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Original Research Article

Development and Evaluation of Nitrogen Fixing and Phosphate Solubilizing Microbial Consortia on Spinach (*Spinacia oleracea*)

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A B S T R A C T

Soil is a very complicated natural ecosystem that acts as a pool for all the plant nutrients that are in fixed and available form. These nutrients are fixed in the soil by chemical reactions and ultimately influencing the non-availability of nutrients to the plants. However, microbial mineral interactions have several implications for the field of agriculture and rhizosphere microbes play an important role in biogeochemical cycling of nutrients and also making the mineral nutrients available to the plant by their metabolic processes. In an attempt to isolate, characterize and screen native associative nitrogen fixing and phosphorus solubilizing bacteria from the rhizosphere soil of spinach from. Eight *Azospirillum* and four phosphate solubilizing bacterial isolates were obtained from five soil and root samples collected from rhizosphere and non rhizosphere soils of Spinach and Amaranthus. Out of eight *Azospirillum* isolates screened for nitrogen fixing potentiality, the isolate number Asp-4 fixed maximum of 5.2 mg of nitrogen per g of carbon source used, Hence Asp- 4 was selected for further studies. On the other hand out of 4 phosphate solubilizing isolates the maximum of inorganic phosphorus (7.40 %) was released by PSB- 1 into the medium, Hence, PSB- 1 is selected. Further, the efficient *Azospirillum* (Asp- 4) and PSB (PSB-1) when evaluated on growth and yield of spinach resulted in better yield. With reference to germination percentage, maximum germination percentage was observed where the combined inoculation of *Azospirillum* and PSB. Similarly, the combined inoculation also resulted in maximum number of leaves, increased fresh and dry weight, chlorophyll content of spinach. Finally, the treatment *i.e., Azospirillum* + PSB + compost + RDF) showed maximum accumulation of nitrogen and phosphorus in the leaves of spinach after harvest. Scale of studies and further required to formulate different formulation of the efficient *Azospirillum* and PSB microbial consortia along with other PGPRs to evaluate in multi-location farm trials.

Keywords

Azospirillum, Bacterial Consortia, Growth and Development, Spinach

Introduction

Biofertilizers are more commonly known as microbial inoculants, which are artificially multiplied cultures of certain soil organisms that can improve soil fertility and crop productivity. Although the beneficial effects of legumes in improving soil fertility was known since ancient times and their role in biological nitrogen fixation was discovered more than a century ago, commercial exploitation of such biological processes is of recent interest and practice in organic agriculture. Biofertilizers have various benefits besides accessing nutrients, for current intake as well as residual, different

biofertilizers also provide growth factors to plants and some have been successfully facilitating composting and effective recycling of solid wastes. By controlling soil borne diseases and improving the soil health and soil impurities. These organisms help not only in saving, but also in effective in utilizing chemical fertilizers and results in higher yield rates.

Now a days, the application of these biofertilizers in the leafy vegetable production is gaining lot of importance because of their cost effectiveness and eco-friendliness. Different microorganisms like *Azospirillum, Azotobacter*, PSB like *Bacillus megaterium* and other biocontrol agents like *Trichoderma harzianum*, *Pseudomonas fluorescence* are used in the leafy vegetables production for increasing the yield and to reduce diseases incidence.

The spinach (*Spinacia oleracea*) belongs to the family of *Amaranthaceae* is one of the ancient and popular leafy vegetable which is grown in South East Asian Countries. Presently, it is grown throughout the tropical regions of Asia, Africa, and America etc., and it has reached Europe by 8th century. Spinach plants are grown for the edible green leaves. This plant needs temperate climate and may survive in temperate regions. The leaves are alternate simple ovate to triangular based very variable in size from about 2-30 cm long and 1-15 cm broad. When allowed to grow this spinach unharvested, this annual plant will go to seeds in late summer allowing the leaves to die off. The maximum duration of spinach is 30-45 days.

Presently, biological means for production of agriculture commodities is gaining lot of importance, among biological means, microorganisms being integral components of soil ecosystem play a prestigious role by making the soil truly living. These organisms have evolved many mechanisms such as antibiosis, competition, parasitism, resistance induction etc., in plants to provide effective disease suspension and plant growth promotion. The significance of plant growth promotion, rhizosphere competence and the suppression of diseases and pests on the plants is much considered research theme in present days, multiple microbial interactions involving bacterial and fungi in the rhizosphere/phyllosphere are shown to provide enhanced biocontrol and plant growth promotional activities than when used singly. There is a growing interest in the presence of certain naturally occurring, beneficial microorganisms in agricultural lands.

Out of sixteen plant nutrients phosphorus is commonly deficient in most of the natural soils, since it is fixed as insoluble iron and aluminium phosphates in acidic soil. (Mc lean, 1976), As a result of the phosphorus fixation some of the micronutrients are unavailable to the plants. Out of these micronutrients Iron (Fe), Manganese (Mn), Aluminium (Al) are the major ones that form complexes with other nutrients and are unavailable to the plants and ultimately affect the yield.

The effective microorganisms including mixed culture of lactic acid bacteria, photosynthetic bacteria, actinomycetes and other fermenting fungi were evaluated for the control of damping of disease in lettuce (Higa, 1994). Similarly, Zahir *et al.,* (2004) reported the effect of different *Azospirillum* strains on growth and yield of some of the leafy vegetables like lettuce, amaranthus and coriander.

Gaur *et al.,* (1972) reported that *Bacillus firmus* and *Bacillus polymyxa* play important role in plant nutrition through increase in

phosphorus and potassium uptake by plants and thereby increasing crop yield.

Based on the past work done by different researchers and in view of greater need for development of phosphate and nitrogen fixing bacterial consortia for healthy production of Spinach.

Materials and Methods

Collection of soil and root samples

A total of 5 rhizosphere soil and spinach root bit samples were collected from the spinach growing farms in the district of Shivamogga for isolation of associative N-fixing and P-solubilizing bacteria.

Isolation of associative N-fixing bacteria (*Azospirillum*) and Phosphate solubilizing bacteria

Fresh root samples were cut into bits of 1cms length then were washed thoroughly in running tap water and surface sterilized by dipping in 0.1 % $HgCl_2$ solution for three minutes followed by dipping in 70 per cent alcohol for one minute. The roots were finally washed in six to eight changes of sterile distilled water. The root bits were then placed at subsurface level in screw cap tubes containing sterilized semisolid N-free malate medium under aseptic conditions. The tubes were inoculated at 300 C for a period of one week and observed for growth of Azospirillum as subsurface white pellicles. This isolates were purified by repeated sub culturing. A loopful of culture was streaked on malate agar plates containing 1 per cent NH4Cl. After a week of incubation, typical small white dense single colonies were picked and transferred to semisolid N-free malate medium in culture tubes. The isolates that formed characteristic subsurface white pellicle in this medium were tentatively considered as *Azospirillum*. (Okon *et al.,* 1977).

Similarly, the phosphate solubilizing microorganisms were isolated from all the rhizosphere soil samples by dilution plate technique on Pikovskaya's agar medium. The plates were incubated at 280 ± 20 C for seven days and colonies with clear zones around were counted. The representative colonies of each type of bacteria with clear zones around were purified, sub cultured and maintained on the slants of Pikovskaya's agar (Pikovskaya, 1948).

Identification of P-solubilizing and N_2 – fixing bacterial isolation

The phosphorus solubilizing and N_2 fixing bacteria isolated from spinach rhizosphere soils and root samples were identified up to generic level based on the morphological and biochemical tests as specified in Bergery's manual as Determinative Bacteriology (Anon, 1957: Barthalomew and Mittewer, 1950)

In vitro screening of phosphorus solubilizing & Associative N fixing bacteria

Agar plate method

All the phosphorus solubilizing bacterial isolates were spotted on Sperber's media for analyzing the phosphate solubilizing potentiality of each isolates. Based on the zone of solubilization of phosphorus on the media the phosphate solubilizing potentiality was interpreted (Gaur *et al.,* 1990).

Chemical method

Isolates of the phosphate solubilizing bacteria (10 ml of the overnight culture were inoculated to 100 ml of Pikovskaya's broth

in 250 ml flask with equal number of uninoculated controls. The flasks were incubated on a mechanical shaker at 280 C for 10 days. The amount of pi released in the broth in flasks was estimated at 10 days after inoculation. The broth cultures of bacteria were centrifuged at 9000 rpm for 20 minutes in a centrifuge to separate the supernatant from the cell growth and insoluble phosphate. The available pi content in the supernatant/filtrate was estimated by phosphomolybdic blue colour method (Jackson, 1973)

Quantitative estimation of nitrogen by N_2-fixers

To 250 ml conical flasks, 100 ml of the N free semi solid sodium malate medium was dispensed for all flasks and autoclaved. One ml of culture was inoculated to each flask. The flasks were incubated at 37^0 C for seven days. After seven days of incubation the culture was homogenized and 10 ml was digested with 5 ml of concentrated H_2SO_4 along with 0.2 g digestion catalyst mixture K_2SO_4 : $CuSO_4$: Selenium (100:10:1). After cooling, volume was made up to 100 ml with distilled water. Later, 10 ml of aliquot was transferred to microkjeldhal distillation unit, for which 20 ml of 40 per cent NaOH was added and distilled. Ammonia evolved was trapped in 4 per cent boric acid mixed indicator (Bromocresal green 0.066 g and methyl red 0.033 g in 100 ml methanol) till the solution turned from pink to green and then titrated against 0.05 N H_2SO_4 till the green colour is turned to pink and total nitrogen content of the culture was determined and results were expressed as mg of N fixed per g of glucose.

$$\text{Percent N} = \frac{\text{Titre value} \times 0.014 \times \text{N of } H_2SO_4 \times \text{vol. made}}{\text{Volume of sample used}} \times 100$$

Evaluation of selected associative N-fixing and phosphate solubilizing bacteria on plant growth nutrient uptake

Based on the amount of per cent nitrogen fixed by the *Azospirillum* and amount of inorganic phosphorous released by the Phosphate Solubilizing Bacteria the efficient isolates were selected and evaluated for their influence on plant growth under field condition using spinach as the test crop.

Treatment details of the field experiment

T1 = Absolute control
T2 = Control (Compost + RDF)
T3 = *Azospirillum* +Compost +RDF
T4 = PSB + Compost + RDF
T5 = *Azospirillum* + PSB + Compost + RDF

All the data obtained during the course of study were analyzed and interpreted by Duncan's multiple range (DMRT) (Steel and Torrie, 1960).

Results and Discussion

Isolation and Characterization of Azospirillum and phosphorus solubilizing bacteria

As many as eight Azospirillum and four PSB isolates were obtained from the soil and root bits of Amaranthus and Spinach and further, they were named as, Asp-1, Asp-2, Asp-3, Asp-4, Asp-5, Asp-6, Asp-7, and Asp-8 for Azospirillum isolates and for PSB isolates PSB-1, PSB-2, PSB-3, and PSB-4 (Plate 1). Further, the isolates were tentatively identified as Azospirillum and Bacillus species based on morphological and biochemical characters (Table 1 and 2). The results are in agreement with the findings of Gaur *et al.*, 1973, who isolated and characterized three strains of *Bacillus* species from soils of mussooriee and merton

rock phosphate capable of solubilizing tricalcium phosphate.

In support of Gaur *et al.*, (1973), Loaw and Webley, 1959, isolated acid producing bacteria from rhizoplane, rhizosphere soils of oat plant for solubilization of phosphate mineral fertilizers and other related compounds.

In vitro screening of associative nitrogen fixing and phosphate solubilizing bacterial isolates

Statistically highest nitrogen fixation was observed in Asp-4 isolate (5.2 mg/gm of 'C' source followed by Asp-6 and Asp-7 (4.10 & 4.80 mg/gm of 'C' source) respectively. Whereas, the isolate number Asp-1 Asp-2, Asp-3, Asp -5 and Asp-7 where having low nitrogen fixing potentiality compare to other three isolates. Hence, the isolate number Asp-4 was selected for further field studies (Table 3). Similarly, with reference to phosphate solubilization, the highest Pi released was observed in PSB-1 at 10th day (7.40 %) followed by PSB-3 (6.30 %). However the lowest Pi released was found in PSB-4 (5.01 %) and PSB-2 (5.30 %) at the 10^{th} day of incubation respectively. Hence, the isolates number PSB-1 has further field experimental studies (Table 4 and Plate 2).

The persual of table 3 clearly defines the nitrogen fixing potentiality of *Azospirillum* isolates. Out of eight isolated tested, the isolate number Asp-4 was superior as it fixed 5.2 mg of nitrogen/g of 'C' source used. The results are in agreement with the findings of Okon, 1985; Tamilvendan and Purushottam (1996) who screened *Azospirillum lifoferum* and *Azospirillum brasilence* in *in vitro* condition and concluded that both the isolates capable of fixing nitrogen in the range of 7.54 – 24.53 mg of nitrogen per g of malic acid after seven days at 28^0C under static conditions. Similarly, Gaind and Gaur, (1981) isolated and screened *Bacillus megatherium, B.brevis, B. cerculiance, Bacillus subtilis* from rhizosphere of Oat and Arhar. Out of the four PSB isolate screened for percent Pi released into the medium the isolate number PSB –1 showed maximum 7.4 % Pi at 10^{th} days after inoculation.

Influence of efficient *Azospirillum* and phosphate solubilizing *Bacillus* isolates on growth parameters of spinach

Germination percentage and Number of Leaves

An evaluation of efficient *Azospirillum* and PSB isolates in single and combination where evaluated to know their effect on germination percentage and number of leaves of spinach (Plate. 3). Statistically, the highest germination percentage (100 %) was observed in the combined application of *Azospirillum* + PSB treatment whereas, the low germination percentage of 80 % was observed in absolute control where no chemical and microbial fertilizers are applied (Table 5). Similarly, With respect to number of leaves per hill is concerned, the maximum number of leaves where observed in the treatment number 5 (*i.e. Azospirillum* + PSB + Compost + RDF). Further, the least leaf population was observed in absolute control treatment.

The findings are in line with the findings of Adesemoye *et al.*, (2008) who evaluated different plant growth promoting *Pseudomonas* and *Bacillus sp.* on growth and yield of three vegetables like tomato, okra and amaranths where the number of leaves increased after 60 days of planting where the consortial application of *Pseudomonas* and *Bacillus*.

Table.1 Morphological and biochemical characteristics of isolates of associative nitrogen fixers

Sl.No.	Isolates	Colony Morphology on N-free maltase medium	Pellicle formation	Cell shape	Motility	Growth on nutrient agar	Growth on potato infusion agar	Utilization of various carbon source				Probable genus
								Sucrose	**Dextrose**	**Maltose**	**Citrate**	
1	Asp-1	White dense and medium	Present	Spiral	Cork screw	White and raised	Pink and curled	+	+++	+	+++	*Azospirillum sp.*
2	Asp-2	White dense and medium	Present	Spiral	Cork screw	White and raised	Pink and curled	+	+++	+	+++	*Azospirillum sp.*
3	Asp-3	White dense and medium	Present	Spiral	Cork screw	White and raised	Pink and curled	+	+++	+	+++	*Azospirillum sp.*
4	Asp-4	Pale, white shiny	Present	Spiral	Cork screw	Pink & curled	Pink and curled	++	++	+++	+++	*Azospirillum sp.*
5	Asp-5	Small, white dense	Present	Spiral	Cork screw	White and raised	White & smooth	+++	++	+++	+++	*Azospirillum sp.*
6	Asp-6	Spindle & transparent	Present	Spiral	Cork screw	Pale, white & transparent	Pale white & transparent	+	++	+++	+++	*Azospirillum sp.*
7	Asp-7	Pale shiny white medium	Present	Spiral	Cork screw	Pale white & raised	White & smooth	+	+	++	++	*Azospirillum sp.*
8	Asp-8	Pale, white shiny & small	Present	Spiral	Cork screw	Pale white & raised	White & smooth	++	++	++	++	*Azospirillum sp.*

Table.2 Morphological and biochemical characteristic of isolates of PSB

Isolates	**Morphological Test**		**Biochemical Test**								
	Colony morphology	Grams relation cell shape	Gelatin liquefaction	Starch hydrolysis	Casein hydrolysis	Catalase hydrolysis	Acid	Gas	H_2S production	Simmon's citrate test	Probable genus
PSB-1	Medium round creamy to Yellowish	+ ve rods	+	+	+	_	+	+	_	+	*Bacillus sp.*
PSB-2	Medium round creamy to Yellowish	+ ve rods	+	+	+	+	+	+	_	_	*Bacillus sp.*
PSB-3	Medium round creamy to Yellowish	+ ve rods	+	+	+	_	+	+	_	+	*Bacillus sp.*
PSB-4	Medium round creamy to Yellowish	+ ve rods	+	+	+	_	+	+	_	_	*Bacillus sp.*

Table.3 N_2-fixing potential of *Azospirillum* isolate under *in vitro* condition

Sl.No	*Azospirillum* Isolates	Nitrogen (mg/gm of 'C' source)
1	Control	0.70 (e)
2	Asp-1	3.50 (d)
3	Asp-2	3.93 b (c)
4	Asp-3	3.97 b (c)
5	Asp-4	5.20 (a)
6	Asp-5	3.40 (d)
7	Asp-6	4.10 (b)
8	Asp-7	4.80 (b)
9	Asp-8	3.70 (bc)
	SEm ± CD at 1 %	0.92 0.29

Note: Means followed by the same letters do not differ significantly

Table.4 Per cent Pi released by P-solubilizing bacterial isolates under *in vitro* condition

Sl. No.	PSB isolates	Pi released (%) at 10th days after inoculation
1	Control	4.10 (d)
2	PSB-1	7.40 (a)
3	PSB-2	5.30 (c)
4	PSB-3	6.30 (b)
5	PSB-4	5.01 (c)
	SEm ± CD @ 1%	0.180 0.740

Note: Means followed by the same letters do not differ significantly

Table.5 Effect of microbial inoculants on germination percentage and number of leaves of spinach under field condition

Sl. No.	Treatments	Germination (%)	No. of leaves per hill		
			15days	30days	45days
1	Absolute control	80.00 (e)	10.00(b)	15 (d)	18 (cd)
2	Control + compost + RDF	94.00 (d)	10.00 (b)	17 (b)	17 (d)
3	*Azospirillum* + compost + RDF	96.00 (c)	13.00(a)	16 (bc)	20 (c)
4	PSB(*Bacillus* sp.) + compost + RDF	98.00 (b)	13.00(a)	16 (bc)	22 (b)
5	*Azospirillum* + PSB (*Bacillus* sp.) + compost + RDF	100.00 (a)	11.00 (b)	18 (a)	25 (a)
SEm ± CD at 1%		0.48 1.80	2.607 0.907	6.186 1.592	6.139 1.586

Note:
1. Absolute control = only soil without compost or fertilizer treatment
2. RDF = Recommended Dose of Fertilizer
3. Means followed by the same letters do not differ significantly

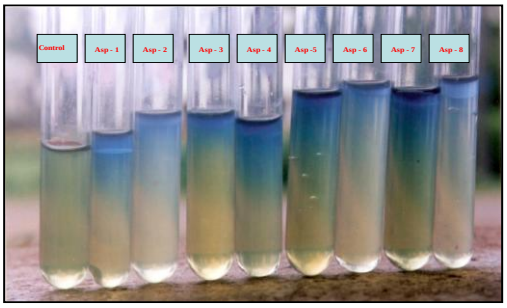
Table.6 Influence of microbial inoculants on total chlorophyll, fresh and dry weight of Spinach

Sl. No.	Treatments	Chlorophyll (mg/g of tissue)	Fresh weight (g/plant)	Fresh weight (g/plant)
1	Absolute control	1.00 $^{(e)}$	25 $^{(e)}$	3.02 $^{(c)}$
2	Control + Compost + RDF	1.52 $^{(d)}$	27 $^{(d)}$	3.12 $^{(bc)}$
3	*Azospirillum* + Compost+ RDF	1.08 $^{(c)}$	30 $^{(c)}$	3.39 $^{(b)}$
4	PSB + Compost +RDF	2.75 $^{(ba)}$	32 $^{(b)}$	3.32 $^{(b)}$
5	*Azospirillum* + PSB (*Bacillus* sp.)+compost +RDF	2.95 $^{(a)}$	35 $^{(a)}$	4.68 $^{(a)}$
	SEM ±	0.03	6.62	0.01
	CD @ 1%	0.14	2.18	0.07

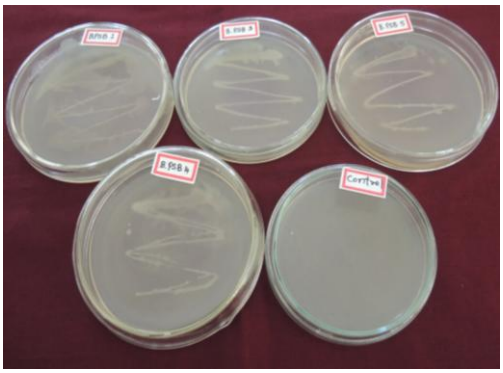
Note: Means followed by the same letters do not differ significantly

Table.7 Effect of microbial inoculants on plant and soil nutrient status after harvest

Sl. No	Treatments	Plant nutrient status (mg/plant)			Soil nutrient status (kg/ha)		
		N	P	K	N	P	K
1	Absolute Control	154.33 $^{(e)}$	150.33 $^{(d)}$	146.33 $^{(e)}$	161.33 $^{(e)}$	20.00 $^{(e)}$	143.67 $^{(c)}$
2	Control + Compost + RDF	254.33 $^{(c)}$	192.66 $^{(c)}$	166.67 $^{(d)}$	206.00 $^{(d)}$	25.67 $^{(d)}$	145.67 $^{(b)}$
3	*Azospirillum* + Compost +RDF	278.00 $^{(b)}$	190.00 $^{(c)}$	158.33 $^{(d)}$	274.00 $^{(b)}$	22.83 $^{(c)}$	150.67 $^{(a)}$
4	PSB +Compost +RDF	231.00 $^{(a)}$	221.00 $^{(b)}$	162.33 $^{(b)}$	225.00 $^{(c)}$	33.67 $^{(b)}$	151.67 $^{(a)}$
5	*Azospirillum* + PSB +Compost +RDF	289.00 $^{(a)}$	284.00 $^{(a)}$	178.33 $^{(a)}$	390.00 $^{(a)}$	35.81 $^{(a)}$	150.33 $^{(a)}$
	SEm ±	16.43	16.52	12.11	13.56	9.05	18.03
	CD (0.05)	2.04	2.61	2.30	2.60	1.99	3.00



***Azospirillum* isolates**



***PSB* isolates**

Plate 1. *Azospirillum* and PSB isolates

Chlorophyll content, fresh and dry weight

Chlorophyll content of spinach leaves was statistically significant at harvest due to various inoculation treatments. At harvest, treatment where *Azospirillum* + PSB + Compost + RDF used recorded the highest chlorophyll content of 2.95 mg/g of tissue. Whereas, the absolute control treatment was having less chlorophyll content (1 mg/g of tissue).

Similarly, significant variations among the treatment where observed in the fresh and dry weight. Among all single and dual inoculation treatment number 5 (Azospirillum + compost + RDF) has found to be statistically superior over the rest of all treatments *ie.,* 35 g/plant of fresh weight and 4.68 g/plant of dry weight followed by treatment number 4 Whereas the absolute control treatment was having fresh and dry weight which indicated the effect of microbial inoculants on the fresh and dry weight of spinach (Table 6). The results of the present study was strongly supported by the findings of Adesemoye *et al.,* (2008) who evaluated effective PGPR microorganisms on amaranths and concluded that the leaf area, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight and chlorophyll content is a more due to combined inoculations.

Influences of microbial inoculants on NPK content of plant and soil

An evaluation of best efficient Azospirillum and PSB isolates where to evaluate the NPK content of the spinach and soil at the time of harvest. The results obtained are presented in Table 7. With references to nitrogen level in plants. The treatments, where Azospirillum imposed resulted in high nitrogen content. But in the other treatments the average nitrogen contents almost less. With reference to phosphorus the result showed increase in phosphorus content where the PSB-1 is imposed *ie.,* 284.00 mg/plant was recorded in the treatment where Azospirillum + PSB + compost + RDF was used. In contrast to potassium concentration of plant was not showed any significant increase but in the treatment 5 the content of potassium was 178.33 mg/plant. With reference to the soil nutrient status similar results were obtained *i.e.,* the treatment 5 recorded highest levels of soil Nitrogen, phosphorus and potassium respectively (Table 7).

Similarly, Ishque *et al.,* (2009) supported the findings of present investigation. Here six different levels of nitrogen along with *Azospirillum* were evaluated under field conditions. The result revealed that nitrogen application at 140 / ha + *Azospirillum* significantly increased number leaves, plant height and also recorded the high nitrogen content in lettuce leaves after harvest.

The result of soil nutrient status confirm the work of Mago and Mukargi (1995); Maheshwari *et al.,* (1991) and Vasanthkumar, (2003) who concluded the maximum accumulation of residual nitrogen and phosphorus is more where the N-fixes and P solubilizers are used in the treatments.

Scale up studies are required to commercialize the formulation of Asp-4 and PSB-1 for large scale application and also standard procedure for the quality control of Asp-4 and PSB-1 formulations for effective usage in the organic farming practices.

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