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NODULATION OF *SESBANIA* SPP., BY INTRODUCED RHIZOBIA IN COMPETITION WITH NATURALIZED STRAINS IN DIFFERENT SOIL TYPES

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Abstract

Seven rhizobial strains were isolated from *Sesbania aculeata* and three from *Sesbania grandifolia* from three different soil types. These strains were characterized for their morphology and carbon source utilization. The affectivity of these strains was confirmed by re inoculating the host and were effective on both *Sesbania* spp. The strains Sa1 and Sa2, however found to be more effective having nitrogenase activity ranging from 3.7-85.2 and from 4.8-77.7 m moles of C_2H_4 produced h^{-1} g^{-1} nodule dry weight respectively. The consortium of Sa1, Sa2, Sg1 and Sg2 was also found to be highly effective in both *Sesbania* spp. In competition with the indigenous population, the strains Sa1 and Sa2 were found to be most competitive among the others. Fluorescent antibody studies showed the nodule occupancy ranged from 30-100% by Sa1 and Sa2 in both species. Response of inoculum was better in soils 1 & 2 which were low in nutrient level than in soil 3.

Introduction

Pulse, pastures and grain legumes are important for maintaining productivity in many agricultural soils. The formation of effective nitrogen symbiosis between legumes and root nodule bacteria is essential. In many situations soil inoculum with effective strains is required for maximizing legume yield. A key strategy to enhance the performance of inoculants is the selection of elite strains with improved characteristics such as greater nitrogen ability, ability to survive stressful edaphic conditions and greater competitive ability (Hara, 2002).

Numerous studies on competition among *Rhizobium* strains for nodulation of their legume host have been emphasized that it is a major limitation to the establishment of the superior nitrogen fixing inoculant strains in the soil (Lupwayi, 2000). It is very likely that all the agricultural soils contain some bacteria capable of nodulating some legumes. However, the particular bacteria present in any soil may not be able to nodulate the specific host or even if they do not form an effective symbiosis that contributes to the availability of nitrogen to the host. This is quite common for situations where new legumes are being introduced to new lands in many areas of the world (Hafeez *et al*., 2000, 2001, Hara, 2002, Naeem *et al*., 2004). Legumes are commonly inoculated with efficient nitrogen fixing rhizobia with an objective to maximize crop productivity. The host bacteria specificity operates at both the nodulation and nitrogen fixation levels and is a function of exchange of specific chemical signals between the symbionts (Perret *et al*., 2001). Formally, the nitrogen fixing ability of *Rhizobium* strains was considered to be the most important criterion in strain selection for inoculum production, it must however be able to survive in the soil and compete with the native rhizobia for nodulation

(Scupham *et al*., 2000; Sadowsky, 2000). Three general scenarios are important when a legume is introduced in a new land (Howieson *et al*., 2000b). One is that the uninoculated legume may form abundant effective nodules indicating the presence of large number of effective rhizobia. Secondly, there is less or even no nodulation at all indicating very little or in some cases no back ground population of rhizobia in soil for the particular host. Thirdly, legume may have inefficient nodules indicating the presence of ineffective rhizobia in the soil. Poor competitiveness can sometimes be overcome by applying high number of inoculant strains, but the number that is required to overcome the indigenous population are often difficult to achieve for commercial production of inoculum. It is therefore important to identify the highly effective strains that are competitive *in situ* (Moawad & Bohlool, 1984).

The benefits of this research can be increased through advances in the application of appropriate inoculation technologies to deliver the new strains to the soil with enhanced survival (Stephan & Rask, 2000). *Sesbania* is considered an important source of green manure, forage fiber, wood pulp and landscape decoration. The tender pods and young leaves are favorite vegetable in India and Thailand. It is best leguminous crop for reclaiming sandy and alkaline soil being rich in nitrogen fixing bacteria (Fazil, 1994). The successful establishment of nodulation in *Sesbania* species requires the introduction of effective and competitive microorganisms in the soil. Therefore, the main aim of this study was to isolate most specific, effective and competitive strains for different *Sesbania* species and to use them as commercial inoculants.

Materials and Methods

Soil samples and host plants: Three soil samples were collected from the different localities of Faisalabad, with different cropping history (Table1). The seeds of *Sesbania grandifolia* and *Sesbania aculeata* were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan.

Isolation and effectiveness of indigenous *Rhizobium* strains: *S. aculeata* and *S. grandifolia* were grown in three different soils in plastic pots and watered with canal water. Seven weeks old plants were harvested and healthy, pink and undamaged nodules were detached. The nodules were surface sterilized with 0.1% $HgCl_2$. The crushed nodule extract was streaked on YEM agar plates having Congo red (Shah *et al*., 1995). After the growth appeared on the plates, the cultures were purified by sub-culturing on separate plates by picking single colony on the basis of morphological differences. The pure cultures were confirmed by Gram's staining technique (Rao, 1999) and then grown in YEM broth media having bromothymol blue (Vincent, 1970). The purified cultures were authenticated by their infectivity to their host plants grown in 500 mL vermiculite and agar glass tubes containing N-free Hoagland nutrient solution (Arnon & Hoagland 1940). Treatments, inoculated and uninoculated were used with three replicates each (Table 2). The glass tubes were kept in growth room at 30°C. The inoculum was given @ 1mL broth culture per tube (10^9 cells ml^{-1}). Four weeks old nodules were picked and incubated with acetylene for 1 hour at room temperature to determine the nitrogenase activity (Hafeez *et al*., 1995). Two controls were used for the assay: 1) without any nodules, containing pure acetylene only; 2) root nodules with acetylene. Trace Gas Chromatograph–GC 2000 (Thermo Quest–C.E instrument Italiana) with a hydrogen flame ionization detector (FID) was used for acetylene reduction assay. The unchanged acetylene and the ethylene produced were calculated as ratio on chrome card software. The nitrogenase activity was expressed as m moles of C_2H_4 produced h^{-1} g^{-1} nodule dry weight.

Table 1. Physiochemical properties of soils, cropping history and the estimation of population of indigenous *Rhizobium* sp. in three soils.

Soil	Indigenous population (cells g^{-1} soil)	Cropping history	pH	E_{ce} (dsm^{-1})	Total N (%)
Sandy loam	5.8×10^3	Wheat field at NIBGE	7.8	1.13	0.052
Loam	3.1×10^3	Chickpea field NIAB	8.0	0.74	0.047
Sandy loam	2.1×10^3	Canal side at NIBGE	7.9	0.42	0.086

Annual rainfall = 310 ± 47.5 mm.

Table 2. Effect of rhizobial inoculation on growth nodulation and N_2-fixation in two *Sesbania* species (Reinoculation experiment).

Strains	Nodule $Plant^{-1}$				ARA (m moles of C_2H_4 h^{-1} g^{-1} nodule dry wt)		Shoot dry weight $plant^{-1}$ (g)	
	Number		Dry wt (mg)					
	I	II	I	II	I	II	I	II
Sa1	5.3abc	5.0b	18.3b	12.4bc	0.27ab	0.24ab	0.27abc	0.14ab
Sa2	8.0ab	3.7bc	22.0b	21.3a	0.27ab	0.26ab	0.20cde	0.23a
Sa3	2.7c	6.7ab	9.7c	16.0ab	0.21ab	0.59a	0.17bcde	0.15ab
Sa4	6.2abc	6.7ab	13.9b	23.7a	0.23ab	0.02b	0.11de	0.27a
Sa5	3.2bc	7.3a	11.7c	16.7ab	0.05b	0.53a	0.28ab	0.24a
Sa6	7.0abc	7.3a	20.0b	18.0ab	0.18b	0.46a	0.17bcde	0.24a
Sa7	9.2a	7.5a	22.7b	16.7ab	0.30ab	0.25ab	0.26abcd	0.16ab
Sg1	6.5abc	5.0ab	15.7b	16.0ab	0.01ab	0.14ab	0.13cde	0.16ab
Sg2	5.0ab	4.8b	12.3b	24.7a	0.16ab	0.28ab	0.10e	0.23a
Sg3	6.5abc	5.5ab	15.5b	19.0a	0.10ab	0.27ab	0.17bcde	0.18ab
Sa1, Sa2, Sg1, Sg2	10a	7.5a	38.0a	20.3a	0.52a	0.24ab	0.38a	0.21a
Uninoculated with N	-	-	-	-	-	-	0.14cde	0.17ab
Uninoculated	-	-	-	-	-	-	0.09e	0.05b

Means followed by the same letters are statistically non significant ($p< 0.05$)
ARA: acetylene reduction assay. N: nitrogen as urea (60 mgN/g)
I: *Sesbania grandifolia*
II: *Sesbania aculeata*

C-source utilization: The rhizobial strains isolated from plants were tested for their capability of utilizing different carbon sources. In yeast-mannitol media (Vincent, 1970), arbinose, xylose, glucose, glactose, raffinose, sucrose, maltose, mannitol and molasses were used as alternative carbon source for mannitol. The concentration of the carbon source was 1% (w/v). Each chemical was filter sterilized by passing through membrane filters 0.2µm size (Millipore Corp). The isolates were streaked on plates in triplicate and the presence or absence of their growth was observed after 3-5 days. Seven isolates from *S. aculeata* and three from *S. grandifolia* were selected for further studies.

Indole acetic acid (IAA) production: For detection of IAA production by the bacterial isolates, cultures were grown in Okon's malate medium (Okon *et al*., 1977). Tryptophane (100mg/L) was added as the precursor of IAA. After one week of growth, qualitative estimation of IAA was performed by Fe-$HClO_4$ and H_2SO_4 reagents (Yasmin *et al*., 2004).

Indigenous population: *Sesbania* seeds were surface sterilized with 0.1% $HgCl_2$ and distilled water. The seeds were germinated on water agar plates for 48 hours at 28±2°C and 14 hours photoperiod in growth room. The uniform seedlings were transferred to growth pouches and water with N free Hoagland nutrient solution (Arnon & Hoagland,

1940). Serial dilutions of each soil sample were prepared. After 3 days of transfer of seedlings to pouches 1 mL of each dilution was applied to the roots of test plants with four replicates for each dilution. Nodule formation was observed daily and data was collected after 30 days of planting. Positive results were compared with a standard most probable number table (Vincent, 1970).

Competition experiment: The competitive experiment was conducted between the indigenous and inoculated strains. The experiment was conducted in pots containing 4 kg soil. Soil was collected from three locations by mining the soil up to 20 cm, after removing litter and top 1cm of soil, the soil was dried and sieved (0.5 cm). The experiment was performed in a Completely Randomized Block Design with four replicates and three treatments i.e., inoculated, uninoculated and nitrogen (N) control. N was applied @ 60 mg N g^{-1}. The number of viable rhizobial cells was 10^4 per seed at the time of sowing. Each pot contained 5 seeds. The experiment was conducted in net house at 30±2°C. The plants were harvested after 8 weeks of plantation and data was recorded for nodule number dry weight and total N.

Nodule occupancy was studied by the method of Schmidt *et al*., (1968) with the respective fluorescent antibodies of the inoculated strains. Fluorescent antibodies were developed against two strains Sa1and Sa2, in six months old female albino rabbits (Hafeez *et al*., 2001).

Results

Ten strains isolated from two *Sesbania* species (Sa1-Sa7 from *S. aculeata* and Sg1-Sg3 from *S. grandifolia*) showed significant growth in 3 days and turned the yeast mannitol media containing bromothymol blue to yellow color. The colonies were gummy, circular, and convex with smooth edges glistening, translucent or white. The isolates were Gram negative and rod shaped and produced indole acetic acid. Sa1, Sa2, Sa3, Sa6 and Sa7 showed high IAA production than other strains. All the strains utilized 6 types of carbon source except Sa1, Sa5 and Sa6 which were unable to utilize glactose. The reinoculation experiment confirmed that the isolates belonged to genus *Rhizobium* as the isolates were effective and formed nodules in both *Sesbania* spp. In *S. grandifolia* the maximum number of nodules was 10 showed by the mix inoculum of Sa1, Sa2, Sg1 and Sg2, whereas, the minimum number of nodules (3) were formed by Sa3. Difference was also recorded in dry weight of nodules formed by different strains. The strain Sa7 showed dry weight 22 mg per plant, whereas the minimum dry weight 2.7 mg per plant was showed by Sa3. The mix inoculum showed 38 mg per plant dry weight that was maximum. The strains were different in their effectiveness. The maximum nitrogenase activity m moles of C_2H_4 produced h^{-1} g^{-1} nodule dry weight (0.53) was detected in the mix inoculum and the minimum (0.06) was in Sa5.

In *S. aculeata* the maximum (7.5) and minimum (3.7) nodules were formed by Sa7 and Sa2 respectively, the mix inoculum also formed 7.5 nodules per plant. The maximum dry weight per plant was observed 24.7 mg by Sg2 and minimum was 16 mg by Sa3 and Sa1. The nodule dry weight observed in mix inoculum was 20.3 mg per plant. The maximum nitrogenase activity was detected in Sa3 (0.59 m moles of C_2H_4 produced h^{-1} g^{-1} nodule weight Table 2).

The antisera developed against Sa1, Sa2 showed fluorescence with their own antigen culture but did not cross react with other rhizobial strains.

Competition experiment: With a low co-efficient of variations (12.4%) all the variables (species, soils and strains) and their interactions remained highly significant at the 0.05 probability level. Both the species varied significantly regarding the number of nodules, nitrogenase activity and nodule dry weight (Table 3). The nodulation response was more frequent in soil 1 and soil 2 which were rich in indigenous population whereas in soil 3 the response of inoculum was less, which don't have any legume cropping history (Table 1). Nodule occupancy by inoculant strains ranged from 30-100%. In soil 1, the strains Sa 1 and Sg2 were found to be more effective whereas in Soil 2 the strains Sa1 and Sa2 were more effective.

Discussion

An essential desired characteristic for the inoculum strains of rhizobia is the highly effective nitrogen fixing ability with the intended host species and in some instances there is a requirement to effectively nodulate a wide range of host legumes. Other beneficial characteristics include stress tolerance, competitive ability against indigenous strain, genetic stability and satisfactory growth and survival during the production of commercial inoculum (Howieson *et al*., 2000b).

This study was undertaken to isolate and characterize the *Sesbania* rhizobial strains from the indigenous population to be used as an inoculum to maximize the efficiency of *Rhizobium* legume symbiosis on the basis of morphological and biochemical studies (Vincent, 1970; Ghosh & Basu, 1998; Chhaya *et al*., 1998). All the strains utilized nearly all carbon sources confirming the findings of Sadowsky *et al*., 2000 and Hafeez *et al*., 1995 that the fast growing rhizobial strains utilize a great assortment of carbohydrates.

Fluorescent antibodies developed against two isolates Sa1 and Sa2 showed that all the strains were different as the antisera did not cross react with other rhizobial strains. This was according to the study of Irisarri *et al*., (1996) who used antisera of three isolates to separate 15 isolates on the basis of cross reactivity.

Inoculum response of rhizobia was different in three soil types which was similar to the findings of other authors (Kuecy, 1989; Theis *et al*., 1991; Hafeez *et al*., 2001; Naeem *et al*., 2004), that the inoculation response are usually high in situation where the population density of effective soil rhizobia is low and level of mineral nitrogen is insufficient for adequate plant growth. This also suggests that why the response of inoculation in soil 3 was so low. This may be due to the high nitrogen contents which hindered the nodulation in this soil (Table 3). In re inoculation experiment, the mixture of strains affected positively all the parameters studied in both the species of *Sesbania*, however, Sa5, Sa6 and Sa7 performed better in *Sesbania aculeata*.

In competition experiment no single treatment was a true indicator of competitive effectiveness. However in general on the basis of different parameters Sa1, Sa2, Sa3, Sg1 and the mixture of strains were better inoculants. All growth parameters along with nitrogenase activity were significantly increased as also reported by Kumar *et al*., (1993), Pandher (1995) and Abdullahi and Giller (2001).

From these experiments there is a clear understanding that why there is a need for inoculation? The study of inoculum response in legumes is relatively a simple task but sometimes it becomes difficult because procedures do not have the appropriate background skills to adequately interpret the results (Date, 2000). In essence, simple experiments using three treatments: uninoculated, inoculated and fertilizer nitrogen can be done to answer the question whether there is a need for inoculation (Brockwell & Bottomley 1995; Brockwell *et al*., 1995; Date, 2000).

Table 3. Comparison of different strains on the basis of nodulation, nitrogenase activity and total nitrogen in three different soils (Competition experiment).

Treatments	Nodule number plant^{-1}		Nodule dry wt (mg plant^{-1})		ARA (m moles C_2H_4 produced h^{-1} g^{-1} nodule)		Total N (mg plant^{-1})		Shoot dry weight (g plant^{-1})	
	I	II	I	II	I	II	I	II	I	II
					Soil 1					
Sa 1	15.6a	12.3c	22.8b	18.0cd	34.3ab	48.1a	34.5a	44.6b	0.85a	0.97b
Sa 2	16.0a	8.6d	51.0a	27.0bc	26.3bc	36.4b	33.3ab	55.0a	0.90a	1.29a
Sa 3	10.6b	20.6a	20.9bc	26.2bc	22.6bc	25.6bc	26.6abc	33.8c	0.83ab	0.89b
Sa 4	14.0a	15.6b	29.9b	20.2cd	20.6cd	34.24b	26.6abc	34.0bc	0.82ab	0.91b
Sa 5	6.6d	15.0b	18.0bc	27.1bc	7.3e	20.6cd	13.2de	19.7de	0.48cd	0.58c
Sa 6	15.0a	8.0de	26.5bc	15.3cde	31.7abc	14.2cde	21.7bcd	31.5c	0.68abc	0.91b
Sa 7	8.0cd	19.0a	14.7b	22.0bcd	23.5bc	9.4de	16.9cd	26.2cd	0.55bcd	0.84b
Sg 1	15.3a	10.0d	23.3bc	13.7de	9.9de	52.93a	27.9abc	12.8ef	0.78ab	0.46c
Sg 2	14.6a	10.0d	23.4bc	13.0de	29.8abc	48.9a	23.2a-d	15.6def	0.67abc	0.52c
Sg 3	10.0bc	3.6g	15.0c	6.0e	6.7e	13.5cde	12.7de	11.2ef	0.46cd	0.36c
Sa1, Sa2, Sg1, Sg2	11.3b	6.0ef	28.9b	49.6a	40.1a	21.7cd	27.7abc	33.9bc	0.86a	1.05ab
Uninoculated	6.0d	4.0fg	5.3c	5.6a	4.6e	5.4e	5.3e	5.68f	0.31d	0.32c
Uninoculated with N	-	-	-	-	-	-	30.3ab	40.4b	0.83ab	0.95b

Means followed by the same letters are statistically non significant ($p < 0.05$)
I: *Sesbania grandifolia*
II: *Sesbania aculeata*
- : No activity

Table 3. (Cont'd.)

Treatments	Nodule number plant^{-1}		Nodule dry wt (mg plant^{-1})		ARA (m moles C_2H_4 produced h^{-1} g^{-1} nodule)		Total N (mg plant^{-1})		Shoot dry weight (g plant^{-1})	
	I	II	I	II	I	II	I	II	I	II
	Soil 2									
Sa 1	7.3de	14.0a	23.6ab	18.2b	85.2a	77.7a	44.1a	45.3b	1.06ab	1.15a
Sa 2	10.6bc	9.3b	17.4bc	18.5b	72.5b	63.9b	39.1b	48.5a	1.13a	1.23a
Sa 3	11.6b	6.3cde	13.8bc	19.0b	50.7c	37.7c	20.7c	35.0bc	0.64cd	0.97abc
Sa 4	9.3cd	12.6a	7.0c	11.4bc	20.5de	27.2cd	26.4bc	21.9de	0.89abc	0.74bcd
Sa 5	9.3cd	9.3b	8.1c	43.3a	11.6ef	8.5ef	22.4bc	25.9cde	0.82bc	0.86bcd
Sa 6	7.6de	8.3bc	15.7bc	37.4a	17.3de	23.4d	28.0abc	30.8cd	085abc	1.01ab
Sa 7	16.3a	4.0c	29.1a	23.2b	28.0d	2.6f	22.2bc	18.4ef	0.76bc	0.70cd
Sg 1	4.0f	7.0cd	15.5bc	13.2bc	0.867f	18.3de	31.2abc	19.5ef	0.94ab	0.73bcd
Sg 2	16.0f	5.0def	10.6c	2.2a	15.5e	9.8f	34.3ab	26.1de	0.92abc	0.82bcd
Sg 3	6.0f	4.3ef	9.3c	3.3c	18.4de	4.9f	21.0c	18.4ef	0.83bc	0.70cd
Sa1, Sa2, Sg1, Sg2	9.0cd	14.3a	18.3abc	12.0bc	44.2c	19.1de	30.8abc	35.9bc	0.99ab	1.02ab
Uninoculated	5.6ef	3.0f	10.0c	7.3bc	1.6f	2.7f	6.7d	9.9f	0.39d	0.60d
Uninoculated with N	-	-	-	-	-	-	36.1ab	43.2b	1.02ab	1.05ab

Means followed by the same letters are statistically non significant ($p < 0.05$)
I: *Sesbania grandifolia*
II: *Sesbania aculeata*
- : No activity

Table 3. (Cont'd.)

Treatments	Nodule number plant^{-1}		Nodule dry wt (mg plant^{-1})		ARA (m moles C_2H_4 produced h^{-1} g^{-1} nodule)		Total N (mg plant^{-1})		Shoot dry weight (g plant^{-1})	
	I	II	I	II	I	II	I	II	I	II
	Soil 3									
Sa 1	8.3a	3.5b	8.4b	1.3a	6.2c	3.7d	16.8ab	27.3a	0.47ab	0.72a
Sa 2	8.0a	1.5bc	8.6b	1.6a	4.8d	4.8d	9.4ab	27.5a	0.44ab	0.77a
Sa 3	-	2.2b	-	1.3a	-	-	8.2ab	20.3abc	0.34ab	0.61ab
Sa 4	-	-	-	-	-	-	9.1ab	17.2a-d	0.21c	0.48ab
Sa 5	-	-	-	-	-	-	6.4b	18.8abc	0.26b	0.53ab
Sa 6	-	-	-	-	-	-	11.5ab	12.0bcd	0.36ab	0.39b
Sa 7	-	5.0a	-	1.7a	-	10.8b	10.1ab	9.4cd	0.40ab	0.37b
Sg 1	5.1b	2.0b	11.0a	1.7a	6.3c	7.6c	12.4ab	12.4bcd	0.58a	0.49ab
Sg 2	8.6a	3.5b	8.7b	1.7a	29.0a	80.2a	11.2ab	23.1ab	0.54ab	0.69a
Sg 3	2.2c	-	2.3d	-	-	-	15.0ab	19.1abc	0.56ab	0.53ab
Sa1, Sa2, Sg1, Sg2	6.0b	-	5.3c	-	13.7b	-	19.7a	19.5abc	0.28b	0.31b
Uninoculated	1.7c	2.0b	1.0d	1.0a	-	-	5.7b	5.0d	0.18cd	0.15c
Uninoculated with N	-	-	-	-	-	-	12.6ab	21.3b	0.42ab	0.65ab

Means followed by the same letters are statistically non significant ($p < 0.05$)
I: *Sesbania grandifolia*
II: *Sesbania aculeata*
- : No activity

Table 4. Relative abilities of inoculated strains to nodulate two *Sesbania* spp., in different soils.

Strain number	Soil 1		Nodule occupancy % Soil 2		Soil 3	
	I	II	I	II	I	II
Sa 1	70	65	100	90	30	25
Sa 2	60	59	85	73	25	20

I: *Sesbania grandifolia*
II: *Sesbania aculeata*

Conclusion

The variability among strains could be utilized to select strains capable of increasing the contribution of N_2 fixation in the legume nutrition. After testing the competition between isolated rhizobial strains and indigenous rhizobial population in soil, Sa1, Sa2 and Sg1 and mixture of strains were found better for inoculum production for commercial use. No host specificity was observed for different strains. Similarly, both *Sesbania* species are equally respondent to inoculum showing that inoculation response is independent of species in *Sesbania* but depend on soil type. While comparing soils, soil 1 and soil 2 were better than soil 3 in nodulation response.

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