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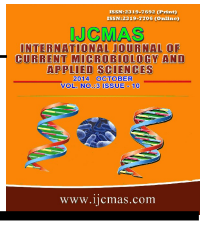

Original Research Article

Efficiency of different nitrogen fixing bacteria with respect to growth and development of legumes

Juhi Sharma*, Tapasuita Gurung, Krittika Nandy and A.K, Mitra

Post-Graduate Department of Microbiology, St. Xavier's College, 30 Mother Teresa Sarani Kolkata- 7000016, India
**Corresponding author*

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A B S T R A C T

The introduction of green revolution has led the scientific community to identify bio-fertilizers as an alternative to chemical fertilizers to increase soil fertility and crop production. In the present research, a comparative analysis of *Rhizobium* and *Azotobacter* biofertilizers were carried out on two legume plants, *Dolichos lablab* (Sheem) and *Phaseolus vulgaris* (Bean) under controlled environment. The study was conducted by planting 20 seeds of each plants distributed into 4 treatments :- (i) Control (ii) Rhizobium only (iii) Azotobacter only (iv) Azotobacter (50%) + Rhizobium (50%). After 1month, the different growth parameters of the individual plants were measured which showed a 5-10% increase in single bio-fertilizer treatment (ii, iii) and a 14-17% increase in combined treatment (iv) of plants when compared to the control ones. A 15% increase in Saturated Fatty Acid (SFA) content was recorded from the *Phaseolus vulgaris* showing high stability of secondary metabolites. In contrast, *Azotobacter* showed a better effect on both the plants when individual bio-fertilizers were considered. Thus, it can be concluded that beside the combinatorial effect of *Azotobacter* and *Rhizobium*, *Azotobacter* is more effective on the legumes, as it showed better response in plant growth / vigor, and provided higher Nitrogen fixing ability as well as better protection against harmful ROS. Thus it can be used in large scale production of the commercially valuable legume plants which will benefit the producers with better quality of vegetables.

Introduction

Biofertilizers are commonly called microbial inoculants which are capable of mobilizing important nutritional elements in the soil from non-usable to usable form through biological processes (Chandrasekar, *et al*, 2005; Selvakumar, 2009). Soil is considered a storehouse of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space. Soil microorganisms play an important role in soil processes that determine plant productivity. There is a continuum of bacterial presence in soil, rhizosphere, rhizoplane, and internal the

plant tissues. Bacteria living in the soil are called free-living as they do not depend on root exudates for their survival. Some bacteria support plant growth indirectly, by improving growth restricting conditions either via production of antagonistic substances or by inducing resistance against plant pathogens (Tilak *et al*, 2005). The interactions among the rhizosphere, the roots of higher plants and the soil borne microorganisms have a significant role in plant growth and development. The organic compounds, released by roots and bacteria, play an important role in the uptake of mineral nutrient. The hormones produced by the rhizosphere bacteria have direct effects on higher plants. The density of PGPB (*Plant Growth Promoting Bacteria*) depends on the soil status and so the human activities (Marianna *et al,* 2005). Biofertilizers can add 20-200kg N ha.1 (by fixation), liberate growth-promoting substances and increase crop yield by 10-50%. They are cheaper, pollution free, based on renewable energy sources and also improve soil tilth (Saeed *et al*, 2004).

The use of biofertilizers effectively enrich the soil and cost less than chemical fertilizers, which harm the environment and deplete non-renewable energy sources. Biofertilizers have definite advantage over chemical fertilizers. Chemical fertilizers supply over nitrogen whereas biofertilizers provide in addition to nitrogen certain growth promoting substances like hormones, vitamins, amino acids, etc., crops have to be provided with chemical fertilizers repeatedly to replenish the loss of nitrogen utilized for crop growth. On the other hand biofertilizers supply the nitrogen continuously throughout the entire period of crop growth in the field under favorable conditions. Continuous use of chemical fertilizers adversely affects the soil structure whereas biofertilizers when applied to soil improve the soil structure. The effects of chemical fertilizers are that they are toxic at higher doses. Biofertilizers, however, have no toxic effects. Biofertilizers are commonly called as microbial inoculants which are capable of mobilizing important nutritional elements in the soil from non-usable to usable form by the crop plants through their biological processes. For the last one-decade, biofertilizers are used extensively as an ecofriendly approach to minimize the use of chemical fertilizers, improve soil fertility status and for enhancement of crop production by their biological activity in the rhizosphere (Contra costa, 2003, Patil, 2010). Chemical fertilizers are expensive; they disturb the equilibrium of agro-ecosystems and cause pollution to the environment. These problems may be avoided by the use of biofertilizers (Al-Khiat, 2006). The utilization of microbial products has several advantages over conventional chemicals for agricultural purposes: (1) microbial products are considered safer than many of the chemicals now in use; (2) neither toxic substances nor microbes themselves will be accumulated in the food chain; (3) self-replication of microbes circumvents the need for repeated application; (4) target organisms seldom develop resistance as is the case when chemical agents are used to eliminate the pests harmful to plant growth; and (5) properly developed biocontrol agents are not considered harmful to ecological processes or the environment (Wua *et al*, 2004).

Materials and Methods

I.a) Isolation and Identification of *Rhizobium* and *Azotobacter*

1) The commercially available biofertilizer *Rhizobium* and *Azotobacter* from PUSA, New Delhi was used for the experiment.1gm of the

biofertilizer was mixed in 10 ml distilled water.

2) A loopful of the soil solution was taken and streaked in YEMA media (Yeast extract mannitol agar) for isolation of *Rhizobium* and Ashby's media for isolation of *Azotobacter* and incubated at 28^0C for 24-48 hours.

I.b) Gram's staining

Gram-staining of isolated colony of *Rhizobium* and *Azotobacter* was carried out utilizing the two colonies which appeared on the individual plates of the YEMA and the Ashby's media plates.

I.c) Motility Test

Bacteria were introduced into a semisoft agar medium by performing a stab with an inoculating needle and were incubated for 24 hours.

I.d) Pot Experiment

Materials required: Plastic glasses, Sand, Soil, Filter paper

Procedure: The present study was carried out in the month of June- August.

1) The experiment consist of four treatments of biofertilizers :
   A = Control (no inoculation).
   B = *Rhizobium* only
   C = *Azotobacte*r only
   D =*Azotobacter* (50%) + *Rhizobium* (50%)
2) The seeds of sheem and bean were allowed to germinate for 2-3 days.
3) The biofertilizer containing *Rhizobium* and *Azotobacter* was weighed 1 gm and mixed in 10 ml distilled water. The solution was filtered using a filter paper. The seeds of sheem and bean was inoculated with the filtrate and sown in the soil.
4) The treatments were placed in controlled environment and watering was performed regularly.
5) The readings of leaf, stem and root lengths were taken for seeds inoculated without biofertilizer (control) and the seeds inoculated with the filtrate of *Rhizobium*, *Azotobacter* and combination of *Rhizobium* and *Azotobacter*.

II) Biochemical(Enzymatic) Assays of the Crude Extracts of the Plants (*Dolichos lablab* and *Phaseolus vulgaris*)

The following enzymatic assays were performed to test the presence or absence of antioxidant activities of the plants. The extracts of the plants were subjected to the following biochemical assays:

II.a)Determination of Glutathione Reductase (GR) by Spectrophotometric assay

Oxidative stress, which is associated with almost all the abiotic stresses, is due to over production of toxic reactive oxygen species (ROS) including superoxide ion, hydrogen peroxide, and hydroxyl radicals. Plants combat the oxidative stress via enzymatic and non-enzymatic machinery. Glutathione reductase (GR) is one of the potential enzymes of the enzymatic antioxidant system, which sustains the reduced status of GSH via Ascorbate–Glutathione pathway and plays a vital role in maintenance of sulfhydryl (–SH) group and acts as a substrate for glutathione- *S* -transferases. GR is a homodimeric FAD-containing enzyme which belongs to the family of NADPH-dependent oxidoreductases.

Glutathione reductase (GR) was assayed by the method of Smith et al; 1988.

1) The enzyme was extracted in extraction buffer (0.1M potassium phosphate buffer-pH 7.5). After extraction, it was centrifuged at 12,000 r.p.m at 4ºC for 10 min. The supernatant was taken for enzyme assay.
2) In the reaction 2.9 ml assay mixture, 1 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DTNB (5, 5 – dithiobis – 2 nitrobenzoicacid), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract were added and finally distilled water was added to make up a final volume upto 2.9 ml. Reaction was initiated by adding 0.1 ml of 2 mM GSSG (oxidized glutathione).
3) The increase in absorbance (by the formation of reduced glutathione-GSH) at 412 nm was recorded at 25ºC over a period of 5 min spectrophotometrically.

II.b) Determination of reduced glutathione content by spectrophotometric assay

According to the method of Stern et al (2006), reduced glutathione (GSH) was assayed.

1) After extraction in extraction buffer,(50 mM sodium phosphate buffer pH 7.6) it was centrifuged at 6000 rpm for 15 min. 5 µL of supernatant was diluted as 1:2 with 5% SSA i.e. 5-sulfosalicylic acid dihydrate solution, further diluting to 1:2 with 400mM sodium carbonate, and 1:8 with phosphate-EDTA dilution buffer (total dilution1:32).
2) The spectrophotometric (UV 2401 PC SHIMADZU, Japan) reading of absorbance was taken at 415nm.
3) By comparing the absorbance with standard curve of glutathione concentration, results were presented in mM of reduced glutathione produced /mg of total protein.

(Reduced glutathione interacts with 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) to form the colored product 2-nitro-5-thiobenzoic acid, which was measured at 415 nm.)

II.c) Determination of Superoxide Dismutase (SOD) activity by spectophotometric assay

Detection of SOD in samples is achieved by the aerobic photoreduction of NBT (nitrobluetetrazolium). Crude extracts from all the embryo samples were assayed for SOD activity photochemically using the assay system consisting of methionine, riboflavin and nitrobluetetrazolium (NBT) as described by Beaucchamp and Fridovich (1971).

1) SOD was extracted from all the stocks by grinding in 2 ml of 0.1 M potassium-phosphate buffer (pH 7.8) containing 2mM EDTA buffer, 1M sucrose, 2mM MgCl2, 5mM NaCl and 3mM β-mercapto ethanol (β-ME). The homogenate was centrifuge at 15,000 rpm for 30min at 2-4 ºC.
2) Salting out of the appropriate protein fraction was done by making the supernatant to 30-90% (NH4)2SO4 (w/v). The precipitate thus obtained was dissolved in 0.5ml of extraction buffer (without β-ME) and used as the crude enzyme extract.
3) The absorbance was taken at 632.8 nm spectrophotometrically .Enzyme

activity was expressed in terms of change in absorbance(A) of the samples at 632.8nm /min/mg of total protein.

II.d) Determination of Catalase (CAT) activity by spectrophotometric assay

Catalase was assayed according to the modified method of Chance and Maehly, 1955. The catalase activity was expressed in units, where one unit of CAT converts one μmol of H_2O_2 equivalence/min /mg of total protein.

1) Catalase was extracted from sample in 50mM potassium phosphate buffer (pH 7.0) containing 2mM EDTA, 2mM MgCl2, 3mM NaCl and 10mM β-ME. The extraction was centrifuged at 10,000 r.p.m for 10 min and the supernatant was taken for catalase assay.
2) 3ml of reaction mixture contained 50mM phosphate buffer (pH 7.0), 15mM H2O2 and 75 μg of crude enzyme extract. The decrease in H_2O_2 by virtue of its decomposition by catalase was followed as the decline in absorbance at 25 ºC at 240nm measured spectrophotometrically.

II.e) Determination Of Acetyl CoA carboxylase activity by spectrophotometric assay

1) The enzyme is extracted (according to modified method of Matshuhashi, 1969) in 0.1M Tris-HCl buffer pH 8.0 containing 20mM beta mercaptoethanol, 1mM EDTA, 0.5% Triton-X 100, 1mM benzamidine, 10mM MgCl2, 20% Glycerol. The extract is centrifuged at 27,500g for 30min at 8oC.
2) The supernatant was precipitated with 35-40% $(NH_4)_2SO_4$ saturation.
3) The pellet was finally re-suspended in 1 ml of buffer containing 0.1M Tricine-KOH pH 8.0, 2.5mM MgCl2, 50mM KCl, 1mM DTT.
4) The spectrophotometric (UV 2401 PC SHIMADZU, Japan) assay was performed according to Matsusashi, 1969. The reaction mixture (200μl volume) contained 50mM Hepes pH 8, 2.5mM MgCl2, 0.5mM phosphoenolpyruvate, 0.2mM NADH, 1.1 U pyruvate kinase, 2.3U lactate dehydrogenase, 11mM NaHCO3, 1mM ATP, 0.5mM DTT, 0.03% (v/v) DMSO and ~3μg protein containing crude extract.
5) The mixture was pre-incubated for 10 min at 30oC. The reaction was started by the addition of acetyl-coA. The time-dependent decrease in 340nm absorbance (absorbance of NADH) was monitored and used to calculate initial reaction velocity.

Results and Discussion

I.a) The *Azotobacter* colonies appeared as flat, soft, milky and mucoid in texture on the Ashby's media and *Rhizobium* colonies appeared pinkish and gummy on YEMA plate.

I.b) On Gram staining, *Rhizobium* showed rod shaped cells red in color hence it is Gram negative. *Azotobacter* appeared oval shaped and are Gram negative.

I.c) After incubation, motility was determined by examining whether or not the bacteria have migrated away from the stab line and throughout the medium. *Rhizobium* and *Azotobacter* both migrated away from stab line; hence they were motile.

The results thus obtained indicate that both the biofertilizers(*Rhizobium* and *Azotobacter*) had significant effects on the growth of both the experimental legume plants *Phaseolus vulgaris* and *Dolichos lablab*.But this effect was magnified when the two were used together. The leaf,stem and root lengths increased from 2.4cm, 9.8cm and 3.2cm to 4.4cm, 23.3cm and 8cm respectively in case of *Phaseolus vulgaris (*Bean) and from 2.8cm,16.7cm and 2.5cm to 5.8cm,45.3cm and 5.5cm incase of the *Dolichos lablab* (Sheem). But compared to *Rhizobium, Azotobacter* was more fruitful as a potential biofertilizer when the two were used individually incase of both the plants(I.d and graphs I and II).

The enzymatic assays revealed that compared to *D.lablab, P.vulgaris* had comparatively higher concentrations of all the four ROS scavenging enzymes(II a,b,c,d,e). But this effect was comparatively more pronounced incase of the combined treatment of the biofertilizers which also held true in case of *D. lablab*. Thus we conclude that *Rhizobium* and *Azotobacter* were agonistic in nature and this relationship led to not only a significant increment of all the ROS scavenging enzymes but also to the promotion of growth in the plants under stressed circumstances. But if the efficacy of the individual biofertilizers were to be assessed, it was found that *Azotobacter* containing plants had comparatively greater concentrations of the scavenging enzymes which contributed to the all-round development of the plants.

A) For *Dolichos lablab* (Sheem) (All lengths measured in cm)

Treatment	**Leaf Length**	**Stem Length**	**Root Length**
Control	2.8	16.7	2.5
Rhizobium	4.7	28.5	3.4
Azotobacter	5.4	33.2	4.5
Rhizo + Azo	5.8	45.3	5.5

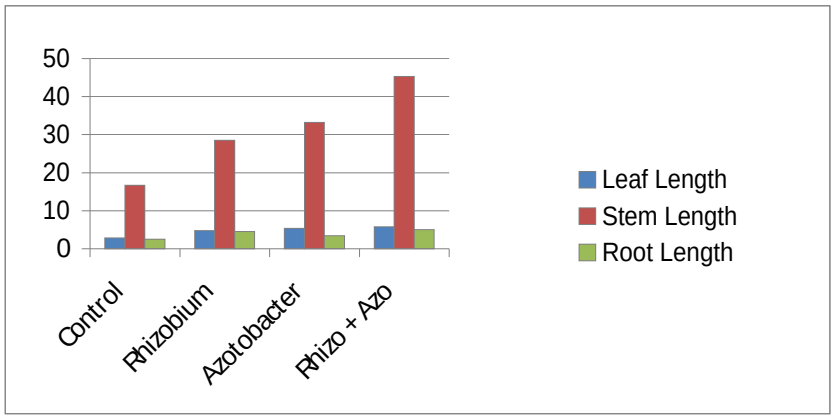

Fig I

Fig.I Graph elucidating the individual and combinatorial effects of the biofertilizers on the leaf,stem and root growth of the seedlings of *Dolichos lablab*(Sheem)

B) For *Dolichos lablab* (Sheem) (All lengths measured in cm)

Treatment	Leaf Length	Stem Length	Root Length
Control	2.4	9.8	3.2
Rhizobium	4	15.2	4
Azotobacter	4.2	18.5	4.4
Rhizo + Azo	4.4	23.3	8

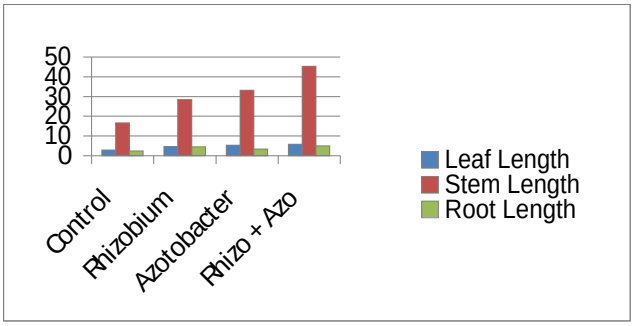

Fig.II Graph elucidating the individual and combinatorial effects of the biofertilizers on the leaf,stem and root growth of the seedlings of *Phaseolus vulgaris*(Bean)

II. Biochemical (Enzymatic) Assays

a) Glutathione Reductase (GR) For *Dolichos lablab* (Shem)

BIOFERTILIZER	Glutathione Reductase (Mg of total protein)
Control	0.20
Rhizobium	0.32
Azotobacter	0.42
Rhizobium+Azotobacter	0.52

For *Phaseolus vulgaris* (Bean)

BIOFERTILIZER	Glutathione Reductase (Mg of total protein)
Control	0.17
Rhizobium	0.39
Azotobacter	0.49
Rhizobium+Azotobacter	0.58

II.b) REDUCED GLUTATHIONE CONTENT

For *Dolichos lablab* (Sheem)

BIOFERTILIZER	**Total Reduced Glutathione(Mg of total protein)**
Control	0.02
Rhizobium	0.09
Azotobacter	0.15
Rhizobium+Azotobacter	0.2

For *Phaseolus vulgaris*(Bean)

BIOFERTILIZER	**Total Reduced Glutathione(Mg of total protein)**
Control	0.01
Rhizobium	0.089
Azotobacter	0.18
Rhizobium+Azotobacter	0.25

IIc) SUPEROXIDE DISMUTASE (SOD)

For *Dolichos lablab*(Sheem)

BIOFERTILIZER	**Net SOD Activity(Mg of total protein)**
Control	0.12
Rhizobium	0.29
Azotobacter	0.36
Rhizobium+Azotobacter	0.79

For *Phaseolus vulgaris* (Bean)

BIOFERTILIZER	**Net SOD Activity (Mg of total protein)**
Control	0.09
Rhizobium	0.37
Azotobacter	0.45
Rhizobium+Azotobacter	0.86

II.d) CATALASE(CAT) ACTIVITY

For *Dolichos lablab* (Sheem)

BIOFERTILIZER	**Total Catalase/CAT activity(mg of total protein)**
Control	0.05
Rhizobium	0.1
Azotobacter	0.25
Rhizobium+Azotobacter	0.29

For *Phaseolus vulgaris* (Bean)

BIOFERTILIZER	**Total Catalse/CAT activity(mg of total protein)**
Control	0.08
Rhizobium	0.15
Azotobacter	0.27
Rhizobium+Azotobacter	0.39

II.e) ACETYL CoA CARBOXYLASE ACTIVITY

For *Dolichos lablab* (Sheem)

BIOFERTILIZER	**Net Acetyl CoA Activity(Mg of total protein)**
Control	0.02
Rhizobium	0.1
Azotobacter	0.21
Rhizobium+Azotobacter	0.18

For *Phaseolus vulgaris* (Bean)

BIOFERTILIZER	**Net Acetyl CoA Activity(Mg of total protein)**
Control	0.02
Rhizobium	0.34
Azotobacter	0.49
Rhizobium+Azotobacter	0.29

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