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Endophytic colonization and *in planta* nitrogen fixation by a diazotrophic *Serratia* sp. in rice

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Nitrogen fixing endophytic *Serratia* sp. was isolated from rice and characterized. Re-colonization ability of *Serratia* sp. in the rice seedlings as endophyte was studied under laboratory condition. For detecting the re-colonization potential in the rice seedlings, *Serratia* sp. was marked with reporter genes (*egfp* and *Km*^{r}) using transposon mutagenesis. The conjugants were screened for re-colonization ability and presence of *nif* genes using PCR. Further, the influence of flavonoids and growth hormones on the endophytic colonization and *in planta* nitrogen fixation of *Serratia* was also investigated. The flavonoids, quercetin (3 μg/ml) and diadzein (2 μg/ml) significantly increased the re-colonization ability of the endophytic *Serratia*, whereas the growth hormones like IAA and NAA (5 μg/ml) reduced the endophytic colonization ability of *Serratia* sp. Similarly, the *in planta* nitrogen fixation by *Serratia* sp. in rice was significantly increased due to flavonoids. The inoculation of endophytic diazotrophs increased the plant biomass and biochemical constituents.

Key words: Endophytes, Flavonoids, Nitrogen fixation, Rice, *Serratia*

Nitrogen is the most frequent limiting nutrient in rice production for which 1 kg of nitrogen is needed to produce 15 to 20 kg of grain[1]. Maximum exploitation of biological nitrogen fixation will significantly contribute to the long term nitrogen nutrient availability to rice crop. The cyanobacteria, heterotrophic aerobic and anaerobic bacteria, both associative symbiotic and free-living are the major contributors of nitrogen in different layers of flooded rice soils. Recently, several endophytic nitrogen fixing organisms have been reported in the rice plant and the exploitation of their potential will be the future strategy for sustained rice production[2]. The successful endophytic diazotrophic associations have already been reported in sugarcane with *Gluconacetobacter diazotrophicus*[3] and in kallar grass (*Leptochola fusca*) with *Azoarcus* sp[4]. Being same family, rice also harbors variety of diazotrophic endophytes like *Gluconacetobacter* sp.[5], *Azoarcus*, *Herbaspirillum*[6], *Burkholderia, Serratia marcescens*[7], *Pantoea*[8] etc. These diazotrophs colonize root, stem, leaves of cereals endophytically and therefore probably suffer much less competition from other microorganisms for carbon substrates than rhizosphere bacteria, and possibly excrete part of their fixed nitrogen directly into the plant[9]. However, the root colonization of endophytic diazotrophs in crop plants has to be optimized for entry and internal colonization so as to get maximum nitrogen contribution. Few attempts have been made to use some amendments and flavonoids to increase the endophytic colonization of rice by *Serratia marcescens* IRBG500[10], *Herbaspirillum seropedicae* Z67[10] and *Azorhizobium*[11,12]. Most of the flavonoids, nodD independently stimulated the root colonization of *Rhizobium* in cereals[12]. Similarly growth regulators like naphthalene-1-acetic acid (NAA), indole acetic acid (IAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) also enhanced the lateral root formation and entry of rhizobia in side the rice root tissues[13]. The present study was undertaken to investigate the extent of endophytic colonization ability of an endophytic diazotroph, *Serratia* sp., isolated from rice and also to evaluate the influence of flavonoids and growth hormones on the endophytic colonization ability and *in planta* nitrogen fixation in rice seedlings.

Materials and Methods

Bacterial cultures — An endophytic diazotroph, isolated from rice (cultivar ADT36) was used in the present study that was identified and authenticated as *Serratia* sp. by standard microbiological tests[14], confirmed the presence of *nifH* by targeted PCR[7]. The host cells used for plasmid amplification were *Escherichia coli* JM109 *(recA endA1 gyrA96 thi*

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hsdR17 supE44Δ (lac proABrelA1) and *E. coli* S17-1 *λpir (Tp*^{r} *Sm*^{r} *recA thi pro hsdR*$^-$ *M*$^+$ *RP4-2-Tc:Mu:Km Tn7)*.

Media and culture conditions — The isolate *Serratia* sp. was maintained in LB medium without antibiotics and *E. coli* strains were maintained in LB medium with 100 μg/ml of amphicillin. The conjugant *Serratia* sp. was maintained in LB + 30 μg/ml kanamycin plates. The rice seedlings (cultivar ADT 36) were grown in N- free Fahraeus medium[7].

Gene manipulation — The plasmid purification, transformation and agarose gel electrophoresis were done as per the standard protocols described by Sambrook and Russel[15].

Development of marker Serratia by transposon mutagenesis — The transposon vector (pUT) conferring promoter less *egfp* gene and Km^r (kanamycin resistance) was first transferred to *E. coli* S17-1 by calcium chloride mediated transformation following the standard procedures of Sambrook and Russel[15]. The pUT vector of *E. coli* S17-1 is then transferred to *Serratia* by bi-parental mating[16]. The conjugants with stable expression of Km^r were purified and used for the studies.

Inoculation study with marker Serratia sp. — Dehulled seeds of rice variety ADT 36 were surface sterilized by immersion in 70% ethanol for 30 sec followed by soaking in 0.2% of mercuric chloride for 30 sec and then washed thoroughly with sterilized distilled water. The sterilized seeds (30 No. per plate) were germinated aseptically in plain agar (1%) plates. The plates were kept in dark for a day and then transferred to normal light next day at room temperature. Three days old seedlings that were free of any visual bacterial and fungal contamination were used for inoculation with marker *Serratia*. The latter were grown in LB broth supplemented with kanamycin (30 μg/ml) until they reached an optical density of 0.6 (approximately 48 hr). The cells were then harvested by centrifugation at 6000 rpm for 5 min at room temperature. The cell pellets were washed twice with 20 ml of phosphate buffer (*p*H 7.0) and resuspended in 1.5 ml of phosphate buffer. Just before placing the seedlings, the medium was inoculated with 100 μl of bacterial suspension. Surface sterilized seedlings were placed in glass tubes (60 ml) with 15 ml of N- free Fahraeus medium[7]. Two seedlings were maintained in each tube. The seedlings were then grown in a growth chamber (14 hr light and 10 hr dark cycle) at 27 °C. For each conjugant culture, 6 replications were maintained. Uninoculated plants served as controls.

Re-isolation of the conjugants — The 15 days old seedlings that were inoculated with marker *Serratia* sp. were surface sterilized in 0.1% mercuric chloride for 1 min. The seedlings were then quickly washed with sterilized distilled water thrice. The seedlings were cut into four parts *viz.*, root, shoot, lower and upper halves of the leaves and then macerated separately in sterile pestle and mortar with sterile distilled water. Then 10 μl of the macerate was placed on LB agar plates containing 30 μg/ml of kanamycin and incubated at 30°C for 2 days.

Flavonoids and growth hormone amendments study — Flavonoids such as quercetin and diadzein were dissolved individually in sterile distilled water (adjusted with sodium hydroxide to approximate *p*H 9.5), filter sterilized and stored at -20°C. IAA and NAA were dissolved in a few drops of ethanol, heated slightly and gradually diluted with double distilled water. Kinetin (kn) and 6-benzylamino purine (BAP) were dissolved in a few drops of 1 N NaOH, heated slightly and gradually diluted with double distilled water. The stocks were filter sterilized and stored at -20°C. The flavonoids and growth hormones were added at appropriate concentrations in nitrogen free Fahraeus medium just before cooling of the medium and surface sterilized dehulled rice seeds were placed. The *Serratia* (Conjugant #8) inoculation was performed as described previously. The plant biomass, protein and chlorophyll content, *in planta* nitrogenase activity of rice seedlings and the population of marked *Serratia* in the plant tissues and endophytic colonization in different parts of rice seedlings were recorded on day 15.

PCR condition — The total genomic DNA of the isolates as well as 2 days old actively grown intact bacterial colonies were used as template DNA. The genomic DNA of the endophytic isolates was isolated and purified following the standard procedures[15]. For colony PCR, the single actively grown *Serratia* sp. was dispersed in 15 μl of sterile water in 1.5 ml Eppendoff tubes, heated in water bath at 95°C for 10 min. After cooling with ice, 5 μl of lysate was used as template DNA. The reaction mixture (30 μl) contains Taq DNA polymerase buffer (3 μl); 2.5 m*M*, $MgCl_2$ (3 μl); 2.5 m*M*, dNTP mixture (3 μl); nifH1 primer[7] (forward; 3 μl); nifH2 primer[7] (reverse; 3 μl);

template DNA (5 µl); Taq DNA polymerase (0.5 µl); dH_2O (9.5 µl). All products were obtained from Bangalore Genei, India. The PCR (Eppendoff Master thermocycler) conditions[7] were as follows — 94°C for 5 min (initial denaturation) and 94°C for 1 min (denaturation) 50°C for 1 min (primer binding) 72°C for 2 min (primer extension) for 35 cycles followed by 72°C for 10 min for final extension and 4°C for storage.

Result

Several endophytic diazotrophs were isolated from different parts of rice and the presence of *nif* genes was confirmed by PCR amplification of *nifH* gene using standard primers[7]. Among the different endophytic diazotrophs, *Serratia* sp. isolate performed better in terms of growth and nitrogenase activity (data not shown). The *Serratia* isolate was further marked with reporter gene (*egfp* + Km^r cassette) using pUT mini transposon vector through conjugation with *E. coli* (S17-1 *λpir*; transposon mutagenesis), which was used to monitor the occurrence in rice tissues. The growth potential of the conjugants with wild strain was also tested, which revealed that the growth was not affected in all the conjugants due to mutagenesis (data not shown).

The re-isolation experiment revealed the variation of colonization by the conjugants. All the conjugants were able to colonize well in stem portion of rice seedling, and very few colonized the leaf and root portion (Table 1). Even though the entry of the endophyte was through root, the colonization was poor in the root region of the rice seedling. The promising conjugants were selected by re-isolation from different parts of inoculated rice and presence of *nifH* gene. Among the conjugants, conjugant #4 and #8 showed very promising result, due to their ability to colonize well in the root, stem and leaves of rice seedlings on 15th day after inoculation and the presence of *nifH* gene was confirmed by PCR[7].

Flavonoids and growth hormones significantly influenced the endophytic colonization ability of *Serratia* sp. (conjugant #8) in rice seedlings. In general, the flavonoids increased the performance of the endophytic colonization ability of the *Serratia* sp., whereas the growth hormones especially 6-benzyl-amino purine (BAP) and naphthalene-1-acetic acid (NAA) reduced the colonization of *Serratia* in rice seedlings (Table 2). This was further confirmed by the enumeration of total population of *Serratia* per plant. The flavonoids, quercetin and diadzein, significantly increased the population of *Serratia* inside the rice plant (3.84 and 3.66 log cfu per g), whereas the growth hormones NAA, indole-3-acetic acid (IAA) and BAP amendment in the growth medium for rice seedlings reduced the population of *Serratia* (2.49, 2.44 and 2.29 log cfu per g, respectively) compare to unamended control (3.54 log cfu per g) (Table 3). Similarly, *Serratia* inoculation in the flavonoids amended plant growth medium recorded significantly higher *in planta* nitrogenase activity, compared to growth hormone amended medium grown rice seedlings. The performance of *Serratia* in terms of nitrogenase activity was significantly increased, which reflected in the rice biomass, root and shoot length and biochemical constituents like protein and chlorophyll content of rice seedlings (Table 3).

Discussion

The recent introduction of the concept of BNF by endophytes[17] has led to investigation on potential of endophytic N_2 fixing bacteria, which colonize graminaceious plants. It has been suggested that these bacteria better express their N_2 fixing potential inside the plant tissues due to lower competition for nutrients and protection against high level of oxygen present on the root surface[18]. Endophytic colonization of rice seedlings by *Herbaspirillum seropedicae* strains was demonstrated by Elbeltagy *et al.*[6] using

Table 1 — Endophytic colonization pattern of the *Serratia* sp. conjugants in the inoculated rice seedlings
[Values are mean of 6 replications]

Conjugants	Colonization pattern* at various parts of rice			
	Root	Stem	Leaf (lower half)	Leaf (upper half)
C 1	-	++	++	-
C 2	-	++	-	+
C 3	-	++	-	-
C 4	++	++	++	++
C 5	-	++	+	-
C 6	+	++	+	+
C 7	++	++	++	+
C 8	++	++	++	++

++ Good; + Moderate and – nil colonization
*Colonization pattern refers the re-isolation of inoculated *Serratia* conjugants in LB+Km (30 µg/ml) plates when appropriate 15 days old rice tissue macerate was placed (10 µl)

gnotobiotic approach. Similarly, the *gusA* marked *Serratia marcescens* was able to colonize endophytically in stem and leaf tissues of rice seedlings[7]. The deep water rice endophyte, *Pantoea agglomerans* colonized throughout the root portion of rice, which was detected by *gusA* reporter gene[8, 19]. In the present experiment, *Serratia* sp. isolated from rice, marked with *egfp+Km*^{r} genes colonized endophytically throughout the rice seedling tissues, which confirmed the re-colonizing ability of the isolate. Further, conjugants expressed variation in the colonization ability in the rice seedlings, which might be due to random mutagenesis during conjugation. All the conjugants were able to colonize in the stem portion of rice and few colonized in the root and leaf portion of rice seedlings. After re-isolation from rice tissues, the presence of *nifH* gene was detected using colony PCR technique. This was mainly done to confirm, whether the *nif* genes in the conjugants were intact or disturbed by transposon mutagenesis.

Table 2 — Endophytic colonization pattern of the *Serratia* conjugant (#8) in the inoculated ADT 36 rice seedlings grown with nutrient medium containing growth hormones and flavonoids [Values are mean of 6 replications]

Treatments	Colonization pattern** in various parts of rice			
	Root	Stem	Leaf (lower half)	Leaf (upper half)
T_1 - Uninoculated control	-	-	-	-
T_2 - *Serratia* sp.*	+	++	+	+
T_3 - *Serratia* sp. + kinetin (3 µg/ml)	+	+	+	+
T_4 - *Serratia* sp. + 6- BAP (5 µg/ml)	+	-	+	+
T_5 - *Serratia* sp. + IAA (3 µg/ml)	-	-	+	+
T_6 - *Serratia* sp. + NAA (3 µg/ml)	-	+	+	-
T_7 - *Serratia* sp. + quercetin (3 µg/ml)	+	++	+	++
T_8 - *Serratia* sp. + diadzein (2 µg/ml)	++	++	++	++

++ Good; + Moderate and – nil colonization

* *Serratia* sp. (conjugant #8) used was marked with *egfp* + *Km*^{r} gene using transposon mutagenesis; The observations were recorded in the 15 days old rice seedlings

** Colonization pattern refers the growth of *Serratia* conjugants in LB+Km (30 µg/ml) plates when appropriate 15 days old rice tissue macerate was placed (10 µl)

Endophytic diazotrophs such as *Serratia marcescens* and *Herbaspirillum seropidicae* Z67 frequently colonized in the root, stem and leaves of rice systematically[20]. So far, few studies have been conducted to identify the factors that influence the interaction of these bacteria with the host plant. Ca^{2+} and Fe^{3+} ions in the medium subsequently reduced the endophytic colonization of *Serratia* and *Herbaspirillum*[10]. Similarly, microscopic analysis of ultra microtrome sections of the roots inoculated with *Azorhizobium caulinodans* in the presence of naringenin, demonstrated that the xylem was colonized by the organism abundantly in rice seedlings[11]. The flavonoids, quercetin and diadzein, with greater antioxidant activity than naringenin have got the potential to increase the endophytic colonization ability[21]. A change in root morphology to nodule like structure (*para* nodules) has been reported to occur in wheat and rice following the addition of auxins and growth hormones like 2,4 D, NAA, BAP, kinetin etc[22]. These structures showed preferential colonization and enhanced nitrogen fixation by *Azospirillum lipoferum* in wheat[23] and *Azorhizobium caulinodans* in rice[13]. Hence, present experiment was performed to compare the flavonoids and growth hormones for influencing the endophytic colonization and nitrogen fixation due to inoculation to rice. The results clearly indicated that flavonoids, quercetin and diadzein, positively influenced the endophytic colonization by *Serratia*, whereas IAA, NAA and BAP reduced the endophytic colonization ability of *Serratia*. Moreover, the short thicken root formation of rice also led to reduce the root length of rice. The same trend was also reflected in the rice seedling biomass and shoot length as well. The population of endophyte was also drastically reduced in IAA, NAA and BAP treated plants, which showed the deleterious effect of these chemicals against endophytes. Similarly, the colonization pattern of the endophyte was also varying due to flavonoids and growth hormones application. Maximum endophytic colonization of *Serratia* inside the rice tissue (root, stem and leaves) with higher nitrogenase activity was noticed due to flavonoids application. This is mainly due to role of flavonoids in inducing the endophytic colonization, whereas the growth hormones always restrict the organism to colonize in the root portion especially in *para* nodules. Higher population and nitrogen fixation by endophytic *Serratia* in the presence of flavonoid, resulted in the higher

Table 3 — Influence of *Serratia* conjugant (#8) inoculation on the growth, biochemical constituents, endophytic colonization and N_2 fixation in ADT36 rice seedlings grown with medium containing growth hormones and flavonoids

Treatments	Plant biomass (g)	Plant height (cm)		Protein content (mg/g of rice tissue)	Total chlorophyll (mg/g of rice tissue)	*In planta* nitrogenase activity (μM of C_2H_4 produced per hr per g dry wt of rice)	Population of *Serratia* in rice (log cfu per g dry wt of rice)
		Root	Shoot				
T_1 - Uninoculated control	59	5.00	15.30	0.49	1.34	0.00	0.00
T_2 - *Serratia* sp.*	73	6.70	11.80	1.22	2.56	0.84	3.54
T_3 - *Serratia* sp. + kinetin (3 µg/ml)	71	7.90	9.30	0.41	2.55	2.49	3.48
T_4 - *Serratia* sp. + 6- BAP (5 µg/ml)	48	3.20	6.20	0.46	1.66	2.14	2.29
T_5 - *Serratia* sp. + IAA (3 µg/ml)	46	3.60	6.90	0.50	1.35	2.16	2.44
T_6 - *Serratia* sp. + NAA (3 µg/ml)	41	2.50	4.00	0.56	1.39	0.19	2.49
T_7 - *Serratia* sp. + quercetin (3 µg/ml)	72	10.70	14.60	1.97	3.26	2.71	3.66
T_8 - *Serratia* sp. + diadzein (2 µg/ml)	78	7.10	12.00	1.33	2.81	2.64	3.84
CD (0.05)	4.0	0.55	0. 93	0.11	0.18	0.02	0.03

* *Serratia* sp. conjugant (#8) used was marked with *egfp* + *Km*^{r} gene using transposon mutagenesis; The observations were recorded in the 15 days old rice seedlings

chlorophyll and protein content of rice seedlings. The results were in accordance with the similar studies conducted in *Herbaspirillum*[6], *Serratia marcescens*[7] and *Burkholderia* sp[24,25].

The present work revealed that substantial quantity of BNF contribution could be derived through endophytic diazotrophic bacterial inoculation. The present results confirmed the earlier findings with different endophytes on rice crop. Although, development of a carrier based inoculum of endophytic diazotroph is still long way, a 20-30% more nitrogen contribution could be possible due to inoculation with these organisms. Identifying putative, obligate, efficient endophytic diazotrophs and their diversity; analyzing the key factors to increase the population and extent of colonization inside the rice plant and understanding the mode of entry of the organism in the host plant are to be given sufficient thrust for further exploitation of the endophytic diazotrophs as bioinoculant for crop productivity.

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