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Symbiotic effectiveness of potential Bradyrhizobium/Ensifer strains on growth, symbiotic nitrogen fixation and yield in soybean [Glycine Max (L.)]

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ABSTRACT

Symbiotic nitrogen fixation (SNF) is a mutualistic interaction between host plant and the root nodule bacteria. The present investigation was carried out to study symbiotic effectiveness of potential Bradyrhizobium (slow growing; IND1, LSBR3, PANT1, SB271, & DS1) / Ensifer (fast growing; IND2, LSER7, LSER8 & PANT2) strains over un-inoculated control on growth and yield in soybean [Glycine max (L.)]. In-vitro thin layer chromatographic (TLC) analysis revealed significantly high amount of flavonoid like compounds from roots of soybean seeds bacterized with LSBR3 (3.44 µg/ml) as compared to control treatment (2.60 µg/ml). Field evaluation of potential rhizobia presented significantly higher germination and plant height with Ensifer strains, LSER8 (94.7% and 36.8 cm), LSER 7 (93.4% and 36.4 cm) and Bradyrhizobium strain LSBR3 (91.5% and 35.6 cm), respectively. Overall microbial activity assayed by dehydrogenase activity was significantly higher for LSBR3 (17.54 µg/TPF/g/soil/h) and LSER7 (16.75 µg/TPF/g/soil/h) at 90 DAS as compared to other treatments. Nodulation significantly enhanced with LSER8 and LSER7 (ranged from 52.2 to 135.7 and 49.9 to 128.7 mg/plant, respectively) treatments over un-inoculated control. Maximum leghaemoglobin and nitrogen content (5.52 mg/g fresh weight of nodules and 2.89 %, respectively) analyzed with LSBR3. Grain yield recorded was significantly higher with LSBR3 (12.3%) followed by PANT1 (12 %) and LSER8 (11.7%) over control and emerged as effective strains for improving growth, SNF and yield in soybean and can be exploited as commercial inoculants of soybean.

Keywords: Biofertilizer, Rhizobia, Soybean, SNF, Yield.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is a third major oil seed crop of India with good quality protein (40 to 44 %). It occupies an area of 10.27 m/ha with an annual production of 11.00 metric tonnes and with an average yield of 1070 kg/ha in India [1]. Soybean being a leguminous crop fixes atmospheric nitrogen (N) through its root nodule bacterium and 50 to 60% of nitrogen is provided by biological nitrogen fixation (BNF). Soybean nodulating rhizobia are genetically diverse and are classified into different genera and species including the slow-growing *Bradyrhizobium* (*B. japonicum*, *B. liaonengense*, and *B. elkanii*) and fast growing *Ensifer* (E. *fredii* and *E. xinjiangense* and other unclassified rhizobia) [2]. *Bradyrhizobium* species considered as a poor root colonizers. Exploitation of fast growing strains has some advantages due to their shorter generation time and better adaptation in ecological condition than bradyrhizobia [3].

Efficiency of symbiotic BNF is dependent on the mutual compatibility of both partners [4]. Better N_2 fixation can be achieved by selecting superior rhizobia. The strains might establish highly effective nitrogen-fixing symbiosis with the soybean cultivars and they must be adapted and show a competitive ability against native rhizobial populations

[5]. Flavonoids in legume seed and root exudates act as chemoattractants and inducers of the structural nod genes of rhizobia that are required for Nod factor synthesis, also known to induce resistance of *B. japonicum* and *S. fredii* against the soybean phytoalexin glycelloin [6]. Selection of strains with improved competitive abilities for nodulation should be exploited for converting potential *Bradyrhizobium/Ensifer* strains into commercial inoculants of soybean. Therefore, the present study was planned to investigate symbiotic effectiveness of potential *Bradyrhizobium/Ensifer* strains for enhanced growth and yield in soybean.

MATERIALS AND METHODS

Procurement of Bacterial cultures

Eight cultures of rhizobia were procured from different locations. IND1 & IND2 (*Bradyrhizobium* sp. & *Ensifer* sp.) were procured from Directorate of Soybean Research, Indore. LSBR3, LSER7 & LSER8 (*Bradyrhizobium* sp., *Ensifer* sp. & *Ensifer* sp.) were procured from Pulses Microbiology Laboratory, Punjab Agricultural University (PAU), Ludhiana, PANT1 & PANT2 (*Bradyrhizobium* sp. & *Ensifer* sp.) from Department of soil Science, G B Pant University of Agricultural & Technology, Pantnagar. DS1 (*Bradyrhizobium japonicum*) was obtained from Division of Microbiology, Indian Agricultural Research (IARI) New Delhi and SB271 (*Bradyrhizobium* sp.) from Department of Microbiology, PAU, Ludhiana. Purity of all procured cultures was tested with gram staining and ketolactose test. Pure cultures of *Bradyrhizobium* and *Ensifer* were maintained on Yeast Extract Mannitol Agar (YEMA) medium and further sub-cultured once a month throughout the period of investigation and stored at 4ºC in refrigerator.

Identification and estimation of flavonoid compounds from *Bradyrhizobium/Ensifer* strains

Extraction of flavonoid like presumptive compounds from soybean seeds bacterized with *Bradyrhizobium/ Ensifer* strains was done with organic solvent (ethyl acetate) [7]. The biomolecules extracted from soybean roots were identified by thin layer chromatography (TLC). TLC plates were developed in solvent-saturated chromatography jars using a hexane: ethyl acetate: methanol (60:40:1) solvent mixture. Fluorescent spots were located and eluted in ethyl acetate. Eluent from each spot was scanned using a Lambda 3b Dual fBeam Perkin Elmer Spectrophotometer between 200 and 400 nm. In order to determine whether the isolates produced similar fluorescent compounds in culture media and from seeds of *Rhizobium* strains of different isolates were extracted with ethyl acetate, and the absorption maxima (289nm) were recorded and quantified using naringenin (an isoflavonoid) as a standard.

Evaluation of *Bradyrhizobium and Ensifer* strains for growth, symbiotic parameters and yield in soybean

Field experiment was carried out the Pulse Research Farm, Pulse Microbiology Laboratory and Quality Research laboratory Department of Plant Breeding and Genetics, PAU, Ludhiana during *Kharif* 2012 in Randomised Block Design (RBD) with three replications in SL 744 chickpea variety using ten bacterial treatments *viz.* Un-inoculated Control, IND1, IND2, LSBR3 (Native *Bradyrhizobium* strain), LSER7 (Native *Ensifer* strain), LSER8 (Native *Ensifer* strain), PANT1, PANT2, SB271(Standard culture) and National reference culture of *Bradyrhizobium* strain (DS1). Seeds of soybean variety SL744 were procured from the Pulses Section, Department of Plant Breeding and Genetics, PAU, Ludhiana. Recommended agronomic practices were followed for raising the soybean crop. Soybean seeds were inoculated with different cultures of *Bradyrhizobium* and *Ensifer* strains as per treatment along with un-inoculated control using 20 g charcoal/Kg seeds, air dried at room temperature under shade before sowing. The observations were recorded on germination count at 7 days after sowing (DAS). Observation for plant height taken at 65 DAS, dry weight of shoot and root, number and dry weight of nodules, leghaemoglobin content of nodules and chlorophyll content of leaves were recorded at both vegetative (65 DAS) and flowering stage (90 DAS) while dehydrogenase activity (DHA) of soil determined at 30 and 60 DAS. Total N of shoot, protein content of seed and grain yield were recorded at the harvesting stage of crop.

Growth parameters

Emergence count was determined by recording number of emerged seedlings per meter row length from central rows of each plot after leaving two border rows on each side. For Plant height the height of shoots from three randomly selected and uprooted plants from each plot was measured from the base in cm after removing roots. Dry weight of shoot and root was observed by weighing the sun dried and then oven dried randomly selected uprooted plants at 60^{o}C for 2 days in grams. Chlorophyll estimation was done by recording the optical density of the chlorophyll extract on UV-Vis spectrophotometer using a solvent blank at 645 nm and 663 nm [8]. Grain yield from each plot (g/plot) was recorded and the final grain yield was expressed in kg/ha.

Dehyrogenase activity in soil

Dehydrogenase activity of soil was assayed at 30 and 60 DAS. The production of Triphenylformazan (TPF) was determined by measuring absorbance at 485 nm after 3 hours of incubation of mixture of soil sample, TTC and glucose [9]. Dehydrogenase activity was calculated as µg of TPF/g of soil/hour.

Symbiotic parameters

The number of nodules per plant was recorded by taking average of nodules carefully detached from three randomly uprooted plants. The detached nodules were oven dried at 60^{o}C for 2 days and the dry weight of nodules per plant was recorded in mg. Leghaemoglobin content was assayed by reading absorbance of clear nodule tissue extract with Drabkin's solution at 540 nm using UV-Vis spectrophotometer [10]. Total nitrogen content of shoot was determined by Kjeldahl's technique with slight modification of McKenzie and Wallace [11] Protein content was determined by NIRT (Near infrared transmittance) by putting sample in whole grain analyser (Foss Infratec 1241 Grain Analyzer). For this 20 healthy seeds were taken and dried in oven for about 1 hour at 50°C and then ground with Cemotec 1090 grinding mill (Foss).

RESULTS

On performing TLC along with naringenin as standard, flavonoid like compounds were extracted from un-inoculated and inoculated roots from soybean seeds. It revealed all the treatments showing a single spot with Rf value similar to that of the flavonoids (naringenin). Further quantification on the basis of absorption maxima (289 nm) using naringenin (isoflavonoids as standard) showed marked increase in flavonoid level of the roots of treated seeds in comparison with untreated seeds (Fig. 1). Maximum amount of flavonoid compounds was produced with soybean roots inoculated with *Bradyrhizobium* and *Ensifer* strains of LSBR3 (3.44 µg/ml) followed by PANT1 (3.28µg/ml) and IND1 (3.22 µg/ml) as compared to control (2.60 µg/ml).

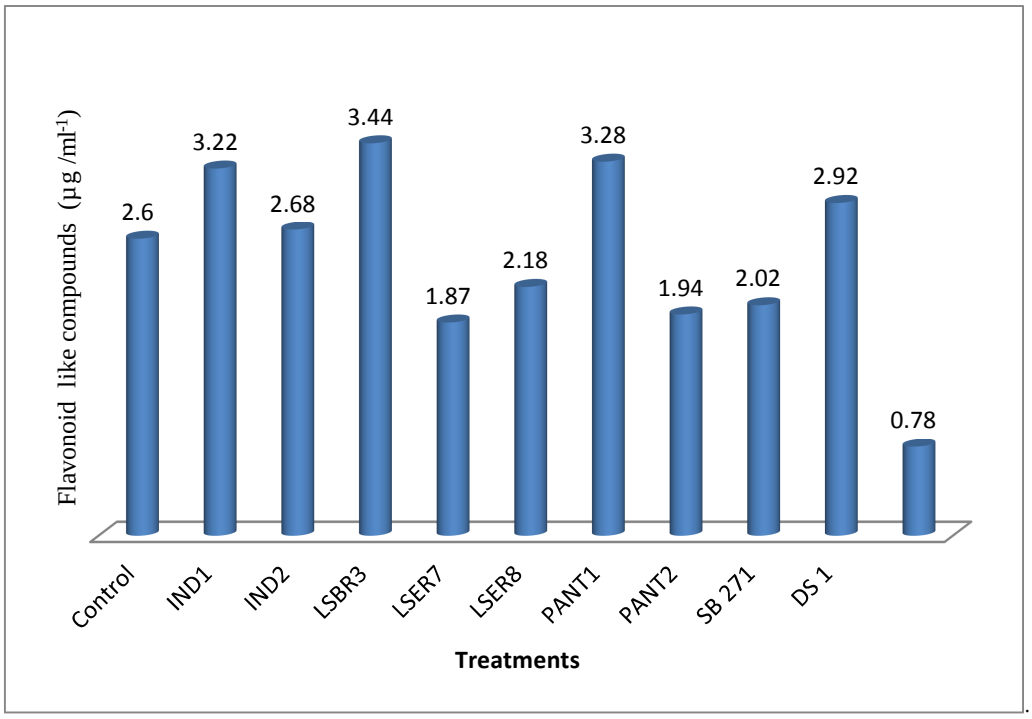

Fig.1: Assessment of *Bradyrhizobium/Ensifer* strains for flavonoid like compounds with inoculated seeds

Further, on evaluation of growth parameters, the data on emergence count and plant height (Fig. 2) revealed significant differences in all treatments as compared to control treatments. Significantly high germination was recorded with *Ensifer* strains LSER8 (94.7%), LSER7 (93.4%) and *Bradyrhizobium* strain LSBR3 (91.5%). Among treatments, plants receiving LSER8 treatment gained maximum height (36.8 cm) followed by LSER7 (36.4 cm) and LSBR3 (35.6 cm).

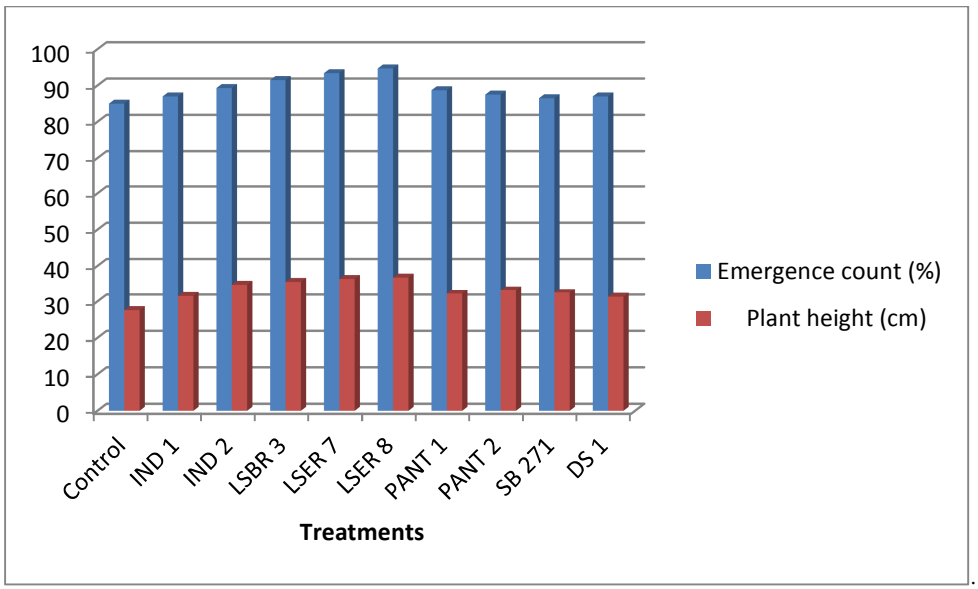

Fig.2: Evaluation of *Bradyrhizobium* and *Ensifer* strains inoculation on Emergence count (7 DAS) and Plant height (65 DAS)

There was an increase in dry weight of shoot with various treatments at flowering stage over vegetative stage. A significant difference for dry weight of shoot was observed for different treatments over un-inoculated control expect SB271 and DS1 treatments (Table 1). Similar trend was followed for dry weight of root. Inoculation with LSER8 gave maximum shoot dry weight (14.9 g/plant) followed by LSER7 (14.4 g/plant) and LSBR3 (14.1 g/plant) whereas LSER7 (0.818 & 2.18 g/plant) and LSER8 (0.811 & 2.19 g/plant) depicted maximum dry weight of root. Increase in chlorophyll content varied with different treatments (Table 1). Significant chlorophyll content was recorded in LSER8 (2.02 mg/g & 2.27 mg/g fresh weight of leaves at vegetative and flowering stage, respectively) followed by LSBR3 (1.96 & 2.35 mg/g) and LSER7 (1.95 & 2.36 mg/g) at vegetative and flowering stage, respectively.

Table 1: Evaluation of *Bradyrhizobium* and *Ensifer* strains inoculation on Growth parameters

Treatments	Dry weight of shoot/plant (g) DAS		Dry weight of root/plant (g) DAS		Chlorophyll content (mg/g fresh weight of leaves) DAS	
	65	90	65	90	65	90
Control	4.2	8.1	0.714	1.54	1.31	1.89
IND1	5.5	13.9	0.780	1.71	1.79	2.05
IND2	5.4	12.9	0.782	1.78	1.88	2.16
LSBR3	6.3	14.1	0.805	2.15	1.96	2.35
LSER7	6.6	14.4	0.818	2.18	1.95	2.36
LSER8	6.8	14.9	0.811	2.19	2.02	2.27
PANT1	5.5	11.8	0.791	1.79	1.88	2.08
PANT2	5.6	13.4	0.801	1.83	1.85	2.18
SB 271	4.7	8.6	0.742	1.62	1.58	1.95
DS 1	5.3	8.9	0.776	1.71	1.76	1.98
CD5%	0.6	0.9	0.001	NS	0.05	0.18

Dehydrogenase activity of soil is a useful indicator of overall microbial activity in soil. At 30 DAS, DHA (Fig.3), of soil was highest in treatment LSER8 (9.98 µg/TPF/g/soil/h) followed by LSER7 (9.29 µg/TPF/g/soil/h) and were superior over other inoculation treatments and control treatment (6.01 µg/TPF/g/soil/h). At 90 DAS significantly high DHA were recorded with LSBR3 (17.54 µg/TPF/g/soil/h) followed by LSER7 (16.75 µg/TPF/g/soil/h) as compared to other treatments.

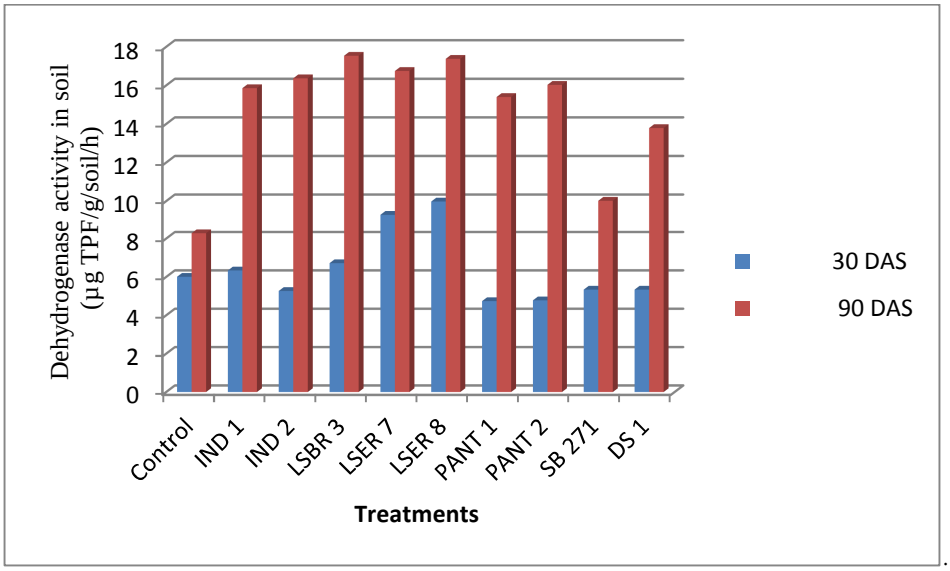

Fig.3: Measurement of Dehydrogenase activity in soil by *Bradyrhizobium/Ensifer* strains

Evaluation of symbiotic efficiency of *Bradyrhizobium* and *Ensifer* strains at vegetative stage, observed significant difference for all the treatments except PANT1 and PANT2 strains over un-inoculated control for number of nodules per plant (Table 2). At flowering stage, the number of nodules was higher than vegetative stage. Inoculation with LSER8 (68.7 NN/plant), LSER7 (65.3 NN/plant) and PANT1 (64.7 NN/plant) showed significant increase in nodule number over un-inoculated control (45.7 NN/plant). *Ensifer* and *Bradyrhizobium* strains inoculation increased the dry weight of nodules significantly in comparison to un-inoculated control at both vegetative and flowering stages. Significant increase in dry weight of nodules were recorded in inoculated plots with inoculation of LSER8 (ranged from 52.2 to 135.7 mg/plant) followed by LSER7 (49.9 to 128.7 mg/plant). Further, all treatments significantly increased leghaemoglobin content over un-inoculated control. Improvement in leghaemoglobin content was significant with LSER8, LSER7 and LSBR3 treatments over the reference culture SB271 and DS1. There was a significant effect of inoculation on nitrogen content in the shoot with maximum nitrogen content being observed in LSBR3 (2.89 %) followed by DS1 (2.80 %) and minimum with un-inoculated control. Significant difference for protein content in different treatments was observed over un-inoculated control with LSER7 (46.40%) and LSBR3 (45.30%) recorded the maximum value.

Table 2: Evaluation of *Bradyrhizobium* and *Ensifer* strains inoculation on Symbiotic parameters

Treatments	Number of nodules/plant (NN) DAS		Dry weight of nodules/plant (mg) DAS		Leghaemoglobin content (mg/g fresh weight of nodules) DAS		*Total Nitrogen content of shoot (%)	*Protein content of seed (%)
	65	90	65	90	65	90		
Control	12.7	45.7	22.8	85.9	3.29	4.27	2.42	42.00
IND1	18.0	60.7	30.2	120.5	3.86	5.12	2.42	43.10
IND2	16.3	59.7	32.0	123.5	3.79	5.15	2.60	44.20
LSBR3	19.0	63.7	45.8	130.7	4.80	5.52	2.89	45.30
LSER7	20.7	65.3	49.9	128.7	4.78	5.42	2.78	46.40
LSER8	23.3	68.7	52.2	135.7	4.75	5.45	2.65	44.90
PANT1	13.3	64.7	25.6	133.7	3.62	5.23	2.65	42.60
PANT2	12.3	60.3	26.2	138.5	3.69	5.50	2.60	42.40
SB 271	15.3	50.3	24.1	98.9	3.77	4.69	2.48	42.40
DS 1	17.3	51.3	32.5	105.5	3.80	5.05	2.80	43.70
CD5%	3.5	7.3	1.9	14.4	0.06	0.09	NS	1.69

**Total N content of shoot, protein content of seed and grain yield were recorded at the harvesting stage of crop.*
On harvesting, Grain yield recorded significantly higher with LSBR3 (12.3%) followed by PANT1 (12 %) and LSER8 (11.7%) over un-inoculated control (Fig. 4).

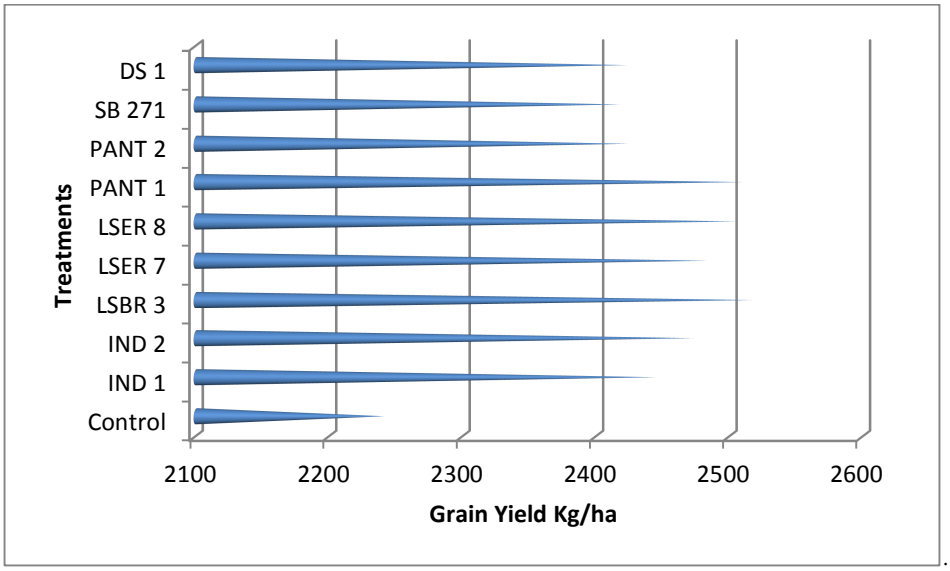

Fig. 4 Evaluation of *Bradyrhizobium* and *Ensifer* strains inoculation on grain yield

DISCUSSION

Flavonoids are low molecular weight compounds involve in the nodulation process. The present study showed a marked increase in flavonoid level from the roots of bacterized seeds in comparison with the roots of untreated seeds (Fig.1). This lead to suggestion that certain rhizobia probably produced molecules which could enhance flavonoid production by plant roots. Thus enhanced flavonoid production in roots might be an additional factor in nodule promotion by rhizobia. These results were coherent with the earlier findings where it was reported that plant roots flavonoid are the inducer of nodulation gene (nod gene) expression in *Rhizobium* [12]. Production of Indole acetic acid (IAA) among rhizobia, has long been assumed to play a role in nodule development. Significantly high germination recorded in the present study with *Ensifer* strains LSER8, LSER7 and *Bradyrhizobium* strain LSBR 3 (Fig.2) might be related to release of IAA as these isolates are known to release IAA in Luria broth amended with tryptophan as precursor. These results corroborated with the findings of Kumar and Ram [13] in mungbean. Plant strand is an important growth index for achieving higher crop productivity. The results summarized in Fig.2 on plant height revealed significant differences over control treatments. Positive effect of

Rhizobium inoculation with an increased height of crop at seedling, flowering and fruiting stages were reported in pea and chickpea [14,15]. Dry weight of shoot is considered as best indirect measurement of nitrogen supplied by rhizobial strain to host cultivar. There was an increase in dry weight of shoot with various treatments at flowering stage over vegetative stage (Table 1). Similar results were also reported in soybean inoculated with *B. japonicum* [16]. The results are in close agreement with who observed that plants inoculated with *B. japonicum* strain ASR011 [17] produced higher plant dry matter accumulation and it emerged as best criteria for selecting most effective legume-*Rhizobium* association in given physical and biological conditions. Sobral *et al.*, [18] explained that *Bradyrhizobium* isolates were able to produce IAA, solubilize phosphate and fix nitrogen which could be used for soybean growth promotion. Similarly, higher shoot dry weight in plants inoculated with rhizobial strains might be ascribed to more N supply to crop through N fixation by bacteria [19]. Enhancement in root dry weight with rhizobial inoculation might be attributed to ability of *Rhizobium* to conserve carbohydrates [20]. Moreover, it was proposed that positive and significant correlation existed between photosynthesis and N_2 fixation [21]. Results summarized in Table 1 depicted the chlorophyll content of leaves attributed improvement in chlorophyll content to increased N uptake by a larger root surface areas associated with additional root hairs and lateral root development and/or to BNF, either directly by the inoculant strains or indirectly by stimulating BNF activity of the associated rhizosphere community [22]. Dehydrogenase activity of soil is a useful indicator of overall microbial activity in soil. Similarly higher levels of DHA at 90 DAS as compared to 30 DAS (Fig.3) are in well agreement with the findings of Meenakshi and Savalgi [23] showing higher DHA in treatment which received seed inoculation with *Methylobacterium* and *B. japonicum* along with foliar spray in soybean.

Nodulation is an important symbiotic trait for effective symbiosis between *Rhizobium* and legume host plant. At vegetative stage, a significant difference was observed for all the treatments except *Bradyrhizobium* and *Ensifer* strains of PANT1 and PANT2 over un-inoculated control (Table 2). At flowering stage, the number of nodules was higher than vegetative stage. Higher nodulation in inoculated plants could be attributed to the availability of large number of effective and infective rhizobia [17] in soybean rhizosphere. These submissions aligned correctly with previous findings of several workers [18, 19]. Greater number of nodules due to inoculation suggested that there is better combining and symbiotic relationship between introduced rhizobia and soybean. Larger response to inoculation and higher number of nodules per plant in comparison to un-inoculated treatments in a field that has no soybean cropping history was also reported by [16] who have reported greater number of nodules due to inoculation treatments and nitrogen. *Bradyrhizobium* and *Ensifer* strains released growth hormone which resulted in root elongation, thus providing more infection sites for nodulation. Dry weight of nodule is indicative of the development of nodules. *Ensifer* and *Bradyrhizobium* strains inoculation increased thedry weight of nodules significantly in comparison to un-inoculated control at both vegetative and flowering stages (Table 2). Treatments differed significantly from each other at both stages over un-inoculated control. Due to the lack of sufficient rhizobia in the soil, plants were having less nodules number and biomass in un-inoculated plots. Whereas significant increase in dry weight of nodules were recorded in inoculated plots. Our results agreed with [19] who depicted significant increase in nodulation and yield due to the inoculation of bradyrhizobial isolates in soybean. Similar results were reported by [24] in soybean due to larger bradyrhizobial population which infected more root hairs enhancing the nodule number, ultimately contributing to the higher dry matter of nodules per plant. Leghaemoglobin pigmentation of central tissues of the nodule represents efficient nitrogen fixation in legumes. Enhanced leghaemoglobin content observed in above mentioned rhizobial treatments (Table 2) might be due to the effective nodulation and symbiotic nitrogen fixation. It was reported that the leghaemoglobin had a positive correlation with N_2 fixation and nitrogenase activity in nodules characteristic of efficient symbiosis [25]. Increase in N content in shoot (Table 2) due to rhizobial inoculation was mainly due to significant increase in nodulation, resulting in higher accumulation of N from atmospheric N_2 fixation. Significant increase in shoot N of soybean inoculated with *B. japonicum* was previously reported [26, 27]. Improvement in protein content found in present study is in close agreement with the findings of (2004) [24] in soybean with *B. japonicum* inoculation. Improvement in grain yield due to *Rhizobium* inoculation has been reported in legumes [19, 24]. The increase in yield and yield attributing characters due to rhizobial inoculation was due to its superiority over native rhizobia in soybean (Fig.4). The plants having higher number of nodules were found to possess high nodule dry matter and better crop yield than un-inoculated plants. It indicates that the degree of root nodulation determines the crop yield in soybean and optimizing the nodulation could maximize the nitrogen fixation [28, 29] According to Gil-Quintana *et al.*, [30], rate of nitrogen fixation in root nodules determines the overall performance of soybean growth, development and yield. Improvement in grain yield due to inoculation with *Rhizobium* might be related to better nodulation of roots and its culmination in more N_2 fixation that in turn had a significant effect on grain yield. Similar genetic variation in biomass production and seed yield has already been reported by earlier workers [17, 19].

CONCLUSION

The present research aimed to investigate potential strains of *Bradyrhizobium/Ensifer* and ability to adapt in prevailing environmental conditions for improving BNF and yield in soybean. It was concluded that LSBR3, PANT1 and LSER8 emerged as effective strains for nitrogen fixation. So selection of strains with improved BNF and yield can be exploited for converting potential *Bradyrhizobium/Ensifer* strains into commercial inoculants of soybean.

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