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Nodulation, nutrient accumulation and yield of rainfed soybean in response to indigenous soybean-nodulating Bradyrhizobia in the Himalayan region of Kashmir-Pakistan

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Abstract

The use of efficient and effective nodulating Bradyrhizobia strains considered as an ecologically and environmentally sound management strategy for soybean production. A 2-yr (2009 and 2010) field experiment was conducted to evaluate the effects of seven indigenous *Bradyrhizobium* strains, one exotic TAL-102 and three N fertilizer rates, i.e., 25, 50 and 100 kg N ha^{-1} on the productivity and N_2 fixation of rainfed soybean [*Glycine max* (L.) Merr.] grown in the Himalayan region of Rawalakot Azad Jammu and Kashmir (AJK), Pakistan. The experiment was conducted in a randomized complete block design with three replications. *Bradyrhizobium* inoculation accelerated plant growth by increasing shoot length (26-47%), root length (45-73%) and shoot dry weight (58-104%). Seed yield in the control was 861 kg ha^{-1} that significantly increased to 1450–2072 kg ha^{-1} with *Bradyrhizobium* strains. Seed yields under indigenous NR_{20} and NR_{22} strains was 24 and 28% higher than that recorded from the exotic TAL-102. Number of nodules, nodules dry weight and acetylene reduction assay with *Bradyrhizobium* strains were 55–123%, 94–178% and 38–103%, (respectively) higher than the non-inoculated control. The higher N rate (N_{100}) depressed nodulation and N_2 fixation. A significant variation in the symbiotic effectiveness and yield potential showed that inoculation response was site/strain specific. Two indigenous strains NR_{20} and NR_{22} were found highly efficient and displayed superiority over the exotic strain TAL-102. Multi-locational trials are required to check the suitability of these isolated isolates for other agro-climatic conditions before using as inoculants or bio-fertilizers.

Keywords: *Bradyrhizobium japonicum*; Inoculation; Indigenous strains; Nodulation; ARA activity; Yield.

Short Title: Soybean production under indigenous *Rhizobium* strains.

Introduction

Soybean [*Glycine max* (L.) Merrill] is recognized as the most important grain legume in the world in terms of total production and international trade (Golbitz, 1995) and used as an important source of protein and oil. In addition to its nutritional value,

soybean plays a major role in the global and agricultural nitrogen (N) input via biological N_2 fixation (BNF) in symbiosis with rhizobia (*Bradyrhizobium* spp.). In general, soybean may be able to fix an average of 175 kg N ha^{-1} yr^{-1} in irrigated and 100 kg N ha^{-1} yr^{-1} in dryland production systems (Unkovich and Pate, 2000; Furseth et al., 2012). Under field conditions, effectively nodulated soybeans acquire a large proportion (60-80%) of their total N from N_2 fixation (Peoples et al., 1995; Hungria et al., 2006). Soybean can also nodulate freely with native rhizobia and able to accomplish a large proportion of its N requirement through BNF once the plants are fully established (Okogun et al., 2004; Singh et al., 2003).

The symbiosis between rhizobia and legumes is an inexpensive and effective mechanism to ensure an adequate supply of N to legume based cropping system (Zahran, 1999) and plays a significant role in improving the fertility and productivity of the soils (Meghvansi et al., 2010). Soils in many part of the world contain an appreciable number of indigenous rhizobial populations which may be effective or may not be effective in N_2 fixation, but have good compatible with environment and competitive ability. However, it is generally believed that soils not previously used for soybean cultivation or soils in non-traditional areas of soybean production seldom contain sufficient population of indigenous strains to ensure satisfactory nodulation or if present are not effective (Rennie et al., 1982; Hatam and Abbasi, 1994). There are reports of a complete absence of soybean nodulation and rhizobium population in many soils of the world including Pakistan (Aslam et al., 1995; Achakzaiet et al., 2002).

In such soils, inoculation with rhizobium strains is an essential agronomical practice (Giddens et al., 1982) to enhance soybean quality and productivity (Wiersma and Orf 1992). Hafeezet et al. (2001) reported that inoculation with rhizobia should be performed in two situations: (i) in soils which are depleted or contain a low indigenous rhizobial population and (ii) when there is an established but inefficient rhizobial population. The effect of inoculation on soybean yield, nodulation and N_2 fixation potential had been studied earlier and remarkable response of soybean to *Bradyrhizobium* inoculation has been reported (Okereke et al., 2001; Egamberdiyeva et al., 2004a; Abbasi et al., 2010). Smith (1992) found a substantial increase in N_2 fixation when soybean seeds were inoculated with efficient *Bradyrhizobium* strains in low N soils without N fertilizer application. Ashraf et al. (2002) reported a specific combination of soybean genotype with rhizobium strains resulting in a many fold increase in the amount of N_2 fixed and grain yield harvested. Egamberdiyeva et al. (2004b) examined the effect of inoculation with *Bradyrhizobium japonicum* on growth, nodulation and yield of soybean in N-deficient soil of Uzbekistan and found a 5-6 fold increase in nodules number, 7-23% increase in shoot dry weight, 56-100% increase in shoot N and 28% increase in seed protein content in the inoculated plant compared with non-inoculated.

In the previous studies conducted at Rawalakot Azad Jammu and Kashmir, exotic *Bardyrhizobium* strains were used which displayed a significant increase in root nodulation, N accumulation and yields of soybean compared to the non-inoculated control (Abbasi et al., 2008; Abbasi et al., 2010). However, use of the exotic strains showed issues related to their availability and prices, thus the technology seemed impracticable in our conditions. Under such conditions, isolation of indigenous *Bardyrhizobium*, their identification and testing for plant growth promoting abilities is

an option for soybean production on sustainable basis. In the earlier study, a total of thirty seven *Rhizobium* strains were isolated from different sites of Rawalakot (unpublished data). The selected isolates were identified and tested for plant growth promoting abilities *in vitro*. This study was performed to explore the efficiency of indigenous *Bardyrhizobium* strains in comparison to an exotic strain and N fertilizer on yield, nodulation, N_2 fixation and nutrient (NPK) accumulation of soybean grown under field conditions at Rawalakot, Azad Jammu and Kashmir, Pakistan.

Materials and Methods

Sites description

The experiment was conducted at the Rawalakot, Azad Jammu and Kashmir (AJK), Pakistan at the Faculty of Agriculture research farm in 2009–2010. The study area lies between the altitudes of 1800 and 2000 m asl and latitude 33 to 36° in the northeast of Pakistan. The soil in the study sites was classified as a Humic Lithic Eutrudepts (Inceptosols). Detailed information about the study area has been given previously (Abbasi et al., 2008). The monthly precipitation and temperature of the experimental area during the growing season are presented in Table 1.

Table 1. Meteorological data of the experimental site during 2009–2010.

Months	Monthly rain fall (mm)		Maximum Temperature (^{0}C)		Minimum Temperature (^{0}C)		Humidity (%)		No. of rainy days	
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
January	103.2	39	12.8	15.6	-0.8	-1.3	84	73	12	3
February	148	295	12.9	11.9	0	0.1	86	86	13	12
March	145	83	17.9	21.7	3.2	5.2	78	80	12	6
April	200	45	21.8	25	5.9	7.7	87	84	12	12
May	41	135	27.5	26	9.5	10.2	82	85	7	13
June	160	59	28.2	27.3	11.4	12	85	88	12	13
July	218	526	28.6	26.5	15.3	16.1	89	93	15	19
August	167	170	27.4	25.4	16.4	17.2	94	95	12	20
September	82	26	27.2	26.2	11.7	13.9	87	92	6	3
October	29	79	23.3	23.5	5.3	7	81	87	2	5
November	39	2	18.7	20.9	1	2.6	75	80	3	2
December	39.3	28	14.9	16.1	-0.8	-1.9	77	70	7	2
	Total 1370	Total 1487	Mean 21.8	Mean 22.2	Mean 6.5	Mean 7.4	Mean 83.6	Mean 84.4	Total 113	Total 110

* Source: Director Regional Meteorological Centre Lahore, Pakistan.

Soil sampling and seedbed preparation

Before the onset of the experiment, soil samples from 0−15 cm were collected from the selected field, air-dried, ground to pass a 2-mm sieve and stored in sealed plastic jars before analysis. The physical and chemical properties of the soil used in the study are presented in Table 2.

For proper seedbed preparation, the selected field was ploughed 2-3 times to a depth of about 20 cm with a simple cultivator attached to a tractor followed by planking with a wooden planker. In the subsequent year of experimentation, fields were plowed (tilled) manually with a spade to avoid any mixing/disturbance of the soil. Amendments were applied to the same allocated plots for the two cropping cycles. The individual plots were prepared according to the treatments and the plot size was 3-m long and 2-m wide.

Table 2. The selected physical and chemical characteristics of the soil used in the experiment.

Soil properties	values
Soil bulk density (Mg m^{-3})	1.34
Particle density (Mg m^{-3})	2.68
Porosity (%)	48.9
Sand (%)	20
Silt (%)	55
Clay (%)	25
Textural Class	Silt loam
Soil pH	7.5
Organic matter (g kg^{-1})	11.0
Organic C (g kg^{-1})	6.4
Total N (g kg^{-1})	0.63
C:N ratio	10:1
Total mineral N (mg kg^{-1})	8.6
Available P (mg kg^{-1})	4.5
Available K (mg kg^{-1})	150

Treatments and experimental design

The experimental set-up was comprised of three N fertilizer levels i.e. 25, 50 and 100 kg N ha^{-1} designated as N_{25}, N_{50} and N_{100}, seven *Bradyrhizobium* strains i.e. NR_4, NR_{13}, NR_{18}, NR_{20}, NR_{22}, NR_{25} and NR_{35} isolated from the native soil, one exotic *Bradyrhizobium japonicum* strain TAL-102 and non-inoculated control. Altogether a total of 12 treatments were used in the experiment. The treatments were assigned to the respective plots, according to randomized complete block design with three replications.

The soybean variety 'William-82' was selected and seeds were collected from the Oil Seed Department, National Agricultural Research Centre Islamabad, Pakistan. The *Bradyrhizobium* strain TAL-102 was collected from National Institute of Biology & Genetic Engineering Faisalabad, Pakistan. All N was applied (to the respective plots) at the time of sowing in the form of urea. A basal dose of 60 kg P ha^{-1} and 60 kg K ha^{-1} (single super phosphate and sulphate of potash) was also applied at the time of sowing. Soybean seeds were inoculated with respective *Bradyrhizobium* strain for half an hour just before sowing. The crop was sown on 15th June 2009 and 11th June 2010. The row to row distance was maintained to 40 cm. After germination, the soybean plant density was adjusted to about 330,000 plants ha^{-1} by removing the smaller, weaker and diseased plants. All standard local cultural practices were followed when required throughout the growth period. No irrigation was provided and manual weeding was carried out on three occasions.

Measurements

A set of three plants from each plot was excavated at full flowering (R_2 stage) for determining root nodulation (number and mass of nodules), shoot, root length, shoot and root dry weights. The yield characteristics were recorded at the crop maturation stage. The selected plants were dipped into the water filled container to loosen the adhering soil and roots were washed to remove soil. Shoots and roots were separated by cutting with fine knife. Nodules were picked and number in each plant was counted followed by their fresh weight. The nodules dry weight (mass) was measured by drying in an oven at 70 ^{0}C to a constant weight. At full maturity (R_8) two central rows from each plot were harvested manually and left for one week for drying. Biological yield was determined by weighing the harvested plants from each plot. Plants were threshed and seed yield was obtained and recorded. Dry matter yield (DMY) was obtained from difference between biological yield and seed yield.

Acetylene reduction assay

Nitrogenase activity (N_2 fixation) was detected by acetylene reduction/ethylene production assay at flowering stage according to the method described earlier (Mirza et al., 2001). The root nodules were detached from the plant and were put into a vacutainer tube, which was closed by a plastic stopper making it airtight. Acetylene, generated from Calcium cyanide (CaC_2) and water was injected into the tube to yield a 10% acetylene atmosphere with hydrogen flame ionization detector. After one hour assay, a 200ul gas sample was drawn from the tube and injected into Gas Chromatograph. Each gas sample was analyzed using a Thermoquest model 3700 gas chromatograph fitted with a porapak N column (80/100 mesh; 200×0.3175 cm) to determine ethylene content. The nitrogenase activity was expressed as μ moles of C_2H_4 produced $plant^{-1}$ h^{-1}.

Plant NPK contents and uptake

The harvested soybean plant samples were dried in an oven at 70^{O}C till constant weight. Samples were ground in a Wiley mill (Polymix PX-MFC 90D; Switzerland) to pass through a 1-mm sieve. Total N in above ground material was estimated by Kjeldhal digestion, distillation and titration methods (Bremner and Mulvaney, 1982). Total P and K were determined by digesting 0.25 g of material with sulfuric acid and hydrogen peroxide. The P in the digests was measured by spectrophotometer (Murphy and Riley, 1962) and K was determined by atomic absorption spectrophotometer (Winkleman et al., 1990). All values are expressed on dry weight basis. The NPK uptake in vegetative tissue was calculated from DMY and NPK concentration in shoots (Abbasi and Tahir, 2012).

Statistical analysis

Analyses of variance (ANOVA) were performed using MSTAT-C (1991) statistical analysis package to determine treatment effects on yield components, nodulation/N_2 fixation and seed chemical characteristics. All statistical comparisons were made at $\alpha = 0.05$ probability level unless otherwise stated, using the least significant difference method for mean separation (Muhammad, 1995). Correlation and linear analyses were used to compare the relationship among different variables using SPSS 12 for Windows (www.SPSS.com).

Results and Discussion

Plant biomass – shoot and root characteristics

The significance level of different treatments applied to soybean over two years is presented in Table 3. Results indicated that the isolated strains generated positive effects on plant growth promotion by increasing shoot and root length (26–47% and 45–73%) and shoot dry weight (58–104%) compared to the non-inoculated control (Table 4) while the root dry weight showed no-significant response. Plants inoculated with indigenous *Bradyrhizobium* strains N_{20} and N_{22} showed significantly higher growth than those inoculated with exotic TAL-102. Growth promotion due to the applied strains may be attributed to the N_2 fixation or due to the production of plant growth promoting hormones in the rhizosphere and other plant growth promoting activities by the strains (Glick, 1995). Previous study indicated that application of *B. amyloliquefaciencs* and *B. japonicum* substantially increased soybean growth due to the production of phytohormones such as indole acetic acid (IAA), Gibberellins (GA3), Zeatin, Ethylene and Abscisic acid (ABA) (Masciarelli et al., 2014). Egamberdiyeva et al. (2004a) reported that *Bradyrhizobium* inoculation increased soybean shoot and root dry weight by 7−23% and 57−78%, respectively compared to the control. In our previous study conducted under field conditions (with the same soil and environmental conditions), application of exotic *Bradyrhizobium* strains increased soybean shoot length by 4–9% and shoot dry weight by 9–25% compared to non-inoculated control (Abbasi et al., 2008). The growth characteristics of soybean displayed significant correlations with yield components (Table 5) suggesting the correlation and positive linkage between plant growth traits and the yield of the crop.

Table 3. Tests of significance (*P* value) and coefficient of variance (CV) for growth, yield, nodulation and NPK accumulation of soybean in response to different treatments (T) i.e. N fertilizer rates, *Bradyrhizobium* strains, years (Y) and their interactions (T×Y) grown under field conditions during 2009 and 2010 at Rawalakot, Azad Jammu and Kashmir, Pakistan.

Sources	D.F.	SL	SDW	RL	RDW	NN	NDW	ARA	Pods	TSW	DMY	SY	Shoot N	N-uptake	Shoot P	P-uptake	Shoot K	K-uptake
T	11	**	**	**	NS	**	**	**	**	**	**	**	**	**	**	**	**	**
Y	1	**	**	**	**	**	**	**	**	**	**	**	NS	NS	NS	NS	NS	NS
T×Y	11	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	NS
C.V		6.0	13.60	9.32	12.91	5.06	6.78	7.50	6.76	4.14	4.0	8.31	3.41	6.30	14.50	17.03	12.74	14.19

* Significant at the 0.05% probability level; ** Significant at the 0.01% probability level; § ns not significant; D.F. = degree of freedom; SL = shoot length; SDW = shoot dry weight; RL = root length; RDW = root dry weight; NN = number of nodules; NDW = nodules dry weight; ARA = Acetylene reduction assay; Pods = number of pods; TSW = thousand seed weight; DMY = dry matter yield; SY = seed yield.

Table 4. Effect of indigenous *Bradyrhizobium* inoculation and N fertilization on plant biomass (shoot and root characteristics) of soybean grown under field conditions at Rawalakot Azad Jammu and Kashmir, Pakistan during 2009 and 2010.

Treatments	Shoot characteristics						Root characteristics					
	Shoot length (cm)			Shoot dry weight (g plant^{-1})			Root length (cm)			Root dry weight (g plant^{-1})		
	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean
Control	57.6^{d}	54.4^{e}	56.0^{D}	13.6^{c}	16.9^{d}	15.2^{D}	20.6^{d}	21.5^{d}	21.0^{E}	2.0	4.1	3.1
N_{25}	61.7^{d}	64.6^{d}	63.2^{C}	14.4^{c}	18.4^{cd}	16.4^{D}	24.7^{d}	22.4^{d}	23.6^{E}	2.1	4.3	3.2
N_{50}	71.8^{c}	71.2^{bc}	71.5^{B}	22.0^{b}	25.5^{b}	23.8^{C}	30.3^{c}	28.3^{c}	29.3^{D}	2.5	4.5	3.5
N_{100}	85.1^{a}	80.5^{a}	82.8^{A}	29.7^{a}	33.4^{a}	31.5^{A}	38.1^{a}	35.0^{a}	36.5^{A}	2.9	4.8	3.8
NR_4	74.6^{bc}	70.6^{bcd}	72.6^{B}	23.3^{b}	24.6^{bc}	24.0^{C}	31.3^{bc}	29.4^{c}	30.4^{CD}	2.2	4.3	3.2
NR_{13}	73.3^{c}	71.4^{bc}	72.3^{B}	23.3^{b}	27.2^{ab}	25.2^{C}	32.1^{bc}	29.6^{c}	30.9^{CD}	2.1	4.5	3.3
NR_{18}	80.5^{ab}	75.1^{ab}	77.8^{A}	27.5^{a}	27.6^{ab}	27.5^{BC}	35.7^{ab}	30.5^{bc}	33.1^{BC}	2.4	4.7	3.5
NR_{20}	89.1^{a}	78.8^{a}	80.9^{A}	29.4^{a}	30.9^{ab}	30.2^{AB}	38.0^{a}	34.8^{ab}	36.4^{AB}	2.4	4.9	3.6
NR_{22}	84.8^{a}	79.5^{a}	82.2^{A}	28.7^{a}	33.3^{a}	31.0^{AB}	38.5^{a}	35.5^{a}	37.0^{A}	2.7	5.0	3.9
NR_{25}	73.3^{c}	66.2^{cd}	69.8^{B}	22.7^{b}	26.1^{b}	24.4^{C}	32.5^{bc}	30.0^{c}	31.3^{CD}	2.3	4.3	3.3
NR_{35}	74.2^{bc}	67.5^{cd}	70.8^{B}	22.0^{b}	26.1^{b}	24.1^{C}	32.7^{bc}	29.5^{c}	31.1^{CD}	2.3	4.6	3.4
TAL102	74.8^{bc}	68.9^{bcd}	71.9^{B}	23.3^{b}	26.4^{b}	24.8^{C}	32.5^{bc}	29.0^{c}	30.7^{CD}	2.5	4.5	3.5
LSD ($P \leq 0.05$)	6.94	6.40	5.07	4.10	6.50	3.93	4.90	4.30	3.35	NS	NS	NS

* N_{100} = 100 kg N ha^{-1}; N_{50} = 50 kg N ha^{-1}; N_{25} = 25 kg N ha^{-1}; TAL-102 = Exotic strain; NR_4, NR_{13}, NR_{18}, NR_{20}, NR_{22}, NR_{25} and NR_{35} are indigenous strains; LSD = Least significant difference.

Table 5. Pearson correlation (R^2) coefficients between plant growth characteristics, yield and yield traits and nodulation components in response to different N fertilizer rates and indigenous *Bradyrhizobium* strains applied to soybean under field conditions at Rawalakot Azad Jammu and Kashmir, Pakistan.

Plant traits	SL	SDW	RL	RDW	NN	NDW	ARA	Pods	TSW	DMY	SY	N-uptake	P-uptake
SDW	0.981**												
RL	0.978**	0.992**											
RDW	0.903**	0.903**	0.887**										
NN	0.636*	0.709**	0.735**	0.514									
NDW	0.636*	0.698*	0.731**	0.494	0.993**								
ARA	0.622*	0.701*	0.732**	0.527	0.992**	0.981**							
Pods	0.982**	0.977**	0.971**	0.929**	0.601*	0.596*	0.594*						
TSW	0.966**	0.980**	0.987**	0.906**	0.681*	0.674*	0.687*	0.983**					
DMY	0.896**	0.950**	0.946**	0.878**	0.737**	0.731**	0.737**	0.927**	0.943**				
SY	0.961**	0.985**	0.982**	0.901**	0.717**	0.710**	0.712**	0.974**	0.980**	0.977**			
N-uptake	0.83**	0.90**	0.89**	0.82**	0.32^{ns}	0.39^{ns}	0.30^{ns}	0.89**	0.91**	0.98**	0.95**		
P-uptake	0.598*	0.609*	0.682*	0.604*	0.525*	0.561*	0.560*	0.630*	0.679*	0.709**	0.665*	0.523*	
K-uptake	0.85**	0.91**	0.88**	0.83**	0.105^{ns}	0.161^{ns}	0.102^{ns}	0.90**	0.90**	0.95**	0.94**	0.98**	0.56*

* Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level; ns, non-significant; SL = shoot length; SDW = shoot dry weight; RL = root length; RDW = root dry weight; NN = number of nodules; NDW = nodules dry weight; ARA = Acetylene reduction assay; Pods = number of pods; TSW = thousand seed weight; DMY = dry matter yield; SY = seed yield.

Nodulation and acetylene reduction assay (ARA)

Analysis of variance revealed that soybean nodulation i.e. number of nodules (NN), nodules dry weight (NDW) and acetylene reduction assay (ARA) were significantly affected by treatments (T) and the years (Y) but the interaction between T×Y was non-significant (Table 3). A significant numbers of nodules (48 per plant) and nodules mass (0.32 g plant^{-1}) were detected in the plants grown in the non-inoculated control (Table 6) showing the presence of indigenous rhizobia capable of forming nodules in plants grown in the soil that had no previous history of soybean cultivation. Similarly, the ARA of the root nodules under non-inoculated control was remarkable i.e. 17.3 µ mol palnt^{-1} h^{-1} reflecting that the observed nodules were effective in N_2 fixation. This finding is in contrast to the general observation that soybean is not able to generate nodules or N_2 fixation in soils that had never been planted with soybean. However, the presence of nodules and ARA in the non-inoculated controls (in the present study) signified that the soybean cultivar was promiscuous, as it was nodulated by indigenous *Bradyrhizobiam* strains present in the soil.

Application of *Bradyrhizobium* strains significantly increased nodule number (NN) (59–108 palnt^{-1}), nodules dry weight (NDW) (0.45–095 g plant^{-1}) and ARA activity (23.9–35.2 µ mol palnt^{-1} h^{-1}) compared to the non-inoculated control (Table 6). Greater nodulation and N_2 fixation due to inoculation suggested that there is better interaction between the microbes (*Bradyrhizobium* strains) and the plant (soybean). Our results are in agreement with the previous findings where soybean growth and nodulation in a field that has no previous soybean cropping history showed higher response to inoculation (Revellin et al., 2000; Abbasi et al., 2008). It has been reported that presence of less than 10^2 cells g^{-1} soil of indigenous rhizobia eliminated inoculation response because of the presence of few effective strains in a soil population (Thies et al., 1991). But in our case, the MPN determined from twenty two sites was substantial ranged between 5.0×10^4 to 8×10^6CFU g^{-1}soil (unpublished data). Results of our study displayed a significant correlation between NN and ARA activity (R^2=0.99) and NDW ARA activity (R^2=0.98) (Table 5) validating these parameters as measures of N_2 fixation efficiency of soybean under our conditions.

Response of NN, NDW and ARA to N fertilization varied with N rates. The lower N rate i.e. N_{25} significantly increased nodulation while N_{50} displayed non-significant difference for NN and NDW but significantly higher ARA level compared to the control. The higher N rate (N_{100}) showed depressing effect on nodulation and N_2 fixation. Although, additions of small N fertilizer to the soil enhance nodulation, yet it is well known that the development of nodules and N_2 fixation is depressed when the plants are exposed to high N concentration (Wahab and Abd-Alla, 1996; Yinbo et al., 1997; Ahmed, 2013). Therefore, only a little or no N fertilizer is preferred for N_2 fixation in soybean.

Table 6. Effect of N Fertilization and *Bradyrhizobium* inoculation on nodulation and nitrogenase activity of soybean grown under field conditions at Rawalakot Azad Jammu and Kashmir in the year 2009–10.

Treatments	Number of nodule plant^{-1}			Dry weight of nodules (g plant^{-1})			ARA (µ mol plant^{-1} h^{-1})		
	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean
Control	46.0^{h}	50.3^{g}	48.2^{H}	0.31^{e}	0.33^{h}	0.32^{H}	16.2^{f}	18.4^{fg}	17.3^{G}
N_{25}	54.6^{g}	62.3^{f}	58.5^{G}	0.41^{d}	0.50^{g}	0.45^{G}	18.6^{e}	20.4^{ef}	19.5^{F}
N_{50}	41.3^{h}	50.9^{g}	46.1^{H}	0.30^{e}	0.36^{h}	0.33^{H}	13.6^{g}	15.1^{g}	14.5^{H}
N_{100}	23.0^{i}	30.7^{h}	26.8^{I}	0.18^{f}	0.22^{i}	0.20^{I}	9.4^{h}	10.4^{h}	9.9^{I}
NR_4	71.0^{f}	78.4^{e}	74.7^{F}	0.60^{c}	0.64^{f}	0.62^{F}	23.4^{d}	24.5^{de}	23.9^{E}
NR_{13}	82.0^{de}	90.2^{c}	86.1^{CD}	0.71^{b}	0.84^{c}	0.78^{CD}	24.3^{cd}	28.0^{cd}	26.2^{D}
NR_{18}	89.3^{bc}	85.2^{cd}	87.3^{C}	0.82^{a}	0.77^{d}	0.80^{C}	27.2^{b}	31.6^{bc}	29.4^{BC}
NR_{20}	91.0^{b}	100.7^{b}	95.8^{B}	0.86^{a}	0.93^{b}	0.89^{B}	28.5^{b}	33.4^{b}	30.9^{B}
NR_{22}	105.6^{a}	109.3^{a}	107.5^{A}	0.91^{a}	0.98^{a}	0.9^{A}	32.8^{a}	37.7^{a}	35.2^{A}
NR_{25}	83.0^{cd}	83.2^{de}	83.1^{CD}	0.73^{b}	0.74^{de}	0.7^{DE}	25.5^{c}	29.5^{bc}	27.5^{CD}
NR_{35}	76.0^{ef}	81.8^{de}	78.8^{EF}	0.67^{bc}	0.70^{e}	0.69^{E}	24.7^{cd}	28.7^{c}	26.7^{D}
TAL102	81.0^{de}	84.6^{d}	82.8^{DE}	0.70^{b}	0.75^{d}	0.73^{DE}	25.5^{c}	29.0^{c}	27.3^{D}
LSD ($P \leq 0.05$)	6.93	5.59	4.29	0.091	0.045	0.049	1.44	4.15	2.09

* N_{100} = 100 kg N ha^{-1}; N_{50} = 50 kg N ha^{-1}; N_{25} = 25 kg N ha^{-1}; TAL-102 = Exotic strain; NR_4, NR_{13}, NR_{18}, NR_{20}, NR_{22}, NR_{25} and NR_{35} are indigenous strains; LSD = Least significant difference.

Yield and yield components

The thousand seed weight (TSW) DMY and seed yield displayed significant responses ($P \leq 0.05$) to treatments (T) and years (Y), but the interaction between the T×Y was non-significant (Table 3). However, T, Y and T×Y was significant for number of pods per plant. Soybean yield and yield components in response to *Bradyrhizobium* strains had shown a significant increase in number of pods, TSW, DMY and seed yield compared to the yield recorded under non-inoculated control (Table 7). These results could be explained by the reported symbiosis efficiency between soybean and *Bradyrhizobium japonicum* (Abbasi et al., 2008). Increased nodulation and subsequent N_2 fixation due to inoculation resulted in the measured increases in yield and yield components of soybean. In addition, increased NPK uptake due to *Bradyrhizobium* inoculation and N fertilizer may also contributed to increase soybean yield and yield components as significant positive correlations existed between these components (Table 5). Increased soybean yields due to inoculation have been reported earlier by many researchers. Egamberdiyeva et al. (2004b) reported a 48% increase in soybean yield after inoculation in Uzbekistan while Okereke et al. (2001) in Nigeria found a significant increase in soybean seed yields after *Bradyrhizobium* inoculation varied between 14–108% compared to the non-inoculated treatment. Zhang et al. (2002) suggested that *Bradyrhizobium japonicum* improved seed yield of soybean largely due to increase in pod and seed number as observed in this study. The correlation coefficient between number of pods per plant and seed yield was $R^2 = 0.97$ and TSW and seed yield was $R^2 = 0.91$ (Table 5). Comparative assessment of the results of the greenhouse and field experiment indicated a significant improvement in nodulation, vegetative growth and seed yield of three soybean genotypes grown in Rajasthan, India (Meghvansi et al., 2010). The experiments conducted here in Pakistan had shown a

substantial increase in soybean yield and yield components after inoculation (Ashraf et al., 2002; Oad et al., 2002; Fatima et al., 2007; Abbasi et al., 2008; Abbasi et al., 2010). In contrast to these results, there are also many reports highlighting non-significant effect of Rhizobium inoculation on soybean yield (Simanungkalit et al., 1995; Achakzai et al., 2002). The yield and yield traits under non-inoculated control were the lowest despite of a remarkable nodulation and ARA (N_2 fixation) reflecting that the native rhizobia was not able to compete with the inoculated rhizobia in an efficiency in colonize the nodules and be effective in the BNF process.

The effect of N fertilization on yield and yield components of soybean was rate specific. N_{25} did not show any positive effects on soybean yield components (except number of pods palnt^{-1}) in spite of its positive effect on nodulation and ARA. Earlier studies suggested that only rarely could seed yield of legumes be enhanced by starter N fertilizer application (Yinbo et al., 1997; Starling et al., 1998). A starter-N fertilizer is effective only when the host plant showed poor nodulation or when rhizobia in soil was ineffective (Kessel and Hartley, 2000). The N fertilizer N_{50} and N_{100} displayed significant increase in yields compared to the control and N_{100} had shown the highest yields compared to all treatments.

Plant nutrient accumulation

Analysis of variance indicated that shoot NPK content and NPK uptake had shown a significant response to the treatments (T) but the effect of years (Y) and the interaction between T×Y were non-significant (Table 3). The results pertaining to NPK contents and NPK uptake in the vegetative tissue (shoot) of the plant in response to indigenous *Bradyrhizobium* inoculation and N fertilization is presented in Figures 1 and 2. According to analysis of variance years had no significant effect on NPK contents therefore, averaged values across the years are presented. The results of NPK contents and NPK-uptake in shoot biomass signified the efficient allocation/uptake of NPK to plants due to the application of *Bradyrhizobium strains* and N fertilizer rates (N_{50} and N_{100}) as reported earlier (Okereke et al., 2000; Egamberdiyeva et al., 2004b; Fatima et al. 2007; Abbasi et al., 2010). The nutrient accumulation in soybean plants by the *Bradyrhizobium* strains was either equivalent to (N and K) or higher than (P) that recorded under higher N fertilizer rate N_{100}. This increase in nutrient uptake in plant tissue is mainly due to a better plant performance due to N_2 fixed and nutritional N-status, which contributes to other traits related to P nutrition, like root branching and total length. Increased NPK accumulation in plant biomass may be a result of increased nodulation and ARA activity by the effective *Bradyrhizobium* strains (Okereke et al., 2000). The highest N and K concentration/uptake was recorded in plants supplemented with isolates NR_{20} and NR_{22} those displayed the highest nodulation and nitrogenase activity (N_2 fixation). In the present study, the overall relationship between ARA and N-uptake is non-significant because of the addition of N fertilizer those exhibited lower ARA activity but higher N-uptake (Table 3). Nevertheless, the correlation co-efficient between ARA and N-uptake under strains treatments was highly significant ($R^2 = 0.85$) indicating a close association between plant N concentration/N-uptake and N_2 fixation. Okereke et al. (2001) reported that the percentage increase in shoot N content and total N-uptake of soybean after *Bradyrhizobium* inoculation ranged between 18–62% and 35–191%, at the Awka site (Nigeria) while the corresponding increase at the Igbariam site was 0–43% and 19–125%, respectively.

Table 7. Effect of indigenous *Bradyrhizobium* inoculation and N fertilization on yield and yield components of soybean grown under field conditions at Rawalakot Azad Jammu and Kashmir, Pakistan during 2009 and 2010.

Treatments	Numbers of pods plant^{-1}			Thousand seed weight (g)			Dry matter yield (kg ha^{-1})			Seed yield (kg ha^{-1})		
	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean
Control	50.7^{d}	52.4^{g}	51.6^{E}	150.0^{e}	159.0^{e}	154.7^{C}	1345^{g}	1353^{e}	1334^{G}	804^{e}	917^{d}	860^{F}
N_{25}	58.3^{d}	64.5^{f}	61.4^{D}	159.0^{de}	164.0^{de}	161.5^{C}	1293^{g}	1305^{e}	1299^{G}	933^{e}	1030^{d}	982^{F}
N_{50}	71.3^{bc}	76.5^{de}	73.9^{BC}	171.3^{cd}	171.7^{cd}	171.5^{B}	1865^{e}	1909^{d}	1887^{E}	1340^{d}	1482^{c}	1411^{E}
N_{100}	83.0^{a}	104.5^{a}	93.7^{A}	188.7^{a}	195.7^{a}	192.2^{A}	2582^{a}	2652^{a}	2617^{A}	2037^{a}	2182^{a}	2110^{A}
NR_4	70.0^{c}	73.7^{e}	71.9^{C}	173.0^{c}	174.3^{cd}	174.0^{B}	1588^{f}	1778^{d}	1683^{F}	1354^{d}	1545^{bc}	1450DE
NR_{13}	70.3^{c}	81.8^{cd}	76.1^{BC}	171.3^{cd}	179.0^{c}	175.2^{B}	2239^{b}	2311^{b}	2275^{B}	1639^{b}	1772^{b}	1706^{B}
NR_{18}	70.0^{c}	86.8^{c}	78.4^{B}	174.3^{bc}	181.7^{bc}	178.0^{B}	2138^{bc}	2127^{c}	2132^{CD}	1609^{bc}	1728^{bc}	1668^{B}
NR_{20}	81.0^{ab}	97.2^{b}	89.1^{A}	187.0^{ab}	192.0^{ab}	189.5^{A}	2555^{a}	2657^{a}	2606^{A}	1962^{a}	2072^{a}	2017^{A}
NR_{22}	82.3^{a}	101.7^{ab}	92.0^{A}	188.0^{a}	195.4^{a}	191.8^{A}	2559^{a}	2635^{a}	2597^{A}	1999^{a}	2145^{a}	2072^{A}
NR_{25}	70.7^{c}	74.5^{e}	72.6^{BC}	173.7^{c}	182.0^{bc}	177.8^{B}	1993de	2167^{bc}	2080^{D}	1440^{cd}	1565^{bc}	1503^{CDE}
NR_{35}	70.0^{c}	73.9^{e}	71.9^{C}	173.3^{c}	176.0^{c}	174.7^{B}	2151^{bc}	2220^{bc}	2186^{BC}	1580^{bc}	1619^{bc}	1600^{BCD}
TAL102	70.3^{c}	75.6^{de}	73.0^{BC}	174.0^{c}	178.3^{c}	176.2^{B}	2082^{cd}	2178^{bc}	2130^{CD}	1590^{bc}	1659^{bc}	1624^{BC}
LSD (P≤0.05)	10.27	6.36	5.93	12.84	10.63	8.48	134	151	96	178	246	153

* N_{100} = 100 kg N ha^{-1}; N_{50} = 50 kg N ha^{-1}; N_{25} = 25 kg N ha^{-1}; TAL-102 = Exotic strain; NR_4, NR_{13}, NR_{18}, NR_{20}, NR_{22}, NR_{25} and NR_{35} are indigenous strains; LSD = Least significant difference.

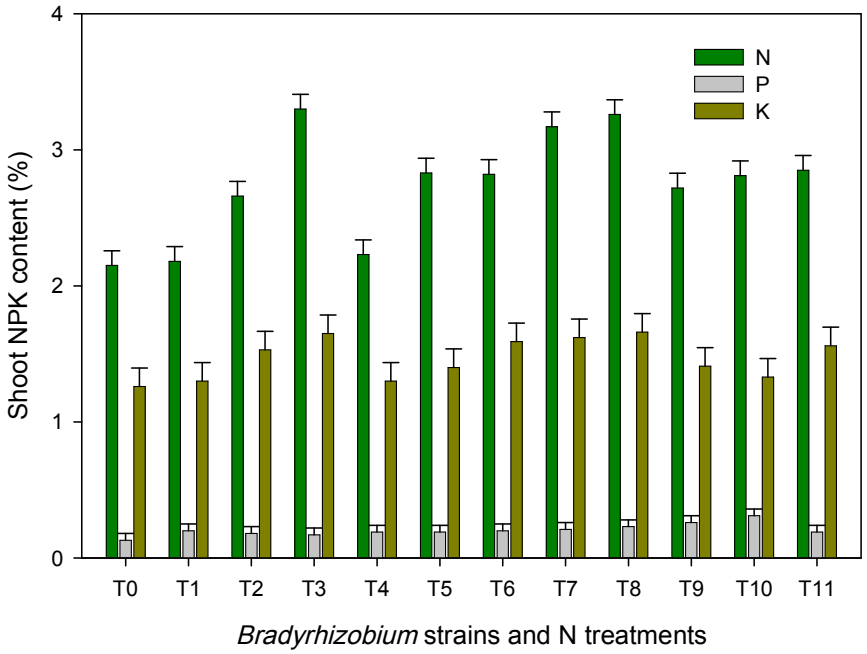

Figure 1. Effect of indigenous *Bradyrhizobium* inoculation and N fertilization on shoot N, P and K contents of soybean grown under field conditions at Rawalakot Azad Jammu and Kashmir, Pakistan during 2009 and 2010; The legend on x-axis representing T_0 = un-inoculated control; $T_1 = N_{25}$; $T_2 = N_{50}$; $T_3 = N_{100}$; $T_4 = NR_4$; $T_5 = NR_{13}$; $T_6 = NR_{18}$; $T_7 = NR_{20}$; $T_8 = NR_{22}$; $T_9 = NR_{25}$; $T_{10} = NR_{35}$; T_{11} = TAL 102. The vertical line on each bar representing the least significant difference (LSD at $P \leq 0.05$) among different treatments for each trait i.e. N, P and K.

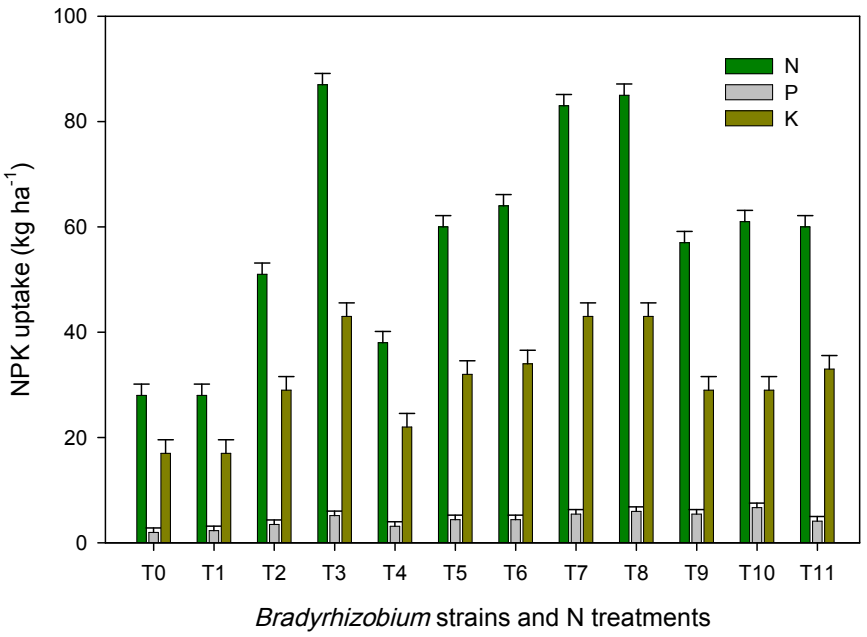

Figure 2. Effect of indigenous *Bradyrhizobium* inoculation and N fertilization on shoot N, P and K uptake of soybean grown under field conditions at Rawalakot Azad Jammu and Kashmir, Pakistan during 2009 and 2010; The legend on x-axis representing T_0 = un-inoculated control; $T_1 = N_{25}$; $T_2 = N_{50}$; $T_3 = N_{100}$; $T_4 = NR_4$; $T_5 = NR_{13}$; $T_6 = NR_{18}$; $T_7 = NR_{20}$; $T_8 = NR_{22}$; $T_9 = NR_{25}$; $T_{10} = NR_{35}$; T_{11} = TAL 102. The vertical line on each bar representing the least significant difference (LSD at $P \leq 0.05$) among different treatments for each trait i.e. N, P and K.

Rhizobium strains used in this study were isolated form 22 different sites located at the same ecological region but displayed a significant variation with regard to their symbiotic effectiveness (root nodulation and ARA activity), NPK accumulation and yield characteristics of soybean. Therefore, site or location may have contributed toward the variability of the isolates with regard to their symbiotic effectiveness and yield potential as suggested earlier (Sanginga et al., 2000; Okereke et al., 2001). Such differences due to site variability was explained earlier by Osunde et al. (2003) those may be attributed to the previous cropping sequence, variation in the symbiotic properties of indigenous rhizobial populations, or the available soil N content. Meghvansi et al. (2010) also found variable response of *Bradyrhizobium* isolates towards inoculation in three soybean genotypes. Such variations may be attributed to the variations in the genomic constitutions of the host or bacteria or both which control symbiosis. The fact that the symbiotic effectiveness and yield potential of soybean varied between isolated strains indicated that the choice of strains for inoculant production should be based on rhizobial screening to identify the best strains.

In the present study, the performance difference between exotic strain TAL-102 and five indigenous strains NR_4, NR_{13}, NR_{18} and NR_{25} was almost the same. Soybean symbiotic effectiveness and yield potential between these strains was non-significant for most of the traits but significantly higher than the un-inoculated control. However, performance of *Bradyrhizobium* strains NR_{20} and NR_{22} was significantly higher than the remaining strains including the exotic TAL-102. Soybean seed inoculation with these two strains caused a 13 and 14%, 22 and 25%, 19 and 21%, 13 and 29%, 16 and 30%, 22 and 305 and 24 and 28% enhancement in SL, SDW, RL, ARA, NN, NDW and seed yield, respectively, compared to the TAL-102. Our result are in accordance with the findings indicating that indigenous rhizobia are generally promiscuous and effective and are usually more effective in nodule formation than introduced strains (Ahmed and McLaughlin, 1985; Salanturet et al., 2005). Low performance of exotic strain compared to NR_{20} and NR_{22} might be attributed to the difference in adapting to local conditions as opposed to indigenous strains which are well adjusted to prevailing conditions at the site and, moreover, can adapt to just about any change in conditions (Vlassak and Vanderleyden, 1997). There are also reports in contrast to our findings highlighting the better performance of non-indigenous commercial strains over indigenous strains in soybean nodulation, N_2 fixation and yield potential (Okereke et al., 2000; Osunde et al., 2003). These two strains NR_{20} and NR_{22} were also able to acquire growth and yield of soybean equivalent to that achieved under N_{100} indicating that the N_2 fixation induced by the indigenous *Bradyrhizobium* isolates supplied N equal to the optimal amounts of N. The performance of strains NR_{20} and NR_{22} during two years study under field conditions suggested to test these two strains under different conditions to use them as bio-inoculant for soybean in the mountain region.

Conclusions

Inoculation of soybean with indigenous strains isolated from twenty two different sites of the mountain ecosystem having no previous soybean history resulted in a significant improvement in symbiotic effectiveness and yields of soybean. Based on the evidences generated from two years field experiments, two isolates NR_{20} and NR_{22} were identified as superior strains in terms of their symbiotic effectiveness and their compatibility with soybean genotype with regards to growth and yield performance.

Effective *Bradyrhizobium* isolates NR_{20} and NR_{22} have potential to be used for inoculants production at large scale and the multi locational trials are required to determine their suitability for other agro-climatic conditions. Analysis of the site variability of rhizobial strains in combination with the symbiotic performance and competitive behavior may allow the identification of strains useful for agricultural purposes. This study showed encouraging results suggesting that among the indigenous populations, successful inoculant strains could be identified and explored for further experiments for their ability to N_2 fixation and crop production. These results are important because the use of indigenous *Bradyrhizobium* spp. as a bacterial inoculants or bio-fertilizers will provide a new technological approach and thought that may reduce the use of expensive chemical fertilizers and help to introduce sustainable agriculture production even under degraded N-poor soil conditions existing in the mountain regions of Himalayan including the state of Azad Jammu and Kashmir.

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