked on nutrient agar slants and stored at 4^0C for further biochemical studies.

Effect of Bio-Inoculants on the Growth of Soybean

Preparation of Mono and Co-inoculants:

In the present investigation the isolated cultures of *Rhizobium* sp and Phosphobacteria species were used as monostrain inoculant and co-inoculants for conducting pot trials. The following combination of biofertilizer and chemical fertilizers were tried to study the effect of bio-inoculants on the growth of Glycine

Pot number	Treatments
I	Phosphobacteria alone
II	Rhizobium alone
III	PSB + Rhizobium
C	Control.

The bio-inoculants were sub cultured in 10 ml of nutrient broth (Seed culture). The

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mono inoculants and co-inoculants were prepared using the seed culture. The mono inoculants (PSB or *Rhizobium* sp were prepared by inoculating 1.0 ml of respective seed culture in 100ml of nutrient broth. The co-inoculants (PSB and *Rhizobium* were prepared by inoculating 1.0ml of each *Rhizobium* sp and *Phosphobacteria* in 100 ml of nutrient broth.

Seed Coating

The seeds of glycine max were taken and surface sterilized using 0.1 % mercuric chloride and 70 % ethanol and washed with distilled water (Subba rao, 1993). The surface sterilized seeds were then transferred into 100 ml conical flask separately containing the different combination of bio-inoculants and kept for one hour. After one hour the seeds were air dried. Then the seeds were sown and allowed to germinate in the respective pots.

The total viable bacterial count and N, P, K contents of the pot sediments were examined before performing the experiment.

Growth Study

After 30 days and 60 days of the experiment, the effect of bio inoculants such as *Phosphobacteria* and *Rhizobium* sp on the growth of Soybean (Glycine max) was investigated. The parameters such as height of the plant, length and breadth of leaf, inter nodal length, grain yield, root length, number of nodules, chlorophyll content (a and b) and the total nitrogen and phosphorus contents were observed in the experimental and control of plant soybean treated with biofertilizers in mono and co-inoculants.

Chlorophyll Estimation

The chlorophyll content of the leaves of the experimental & control plants were estimated by the method of Sadasiva and Manikkam (1991). In the present investigation chlorophyll a & b were measured in the control and experimental plants. The chlorophyll content was estimated on the basis of dry weight and wet weight.

One gram of fresh leaf was weighed, and transferred into clean mortar and pestle. For chlorophyll estimation, on the basis of dry weight of the leaf was dried at 105^0C for 24 hours. The fresh dried leaf sample was transferred into mortar and pestle, 20 ml of 90 % acetone was added, and the leaf contents were thoroughly macerated. The ground sample was poured into screw capped tube. The mortar and pestle was rinsed with sufficient acetone and 0.2ml $MgCo_3$ suspension.

The sample was kept at 4^0C for 4-6 hours for pigments to elute. Then the samples were centrifuged at 3000 rpm for 20 minutes. The supernatant of the samples were collected in a volumetric flask. Then these samples were made up to a volume of 100 ml with 90 % acetone. The absorbance was taken for that sample at 663, 645 nm. The chlorophyll a and b values of the control and experimental plants were calculated using the following formula.

mg chlorophyll a /g tissue= 12.7(A663) - 2.69(A645) X V / (1000 X W)
mg chlorophyll b /g tissue = 22.9 (A645) – 4.68 (A663) X V/ (1000X W)
mg total chlorophyll / g tissue = 20.2 (A645) + 8.02 (A663) X V / (1000 X W).
Where,
A – absorbance at specific wave length
V - Final volume of chlorophyll extract in 90% acetone
W – Fresh weight of tissue extracted.

Estimation of Total Nitrogen

The total nitrogen in the leaves of the control and experimental plant samples were estimated by Kjeldahl method.

The method involved in the digestion of the sample is to convert organic nitrogen to ammonia and then determinations of ammonium content in digested sample by a titrimetric procedure. Accurately, weighed 100 mg of dried leaf sample was taken in a boiling test tube. To this, 3ml of con H_2SO_4 was added and contents were boileld for 15 minutes. During boiling few drops of perchloric acid were added and the content in the boiling tube was made to 50 ml using distilleld water. From this 2 ml of sample was taken in a microkjeldahl flask along with 4 ml of 40 % NaOH. Steam was passed for five minutes and the liberated ammonia was collected in a conical flask containing 5 ml of 2 % boric acid with double indicator (Mau-Zazuza indicator –

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Bromo cresol green & Methyl red). The colour of the boric acid was changed from pink to blue which was titrated against N/70 H_2SO_4 . The end point was the appearance of pink colour. The blank was also titrated without adding sample.

Estimation of Phosphorus

Phosphorus reacts with molybolic acid to form phosphor molybolic acid. On treatment with 1,2,4 – aminonaphthol sulponic acid it is relatively reduced to produce a blue colour (molybdenum blue) which is probably a mixture of lower acids of molybdenum. This colour is then compapred in " Spectronic 20" at 660 nm with that of obtained from a suitable standard phosphorus, (KH_2PO_4) treated in the same way. 1 ml, 2 ml,3ml,4ml and 5ml of the working standard phosphorus solution were pipettedout in to a series of the test tubes. The concentration of the above were 8,16,24,32,40 mg phosphorus respectively, the standard was prepared.

1ml of the digested leaf sample was pipettedout into a test tube. To this 0.4 ml of ANSA and 1 ml of molybdate solution. 1 and 1 ml of molybdate solution II were added and made upto 10 ml using distilled water. The contents were mixed well and the colour developed was read at 660 nm after 20 minutes. The phosphorus content of the unknown sample was calculated(Fiske and Subba Rao,1925).

Results and Discussion

Enumeration of Total Viable Counts of Psb and Rhizobium sp

The total viable counts of rhizosphere varied from 2.8 x 10^7cfu/gm to 4.6 x 10^7cfu/gmI. (Table. 1).

Table-1: Enumeration of bacteria from the soil

Organism isolated	Total Viable Count
Phosphobacteria sp *Rhizobium* sp (Nitrogen fixer)	2.8 X 10^7 CFU/gm 4.2 X 10^7 CFU/gm

For the enumeration of PSB the pikovyskaya's agar medium was used, the plates were incubated for 3 days. After three days incubation, hallow zone formation was observed around the colonies.

The surface sterilized nodules were homogenized using sterile mortar and pestle. One ml of homogenized was serially diluted up to 10^8 using 9ml of sterile distilled water. From these dilutions, 0.1ml of the sample was pipetted out and spread plated on Yeast extract mannitol agar medium (YEMA). The inoculated plates were incubated at room temperature, for 3-10 days on the nature of the Rhizobium. The population of the Rhizobium in the rhizosphere soil was 4.2 X 10^7 CFU/gm.(Table 2).

Biochemical Characteristics of PSB and Rhizobium sp

The isolated strains of *Phosphobacteria* and *Rhizobium* sp were identified up to genus level. The Bergey's manual Determinative Bacteriology (1989) was also referred in the identification procedures. The results were tabulated (Table-3).

Effect of Bio inoculant and Co-Inoculant on the Growth of Soybean

In the present investigation the isolated cultures of *Phosphobacteria* and *Rhizobium* sp were used as mono strain inoculant and co-inoculant for conducting pot trials. The results showed that the synergistic growth effect on the Soybean, other than the mono inoculant.

Effect of Bio Inoculant on the Seedling Growth of the Plant

The comparative study were conducted to observe the seedling growth of the plant, and to observe the length of the leaf, stem and internode. The other contents like phosphorus, Nitrogen, Potassium showed increasing value in the Co-inoculant than the mono inoculant.

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Table- 2: Biochemical Characteristics of *Phosphobacteria* and *Rhizobium* Sp

Biochemical Tests	Phosphobacteria (*Pseudomonas* sp)	Nitrogen fixers (*Rhizobium*sp)
Motility	+ve	+ve
Gram's Staining	-ve	-ve
Oxidation-Fermentation test		
a) Acid production	+ve	+ve
b) Gas production	-ve	-ve
Penicillin sensitivity test	Resistant	
Hydrolysis test		
a) Starch	+ve	-ve
b) Casein	-ve	+ve
c) Gelatin	+ve	+ve
Nitrate reduction test	+ve	--
H_2S production	-ve	--
Indole	-ve	--
MR	-ve	--
VP	+ve	--
Citrate	+v3	--
Catalase		--
Mannitol	--	
Litmus milk test	--	+ve
a) Acid production	--	-ve
b) Alkaline production		-ve
c) Curd formation	--	
Sugar fermentation test	--	+ve
a) Glucose	--	+ve
b) Mannitol	--	+ve
c) Sucrose	--	+ve
d) Maltose		+v3
e) Lactose		

Table- 3: Effect of bio-inoculant in the growth of soybean after 60 days in various treatments

Treatment	Stem length (cm)		Leaf Ares (Leaf length cm)		Internode Length (cm)	
	PSB alone	Rhizo & PSB	PSB alone	Rhizo & PSB	PSB alone	*Rhizobium* & PSB
A	45	52	8.6	9.2	5.6	5.8
B	40	46	9.0	10.8	5.8	6.0
C	64	56	10.5	12.0	6.4	6.8
D	38		9.0cm			

Treatment A- Phosphobacteria alone; B- Rhizobium alone
C- PSB + Rhizobium; D - Control.

Table - 4: Growth Rate of the Plant "Soybean" in various treatments

Sample	Nitrogen Content (mg/g)		Phosphorus content (mg/g)		Potassium content (mg/g)	
	30^{th} day	60^{th} days	30^{th} day	60^{th} days	30^{th} day	60^{th} days
A	0.16	0.20	0.380	0.472	92	94
B	0.18	0.24	0.392	0.488	98	97
C	0.24	0.30	0.448	0.556	110	102
D	0.14	0.14	0.360	0.360	88	78

Table -5: Effect of Bioinoculant on the Chlorophyll content at 60th Days

Sample	Chlorophyll a	Chlorophyll b	Total Chlorophyll
A	0.2438	0.1988	0.4426
B	0.2086	0.1782	0.3868
C	0.2672	0.2012	0.4684
D	0.2260	0.2066	0.4326

Conclusion

Phosphorus is one of the major nutrients besides nitrogen and potassium adequate amounts in available forms for the growth and reproduction of plants. Huge amounts of phosphorus end up as sedimentary rock in the form insoluble land inoroganic phosphorus. This insoluble phosphorus, cannot be used up by the plants directly. Chemical fertilizers have come up today posing several health hazards caused through pollution. Depleting fertilizers source and the residues left off by the pesticides has compelled man to divert his attention to another alternative, the so called biofertilizers. The autochthonous microbial population offer the fond alternative to dissolve our problem. In addition to bacteria, actinomycetes belonging to the genera *Micromonospora, Nocardia, Pseudomonas*and *Streptomyces* have also been reported to solubillize insoluble phosphates. In this research project, it was identified that the bio-inoculant of the Phosphobacteria, and *Rhizobium* sp showed the higher plant growth than the control. The co-inoculant of the Phosphobacteria and *Rhizobium* sp showed the symbiotic associated growth on the plant of "Soybean"

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