

## The Role of Some PGPR Strains to Biocontrol *Rhizoctonia Solani* in Soybean and Enhancement The Growth Dynamics and Seed Yield

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**D**AMPING off is a serious disease that frequently targets the roots of seedlings of many crops and finally brings them to the death. *Rhizoctonia solani* is one of the most famous plant pathogenic fungi that causes damping off especially in damp environments and consequently reduces significantly the productivity of infected plants. Biocontrol, therefore, is a promising and urgent tool, particularly in organic farming, to minimize the using of fungicides in agricultural sector and keep the environment safe. The current study aimed to evaluate the efficiency of combined inoculation with *Methylobacterium* strains (*Methylobacterium aminovorans* and *Methylobacterium rhodinum*); *Bradyrhizobium japonicum* (St. 110); *Bacillus megaterium* var. *phosphaticum*; and *Trichoderma viride* to biocontrol *Rhizoctonia solani* in soybean seedlings. All treatments decreased damping off and increased the healthy plants. T5 treatment (inoculation with *B. japonicum* (St. 110) + *M. aminovorans* + *B. megatherium* var. *phosphaticum* (B6) + *T. viride* + foliar spray with *M. aminovorans*) and T6 treatment (inoculation with *B. japonicum* (St. 110) + *M. rhodinum* + *B. megatherium* var. *phosphaticum* (B6) + *T. viride* + foliar spray with *M. rhodinum*) showed increased nodule numbers and dry weight of nodules at 45 and 60 days compared to un-inoculated chemical fertilized control. Significant increase in nitrogen%, phosphorus%, potassium %, number of pods, seed index (g 100 seeds<sup>-1</sup>) and seed yield (ton ha<sup>-1</sup>) of soybean plants was observed due to T5 treatment when compared with the control treatment at 30, 60 and harvest. Soil dehydrogenase, urease, and phosphatase activities were also significantly increased due to the treatments T5 and T6.

**Keywords :** Soybean, Damping off, *Rhizoctonia solani*, Biocontrol, Enzyme activities.

### Introduction

In Egypt, soybean acreage has declined during the last 20 years from about 42016 ha in 1991 to about 8403 ha in 2016. Also, decreasing in soybean area may be refer to competition with other summer crops, increased production cost, reduced net return per unit area and difficulties in marketing channels according to Egyptian Ministry of Agriculture and Land Reclamation 2016. The total production, consequently, became insufficient for national consumption. Therefore, it is necessary to introduce the crop to new land regions, reduce production costs and increase productivity per unit area in order to increase soybean total production at national level. This can be achieved through growing high yielding

cultivars and improve the agronomic treatments using novel techniques such as biofertilizers and biological control (EL-Harty et al. 2010).

Plant growth promoting rhizobacteria (PGPR) have the ability to enhance the plant growth and yield through multitudinous mechanisms which include nitrogen fixation, phosphate solubilization, biosynthesis of indole acetic acid (IAA), antimicrobial compounds and siderophores productions (Karthik et al. 2016; O'Callaghan 2016; Desai et al. 2016). Plant-microbe interactions, in rhizosphere zone, interactions depend on the function of the associated microorganisms

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Many microorganisms have been proved their efficiencies to control plant disease caused by fungal pathogens via different mechanisms such as competing for nutrients and niches, producing antibiotic metabolites and suppressing plant pathogens (Egamberdieva *et al.* 2011). The beneficial effect of *Bradyrhizobium*, *B. megaterium* and *methylobacterium*, particularly in legumes, promote the plant growth to increase the productivity and also inhibit the growth of certain pathogenic fungi *i.e.* *Macrophomina phaseolina*, *Rhizoctonia* spp, *Fusarium* sp. and *Pythium* spp. in both leguminous and non-leguminous plants. In several cases direct biocontrol potential has been demonstrated, especially for plant diseases caused by *Phytophthora*, *Rhizoctonia* and *Fusarium* pathogens (Thijs *et al.* 2014; Keswani *et al.* 2014; Armada *et al.* 2015; O'Callaghan 2016; Sekar *et al.* 2016).

Damping off caused by *Aphanomyces euteiches*, *Rhizoctonia solani*, *Fusarium spp.*, *Sclerotium rolfisii* are the most destructive soil-borne diseases of soybean, pea, chickpea, lentil faba bean and lupine (Cook and Baker, 1983). Regarding to environmental and health concerns about extended use of chemicals; there is considerable interest in finding alternative control approaches for biological control strategies for crop diseases (Pathak and Kumar 2016; Egamberdieva 2016). Poorniammal *et al.* (2009) showed that *Methylobacterium* isolates significantly reduced the growth of plant pathogens like *Sclerotium rolfisii*, *Pythium*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium udum*, *Macrophomina* and *Phytophthora*.

The present study are, therefore, aimed to evaluate some strains of PGPR for growth promotion of soybean plants and their effect to control soybean damping off under field conditions.

## **Materials and Methods**

### *Microbial strains source*

*Bradyrhizobium japonicum* (st. 110), *Bacillus megaterium* var. *phosphaticum* (B6), *Methylobacterium aminovorans* and *Methylobacterium rhodinum* strains were provided from Bacteriology Laboratory, Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt. Pure cultures were routinely maintained on Yeast Extract Mannitol Agar (YEMA) medium (Vincent

1970), Nutrient Agar (NA) medium (Atlas 1997) and Ammonium Mineral Salt (AMS) medium (Whittenbury *et al.* 1970), respectively.

*Trichoderma viride* and *Rhizoctonia solani* were also used in this study and provided by Department of Microbiology, Faculty of Agriculture, Mansoura University, Egypt and Department of Plant Pathology, Sakha Agricultural Research Station, Egypt, respectively. Pure cultures were routinely maintained on Potato Dextrose Agar (PDA) medium (Okon *et al.* 1977).

### *Experiment design*

The field trial was conducted at Teida village location 30° 56' 49.652" E Longitude and 31° 05' 38.543" N Latitude, Sidi Salem region, Kafr El-Sheikh Governorate, Egypt. The field experiment was conducted in loam soil in texture having the following characteristics: pH, 7.71; EC, 0.170 dSm<sup>-1</sup>; organic matter (%), 1.48; particle size distribution sand, silt and clay (%), 48.15, 34.50 and 17.35, respectively; soluble cations Ca<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup> (meq L<sup>-1</sup>), 0.83, 0.45, 0.46 and 0.14, respectively; soluble anions CO<sub>3</sub><sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup> and SO<sub>4</sub><sup>-</sup> (meq L<sup>-1</sup>), 0.0, 1.0, 0.71 and 0.17, respectively; available N (mg Kg<sup>-1</sup>), 7.55; available P (mg Kg<sup>-1</sup>), 6.30; available K (mg Kg<sup>-1</sup>), 381.9; Also, total count of bacteria, 197 x 10<sup>6</sup> CFU/g; total count of fungi, 88 x 10<sup>4</sup> CFU/g and total count of actinomycetes 54 x 10<sup>5</sup> CFU/g according to Allen 1959.

The experiment was carried out in randomized complete block design with four replicates. Area of the experimental plot was 3×3 m with 5 rows, and 225 seeds were sown in each plot (45 seed per row); soybean seeds were planted at the rate of 3 seeds per hole with 20cm space. The used seeds of soybean (*Glycine max* L.) C.V. Giza 111 in the current field experiment were kindly supplied by Field Crops Research Institute, Department of Leguminous Crops, Sakha, Agricultural Research Station, Egypt. It comprised 2 treatment (uninfested and infested soils) and 6 inoculation treatments. The inoculation treatments were prepared as peat-based inoculums, 15 ml of culture per 30 g of sterilized carrier and mixed with the seeds before sowing using a sticking material. Also, the foliar spray was undertaken with *Methylobacterium* broth cultures about (1 × 10<sup>9</sup> CFU ml<sup>-1</sup> of the culture), then diluted to 1:100 and sprayed at 30 and 60 days from sowing (Meenakshi and Savalgi, 2009).

The present experiment tested 6 different biocontrol agents for *R. solani* under infested and uninfested soil with *R. solani* (Treatments from

1-6 are uninfested and from 7-12 are infested) as follow :

Treatment Symbol	Description
T1	Control (no inoculation, no foliar spray)
T2	Inoculation with <i>B. japonicum</i> (St. 110)
T3	Inoculation with <i>B. japonicum</i> (St. 110) + <i>M. aminovorans</i> + <i>B. megatherium</i> var. <i>phosphaticum</i> (B6) + <i>T. viride</i>
T4	Inoculation with <i>B. japonicum</i> (St. 110) + <i>M. rhodinum</i> + <i>B. megatherium</i> var. <i>phosphaticum</i> (B6) + <i>T. viride</i>
T5	Inoculation with <i>B. japonicum</i> (St. 110) + <i>M. aminovorans</i> + <i>B. megatherium</i> var. <i>phosphaticum</i> (B6) + <i>T. viride</i> + foliar spray with <i>M. aminovorans</i>
T6	Inoculation with <i>B. japonicum</i> (St. 110) + <i>M. rhodinum</i> + <i>B. megatherium</i> var. <i>phosphaticum</i> (B6) + <i>T. viride</i> + foliar spray with <i>M. rhodinum</i>

#### Growth parameters

At thirty and sixty days after sowing, plant samples were taken to determine shoot and root dry weights (g plant<sup>-1</sup>), total chlorophyll, nitrogen, phosphorus and potassium contents (%) in shoot and root systems. Also, number of nodules and dry weight of nodules (mg plant<sup>-1</sup>) at 45 and 60 days, while number of pods, test weight (g 100 seeds<sup>-1</sup>), seeds yield (ton ha<sup>-1</sup>).

Total carbohydrate contents were extracted from dry finely ground soybean seeds (powdered), and extracted according to Herbert et al. (1971) and estimated colourimetrically by the phenol-sulphuric acid method as described by Montgomery (1961). For protein content was calculated as total N × 6.25 (Allen, 1953), were also recorded but at harvest. On the other hand, enzyme activity of dehydrogenase, urease, and phosphatase was recorded at 30, 60 and harvest.

#### Dehydrogenase activity

Dehydrogenase activity in the soil samples was determined by following the procedure as described by Casida et al. (1964). Ten grams of soil and 0.2 g CaCO<sub>3</sub> were thoroughly mixed and dispensed in test tubes. To each tube, one ml of 3% aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC), one ml of 1% glucose solution and eight ml of distilled water which was sufficient to leave a thin film of water above the soil layer were added. The tubes were stoppered with rubber cork and incubated at 30°C for 24 h. At the end of incubation period, the contents of the tube were rinsed down into a small beaker

and slurry was made by adding 10 ml methanol. The slurry was filtered through whatman No. 50 filter paper. Repeated rinsing of soil with one ml methanol was continued till the filtrate ran free of red colour. The filtrate was pooled and make up to 50 ml with methanol in a volumetric flask. The intensity of red colour was measured at 485 nm against a methanol blank using UV/Visible Spectrophotometer (Model 6705). The concentration of formazan in soil samples were determined by reference to a standard curve prepared by using graded concentration of formazan. The results were expressed as mg of triphenyl formazan (TPF) formed g<sup>-1</sup> soil per day.

#### Urease activity

The procedure used to determine the urease activity of soil was essentially the same as adopted by Pancholy and Rice (1973) except the ammonia liberated due to hydrolysis of urea in the reaction mixture was determined by Nesslerization as described by Jackson (1973). five grams each of freshly collected soil samples were placed in 100 ml capacity Erlenmeyer flasks to which 0.5 ml toluene was added and allowed to stand for 15 min to permit complete penetration into soil. Each of these flask were added with 10 ml of phosphate buffer (17.85 g KH<sub>2</sub>PO<sub>4</sub> per 500 ml added to 500 ml solution of K<sub>2</sub>HPO<sub>4</sub> containing 20.66 g, pH 7.6) and 10 ml of 10% urea solution. For control flasks, urea solution was replaced by equal quantity of distilled water. The contents of the flask were well shaken for five minutes and incubated at 30°C for 24 h. After incubation, the contents of the flasks were filtered through filter

paper Whatman No. 42. The remaining soil in the flask was added with 15 ml of 1 N KCl solution shaken for five minutes and filtered. The volume of the total filtrate was made up to 100 ml in the volumetric flask using distilled water. One ml filtrate of each sample was transferred to a 50 ml volumetric flask, to which one ml of 10% sodium and potassium tartrate and one ml of 1% gum acacia solution and 5 ml of Nessler's reagent was added (Hg 3%, KI 3.5%, NaOH 12% and Water 81.5%). The volume was made to 50 ml with distilled water. The yellow colour developed after 30 minutes was measured at 410 nm using UV/Visible spectrophotometer (Model 6705) against the reagent blank. The results obtained were expressed as mg of ammonia liberated per gram soil per day with reference to a standard curve outlined by using graded concentrations of  $(\text{NH}_4)_2\text{SO}_4$  solution and developing the colour by nesslerization.

#### Phosphatase activity

Phosphatase activity of soil samples was determined by following the procedure of Tabatabai (1982). One gram of soil sample was placed in 50 ml Erlenmeyer flask to which 0.2 ml toluene followed by 4 ml modified universal buffer (MUB) solution. One milliliter of P-nitrophenyl phosphate solution was added which dissolve 0.42 g of disodium p- nitrophenyl phosphate tetrahydrate in 40 ml of (MUB) pH11 and dilute the solution to 50 ml with MUB of the same pH and store the solution in a refrigerator, mixed well, and the flask was capped with a rubber. This mixture was incubated at 37°C for 1 h. After incubation, 1 ml of 0.5 M  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$  and 4 ml of 0.5 M NaOH were added and the solution was mixed thoroughly. This mixture was filtered through filter paper Whatman no. 42. The intensity of yellow colour developed was measured at 420 nm against the reagent blank using UV/visible Spectrophotometer (model 6705). Control were maintained for each soil sample and were analyzed by following the same procedure described above except that the paranitrophenol phosphate solution was added after the addition of 0.5 M  $\text{CaCl}_2$  and 0.5 M NaOH and just before filtration. The phosphatase activity in the soil samples was expressed as  $\mu\text{g}$  paranitrophenol formed per gram soil per hour with reference to the standard curve prepared by using graded concentrations of P-nitro phenol phosphate.

#### Disease assessment

Percentage of pre-emergence damping off was determined after 15 days as (Nawar, 2007):

$$\% \text{ pre-emergence} = \frac{\text{No. of ungerminated seeds}}{\text{No. of total sown seeds}} \times 100$$

Percentage of post-emergence damping off was determined after 30 days (Nawar, 2007):

$$\% \text{ post-emergence} = \frac{\text{No. of died seedlings}}{\text{No. of survival plants}} \times 100$$

Percentage of diseased plants was determined after 60 days (Nawar, 2007):

$$\% \text{ diseased plants} = \frac{\text{No. of infested plants with root-rot}}{\text{No. of survival plants}} \times 100$$

#### Statistical analysis

Data obtained from experiment treatments were subjected to the analysis of variance and treatments means were compared using the L.S.D. method according to Steel and Torrie (1980).

## Results and Discussion

#### Disease assessment

Data of Table 1 showed that all treatments decreased damping off % and increased number of healthy plants as compared to control (T1). In the infested soil, the inoculation with the different bio-inoculants revealed variable decreases in damping off plants. The lowest pre-emergence damping off plants (15 days), were recorded for the treatments T3, T4, T5 and T6 (33.33 %), compared to un-inoculated control (66.66 %). Under uninfested soil with *R. solani*, the highest percentage of pre-emergence damping off was 33.33 % in T1 treatment compared with the lowest percentage (0.00%), for all treatments under study. Also, the post-emergence percentages of damping off plants (30 days), showed notable decreases due to the effect of different studied bio-inoculants, all treatments increased healthy plants, otherwise all germinated seeds in infested soil with *R. solani* were died in control plots. Concerning the percentage of diseased plants (60 days), all treatments recorded 0.00% which

meant damping off % is decreased due to different bio-inoculants under study. Ashour and Afify (1999, 2000) studied that *Bacillus* strains and *Pseudomonas* spp. mixtures to improve biological control in both cotton and flax seedlings under field conditions. Data showed increasing stand and yield however; their efficiency was much higher when they were applied in a mixture of *Bacillus* strains in cotton. As well as the mixture were more effective in increasing seed yield and straw yield in field trails in flax. Gupta et al.

(2002) found that bacterization of peanut seeds with fluorescent *Pseudomonas* GRC2 increased seed germination, early seedling growth, fresh nodule weight, grain yield and reduced charcoal rot disease of peanut in *M. phaseolina*- infested soil as compared to control. Landa et al. (2004) proved that seed and soil treatments with biocontrol agents *Bacillus megaterium*, *B. subtilis* and *Pseudomonas fluorescens* significantly reduced chickpea *Fusarium* wilt disease intensity and increased chickpea seed yield.

**TABLE 1. Effect of different bio-inoculant treatments on the percentage of damping off disease of soybean plants under uninfested and infested soils.**

Treatments	Pre-emergence %		Post-emergence %		Diseased plants %	
	Uninfested	Infested	Uninfested	Infested	Uninfested	Infested
T1	33.33	66.66	0.00	100.00	0.00	0.00
T2	0.00	66.66	0.00	0.00	0.00	0.00
T3	0.00	33.33	0.00	0.00	0.00	0.00
T4	0.00	33.33	0.00	0.00	0.00	0.00
T5	0.00	33.33	0.00	0.00	0.00	0.00
T6	0.00	33.33	0.00	0.00	0.00	0.00

T<sub>1</sub>: Control; T<sub>2</sub>: inoculation with *B. japonicum*; inoculation with *B. japonicum* + *B. megatherium* + *T. viride* + *M. aminovorans*; T<sub>4</sub>: inoculation with *B. japonicum* + *B. megatherium* + *T. viride* + *M. rhodinum*; T<sub>5</sub>: T<sub>3</sub> + foliar spray with *M. aminovorans* , T<sub>6</sub>: T<sub>4</sub> + foliar spray with *M. rhodinum*

#### Number of nodules

As shown in Fig. 1 and 2, the inoculation with the varied bio-inoculants gave significant differences than un-inoculated plants in both infested and non-infested soils. At the same time, the infestation with *R. solani* relatively decreased number of nodules compared to those of uninfested soil. At 45 days, the treatments T<sub>4</sub>, T<sub>5</sub> and T<sub>6</sub> recorded the highest number of nodules (62.3, 65.0 and 63.0 nodule plant<sup>-1</sup> in case of uninfested soil, and 46.7, 44.0 and 49.7 nodule plant<sup>-1</sup> for infested soil, respectively).

At 60 days, the increase of nodules number was observed in all treatments, seed inoculation with *M. rhodinum*, *B. japonicum* , *B. megatherium* , *T. viride* and foliar spray with *M. rhodinum* recorded maximum number of nodules 73.3 plant<sup>-1</sup> and 56.3 plant<sup>-1</sup> for uninfested and infested soils compared to control, respectively. The increase in the number of nodules can be attributed to the presence of rhizobia in the legume rhizosphere influencing the legume roots to release growth promoting substances. The maximum number

of nodules due to nodulation of two or more beneficial organisms over single inoculation and un-inoculated control has been reported (Rao and Dhir 1993; Balachander and Nagarajan 1999).

Radha (2007) and Meenakshi and Savalgi (2009) studied soybean plant and observed that inoculation of soybean with Bradyrhizobium increased the nodulation parameters, nitrogen fixation and yield of crop. Also, inoculation with *B. japonicum*, *Bacillus cereus* (BC), *Bacillus megatherium* (BM) and *Pseudomonas fluorescens* (PF) increased nodulation parameters and healthy plants of soybean under field conditions compared to control (Attia et al. 2011).

#### Dry weight of nodules (g plant<sup>-1</sup>):

Figure 3 and 4 Show significant variations in the nodules dry weight due to combined inoculation of seed with *M. aminovorans*, *M. rhodinum*, *B. japonicum*, *B. megatherium*, *T. viride* and foliar spray with *Methylobacterium* isolates at 45 and 60 day under uninfested and infested soils with *R. solani*. No nodules were found in control

plants grown in infested and uninfested soils with *R. solani*. At 45 day, significantly higher nodules dry weight of 0.63 g plant<sup>-1</sup> and 0.51 g plant<sup>-1</sup> were recorded for T5 (*M. aminovorans*, *B. japonicum*, *B. megaterium*, *T. viride* and foliar spray with *M. aminovorans*) under uninfested and infested soils compared to T2 treatment 0.29 to 0.23 g plant<sup>-1</sup>, respectively.

Similar trend for dry weight of nodules (g plant<sup>-1</sup>) is measured at 60 days after sowing. The treatments T5 and T6 were the superior in this context under either infested or uninfested circumstances, where T5 recorded 0.76 and 0.54 g

plant<sup>-1</sup>, whereas T6 recorded 0.73 and 0.52 g plant<sup>-1</sup> for uninfested and infested soils, respectively. This increase in dry weight of nodules may be due to the effective *Bradyrhizobium* and methylotrophs strains used in the applied inoculum as well as *B. megaterium*. Our results are in agreement with those reported by Holland and Polacco (1994) on soybean, they attributed the improvement in dry weight of nodules to the presence of rhizobia in the rhizosphere. Also, Radha (2007) and Meenakshi and Savalgi (2009) observed that inoculation of soybean with *Bradyrhizobium* increased the nodulation parameters, dry weight of nodules, nitrogen fixation and yield of crop.

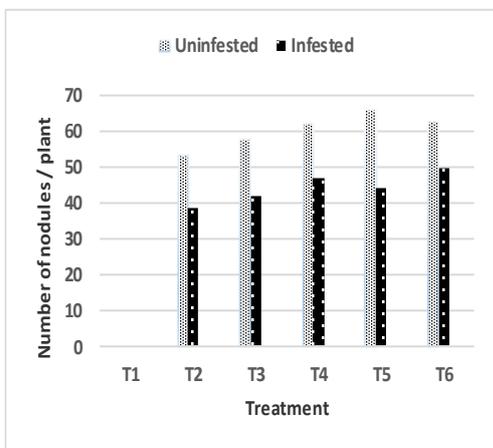


Fig. 1. Effect of different bio-inoculant treatments on number of nodules plant<sup>-1</sup> of soybean plant under uninfested and infested soils at 45 day.

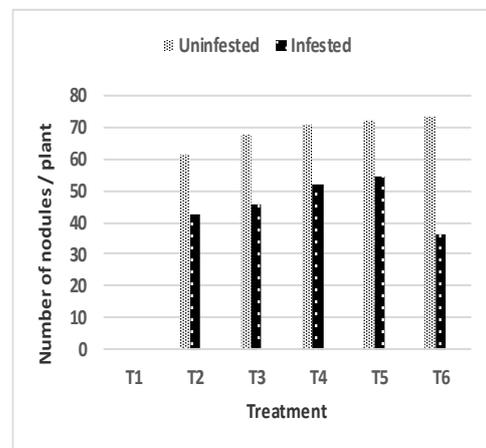


Fig. 2. Effect of different bio-inoculant treatments on number of nodules plant<sup>-1</sup> of soybean plant under uninfested and infested soils at 60 day.

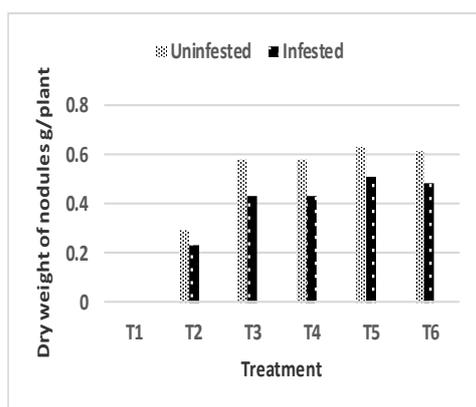


Fig. 3. Effect of different bio-inoculant treatments on dry weight of nodules plant<sup>-1</sup> of soybean plant under uninfested and infested soils at 45 day.

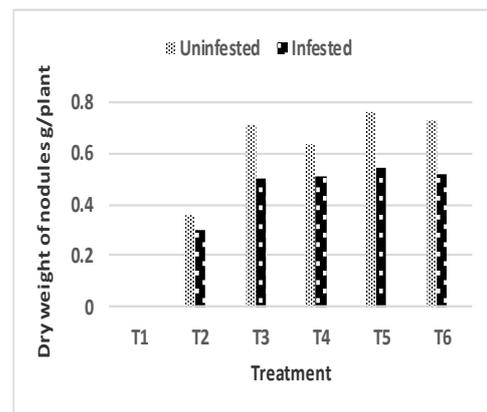


Fig. 4. Effect of different bio-inoculant treatments on dry weight of nodules plant<sup>-1</sup> of soybean plant under uninfested and infested soils at 60 day

*Nitrogen, phosphorus and potassium (%) in shoot and root*

Data in Table 2 revealed that there is an increase in nitrogen, phosphorus and potassium percentage with the application of the different microbial strains under either uninfested or infested soils with *R. solani*. In general, the treatments T5 and T6 recorded the highest value of nitrogen, phosphorus and potassium percentage for shoot and root at different growth stages. The highest N content was found in treatments 5 and 6, where it recorded 1.047 and 1.043% for non-infested soils

and 0.940 and 0.963 for infested soils at 30 days; 3.147 and 3.140 for non-infested soils and 2.870 and 2.827 for infested soils at 60 days. But for uninfested and infested soils for root at 30 days, the seed inoculated with combined bio-inoculants along with foliar spray (T5 and T6 treatments) showed the highest records of N% (0.657 and 0.637) and (0.553 and 0.553), respectively. On the other hand, the highest value was recorded at 60 days for T5 treatment (1.983 and 1.817) for uninfested and infested soils compared to control and other treatments.

**TABLE 2. Effect of different bio-inoculant treatments on nitrogen, phosphorus and potassium contents of shoot and root of soybean plant under uninfested and infested soils at 30 and 60 days from sowing.**

Treatments	Shoot N (%)				Root N (%)			
	30 days		60 days		30 days		60 days	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
<b>T1</b>	0.683 c*	0.550 e	2.057 c	1.723 d	0.460 e	0.370 d	1.387 e	1.163 e
<b>T2</b>	0.923 b	0.677 d	2.783 b	2.263 c	0.537 d	0.433 c	1.620 d	1.340 d
<b>T3</b>	0.950 b	0.777 c	2.867 b	2.503 b	0.587 c	0.490 b	1.763 c	1.447 c
<b>T4</b>	1.017 a	0.877 b	3.057 a	2.600 b	0.613 bc	0.487 b	1.860 bc	1.577 b
<b>T5</b>	1.047 a	0.940 a	3.147 a	2.870 a	0.657 a	0.553 a	1.983 a	1.817 a
<b>T6</b>	1.043 a	0.963 a	3.140 a	2.827 a	0.637 ab	0.553 a	1.927 ab	1.750 a
	Shoot P (%)				Root P (%)			
	30 days		60 days		30 days		60 days	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
<b>T1</b>	0.153 c	0.120 d	0.323 d	0.253 c	0.100 e	0.090 d	0.230 d	0.180 d
<b>T2</b>	0.183 b	0.147 c	0.370 c	0.313 b	0.123 d	0.103 cd	0.277 c	0.210 cd
<b>T3</b>	0.212 a	0.170 bc	0.427 b	0.357 a	0.140 c	0.110 c	0.330 b	0.237 bc
<b>T4</b>	0.213 a	0.167 bc	0.433 b	0.367 a	0.157 b	0.127 b	0.330 b	0.270 ab
<b>T5</b>	0.217 a	0.183 ab	0.450 b	0.387 a	0.167 b	0.140 ab	0.350 b	0.297 a
<b>T6</b>	0.233 a	0.197 a	0.490 a	0.387 a	0.193 a	0.150 a	0.387 a	0.290 a
	Shoot K (%)				Root K (%)			
	30 days		60 days		30 days		60 days	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
<b>T1</b>	0.153 c	0.120 d	0.323 d	0.253 c	0.100 e	0.090 d	0.230 d	0.180 d
<b>T2</b>	0.183 b	0.147 c	0.370 c	0.313 b	0.123 d	0.103 cd	0.277 c	0.210 cd
<b>T3</b>	0.212 a	0.170 bc	0.427 b	0.357 a	0.140 c	0.110 c	0.330 b	0.237 bc
<b>T4</b>	0.213 a	0.167 bc	0.433 b	0.367 a	0.157 b	0.127 b	0.330 b	0.270 ab
<b>T5</b>	0.217 a	0.183 ab	0.450 b	0.387 a	0.167 b	0.140 ab	0.350 b	0.297 a
<b>T6</b>	0.233 a	0.197 a	0.490 a	0.387 a	0.193 a	0.150 a	0.387 a	0.290 a

\* In a column means followed by a common letter are not significantly different at 5% level by DMRT.

In case of P content (%), the results showed that the influence of the studied bio-inoculants on the shoot P content had a similar trend whether in infested or uninfested soils. T6 treatment gave, 0.233 and 0.197 followed by T5, 0.217 and 0.183 with regard to control (T1) which exhibited 0.153 and 0.120 at 30 days under uninfested and infested soils, respectively. However, the highest value was recorded at 60 days for T6 treatment

(0.490 and 0.387) for uninfested and infested soils compared to control and other treatments. On the other hand, the results of root P content had a similar trend as shoot P content except the high values of P content of shoot compared to root. Values of P content in plants of treatment (T6) were 0.193 and 0.150 at 30 days; and 0.387 and 0.290 at 60 days under uninfested and infested soils.

In case of K content (%) the results showed an increase of K content of shoot ranged from 0.097 and 0.057 for T6 to 0.067 and 0.047 for T2 compared to 0.057 and 0.037 for control (T1) at 30 days. Moreover, the highest value was recorded at 60 days for T6 treatment (0.197 and 0.163) for non-infested and infested soils. On the other hand, the highest percentages of K content in roots were noted for T4, which recorded 0.063 and 0.040 followed by T5 which recorded 0.060 and 0.040 at 30 days for uninfested and infested plants. At 60 days, the highest value was found in T6 (0.130 and 0.107) for uninfested and infested plants.

The increase in N, P and K uptake due to combined inoculation of two or more microorganisms has been documented by several researchers (Hoflich *et al.* 1995; Biswas *et al.* 2000 and Senthilkumar 2003) they discussed the increase in nutrient use efficiency when inoculated with *Rhizobium leguminosarum* bv. trifolii in wheat, corn, radish, mustard, rice and by ((insert full name of PPFMs)) PPFMs in soybean, respectively.

Meenakshi and Savalgi (2009) and Radha *et al.* (2009) who worked on soybean plant and they observed that inoculation of soybean plants with *Bradyrhizobium* and PPFMs has been reported to increase the uptake of N, P and K in shoot and root, nodulation and yield of crop as compared to control. Root colonizing and plant growth promoting bacteria affect plant growth by increasing nutrient cycling, suppressing pathogens and producing biologically active compounds (Khalid *et al.* 2004). Additionally, Meenakshi and Savalgi (2009) showed that the increase in nitrogen, phosphorus and potassium percentages were found in soybean plants due to different inoculations with *B. japonicum*, *B. megaterium* and methylotrophs isolates.

#### *Number of pods, test weight (g 100 seeds<sup>-1</sup>) and seed yield (ton ha<sup>-1</sup>)*

Data presented in Table 3 indicated that inoculation with the different bio-inoculants variably increases number of pods, test weight and seed yield of soybean plants.

Number of pods plant<sup>-1</sup> was significantly increased due to combined inoculation with *Methylobacterium* and *B. japonicum* and further foliar spray with *Methylobacterium* isolates. Statistically, significant results were recorded in T5 treatment (seed inoculation with *B. japonicum*, *B.*

*megaterium*, *T. viride*, *M. aminovorans* and foliar spray with *M. aminovorans*) showing more number of pods (172.3 and 140.0) which was significantly superior over control (81.7 and 83.7) for uninfested and infested soils. On the other hand, the maximum value of test weight of 100 seeds was observed in T5 followed by T6 and T4 (22.20, 21.92 and 21.28 g 100 seeds<sup>-1</sup>) and (21.20, 20.85 and 20.30 g/100 seeds) for uninfested and infested soils, respectively compared to chemical fertilizer (control).

Generally, all treatments under uninfested soils recorded the higher values of seed yield (kg plot<sup>-1</sup>) compared to infested soils. The treatments T5 and T6 recorded the highest seed yield (4.700 and 4.600 ton ha<sup>-1</sup>) in case of uninfested soils, and 4.272 and 4.260 ton ha<sup>-1</sup> for infested soils) compared to T1 (control), 3.940 and 3.468 ton ha<sup>-1</sup> in uninfested and infested soils, respectively. The maximum increase in yield parameters due to dual or combined inoculation of PGPRs was documented. The increase in the yield due to compatible nature of *Methylobacterium* and *Bradyrhizobium* which was established by (Senthilk *et al.* 2002; Madhaiyan *et al.* 2004 and Radha *et al.* 2009) due to their compatible nature, combined influence and foliar spray by methylotrophs which are PGPR bacteria and on rhizosphere by *rhizobium* which is nitrogen fixing bacterium might have resulted in increased plant growth and yield parameters.

#### *Percent of total carbohydrate and protein in seeds*

Table (4) depicted the results of total carbohydrate and protein content in seeds of soybean. Significant variations in total carbohydrate content in seeds were observed under uninfested soils which recorded the higher value in T5 and T6 treatments *i.e.*, 32.400 and 30.627% compared to 20.743% for T1 (control). But under infested soils, higher carbohydrate content was measured in plants of treatment T6 (26.983%) followed by treatment T5 (26.423%) as compared to 18.760% for T1 (control).

The results showed an increase in protein content with the application of different microbial strains under uninfested and infested soils with *R. solani*. Although, the reading of infested treatments obviously lower than those of uninfested ones. The treatments T5 and T6 recorded the highest values which they were 41.020 and 40.170% for uninfested soils and 39.330 and 38.627 for infested soils compared to T1 (control) 37.607 and 36.670% under uninfested and infested plants.

**TABLE 3. Effect of different bio-inoculant treatments on number of pods, test weight and seed yield of soybean plants under uninfested and infested soils.**

Treatments	Number of pods		Test weight (g100 seeds <sup>-1</sup> )		Seed yield (ton ha <sup>-1</sup> )	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
T1	81.7 e*	83.7 c	19.28 d	19.13 d	3.940 f	3.468 e
T2	105.7 d	93.3 c	19.86 c	19.69 c	4.166 e	3.741 d
T3	144.0 b	120.7 b	20.26 c	19.79 c	4.382 d	3.873 c
T4	131.0 c	112.7 b	21.28 b	20.30 b	4.430 c	4.072 b
T5	172.3 a	140.0 a	22.20 a	21.20 a	4.700 a	4.272 a
T6	149.0 b	137.3 a	21.92 a	20.85 a	4.600 b	4.260 a

\* In a column means followed by a common letter are not significantly different at 5% level by DMRT.

**TABLE 4. Effect of different bio-inoculants on total carbohydrate and protein content in seeds of soybean plants under uninfested and infested soils.**

Treatments	Total carbohydrate (%)		Protein (%)	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
T1	20.7 d*	18.8 d	37.7 c	36.7 d
T2	22.8 cd	21.0 cd	38.9 b	37.4 cd
T3	23.7 c	21.4 c	40.0 a	38.2 bc
T4	27.5 b	24.4 b	40.2 a	38.7 ab
T5	32.4 a	26.4 ab	41.0 a	39.3 a
T6	30.7 a	27.0 a	40.2 a	38.6 ab

\* In a column means followed by a common letter are not significantly different at 5% level by DMRT.

In general, it is important to estimate carbohydrate and protein contents in the yielded seeds of soybean plant for seed quality assessment. These results coincided with those of other workers who reported that increasing growth, total carbohydrates, protein and oil contents in the yielded seeds with using different biofertilizers (Abdelhamid and El-Metwally 2008; El-Rokiek et al. 2010). Also, Raj et al. (2004); Meenakshi (2008) and Radha et al. (2009) who reported an increase of protein content due to different inoculations with *B. japonicum*, *B. megaterium* and methylotrophs isolates.

#### *Dehydrogenase, urease and phosphatase activities*

Data of Table 5 revealed an increase in dehydrogenase, urease and phosphatase activities with the application of the different treatments under uninfested and infested soils with *R. solani*. The dehydrogenase activity was noted to decline with increasing plant age. In general, the treatments T5 and T6 recorded the highest values at all growth stages, they recorded 250.630 and 231.550 mg TPF g<sup>-1</sup> soil day<sup>-1</sup> for uninfested soils

and 149.350 and 171.537 mg TPF g<sup>-1</sup> soil day<sup>-1</sup> for infested soils at 30 days; 206.737 and 206.200 mg TPF g<sup>-1</sup> soil day<sup>-1</sup> for uninfested soils and 177.943 and 175.097 mg TPF g<sup>-1</sup> soil day<sup>-1</sup> for infested soils at 60 days; 148.003 and 143.687 mg TPF g<sup>-1</sup> soil day<sup>-1</sup> for uninfested soils and 117.690 and 124.023 mg TPF g<sup>-1</sup> soil day<sup>-1</sup> for infested soils at harvest.

On the other hand, urease activity was shown to rise due to inoculation treatments compared to the respective control for each plant age under uninfested or infested circumstances. Also, it is clearly showed that urease activity levels decreased with the increase in plant age. T6 treatment showed significant maximum urease activity (115.190 and 105.420 mg g<sup>-1</sup> d<sup>-1</sup>) followed by T5 (113.647 and 103.037 mg g<sup>-1</sup> d<sup>-1</sup>) compared to control (93.563 and 84.330 mg g<sup>-1</sup> d<sup>-1</sup>) for uninfested and infested soils at 30 days. But for uninfested and infested soils at 60 days, the seed inoculated with combined bio-inoculants along with foliar spray (T5 and T6 treatments) showed the highest records of urease activity (203.337

and 200.330 mg g<sup>-1</sup> d<sup>-1</sup>) and ( 166.797 and 167.213 mg g<sup>-1</sup> d<sup>-1</sup>). On the other hand, the highest value was recorded at harvest for T5 treatment (125.803 and 111.113 mg g<sup>-1</sup> d<sup>-1</sup>) for uninfested and infested soils compared to control and other treatments.

At 30 days after sowing, phosphatase activity of soil recorded the highest values (8.011 and 5.632 µg g<sup>-1</sup> h<sup>-1</sup>) as a result of inoculation with *B. japonicum*, *B. megaterium*, *T. viride*, *M. aminovorans* and foliar spray with *M. aminovorans*) significantly superior over all seed inoculation treatments and control (3.131 and 2.915 µg g<sup>-1</sup> h<sup>-1</sup>) for uninfested and infested soils. While, at 60 days, phosphatase activity of soil showed that T5 and T6 treatments recorded 11.021 and 10.966 µg g<sup>-1</sup> h<sup>-1</sup> for uninfested soils and 6.940 and 6.515 µg g<sup>-1</sup> h<sup>-1</sup> for infested soils. Phosphatase activity in soil generally reduced with the aging of the plant. In the entire crop period, the enzyme activity increased initially at 30 and 60 days and then declined with the age of the crop (Singaram and Kamalakumari 1995). These observations are in accordance with the findings of

the present investigation. More than the microbial population and the enzyme activities are regulated by the soil characters like organic carbon, pH and nutrient status (Nagaraja *et al.* 1998). Chendrayan *et al.* (1980) showed that increases in dehydrogenase activity has mainly due to the higher microbial population, they also claimed that the earlier studies revealed that the enzyme activities are often used as indices of microbial growth rather than the microbial number, which further may reflect the microbial respiration and the potential capacity of soil to perform biological transformations of importance to soil fertility. Meenakshi (2008) reported that urease activities in rhizosphere soil of soybean were significantly higher in treatment that inoculated with *B. japonicum* and *Methylobacterium* isolates at all the stages of crop growth when compared with control (recommended dose of N,P and K) and all other treatments. Additionally, Meenakshi and Savalgi (2009) found increase in phosphatase activity was found in rhizosphere soybean plants inoculated with different inoculations of *B. japonicum*, *B. megaterium* and methylotrophs isolates.

**TABLE 5. Effect of different bio-inoculants on dehydrogenase, urease and phosphatase activities in rhizosphere of soybean plants under uninfested and infested soils at different soybean growth stages.**

Treatments	Dehydrogenase activity (mg TPF g <sup>-1</sup> soil d <sup>-1</sup> )					
	30 days		60 days		At harvest	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
T1	103.723 d*	87.867 e	133.760 e	117.550 d	97.033 d	79.507 e
T2	117.183 d	99.097 de	167.943 d	140.333 c	103.617 d	89.483 d
T3	152.837 c	107.127 d	180.343 c	141.450 c	117.633 c	98.633 c
T4	151.873 c	130.420 c	196.697 b	160.760 b	124.957 b	110.587 b
T5	250.630 a	149.350 b	206.737 a	177.943 a	148.003 a	117.690 ab
T6	231.550 b	171.537 a	206.200 a	175.097 a	143.687 a	124.023 a
	Urease activity (mg NH <sub>4</sub> <sup>+</sup> -N g <sup>-1</sup> soil d <sup>-1</sup> )					
	30 days		60 days		At harvest	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
T1	93.563 d	84.330 d	155.937 e	132.090 c	105.443 d	99.670 c
T2	103.377 c	92.357 c	175.230 d	155.817 b	113.535 c	103.910 b
T3	107.810 b	100.513 b	186.013 cd	172.353 a	113.313 c	104.747 b
T4	107.367 b	102.870ab	190.487 bc	166.613 ab	118.197 b	108.857 a
T5	113.647 a	103.037ab	203.337 a	166.797 ab	125.803 a	111.113 a
T6	115.190 a	105.420 a	200.330 ab	167.213 ab	124.650 a	111.083 a
	Phosphatase activity (µg pnp g <sup>-1</sup> soil h <sup>-1</sup> )					
	30 days		60 days		At harvest	
	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>	<i>Uninfested</i>	<i>Infested</i>
T1	3.131 d	2.915 d	4.321 d	3.866 c	1.600 e	1.117 d
T2	4.847 c	3.496 cd	6.302 c	4.208 c	2.123 d	1.331 cd
T3	5.866 b	4.218 bc	7.137 bc	5.381 b	2.553 c	1.490 bc
T4	6.272 b	4.656 b	7.773 b	5.451 b	2.633 c	1.675 b
T5	8.011 a	5.632 a	11.021 a	6.940 a	3.344 a	2.121 a
T6	7.682 a	4.823 ab	10.966 a	6.515 ab	3.063 b	2.094 a

\* In a column means followed by a common letter are not significantly different at 5% level by DMRT.

## Conclusions

Application of inoculation with *B. japonicum* (St. 110) + *M. aminovorans* + *B. megatherium* var. *phosphaticum* (B6) + *T. viride* at the time of planting and foliar spray with *M. aminovorans* at 30 and 60 days as described herein could be recommended for controlling damping-off and wilt of soybean plants as well as increased the activities of most soil enzymes, especially dehydrogenase, urease, and phosphatase and enhancement the vegetative growth and seed yield.

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