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VARIABILITY IN THE GROWTH AND NODULATION OF SOYBEAN IN RESPONSE TO ELEVATION AND SOIL PROPERTIES IN THE HIMALAYAN REGION OF KASHMIR-PAKISTAN

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Abstract

This study was conducted to examine the variability of soybean nodulation and growth in relation to elevation and soil properties across the slopping uplands of the Himalayan region of Rawalakot Azad Jammu and Kashmir (AJK), Pakistan in order to find efficient native N_2 fixing bacteria adapted to local soil and climatic characteristics. Soils from twenty two different sites with variable altitude were collected and analyzed for different physico-chemical characteristics including the quantitative estimation of rhizobium population through most probable number (MPN) technique. Soybean cultivar William-82 was grown in these soils under greenhouse condition for determining the nodulation potential (number and mass) and plant growth characteristics. Morphology of the nodules were observed through optical and transmission electron microscopy. Principal component analysis (PCA) and Biplot graph were used to jointly interpret the relationship between variables and soils (treatments). Soil altitude ranged from 855 m to 3000 m while organic matter content varied between 0.8% to 3.5% and pH from 6.0 to 8.1. The indigenous rhizobia population varied between 5.0 $x10^4$ to 8.0 $x10^6$ CFU g^{-1} showing the existence of a substantial rhizobial population in these soils. The number of nodules per plant varied from 7 to 40 (CV 38%) suggesting site/location as an important factor contributing towards rhizobia population and impacting root nodulation. The electron microscopy of green plant nodules showed densely populated bacteria in these cells and nodule tissue cells were completely infected with bacteria. The growth characteristics of soybean i.e. shoot length, shoot fresh and dry weight, root length, root *fresh* and dry weight varied among the sites but in general a vigorous and healthy plant growth was observed reflecting N assimilation from native soils. Results showed a substantial variability between sites and this is likely to be due to inter/intra species diversity, as well as changes in microbial community composition/structure. The study suggests that soybean could be planted in this region without prior inoculation, and that native N_2-fixing bacteria might be isolated from these soils and used as biofertilizers (or inoculants).

Key words: Bio-fertilizer, *Glycine max*, Isolation, Indigenous rhizobia, Nodulation, Principal component analysis.

Introduction

Introduction and exploitation of soybean [*Glycine max* (L.) Merr.] in our cropping systems could be a promising management strategy in many aspects. Firstly, soybean is an important oilseed crop with high nutritional value. Soybean protein contains essential amino acids and its oil contains 85% unsaturated fatty acids and is free of cholesterol so it is highly desirable for human consumption (Raei *et al*., 2008).It is also a source of carbohydrates, minerals (Ca and P) and vitamins A, B and D (Hymowitz *et al*., 1998; Abbasi *et al*., 2012). The composition of soybean, as well as the ease and geographical range of its agricultural production, makes it an inexpensive source of oil and protein used as food and animal feed. Secondly, soybean plays an important role in the global and agricultural N cycles by facilitating biological N_2 fixation (BNF) into plant-available N. Soybean has been shown to fix an average of 175 kg N $ha^{-1}yr^{-1}$ in irrigated production, and 100 kg N ha^{-1} y^{-1} in dry land production (Unkovich & Pate, 2000; Furseth *et al*., 2012).

Inoculation of soybean seeds with highly effective rhizobia is a common practice in agricultural production, but the success of this technology requires survival and establishment of the inoculated rhizobia in the soil environment (Da & Deng, 2003). Similarly, Wani *et al.* (1995) stated that despite the considerable capacity for acquiring N from BNF, the inoculation of soybean with rhizobial strains does not necessarily result in yield increase. Soybean can also nodulate freely with native rhizobia strains and is able to fulfil its N requirement through BNF once the plants are established (Okogun *et al*., 2004; Singh *et al*., 2003). Many soils contain an appreciable number of indigenous rhizobia which may or not be effective for BNF despite competitive adaptability to their local environment (Zahran, 1999). Soybean cultivation in many parts of India has shown existence of native rhizobia besides other exotically adapted strains (Sharma *et al*., 2010). However, selection of niche-based new elite strains adapted to local environmental conditions and to newly bred plant lines is in its infancy (Appunu *et al*., 2008).

The potential for improving BNF through rhizobium inoculation requires knowledge of the abundance and effectiveness of the indigenous rhizobial populations in the soil. The success of soybean crop depends among other factors on the availability of efficient bradrhizobia in soil. The population of rhizobia is reported virtually absent or very low in many zones of Pakistan and in some cases nodulation was completely absent in soybean roots (Aslam *et al*., 2000; Achakzai *et al*., 2002). Javid & Mahmood (2010) conducted a field experiment on soybean in a loamy textured soil at Lahore Pakistan and found a maximum of 8-10 nodules in plant roots. However, a substantial nodulation of soybean (6–31 per plant) was reported in the northeast of Pakistan (Khyber Pakhtunkhwa , KPK) where three indigenous land races and two improved varieties were tested at different sowing timings (Muhammad *et al*., 2012).

The objective of this study was to determine the variability of selected soils/sites characteristics and to evaluate the relative influences of these characteristics on the occurrence of indigenous rhizobial population, nodulation and growth potential of soybean in soils collected from variable altitude from sub-division Rawalakot Azad Jammu and Kashmir, Pakistan. The study will help is to assess whether native N_2-fixing bacteria will effectively colonize and perform with planted soybeans without the need for prior inoculation.

Materials and Methods

Soil sampling and processing: Twenty two different sites from sub-division (Tehsil) Rawalakot Azad Jammu & Kashmir were selected from undisturbed native grassland on the basis of spatial and temporal variation in the year 2012. Fig. 1 shows the geographical location of the study sites. Rough meadow grass (*Poatrivialis* L.), /Bermuda grass (*Cynodondactylon* L.) and orchard grass (*Dactylisglomerata* L.) dominated the vegetation community at all sites. Within each site, soil samples from the surface 0–20 cm were collected from five points by soil auger and mixed as one composite sample. Before sampling, grass, forest litter or any other material on the soil surface were removed. All the samples were taken from topographically similar locations which were approximately within 100 meter from each other. After being brought into the laboratory, soil samples were air-dried for 2-3 days, partially milled and subsequently sieved. Samples were lightly ground and subsequently sieved through a 2 mm mesh to remove stones, roots and large organic residues. About one kg soil was taken, sealed in the plastic bags and stored in a refrigerator at 4 ^{0}C prior to analysis. Detail meteorological data of selected sites were also recorded during sampling (Table 1).

Laboratory analysis: Sub-samples (triplicate) from each site were taken and analyzed for soil texture, organic matter, total N and most probable number (MPN). Soil texture was determined after the organic matter was removed with 20% H_2O_2, by the Bouyoucos hydrometer method (Bouyoucos, 1962).Soil pH was determined in distilled water with a glass electrode (soil/H_2O ratio 1:2.5 w/v). Soil organic matter (SOM) was determined using a modified Mebius method (Nelson & Sommers, 1982). Briefly, 0.5 g soil was digested with 5 ml of 1N $K_2Cr_2O_7$ and 10 ml of H_2SO_4 at 150 ^{0}C for 30 min, digests were then titrated with standardized $FeSO_4$. Total N was determined by the Kjeldahl's digestion, distillation and titration method (Bremmer & Mulvaney, 1982).

The rhizobial population in the soil samples was determined by serial dilution and colony forming unit (CFU) on YEM agar plates by spread plate method (Somasegran & Hoben, 1994). Briefly, one gram of soil from each sample was added to 9 mL of 0.89 % saline solution and serial dilutions were made. From each dilution (10^{-5}, 10^{-6}, 10^{-7}) 100 µL was spread using sterile glass spreader on YEM agar plates. The plates were incubated at 28ºC for 3 to 5 days and CFU was counted.

Soybean growth under greenhouse conditions: A pot experiment was conducted to assess soybean growth and nodulation in soils collected from each of the 22 sites. Six soybean seeds (cultivar William-82) were planted in pots (18 cm width x 17 cm height) in May 18, 2012. The pots were arranged in a randomized complete block (RCB) design. After germination, the pots were thinned to keep only the 3 healthiest seedlings. No fertilizer was applied in any pot, however, irrigation was given when needed. Pots were kept in the greenhouse of the Faculty of Agriculture, Rawalakot.

Table 1. Meteorological data of the selected sites for the year 2012.

Sampling sites	Altitude (m)	Air Temp. (ºC)	Soil Temp. (ºC)	Humidity (%)
Baikh	1747	30.8	22	62.2
Parat	1379	32.5	24	59.2
PothiBala	1849	27.5	22	65.4
HurnaMera	2037	30.5	22	57.2
Paniola	1341	33.0	23	58.0
Tain	855	31.0	30	53.8
Thorar	1646	26.0	23	73.8
Dar	1090	29.4	29	66.5
Rawalakot	1594	29.0	27	55.4
Tarar	1812	27.6	22	48.5
Chak	1647	28.0	25	49.5
Hussainkot	1818	26.0	22.5	56.0
Banjosa	1779	26.0	23.0	73.0
Jandali	2026	24.0	22.5	73.0
Dothan	1774	25.0	23.0	68.0
Namnota	1763	26.2	24.0	66.0
Khukot	1631	27.5	24.0	59.8
Ali Sojal	1700	28.0	24.0	58.4
KhyusaneBaik	2274	23.0	22.0	71.0
Tolipir	3000	22.0	20.0	75.0
Topa	2008	24.6	22.0	53.3
Rehara	1544	28.5	26.0	68.0

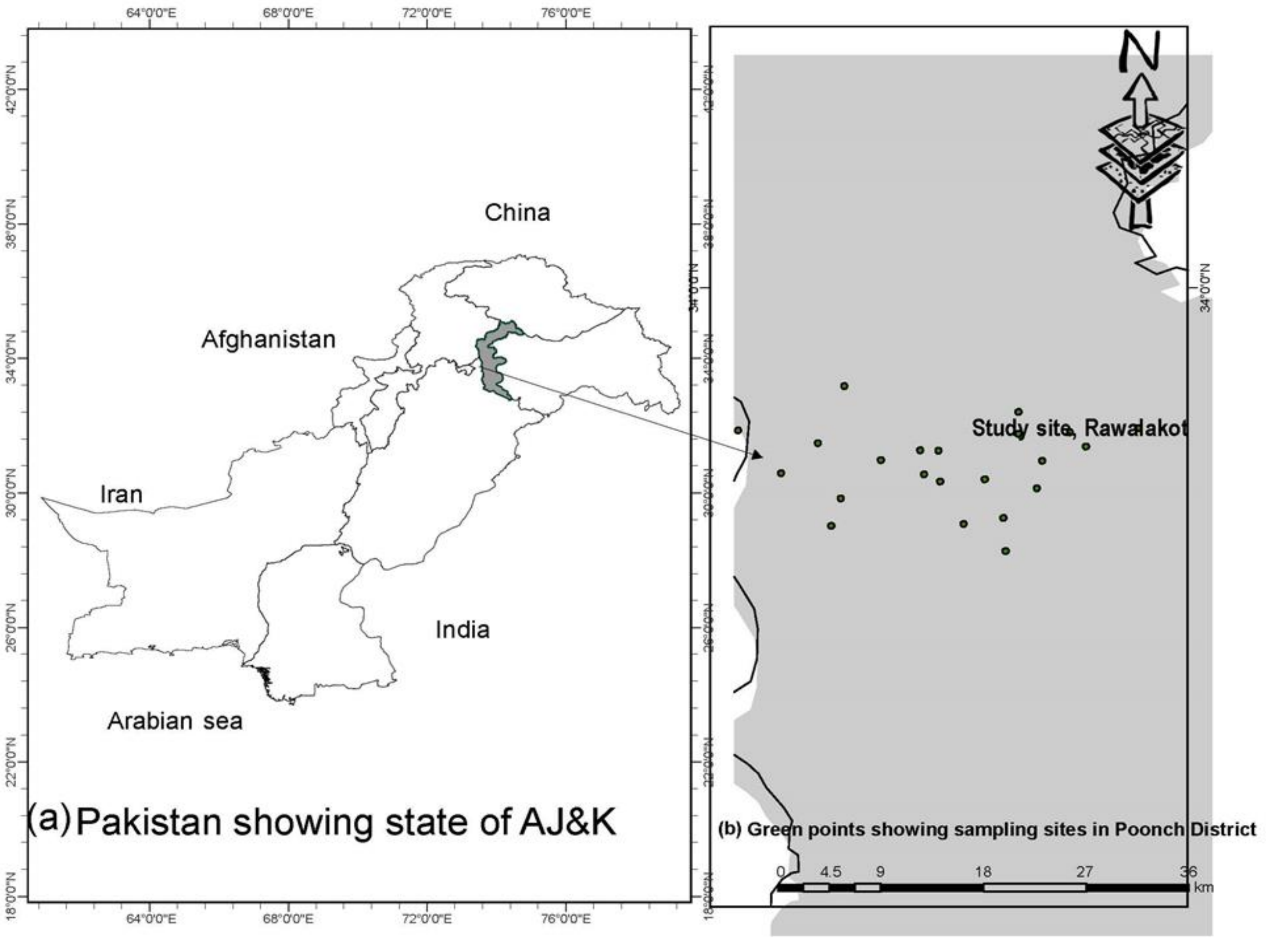

Fig. 1. Geographical representation of the state of Azad Jammu and Kashmir (a) and points on right side (b) representing the sampling sites located at Rawalakot Azad Jammu & Kashmir.

At harvest, plants were carefully taken out of the soil to avoid root damage. Roots were then separated from the shoots and all adhering soil particles were washed away with water. The nodules were separated from the roots, counted and weighted fresh. Plant shots, roots and a subsample of nodules were oven-dried at 70°C for 48 hours to measure dry weight.

Optical and transmission electron microscopy: Bacterial occupancy in the root nodules was examined using light and transmission electron microscope. Nodules samples were collected from soybean plants at flowering. Nodules were cut into 1-3 cm pieces and embedded in water agar to cut 2-3 mm small cubes. These small cubes were fixed in 5% gluteraldehyde for 16-18 h. The samples were then washed two times in 0.2 M PIPES buffer and treated with 0.2% osmimiumtetraoxide prepared in 0.2 M PIPES buffer (pH6.8) for 16-18 h. After washing samples were treated with 5% aqueous uranyl acetate for 16-18 h and again washed with sterilized distilled water. After dehydration with absolute alcohol and then with 100% propylene oxide, samples were transferred to flat embedding moulds containing pure resin and polymerized at 65ºC for 72 h. After polymerization the resin blocks were left at room temperature for at least 24 h before sectioning. Sectioning was performed at ultra-microtone (RMC-7000) to slice the section of 120-200 nm thickness. The sections were mounted on copper grids and stained with uranyl acetate for 30 minutes and lead acetate for 10 minutes. After staining the grids were washed with distilled water. The observations of samples were taken at light microscope (LEICA DM LS) and transmission electron microscope JEOL JEM-1010 (Hameed, 2003).

Principle component analysis: To find out variation in growth and nodulation potential of different soils, principle component analysis (PCA) was done. Eighteen principle components (PCs) were extracted. The Jolliffe cut-off value was 0.7 was used as an indication of significant principal components (Jolliffe, 1986).

Statistical analysis: One way analyses of variance was performed using Anon., (1991) statistical analysis package to determine sites effect on the nodulation and growth components of soybean. All statistical comparisons were made at $\alpha = 0.05$ probability level using the least significant difference method for mean separation (Muhammad, 1995).Average values of each trait were analyzed for multivariate analysis (principal component analysis) by using the SPSS-12 for Windows (www. SPSS. com) as described earlier (Iqbal *et al*., 2008).Correlation and linear analyses were used to examine the relationship among different variables by using SPSS 12 for Windows (www. SPSS.com).

Results

Locations and sites characteristics: Soil samples collected from twenty two sites of the same ecological zone of Rawalakot Azad Jammu and Kashmir had shown a wide variation in altitude ranged between a minimum of 855 m at Tain to a maximum of 3000 m at Tolipir site (Table 1). Soil temperature of these soils at the time of sampling was between 22^0C^0 to 30^0C while the humidity of the selected sites varied between 48.5 % to 75 % maximum at Tolipir and minimum at Trar site. A significant negative correlation existed between altitude and air temperature (r=–0.74) and between altitude and soil temperature (r=–0.81) while the correlation between altitude and humidity was non-significant (r=0.38) (Table 2).Coefficients of variation (CV%) for elevation was high (24%) reflecting the rolling topography typical of this resource area while temperature and humidity showed less variability (10 and 13%) showing that both traits were relatively stable in this ecological zone.

Variability in soil physico-chemical characteristics: Data regarding the physico-chemical characteristics of different sites are presented in Table 3. These soils varied in texture, seven soils are loam, six soils are sandy loam, five soils have silt loam texture, three soils have silty clay loam texture and one soil belong to the class sandy clay. The clay content of these sites varied between 14–35%, sand and silt 15–55%. The pH of the soils varied from 5.89 – 8.08 showing acidic to alkaline nature of the soils. Out of 22 soils, two soils having pH 6 or below (acidic), eleven soils pH between 6.5 -7.5 (neutral), three soils pH .8.0 and six soils pH >7.5 and <8.0 (alkaline). Soils across all the sites showed a wide variation in SOM ranged between a minimum of 0.8% at Tain to a maximum of 3.5% at Tolipir site (Table 3). Two soils had SOM below 1% considered as poor soils while the remaining twenty soils had SOM above 1.29% (critical level) grouped under the category of rich soils. Most of the soils collected from the high altitude with dense vegetation, high rainfall and low temperature that may be the reasons for high organic matter content of soils. The correlation coefficient between SOM and altitude showing significant correlation (r=0.566) showing that variation in SOM may be due to the altitude of the sites. Total soil N (TSN) also showed similar trend that recorded for SOM and varied between 0.06% – 0.195%. Coefficients of variation (CV) for the chemical properties were lowest for pH (9%) and highest for sand content (35%), clay (32%), SOM (33%) while CV for TSN was also highest (27%).

Indigenous rhizobial populations: The population of indigenous rhizobia in soils obtained from 22 sites was variable, ranging from 5.0 x10^4 to 8.0 x 10^6 CFU g^{-1} soils (Table 4). These values showed a substantial population of rhizobia in these mountainous and hilly soils located in the Hindu Kush Himalayan (HKH) region.

Nodulation: In the present study, number, fresh and dry weight of root nodules showed a significant variation among different sites (Table 4). The number of nodules (NN) per plant varied from a minimum of 7 at Tain to a maximum of 40 at Hussainkot. The coefficient of variance (CV %) among different site was high i.e. 38%. Among 22 soils collected from different locations, eleven soils showed nodules number (NN) higher than 20, ten soils had NN 10-20 while one soil had NN below 10. Nodules mass (NM) i.e. nodules fresh weight (NFW) and nodules dry weight (NDW) in response to different sites/soils showed similar response to that recorded for NN (Table 4). The CV for NFW and NDW was 28 and 23%, respectively indicating a substantial variation in nodules mass among different soils/sites located in the same ecological zone.

Light microscopic and ultrastructure studies: Variability and the morphology of the soybean nodules was further studied by optical and transmission electron microscopy. It was observed that nodule from yellow leaves plant bear less bacterial occupancy (Fig. 2A, 2B) while nodules cells were partially infected with bacteria and uninfected cells were also observed in this nodule tissue. Light micrographs studies revealed that nodules obtained from green plant had more bacterial occupancy compared to yellow leaves plant nodule and nodule cells (from green plant) were completely infected with bacteria and very few uninfected cells were observed (Fig. 2A, 2B). The electron microscopy of green plant nodules showed densely populated bacteria in these cells and nodule tissue cells were completely infected with bacteria (Fig. 3) while yellow leaves plant nodule showed low number of bacterial cells and partially infected nodule cells were observed. Unknown big grey round spots were also observed in this nodule tissue.

Plant growth characteristics: Soybean growth performance in response to location/site is presented in Table 5. Significant differences were observed for growth traits among different sites. Shoot length (SL) varied between a minimum of 25.0 cm to a maximum of 46.5 cm while the root length (RL) ranged between 19.2 cm to 29.5 cm. Similarly, shoot fresh (SFW) and dry weight (SDW) were in the range of 2.6 g to 6.7g and 1.04 g to 2.93 g per plant, respectively. Root fresh weight and root dry weight (RDW) varied between 0.31g to 0.97 g and 0.20 g to 0.76 g per plant, respectively. The coefficient of variance (CV %) for these growth traits at different locations was medium to high range i.e. CV% for SL, RL, SFW. SDW, RFW and RDW was 14, 13, 23, 31, 22, 36%, respectively. Both SFW and SDW showed significant (negative) correlations with soil pH (r =–0.58; –0.61), soil organic matter (r =0.57; =0.51) and total soil N (r =0.57; =0.51).

Principle component analysis: On the basis of eigenvalues of principle components, five principle components were found significant having greater than one eigenvalues (Table 6). These significant PCs accounted for a cumulative variance of 79.13%. It was found that principal component PC_1contributed 32.97%, whereas PC_2 and PC_3 contributed 15.12% and 13.67%, respectively of the total variation. The proportion of variance shared by the remaining two PCs PC_4 and PC_5 was 10.16 and 7.2%, respectively. Altogether a cumulative variance of 79.13% was accounted for by these three PCs.

Table 2. Pearson correlation coefficients (*r*) among different soil and plant parameters i.e. altitude, textural class, soil pH, organic matter and total N, and plant characteristics i.e. shoot length, root length, shoot fresh weight, root fresh weight and shoot dry weight in the mountainous region of Rawalakot, Azad Jammu and Kashmir, Pakistan.

Characteristics	Altitude	sand	silt	clay	pH	OM	N	SL	RL	SFW	RFW	SDW
Air temp	-0.74											
Soil temp	-0.81											
Humidity	0.383											
Sand	0.136											
Silt	-0.068	-0.865**										
Clay	-0.155	-0.518*	0.018									
pH	-0.753**	-0.130	0.043	.186								
OM	0.566**	0.214	-0.207	-073	-0.521*							
N	0.565**	0.215	-0.209	-072	-0.522*	1.00**						
SL	0.087	0.205	-0.353	.193	-0.187	0.200	0.200					
RL	0.153	0.252	-0.163	-224	-0.016	0.245	0.247	-0.251				
SFW	0.532*	-0.072	0.102	-030	-0.577**	0.573**	0.572**	0.388	0.002			
RFW	0.548**	0.437*	-0.409	-174	-0.316	0.429*	0.430*	0.261	0.575**	0.461*		
SDW	0.451*	-0.106	0.075	.083	-0.606**	0.506*	0.506*	0.425*	-0.134	0.937**	0.294	
RDW	0.391	0.553**	-.506*	-239	-0.230	0.343	0.344	0.074	0.686**	0.280	0.904**	0.152

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed), The abbreviation are OM, organic matter; N, nitrogen SL, shoot length; RL, root length; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight

Table 3. Physico–chemical properties of soils collected from 22 sites of sub-division Rawalakot Azad Jammu and Kashmir.

Sampling sites	Soil textural group	Sand (%)	Silt (%)	Clay (%)	pH	O.M. (%)	Total N (%)
Baikh	Sandy loam	55	30	15	6.98	1.9	0.115
Parat	Loam	40	35	25	7.6	2.1	0.125
Pothi Bala	Silty clay loam	15	50	35	7.07	1.40	0.09
Hurna Mera	Loam	45	35	20	7.56	1.52	0.096
Paniola	Loam	35	47	18	7.98	1.38	0.089
Tain	silt loam	35	50	15	8.08	0.80	0.06
Thorar	Loam	45	30	25	7.93	1.57	0.098
Dar	Sandy clay	50	15	35	8.02	2.02	0.121
Rawalakot	Loam	40	35	25	7.34	2.06	0.123
Tarar	Sandy loam	55	30	15	6.67	0.86	0.063
Chake	Silty clay loam	15	55	30	8.03	1.43	0.091
Hussainkot	Silt loam	30	50	20	7.68	1.51	0.095
Banjosa	Sandy loam	53	32	15	6.41	2.56	0.148
Jandali	Silt loam	30	53	17	6.72	2.82	0.161
Dothan	Sandy loam	55	31	14	7.49	1.43	0.091
Namnota	Sandy loam	54	29	17	7.32	2.6	0.15
Khukot	Silt loam	35	53	12	7.73	2.13	0.126
Ali Sojal	Silty clay loam	17	53	30	7.02	2.24	0.132
Khyusane Baike	Loam	30	45	25	6.00	1.92	0.116
Tolipir	Sandy loam	55	28	17	5.89	3.5	0.195
Topa	Loam	35	45	20	6.86	1.95	0.117
Rehara	Silt loam	25	55	20	6.72	1.67	0.103

The soil and plant traits, which contributed more positively to PC_1 were altitude (0.844), soil organic matter (OM) (0.812) and soil N (0.812) whereas growth parameters like shoot fresh weight (SFW) (0.751), shoot dry weight (SDW) (0.673), root dry weight (RFW) (0.673), and root dry weight (RDW) (0.531) exerted a positive load on this PC. This PC showed that elevation, OM and N contents were positively correlated with growth traits of soybean and the populations with greater PC_1 values are high growth promoting features and formed by having more shoot and root characteristics.

The second PC (PC_2) was dominated by positive influences of nodulation and exhibited a15.11% variation. The maximum genetic variance to PC_2 was contributed by number of nodules (NN) (0.712), nodules fresh weight (NFW) (0.918) and nodules dry weight (NDW) (0.877). The third PC (PC_3) was dominated by positive influences of root growth characteristics and revealed a 13% variability. The RL (0.819), RDW (0.714) and RFW (0.532) were positively loaded on this PC. The shoot characteristics SDW (-0.463), SL (-0.409) and SFW (-0.322) were loaded negatively to this PC. The fourth PC (PC_4) (7.22% of variance) was dominated by positive influences of most probable number (MPN) (0.791) and shoot length (SL) (0.676), which showed that MPN was positively related with SL. This PC elucidated a 10.16% of the total variance. The fifth PC (PC_5) was positively loaded by soil characteristics N (0.507), O.M. (0.505), soil temperature (0.46) and humidity (0.44). The elevation (-0.300) had a maximum negative load to this PC, indicating a negative relationship of elevation with these soil characteristics.

The Biplot showed a wide variation in the potential of different soils for growth and nodulation of soybean based on the first two PCs (Fig. 4). The biplot depicted that out of 22 sites selected, 07 sites i.e. site 20, 17, 6, 8, 2, 12 and 13 had shown inclusive variation in soil characteristics, growth and nodulation potential of soybean on the basis of the fact that they were found on the edges of scattergram. Most of the parameters were loaded positively to the PC_1 and PC_2 indicating that parameters are positively correlated with each other.

Table 4. Nodulation potential (nodules number, fresh and dry weight of root nodules) of soybean grown under greenhouse conditions in soils collected from different sites at sub-division Rawalakot Azad Jammu and Kashmir.

Sampling sites	MPN CFU g^{-1} soil	Number of nodules $plant^{-1}$	Nodules fresh weight (mg $plant^{-1}$)	Nodules dry weight (mg $plant^{-1}$)
Baikh	2.0×10^5	12.4	230.0	70.0
Parat	5.0×10^5	25.9	426.7	90.0
PothiBala	5.0×10^4	20.5	333.3	96.7
HurnaMera	4.0×10^6	21.8	266.7	73.3
Paniola	8.0×10^5	20.1	256.7	70.0
Tain	2.5×10^6	6.9	173.3	56.7
Thorar	5.0×10^5	36.6	266.7	66.7
Dar	2.5×10^6	26.2	436.7	96.7
Rawalakot	8.0×10^6	13.1	230.0	50.0
Tarar	3.1×10^6	24.2	256.7	73.3
Chake	3.0×10^6	19.1	343.3	90.0
Hussainkot	2.0×10^5	39.9	326.7	110.0
Banjosa	1.0×10^5	23.8	316.7	103.3
Jandali	1.5×10^5	15.0	210.0	66.7
Dothan	1.2×10^6	22.0	170.0	63.3
Namnota	1.2×10^6	26.9	250.0	100.0
Khukot	4.0×10^6	9.8	110.0	43.3
Ali Sojal	$1.5 x10^6$	16.8	240.0	80.0
Khyusane Baike	1.2×10^6	13.8	203.3	63.3
Tolipir	1.5×10^6	16.7	266.7	73.3
Topa	4.0×10^5	15.2	250.0	103.3
Rehara	1.5×10^6	18.7	303.3	96.7
LSD ($p \leq 0.05$)	--------	6.57	45.6	19.82

Table 5. Growth characteristics of soybean grown under greenhouse conditions in soils collected from different sites at sub-division Rawalakot Azad Jammu and Kashmir.

Sampling sites	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g $plant^{-1}$)	Root fresh weight (g $plant^{-1}$)	Shoot dry weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)
Baikh	31.5	25.1	3.83	0.38	1.76	0.38
Parat	38.1	29.5	5.21	0.69	2.13	0.57
Poth Bala	30.3	22.0	3.75	0.37	1.70	0.28
Hurna Mera	35.8	20.3	4.47	0.68	1.86	0.47
Paniola	25.0	27.8	2.64	0.52	1.25	0.48
Tain	40.4	22.6	2.57	0.34	1.04	0.26
Thorar	44.3	22.3	3.17	0.44	1.55	0.38
Dar	41.8	19.8	3.03	0.39	1.39	0.27
Rawalakot	43.8	23.3	4.66	0.64	2.06	0.54
Tarar	39.2	20.8	4.35	0.48	2.01	0.41
Chak	36.9	19.3	4.51	0.37	2.01	0.24
Hussainkot	38.9	26.6	5.14	0.70	1.84	0.43
Banjosa	43.9	20.3	5.57	0.64	2.47	0.45
Jandali	32.5	23.6	4.53	0.40	1.97	0.27
Dothan	39.7	22.8	4.40	0.59	1.82	0.41
Namnota	40.5	24.7	5.06	0.53	2.22	0.42
Kahukot	32.0	20.9	4.07	0.31	1.61	0.20
Ali Sojal	46.57	19.3	6.67	0.41	2.93	0.20
Khyusane Baik	42.7	23.2	4.36	0.51	2.10	0.29
Tolipir	41.0	27.9	6.25	0.97	2.26	0.76
Topa	39.3	19.2	4.30	0.39	1.75	0.22
Rehara	35.1	19.7	5.75	0.36	2.43	0.32
LSD ($p \leq 0.05$)	9.07	5.22	1.01	0.37	0.43	0.11

Table 6. Principle component analysis of different soils for soil characteristics and growth parameters of soybean.

PC	Eigenvalue	Variance (%)	Cumulative variance (%)
1	5.93427	32.968	32.968
2	2.72111	15.117	48.085
3	2.45908	13.662	61.747
4	1.82902	10.161	71.908
5	1.30009	7.2227	79.1307

Jolliffe cut-off value: 0.7

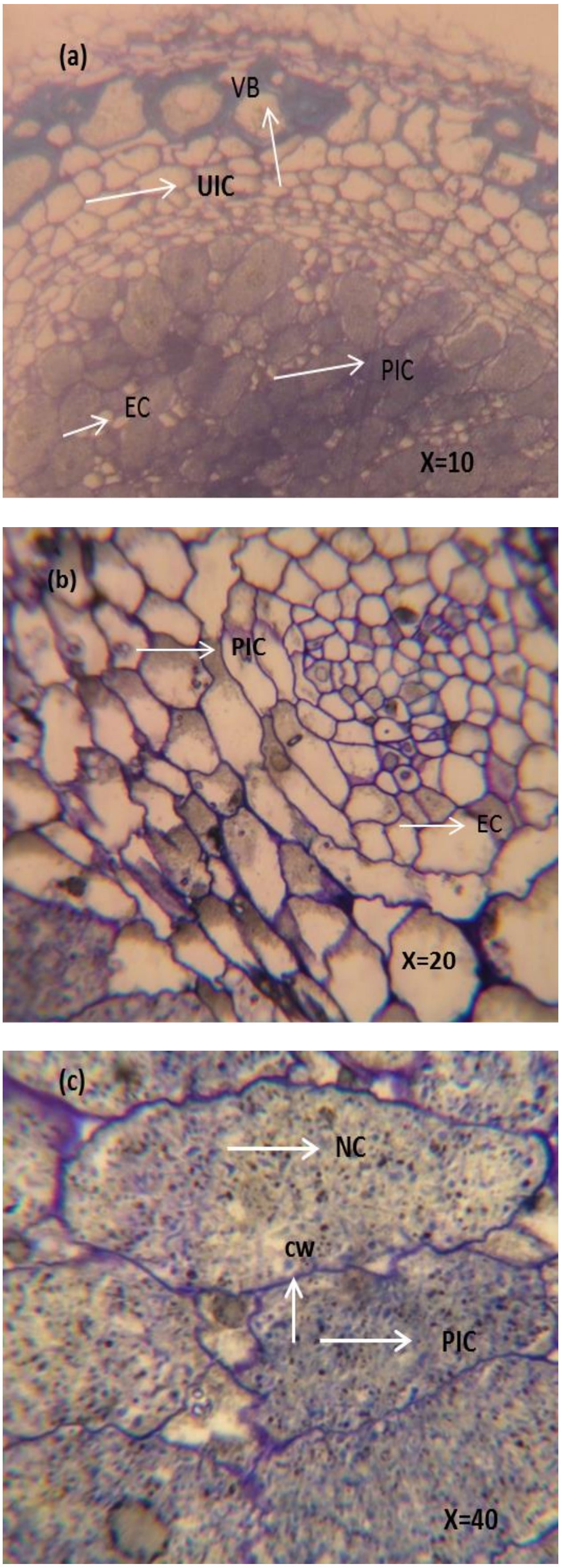

Fig. 2A. Light micrographs showing the less colonization of bacteria in the nodule cells of site 08 yellow leaved plant VB=Vascular bundle, UIC=Un-Infected cells, EC= Empty cells, PIC= Partially infected cells, NC=Nodule cell, CW=Cell wall.

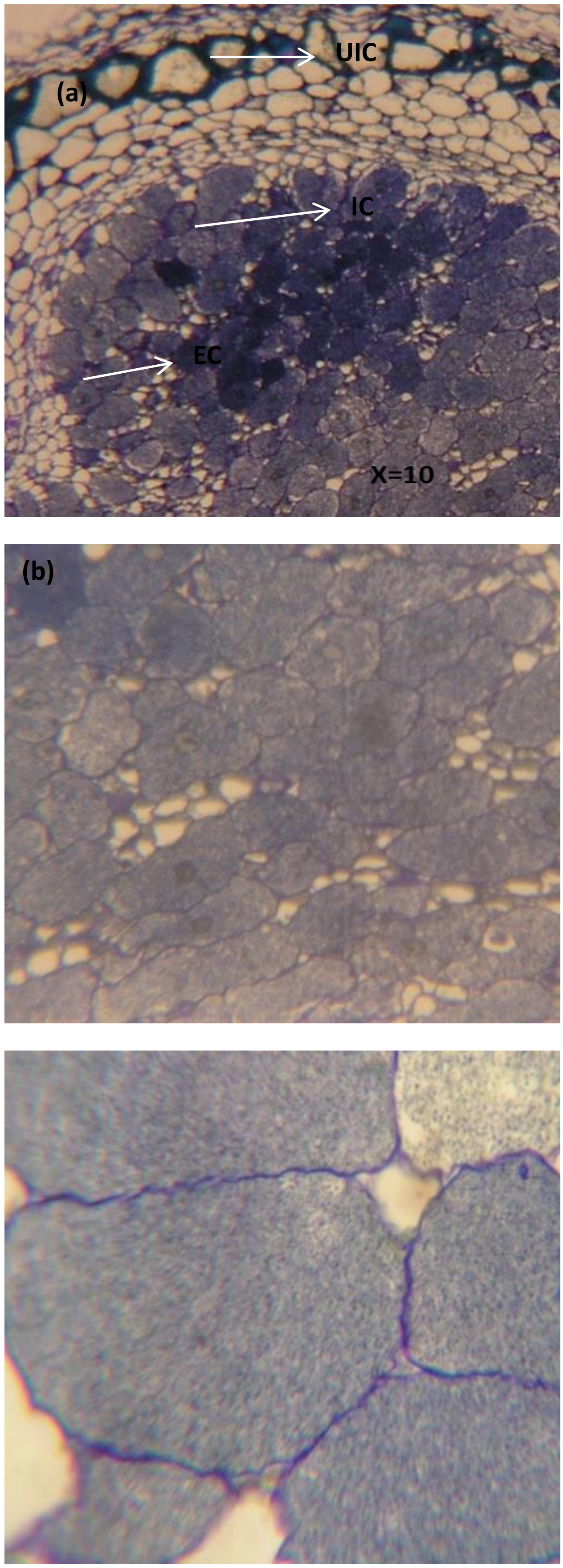

Fig. 2B. Light micrographs showing the bacterial occupancy in nodule cells of site 10 green leaved plant. UIC= Un-infected Cell, IC= infected Cell, EC=Empty cells, NC=Nodule cells, FOC=Fully occupied cells, ICS= Inter cellular spaces.

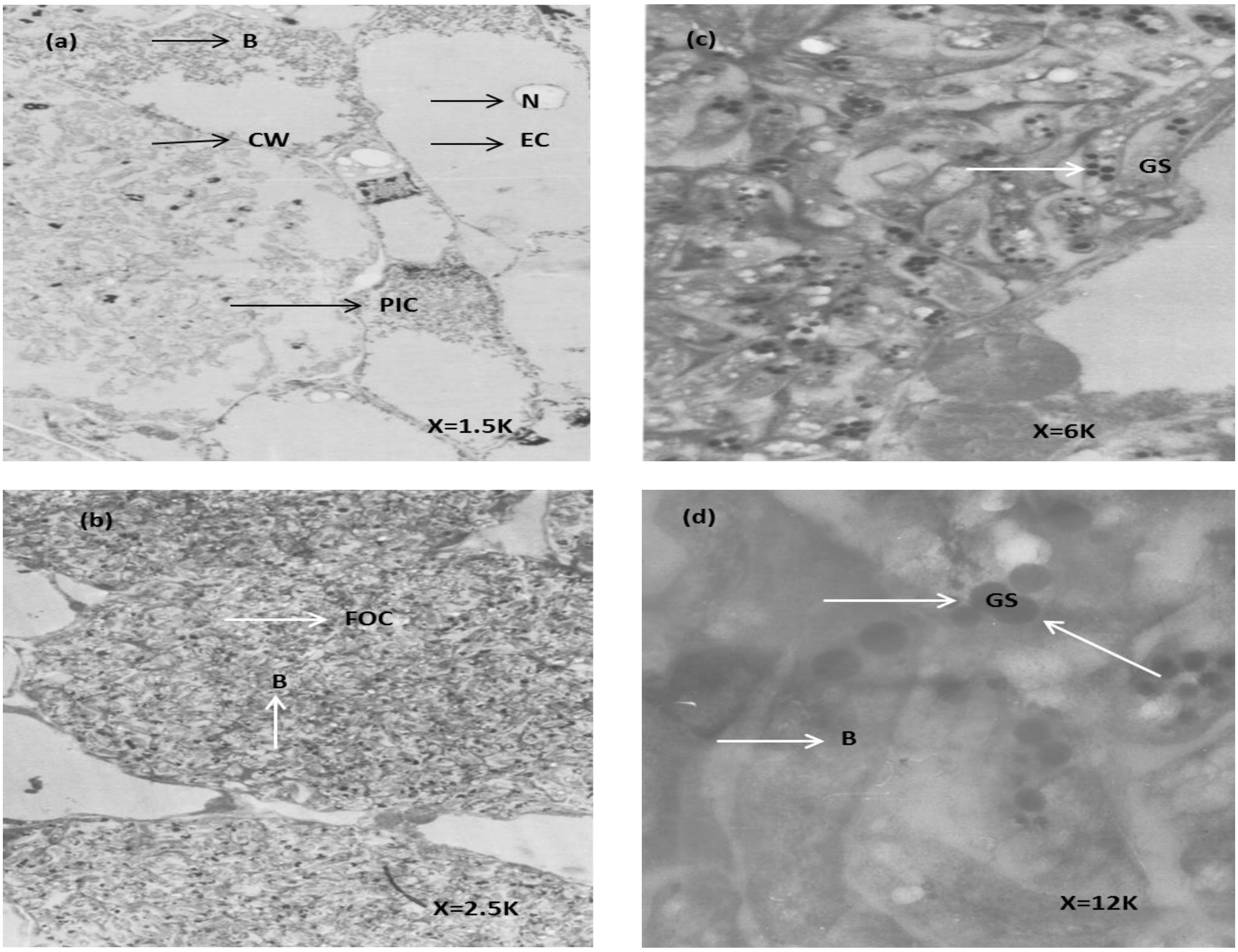

Fig. 3. Transmission electron micrographs showing the colonization of bacteria in root nodules of soybean plant B=Bacteria, N= Nucleus, CW= Cell wall, EC= Empty cell, PIC= Partially Infected cell, FOC= Fully occupied cell, GS= Grey spots

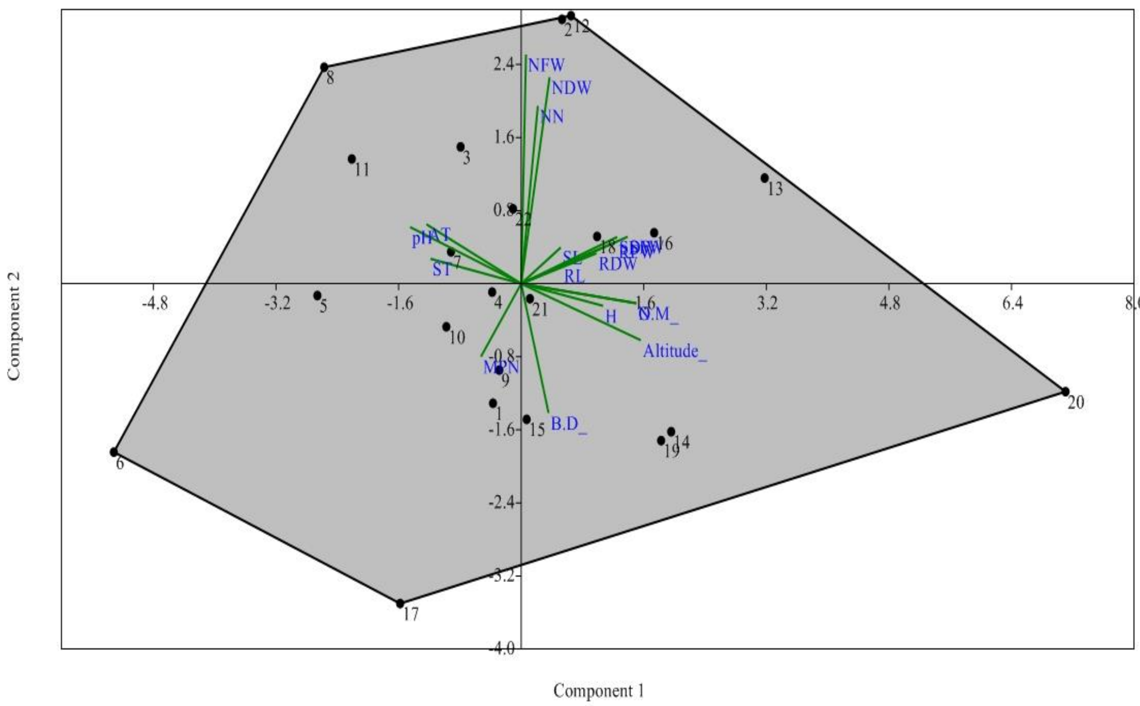

Fig. 4. Scattergram with Biplot of variables of first two principal components (PC_1 and PC_2) on the basis of soil characteristics and growth and nodulation in soybean.

Discussion

The rhizobial population of soil collected from twenty two sites varied between $5.0x10^4$ to 8.0 $x10^6$ g^{-1} soil and the coefficient of variation (CV) among the sites/most probable number (MPN) was 87%, indicating a substantial variation and diversity of rhizobia present in these soils collected from different elevation but of the same region. Soybean had never been grown in this region and even growing history of other leguminous crops seldom existed. But still the presence of enormous rhizobium population is phenomenon. It has been reported that microbial population density of about 10^3 *Bradyrhizobium japonicum* cells g^{-1}soil is required for maximum nodulation and efficient N_2 fixation to satisfy the N demand of soybean (Weaver & Frederick, 1974). The *Rhizobium* population observed in this study was poorly correlated with such soil/site characteristics as altitude, temperature pH, SOM, N (R^2 values below 0.32). Our results were in accordance with previous findings where rhizobial population detected from wetter agro-ecological zones of Zimbabwe was not positively correlated (r values all below 0.2) with pH, CEC, TEB, P, %N and %C (Musiyiwa *et al*., 2005).

The pH of the soil showed significant correlation (negative) with the altitude (*r*=–0.75) (Table 2), demonstrating that by increasing the altitude, pH of the soil tended to decline. This is probably because of the effect of soil organic matter and microbial population. A significant correlation existed between organic matter and pH (*r*=–0.52) (Table 2), confirming that increased soil organic matter decreased soil pH because of the acidifying effect of organic matter. In our previous study (Abbasi *et al*., 2007) the correlation coefficient between soil organic matter (SOM) and pH was significant (r =−0.61) showing that SOM contributed significantly toward the change in pH.

Generally it is believed and reported that compatible populations of Bradyrhizobiaare seldom available in soils where the soybean crop has not been grown previously (Abaidoo *et al*., 2007). This explains the importance of *Bradyrhizobium japonicum* inoculation to achieve adequate and effective nodulation and yield of soybean when the crop is first introduced. However, in this study both soybean nodulation (number and mass) and rhizobium population in soils without soybean growing history was remarkable. There are few reports indicating effective population of bradyrhizobia in the soils where soybean has not been grown previously (Sanginga *et al*., 1996; Javaheri, 1996; Mpepereki *et al*., 2000; Sharma *et al*., 2010).

The presence of indigenous *Rhizobia* in soil has a substantial effect on nodulation, N_2 fixation and finally on the growth and yield of the crops. In the present study, number, and mass (fresh and dry weight) of root nodules showed a significant variation among different sites. This variation in number and mass of nodules among different soils may be due to the variation in the indigenous rhizobium population or some other unknown factors as elevation did not show any correlation with root nodulation. The regression analysis showed that nodulation did not show any significant correlation with altitude, air and soil temperature, soil pH and soil organic matter (SOM) indicating that the presence of indigenous *Rhizobium* is not affected by any of these factors. In contrast to our findings, Adhikari *et al*. (2012) reported that the genetic diversity of soybean-nodulating Bradyrhizobia in Nepal depending on altitude and soil properties, such as soil pH. Shiro *et al*. (2013) also found a positive correlation between the geographic distribution of indigenous soybean-nodulating rhizobia, soil temperature (and its variations due to latitude and altitude), and soil pH. Our results suggested that the site/location is an important factor/component for the development and existence of Rhizobial population that effect on root nodulation.

Light and electron microscopic studies revealed that nodules obtained from green plant had more bacterial occupancy compared to yellow leaves plant nodules, and nodule cells (from green plant) were completely infected with bacteria and very few uninfected cells were observed. These results clearly indicate that in site 08 plant having yellow colored leaves was due to low N_2 fixation and low N availability to plant as low number of bacteria was found in nodules. Whereas plants grown at site 10 having green color and this green leaves color was due to more N_2 fixation and more N availability to plants as more number of bacteria was found in the nodules of these plants. These results were in accordance with the previous studies conducted on different legumes showing a substantial variation in nodules occupancy potential. Iqbal & Mahmood (1992) studied the ultra-structure of *Leucaenaleucocephala* nodule and found 43% uninfected cells. Qadri *et al*. (2007) studied the ultra-structure of root nodule of *Samaneasaman* and reported that bacterial region occupied the central region of nodule and bacteroid tissue of nodules contained both infected and uninfected cells. Yasmin *et al*. (2012) studied the ultra-structure of *Vignaradiata* root nodule by applying *Bradyrhizobium* and *Agrobacterium* in combination with *mycorrhizae* as inoculum and reported the presence of both infected and uninfected cells in the root nodules tissue.

Results indicated that in general soybean showed a higher and vigorous vegetative growth, evident by plant height and shoot fresh and dry weights. The increased vegetative growth of soybean apparently is due to substantial nodulation potential by indigenous rhizobia that may affect N_2 fixation and N assimilation by plants. The significant correlation between plant biomass and soil organic matter/soil total N indicating that the presence of soil organic matter and total N contributed towards the growth characteristics of soybean.

Principal component PC_1contributed 32.97%, whereas PC_2 and PC_3 contributed 15.12% and 13.67%, respectively of the total variation. The proportion of variance shared by the remaining two PCs PC_4 and PC_5were 10.16 and 7.2%, respectively. Altogether, a cumulative variance of 79.13% was accounted for by these three PCs. Our results were in accordance to the previous studies where principle component analysis for soybean had shown wide variation. Aondover *et al*. (2013) performed PCA to find out variation among 17 genotypes of soybean and revealed that the 3 PCs contributed 69.77% of the total variation. Among the 03 PCs, PC_1 contributed 35.8%, whereas PC_2 and PC_3 contributed 22.4 and 18.2%, respectively of the total variation. Iqbal *et al*. (2008) evaluated 139 soybean genotypes by using multivariate techniques. The first 3 PCs contributed 69.77% of the total variation among the soybean genotype and found that PC_1 contributed 35.15%, whereas PC_2 and PC_3 contributed 23.77% and 10.85%, respectively of the total variation. Okugun *et al*. (2004) conducted PCA to find out variation in farmers' fields and revealed that soil characteristics were important factors responsible for yield differences in soybean. Ruiz-Valdiveizo *et al*. (2009) found 54% variation in PC_1 with nitrogenase activity and number of nodules, PC_2 19% with shoot and root weight and PC_3 14% from shoot and root weight and shoot length.

Conclusions

The results show that the highly variable elevation and soil characteristics of the same ecological zone at Rawalakot Azad Jammu and Kashmir might be conducive to the existence of substantial soybean rhizobial population. The relative rhizobial populations varied along with the altitude and sites. The study described here assesses the native nodulation potential of undisturbed soils in the Himalayan region of Kashmir-Pakistan, with the aim to plant soybeans without prior inoculation or isolate efficient, competitive and locally adapted N_2-fixing bacteria from these soils. Variability in nodulation characteristics and soil properties suggests variability in N_2-fixing bacterial population. Isolation of effective strains from these dense rhizobial populated soils could also be used in the development of inoculants for soils with small rhizobial populations. However, their competitiveness in the presence of other commercial and indigenous strains needs to be determined. Introduction and exploitation of legumes especially the soybean on these soils is highly recommended because of the existence of indigenous rhizobia in these soils. The native N_2-fixing bacteria might be isolated from these soils and used as biofertilisers (or inoculants).

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