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Competitiveness Does Not Correlate With Siderophore Production in *Rhizobium-Cajanus cajan* Symbiosis

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Abstract

Twenty five Tn5 mutants of *Rhizobium* sp. *Cajanus* strain PP-18 varying in the amount of siderophore production were used to determine the nodulation competitiveness under pot culture conditions. Generally, low siderophore producing (LSP) mutants produced less nodule biomass, ARA (acetylene reduction assay) activity, root, shoot dry weights, shoot weight ratio, nitrogen and iron contents of pigeonpea plants as compared to moderate siderophore producing (MSP) and siderophore over-producing (HSP) group of mutants. Maximum nodule occupancy of 71 per cent was observed with the inoculation of mutant LSP-19 while minimum of 16 per cent with mutant HSP-5. The parent strain formed 44 per cent of the total nodules. The overall nodule occupancy of low, moderate and siderophore over-producing mutants was 42, 43 and 42 per cent, respectively, indicating, that there is no extra advantage of siderophore over-producing mutants in nodulation competitiveness.

Keywords: *Rhizobium* sp. (*Cajanus*), pigeonpea, nodulation, nodule occupancy, siderophore

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1. Introduction

The presence of native rhizobia limits inoculant performance under agricultural field conditions. Inoculation would be beneficial only when the inoculant strain is able to displace the native rhizobia and form nodules. Nodulation competitiveness appears to be a complex process and is the result of many interacting mechanisms. The attributes of possible relevance to the success of a strain includes: strain, host, strain host interactions and environment. To solve the problem of competitiveness, one of the approach is to select highly competitive strains. Highly effective strains varied greatly in their degree of competitiveness (Dudeja and Khurana, 1988; Gaur and Lowther, 1982a; 1982b; May and Bohlool, 1983; Moawad et al., 1984; Rafique Uddin et al., 1984). Schmidt and Robert (1985) showed that the interactions which occur in the rhizosphere during early plant growth are critical in determining the outcome of competition among *Bradyrhizobium japonicum* strains. Some possible characteristics of a rhizobial strain which may directly or indirectly determine the competitiveness include: motility, polysaccharide production, alternation in the nodule formation efficiency genes *nfe* (Sanjuan and Olivares, 1989; Toro and Olivares, 1986); rhizopine production (Murphy and Saint, 1992); bacteriocin production (Breil et al., 1993; Triplett, 1988; Triplett et al., 1994); proline dehydrogenase (Jimenez-Zurdo et al., 1995; Kohl et al., 1994; Suman, 1998) and the hydrogenase uptake system (Dudeja et al., 1995). Perhaps the ability of a *Rhizobium* strain to produce siderophores in the rhizosphere may have competitive edge over other rhizobial strains as these were positively correlated with the efficacy of N_2 fixation in symbiotic association with pigeonpea (Duhan et al., 1998).

Nodulation of pigeonpea [*Cajanus cajan* (L) Millsp.], a major *Kharif* pulse crop, is generally poor in arid and semi-arid regions of India (Khurana and Dudeja, 1981). Inoculation with efficient strains does not improve its nodulation (Dudeja and Khurana, 1983) and inoculant strains show poor nodule occupancy under field conditions as compared to pot culture conditions (Dudeja and Khurana, 1988). In the present study, siderophore over-producing mutants developed through transposon-mediated mutation of a pigeonpea *Rhizobium* sp. (*Cajanus*) strain PP-18 were evaluated under pot culture conditions for nodule occupancy in *Cajanus cajan* L. Millsp.

2. Materials and Methods

A hydroxamate type of siderophore producing *Rhizobium* sp. *Cajanus* strain PP-18 was selected and mutagenized with Tn5. Tn5 mutagenesis was performed

using the broad host range mobilizable vector pSU 2021 (Simon et al., 1983). PP-18 was grown in tryptone yeast extract (TY) broth at 28±1°C for 24 h and was mated with *E. coli* strain SM-10 grown in Luria Bartini (LB) broth at 37°C for 12 h on shaker. Cultures were centrifuged in 1.5 ml Eppendorf tubes, washed with TY broth and resuspended in 200 µl of TY broth. Cultures were mixed in the ratio of 5:1 (*Rhizobium*:*E. coli*) centrifuged and resuspended in 30–40 µl of TY broth. The mating mixture (25 µl) was spotted on a TY plate and incubated for 16–24 h at 28±1°C. Cells were removed from the spot, resuspended in 5 ml TY broth and vortexed. Serial dilutions were plated on yeast extract mannitol agar (YEMA) medium plates supplemented with kanamycin (50 µg ml^{-1}) and nalidixic acid (25 µg ml^{-1}). Plates were incubated at 28±1°C for 72 h.

As controls, *Rhizobium* and *E. coli* were also plated on YEMA and TY medium containing both the antibiotics. About 1,500 transconjugants were screened for siderophore production using the Universal Chemical Assay on CAS (Chrome Azurol S) agar plates and CAS assay solution (Schwyn and Neilands, 1987). Mutants showing maximum and minimum halos were selected as siderophore mutants. The amounts of hydroxamate type siderophores in these mutants were quantified using the method of Csaky (1948) which determines bound hydroxalamine. Siderophore mutants and the wild type strain were grown in broth as by Modi et al. (1985). Protein contents were estimated following the method of Lowry et al. (1951) after digestion of cells with 2 ml of 0.1 M NaOH for 1/2 h at 90°C.

A pot experiment was conducted to monitor the nodule occupancy by siderophore over-producing mutants of PP-18. Sandy soil from a farmer's field of the nearby village Gangwa was used (soil pH 8.0, electrical conductivity 0.28 m mhos cm^{-1}, organic C 0.43 per cent, total N 0.039 per cent and P 885 ppm). About 8 kg of soil was filled in the earthern pots. Seeds of *Cajanus cajan* L. Millsp. cv. Manak were treated with 2.0 ml of inoculum containing 10^6–10^7 cells ml^{-1}. After germination four plants per pot and three replicates were maintained and were irrigated with water daily.

Plants were uprooted after 60 days of growth and observations were made on nodule biomass, acetylene reduction activity (ARA), nodule occupancy, root and shoot dry weight, total N and iron contents were recorded. All the nodules were used to determine nodule occupancy using multiple antibiotic resistance markers to identify the inoculants. Nodules were detached and surface sterilized by immersing in 0.2 per cent acidic mercuric chloride for 3–5 min followed by 95 per cent ethanol for 2 min (Vincent, 1970). Nitrogenase activity was expressed as nM of acetylene reduced h^{-1} $plant^{-1}$.

Total nitrogen contents were estimated by Kjeldahl's steam distillation method (Bremner, 1960) and iron contents by the method of Piper (1986).

Table 1. Efficacy of siderophore over-producing mutants of *Rhizobium* sp. *Cajanus* strain PP-18 under pot culture conditions

Tn5 siderophore mutants	Hydroxamate contents (μg N mg^{-1} protein)	Nodule biomass (mg $plant^{-1}$)	ARA nM of C_2H_2 reduced (h^{-1} $plant^{-1}$)	Root dry weight (mg $plant^{-1}$)	Shoot dry weight (mg $plant^{-1}$)	Shoot* weight ratio
Control	–	6	136	249	327	–
PP-18	2.21	**37**	**423**	350	532	1.6
PP-18 LSP-15	ND	**27**	**286**	333	534	1.6
PP-18 LSP-17	ND	**36**	**300**	313	468	1.4
PP-18 LSP-23	0.68	**22**	178	256	448	1.4
PP-18 LSP-24	0.88	**29**	205	230	518	1.6
PP-18 LSP-19	0.85	**23**	184	286	447	1.4
PP-18 LSP-14	0.92	**29**	**396**	413	587	1.6
PP-18 LSP-25	1.00	**18**	197	279	524	1.6
PP-18 LSP-16	1.04	**28**	**327**	301	530	1.6
PP-18 LSP-20	1.06	**30**	**272**	258	503	1.5
PP-18 LSP-18	1.16	**34**	218	349	514	1.6
PP-18 LSP-21	1.27	**29**	177	290	534	1.6
PP-18 LSP-22	1.50	**27**	191	255	489	1.5
Mean	0.97	**28**	249	297	496	1.6
S.E.	–	5	59	51	65	–
P = 0.05	–	10	121	NS	NS	–
PP-18 MSP-12	1.61	**31**	**396**	431	**534**	1.6
PP-18 MSP-13	1.76	**33**	**409**	348	**571**	1.7
PP-18 MSP-11	1.92	**29**	327	365	**558**	1.7
PP-18 MSP-2	2.83	**49**	223	382	**623**	1.9
Mean	2.03	**36**	**464**	382	**572**	1.7
S.E.	–	5	103	59	66	–
P = 0.05	–	10	224	NS	143	–
PP-18 HSP-9	4.27	**85**	491	**436**	**824**	2.5
PP-18 HSP-5	5.09	**53**	477	**420**	**742**	2.3
PP-18 HSP-7	5.43	**44**	641	344	**627**	1.9
PP-18 HSP-1	5.65	**56**	**778**	**418**	**622**	1.9
PP-18 HSP-3	6.63	**60**	**846**	**391**	**775**	2.4
PP-18 HSP-4	6.89	**70**	**900**	**386**	**722**	2.2
PP-18 HSP-8	7.78	**97**	**900**	**458**	**1160**	3.5
PP-18 HSP-6	7.95	**105**	**955**	**372**	**769**	2.4
PP-18 HSP-10	8.05	**93**	**1036**	**445**	**1154**	3.5
Mean	6.41	**74**	**780**	**408**	821	2.5
S.E.	–	11	220	53	95	–
P = 0.05	–	22	456	110	196	–

ND = Not detectable; Dark values are significant at 5% level.

$$\text{*Shoot weight ratio} = \frac{\text{Shoot weight of inoculated plant}}{\text{Shoot weight of uninoculated plant}}$$

Table 2. Nodule occupancy in pigeonpea host inoculated with Tn5 siderophore mutants of *Rhizobium* sp. *Cajanus* strain PP-18 under pot culture conditions

Tn5 siderophore mutants	Nodule occupancy (%)	Mean
Control	0	
PP-18	44	44
PP-18 LSP-15	25	
PP-18 LSP-17	34	
PP-18 LSP-23	35	
PP-18 LSP-24	44	
PP-18 LSP-19	71	42
PP-18 LSP-14	44	
PP-18 LSP-25	48	
PP-18 LSP-16	36	
PP-18 LSP-20	47	
PP-18 LSP-18	34	
PP-18 LSP-21	47	
PP-18 LSP-22	41	
PP-18 MSP-12	37	
PP-18 MSP-13	39	43
PP-18 MSP-11	60	
PP-18 MSP-2	34	
PP-18 HSP-9	46	
PP-18 HSP-5	16	
PP-18 HSP-7	47	
PP-18 HSP-1	29	42
PP-18 HSP-3	50	
PP-18 HSP-4	57	
PP-18 HSP-8	31	
PP-18 HSP-6	57	
PP-18 HSP-10	50	

3. Results and Discussion

Quantification of hydroxamate contents of all the 25 mutants of PP-18 showed large variation in the quantity of hydroxamate produced by different mutants and this ranged from 0.68 to 8.05 μg N mg^{-1} protein (Table 1). Nine mutants over- produced(high siderophore producing, HSP) siderophores (>3.0 μg of hydroxamate N mg^{-1} protein); 4 mutants produced between 1.5–3.0 (MSP), and 10 mutants produced between 0–1.5 μg of hydroxamate N mg^{-1} protein (LSP). In two mutants hydroxamate was not detectable. Results after 60 days of plant growth showed that maximum nodule biomass was produced by mutant HSP-6, while minimum by mutant LSP-15 (Table 1). Overall means of low or non-siderophore producing mutants were 28 mg $plant^{-1}$ of nodule biomass, while

Table 3. Nitrogen and iron contents of pigeonpea host inoculated with siderophore over-producing mutants of *Rhizobium* sp. *Cajanus* strain PP-18 under pot culture conditions

Tn5 siderophore mutants	Total N contents (mg plant^{-1})	Iron contents (ppm)
Control	6.6	**374**
PP-18	13.7	**1,032**
PP-18 LSP-15	12.7	**502**
PP-18 LSP-17	12.5	**479**
PP-18 LSP-23	8.2	**592**
PP-18 LSP-24	10.0	**682**
PP-18 LSP-19	9.8	**654**
PP-18 LSP-14	14.2	**754**
PP-18 LSP-25	11.6	**743**
PP-18 LSP-16	11.7	**783**
PP-18 LSP-20	9.5	**628**
PP-18 LSP-18	12.6	**832**
PP-18 LSP-21	12.7	**818**
PP-18 LSP-22	10.9	**832**
Mean	11.2	**691**
S.E.	1.1	59
P = 0.05	NS	122
PP-18 MSP-12	15.2	**884**
PP-18 MSP-13	15.1	**958**
PP-18 MSP-11	13.8	**1,030**
PP-18 MSP-2	17.1	**1,163**
Mean	15.3	**1,008**
S.E.	1.6	850
P = 0.05	NS	108
PP-18 HSP-9	**21.9**	**1,056**
PP-18 HSP-5	**23.0**	**1,314**
PP-18 HSP-7	**22.6**	**1,189**
PP-18 HSP-1	**18.0**	**1,537**
PP-18 HSP-3	**21.5**	**1,755**
PP-18 HSP-4	**19.3**	**1,756**
PP-18 HSP-8	**29.2**	**2,113**
PP-18 HSP-6	**21.1**	**1,885**
PP-18 HSP-10	**30.5**	**2,207**
Mean	**22.9**	**164.5**
S.E.	2.1	71
P = 0.05	2.0	147

Dark values are significant at 5% level.

moderate and siderophore over-producing mutants produced 46 and 49 mg plant^{-1} of nodule biomass. Maximum ARA (1,036 nM of C_2H_2 reduced h^{-1} plant^{-1}) was recorded by the mutant HSP-10 while minimum by mutant LSP-21.

Overall means for low, moderate and siderophore over - producing mutants were 249, 264 and 780 nM of C_2H_2 reduced h^{-1} $plant^{-1}$, respectively. Maximum root and shoot biomass and shoot weight ratio was observed in case of plants which were inoculated with mutant HSP-8 (Table 1). Root and shoot biomass produced by different rhizobial mutants was higher than the uninoculated control. Overall means of the root biomass in case of low, moderate and siderophore over-producing mutants was 297, 382 and 408 mg $plant^{-1}$. Similarly shoot biomass was 496, 572 and 821 mg $plant^{-1}$. Corresponding values for shoot weight ratio was 1.6, 1.7 and 2.5, respectively.

Maximum nodule occupancy of 71 per cent was observed following inoculation with mutant LSP-19, and the minimum 16 per cent with mutant HSP-5 (Table 2). The wild type formed 44 per cent of the total nodules. In case of low, moderate and siderophore over-producing mutants the nodule occupancy ranged from 34–71, 34–60 and 16–57 per cent, respectively. Overall means for nodule occupancy were 42, 43 and 42 per cent, respectively. In other words siderophore production was unrelated to nodule occupancy. Using *R. fredii* almost similar results have been reported elsewhere (Manjanatha et al., 1992).

Maximum nitrogen (30.5 mg $plant^{-1}$) and iron (2,207 ppm) contents were produced by the plants inoculated with mutant HSP-10 (Table 3). However, minimum nitrogen and iron contents were recorded following inoculation with LSP-23 and LSP-17 which in turn was higher than in the uninoculated control. Overall means of nitrogen contents for LSP, MSP and HSP mutants were 11.2, 15.3 and 22.9 mg $plant^{-1}$, respectively and the corresponding iron contents were 691, 1,008 and 1,645 ppm. Similarly, positive correlations of siderophore production and nitrogen fixation has been reported elsewhere by selecting insertion mutants of *R. meliloti* 1021 (Gill et al., 1991). Siderophore over-producing Tn5 mutants of *R. fredii* produced more mature and pink nodules on soybean plants than the wild type (Manjanatha et al., 1992). This production of higher amounts of siderophores by a strain or mutant enhances N_2 fixation and iron acquisition by pigeonpea plants but does not affect nodulation competitiveness.

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