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Tn5 siderophore producing mutants of *Rhizobium* and its role in nitrogen fixation and iron uptake in pigeonpea

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Pigeonpea is an economically important kharif grain legume crop that nodulates poorly in the northern part of India. The role of siderophore production in nitrogen fixation and iron uptake in pigeonpea (*Cajanus cajan*) was assessed. 25 Tn5 siderophore producing mutants of pigeonpea-*Rhizobium* strain PP-18 were selected using Chrome Azurol S (CAS) agar plate as well as CAS assay solution. Finally, the efficacy in pigeon pea host was assessed under controlled conditions. PP-18 LSP-15 and PP-18 LSP-17 mutants did not produce detectable quantity of hydroxamate type of siderophore, while nine mutants over produced siderophores (HSP) as compared to wild type. The rhizobial mutants produced from 0.68 to 8.05 µg of hydroxamate N mg-1 protein, whereas wild type produced 2.21 µg of hydroxamate N mg^{-1} protein. On overall mean basis, low siderophore producing group of mutants produced less nodule biomass, root, shoot dry weight and shoot weight ratio as compared to moderate siderophore producing and siderophore over-producing mutants. When pigeonpea plants were inoculated with HSP, they achieved the highest nitrogen and iron contents; 16.2 mg $plant^{-1}$ and 1408 ppm respectively. Hydroxamate type of siderophore production was highly correlated with N_2 fixing efficiency and iron contents of pigeonpea plants.

Key words: Rhizobium sp. (*Cajanus*), pigeonpea, nitrogen fixation, iron uptake, siderophore, nodulation.

INTRODUCTION

Pigeonpea is an economically important kharif grain legume crop that nodulates poorly in the northern part of India and this is known as an important handicap to improve its yield. In nitrogen fixing root nodules, a large quantity of iron is present in many enzymes and proteins. Plants bacteria and bacteroids therefore, need an adequate supply of iron. Most of the microorganisms have evolved specific, high affinity mechanism to acquire iron by producing extra cellular siderophores (Koo et al., 2010; Deshwal et al., 2003). Broadly, these are of two types; hydroxamates and catecholates. *Bradyrhizobium* and *Rhizobium* spp. infecting different legume hosts have been reported to produce siderophores (Dudeja, 1996). Rhizobia are known to excrete catecholates (Modi et al.,1985; Suneja et al., 1994; Duhan et al., 1998). On the other hand, hydroxamate type of siderophores is produced by chickpea, clover and pigeonpea rhizobia (Carson et al., 1992; Suneja et al., 1992; Duhan et al.,1998).Specific type of siderophores like rhizobactin, pseudobactin, schizokinen and some unknown types of siderophores are also known to be produced by different rhizobial and other bacterial strains (Reigh and O'Connell,1989; Lesueur et al., 1993; Schwyn and Neilands 1987; Storey et al., 2006; O'Hara, 2001).

In vitro studies of iron nutrition of growing rhizobial cells may only be related to saprophytic growth in soil and not

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to functioning of the bacteroids in the root nodules. Bacteroids inside the nodules must receive an adequate supply of iron from the host legume for proper nodulation and efficient N_2 fixation. Improved iron scavenging properties of the rhizobia positively correlate with rhizosphere growth and nodulation effectiveness in groundnut and pigeonpea (O'Hara, 2001; Carson et al., 1992; Duhan and Dudeja, 1998).

The objectives of the present study were to pin point the role of siderophore production in nitrogen fixation and iron uptake in pigeonpea. To select Tn5 siderphore producing mutants of pigeonpea-*Rhizobium* and to assess the efficacy of these mutants in pigeonpea host.

MATERIALS AND METHODS

Tn5 siderphore producing mutants of pigeonpea-*Rhizobium*

A hydroxamate type of siderophore producing pigeonpea-*Rhizobium* strain PP-18 was selected and mutagenized with Tn5. Tn5 mutagenesis was carried out with a broad host range mobilizable vector pSU2021 (Simon et al., 1983). *Rizobium* strain PP-18 was grown in tryptone yeast extract (TY) broth (Beringer, 1974) at 28± 1°C for 24 h and was mated with *Escherichia coli* strain SM-10 grown in Luria Bertani broth (Sambrook et al., 1989) 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. Conjugal mixture (25 µl) was spotted on TY plates and incubated for 16 to 24 h at 28±1°C. Cells from the spot were removed and suspended 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 control, *Rhizobium* and *E. coli* were also plated on YEMA and TY medium containing both the antibiotics. About 1500 transconjugants along with the wild type PP-18 were screened for siderophore production using Chrome Azurol S (CAS) agar plate and CAS assay solution (Schwyn and Neiland 1987). 25 mutants showing variable size of halos were selected as siderophore mutants. Amount of hydroxamate type of siderophore in these mutants was quantified.

Assay for hydroxamate estimation

Hydroamate type of siderophore was assayed and estimated by Csaky (1948) test with some modification which determines bound hydroxalamine. Siderophore mutants and wild type were grown in a broth (Modi et al., 1985) and hydroxamate was estimated as detailed by other work such as that of Duhan et al. (1988). 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 ½ h at 90°C.

Effectiveness of Tn5 siderophore producing mutants in pigeonpea plant

The effectiveness of Tn5 siderophore producing mutants of pigeonpea *Rhizobium* strain PP-18 was determined under sterilized chillum jar assemblies (Dahiya and Khurana,1981) containing acid washed sand and autoclaved for 3 h at 15 psi unit pressure.

Surface sterilized seeds of pigeonpea cv. Manak were treated with 1 ml each of the pigeonpea rhizobial mutants and wild type containing 10^8 - 10^9 cells ml^{-1} in each jar. In each chillum jar, four plants were maintained. Sloger's nitrogen free nutrients solution (1/4 strength) without $FeCl_3$ was added as and when required (Sloger, 1969).Observations on nodule biomass, root and shoot dry weights, total N and Fe contents were determined after 60 days of growth in screen house.

The nodules were detached from the roots and were dried in the fold of filter papers. Dry weight of the nodules, roots and shoots were determined after the samples were dried at 80°C until constant weight. Total nitrogen content of the pigeonpea plants was estimated by Kjeldahl's steam distillation method (Breminer, 1960) using 200 mg of finely ground plant material. Plants samples used for nitrogen determination were also used to determine the iron contents. A weighed amount (1 g) of ground plant sample was taken in 100 ml conical flask. To this, diacid mixture (HNO_3: $HClO_4$; 4:1 v/v) was added and kept over-night. The contents were digested by heating until clear white precipitates settled down at the bottom (Piper, 1986). The contents were filtered through Whatman filter paper No. 42. The volume of filtrate was made to 50 ml with double distilled water and used for determination of total iron by Atomic Absorption Spectrophotometer (Perkin-elemer, Model 2320) at 240 nm.

To evaluate the efficacy of Tn5 siderophore producing mutants in nitrogen fixation and iron uptake, all the pigeonpea rhizobial mutants were categorized into three groups depending on the quantity of siderophores produced. In low siderophore producing group mutants producing 0.0 to 1.5 µg of hydroxamate N mg^{-1} protein were included while in moderate and siderophore over producing (high) group 1.5 to 3.0 and >3.0 µg of hydroxamate N mg^{-1} protein, respectively were included.

Statistical analysis

One way ANOVA was used to test the significance of the data.

RESULTS AND DISCUSSION

Screening of all the 25 mutants for hydroxamate type of siderophore production showed that 23 mutants which were found to be siderophore positive by CAS assay showed the presence of hydroxamate type of siderophores and in two mutants, PP-18 LSP-15 and PP-18 LSP-17 no hydroxamate type of siderophore was detected. Quantification of hydroxamate showed large variation in the quantity of hydroxamate produced by different rhizobial mutants and this ranged from 0.68 to 8.05 µg N mg^{-1} protein. Mutant PP-18 HSP-10 produced the maximum amount of hydroxamate type of siderophore followed by PP-18 HSP-6 and PP-18 HSP-8. Nine mutants were HSP. Minimum quantity of siderophore was observed in mutant PP-18 LSP-23. Two mutants PP-18 LSP-15 and LSP-17 did not produce detectable amount of hydroxamate. These mutants were also screened for the presence of catechol type of siderophore and none of the mutant produced this type of siderophore as the wild type was a hydroxamate type of siderophore producer (Figure 1).

On overall, mean basis maximum nodule biomass (69±8.5 mg $plant^{-1}$) and nitrogen contents (16.2±1.4 mg $plant^{-1}$) was found in high siderophore producing mutants (Figure 2). Nodule biomass and nitrogen content

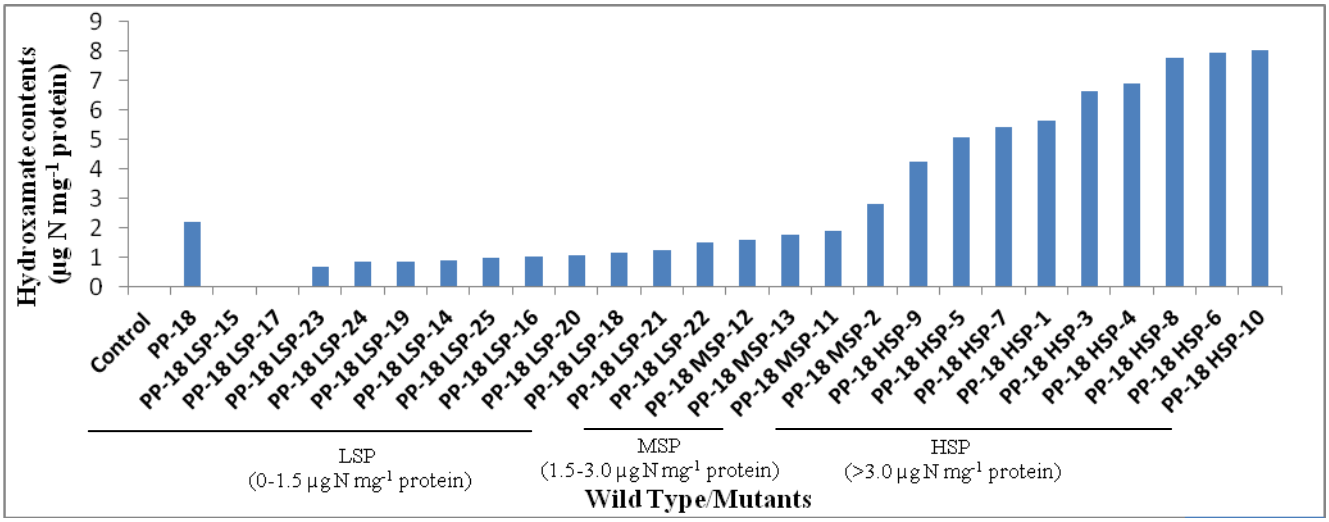

Figure 1. Screening, quantification and categorization of siderophore producing Tn5 mutants of pigeonpea-*Rhizobium* strain PP-18.

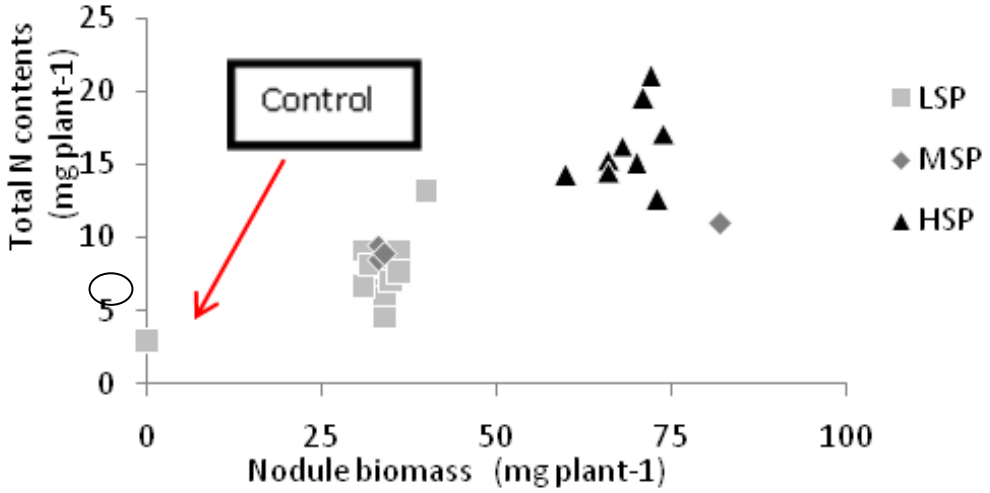

Figure 2. Nodule biomass and total nitrogen contents of Tn5 siderophore producing mutants of pigeonpea-*Rhizobium* strain.

produced by other mutants was also higher and comparable to t he parent strain. Low siderophore producing (LSP) mutants produced 33±8.1 mg plant^{-1} of nodule biomass, moderate (MSP) mutants formed 46±8.4 mg plant^{-1} while corresponding values of nitrogen contents were 9.5±1.1 and 7.5±0.9 mg plant^{-1}, respectively. High nodule biomass can be correlated with the nitrogen contents.

Likewise, on overall mean basis, the root and shoot biomass produced by LSP mutants was 174 ±28.8 and 299±80.4 mg plant^{-1} while corresponding values for MSP and HSP were 177±43.2, 278±90.3 and 251±46.6, 488±73.8 mg plant^{-1}, respectively indicating a progressive increase. Shoot weight ratio in the plants that received inoculation with LSP, MSP and HSP mutants was 1.5, 1.8, and 2.5, respectively (Figure 3).

Nitrogen content as well as iron content was found maximum on over all basis in high siderophore over-producing mutants (HSP) that is 16.2±1.4 mg plant^{-1} and 1408 ppm. It decreased up to 9.5±1.1 mg plant^{-1} (N content) and 852 ppm (Fe content) in moderate (MSP) and recorded lowest that is 7.5±0.9 mg plant^{-1} and 636 ppm in low siderophore producing (LSP) mutants (Figure 4).

Figure 5 shows that hydroxamate production by pigeonpea rhizobial mutants was highly correlated with nitrogen fixing efficiency. As HSP mutants produced maximum hydroxamate contents as well as nitrogen

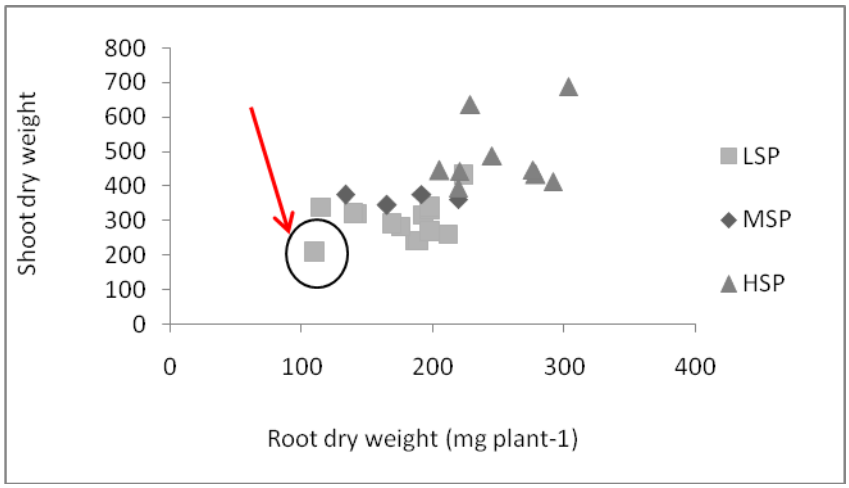

Figure 3. Shoot dry weight and root dry weight of Tn5 siderophore producing mutants of pigeonpea-*Rhizobium* strain PP-18.

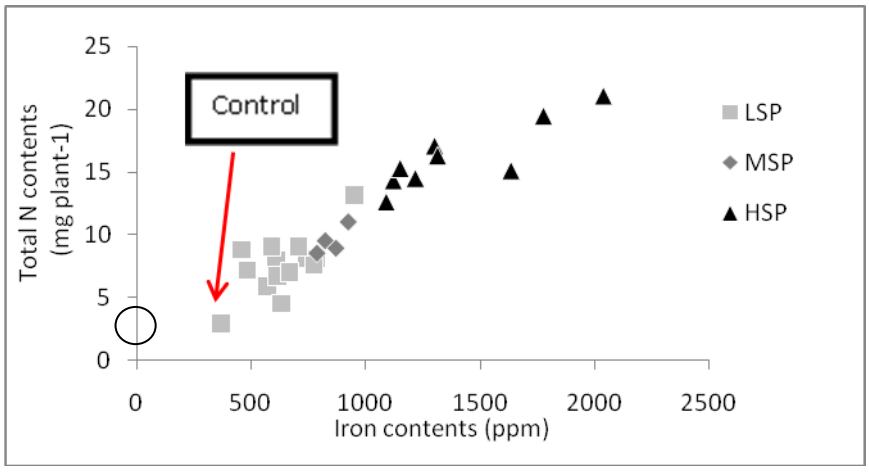

Figure 4. Total nitrogen and iron contents of Tn5 siderophore producing mutants of pigeonpea-*Rhizobium* strain PP-18.

contents that is 6.41±1.37 µg N mg^{-1} protein and 16.2±1.4 mg $plant^{-1}$. Both the contents decreased in case of MSP up to 2.03±0.55 µg N mg^{-1} protein and 9.5±1.1 mg $plant^{-1}$. It was recorded least in LSP mutants that is 1.43±0.57 µg N mg^{-1} protein in hydroxamate contents and 7.5±0.9 mg $plant^{-1}$ nitrogen content.

In general, HSP of pigeonpea rhizobia produced more nodule biomass, root and shoot biomass, shoot weight ratio, plant nitrogen and iron contents as compared to LSP and MSP, indicating a positive correlation between the amount of siderophore produced by different mutants and quantity of nitrogen fixed (r =0.93) and iron (r =0.96) taken up by pigeonpea plants. Similar positive correlation between the high affinity transport system, the siderophore production and nitrogen fixation was reported in *Rhizobium* sp. *cicer* infecting chickpea (Dhull,1996).Similarly, siderophore controlled iron assimilation was reported in enterobacterium *Erwinia chrysanthemi* (Expert et al., 2008). High iron scavenging *Bradyrhizobium* strains were more effective in nodulating the groundnut (O'Hara, 2001).Gill et al. (1991) also supported this view after selecting single site insertion mutants of *Rhizobium meliloti* 1021 isolated from alfalfa using Tn5 mutagenesis. Siderophore over-producing mutants selected by Tn5 mutagenesis of *R. fredii* produced more mature and pink nodules on soybean plants as compared to parent strain (Manjanatha et al., 1992). Similarly, role of the *sit* gene in managanese acquisition has been shown in *Sinorhizobium meliloti* (Platero et al., 2003).In contrast to this, Fabiano et al. (1996) reported that siderophores produced by a *Rhizobium* strains are not related to effectiveness.

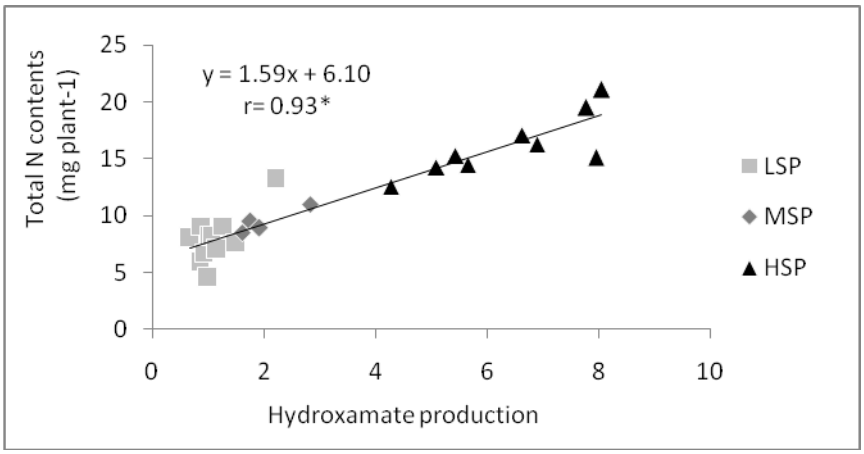

Figure 5 .Hydroxamate production and total nitrogen contents of Tn5 siderophore producing mutants of pigeonpea-*Rhizobium* strain PP-18

Conclusion

It can be concluded from this study that siderophore production was highly correlated with N_2 fixing efficiency and iron uptake of pigeonpea plants. But, how siderophore over-production helps the plants to fix more nitrogen and uptake more iron is still not very clear. Probably after entering the root nodules, bacteriods form of rhizobia make the availability of iron more to the required components of nodules by excreting siderophores in the nodule cytosol (Wittenberg et al., 1996). Secondly, free living rhizobia left in the soil may also help the plants to acquire more iron (Duhan and Dudeja, 1998). Labelled iron studies are required to strengthen these views.

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