

Nutrient Amendments Influence Endophytic Colonization of Rice by *Serratia marcescens* IRBG500 and *Herbaspirillum seropedicae* Z67

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Abstract *Serratia marcescens* IRBG500 and *Herbaspirillum seropedicae* Z67 grow endophytically in rice. The ability of these bacteria to colonize rice grown under increased nutrient availability was assessed in variety IR72 using strains marked with transposon-based *gusA*. The endophytic colonization was monitored via bacterial enumeration and histochemical visualization of GUS expression of bacteria in plant tissues. Rhizoplane and endophytic colonization by both bacteria was significantly inhibited in the rice plants grown in the presence of 10 mM NH_4Cl . In contrast, the addition of 10 mM KNO_3 showed no adverse effect on colonization. Increasing the concentration of Ca^{2+} to 5 mM significantly reduced endophytic colonization by both bacterial strains, whereas the addition of 0.5 mM Fe^{2+} substantially lowered the colonization of roots by *S. marcescens* IRBG500 but showed no effect on colonization by *H. seropedicae* Z67. Taken together, these findings suggest that, like in legume-rhizobial symbiosis as well as plant-pathogen interactions, nutrient status, particularly NH_4^+ and Ca^{2+} concentrations in the surrounding medium, plays an important role in the regulation of endophytic infection and colonization processes in rice.

Key words: Rice, endophytes, colonization, nutrients, pH, *gusA*

Endophytic diazotrophs such as *Acetobacter diazotrophicus* and *Herbaspirillum* spp. are postulated to be responsible for significant nitrogen fixation observed in certain sugarcane varieties [14]. Endophytic bacteria have an advantage over free-living rhizospheric bacteria because they can have better access to the carbon sources inside the plant and have less competition with the native root-associated microbes [11]. Rice, an important cereal crop, also harbors a variety of N_2 -fixing endophytic bacteria [2]. The contribution

of the putative endophytic bacteria for the nitrogen economy of rice, however, is low [16, 19, 23]. Enhancement of N_2 fixation by using efficient endophytic bacteria can reduce the dependency of rice on N-fertilizer input [17].

Endophytes such as *Serratia marcescens* IRBG500 and *Herbaspirillum seropedicae* Z67 frequently colonize roots, stems, and leaves of rice systemically [13]. So far, however, no studies have been conducted to identify the factors that influence the interaction of these bacteria with the host plant. In the case of legumes, the presence of fixed nitrogen in the form of nitrate in the root environment inhibits infection and colonization by rhizobia [4]. Similarly, recent studies with sugarcane have shown that nitrogen fertilization drastically reduces the colonization of sugarcane by *A. diazotrophicus* [10]. In addition, Ca^{2+} plays an important role in regulating colonization of legume roots by *Rhizobium* [18, 24], and Fe^{2+} , which has a role in regulation of the expression of hydrolytic enzymes, was shown to have a profound effect on the invasion of plants by pathogenic bacteria such as *Erwinia chrysanthemi* [9]. The present study, therefore, was undertaken to understand factors that could affect colonization of rice by endophytic bacteria. Since different forms of nitrogen differently affect colonization [20], the study was carried out with two major forms of nitrogen (nitrate and ammonium). In addition to the effect of nitrogen on colonization, the roles of Ca^{2+} , Fe^{2+} , and pH were also investigated.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Serratia marcescens IRBG500 was isolated from surface-sterilized tissues of four different rice varieties while *Herbaspirillum seropedicae* Z67 was isolated from surface-sterilized roots of Brazilian rice variety [13]. Both these bacteria have been shown to be aggressive endophytic colonizers of rice roots and stems under axenic conditions,

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and mainly localized in the intercellular spaces, aerenchyma and xylem vessels [13]. *S. marcescens* IRBG500 and *H. seropedicae* Z67 were tagged with transposon-based *gusA* under the control of the constitutive promoter of the kanamycin resistance gene [26]. Colonization abilities of both these *gusA*-marked strains were similar to those of their respective wild-type strains [13, 2]. The bacteria were maintained on Luria-Bertani (LB) agar plates containing spectinomycin ($100 \mu\text{g ml}^{-1}$), because the transposon also codes for resistance to spectinomycin [26]. The medium was also supplemented with tetracycline ($10 \mu\text{g ml}^{-1}$) for *S. marcescens* IRBG500 and nalidixic acid ($10 \mu\text{g ml}^{-1}$) for *H. seropedicae* as these bacteria have natural resistance to the respective antibiotics.

Inoculation of Rice with *S. marcescens* IRBG500-*gusA* and *H. seropedicae* Z67-*gusA*

Seeds of rice variety IR72 were dehulled and surface-sterilized with 70% ethanol for 5 min, followed by 0.2% mercuric chloride treatment for 1 min and washing with sterile distilled water three times. The seeds were germinated on 0.1% tryptic soy agar plates and the seedlings free of visual bacterial and fungal contamination were transferred to glass tubes containing Fahraeus medium (FM) [8] with or without various supplements as indicated below. Single seedling was planted in each tube. The *gusA*-marked strains of *S. marcescens* IRBG500 and *H. seropedicae* Z67 were grown in LB broth until early stationary phase, harvested by centrifugation at $2,000 \times g$, washed two times with normal saline, and then resuspended in an equal volume of saline. One ml of this suspension (approx. 10^6 cfu ml^{-1}) was used to inoculate the medium containing the plants at 5 days after germination.

Effect of Nitrogen, Fe^{2+} , and Ca^{2+} on Colonization of Rice

To study the effect of various factors on colonization, FM free of nitrogen was supplemented with NH_4Cl (10 mM), KNO_3 (10 mM), CaCl_2 (5 mM), or FeSO_4 (0.5 mM), and the pH was adjusted to 7.0. To study the effect of pH on colonization, the pH of FM medium was adjusted to 5–9 by 1 N HCl or 1 N KOH. At 7 days after inoculation (DAI), plants were removed from the medium, and the bacteria colonizing the surface and internal tissues of the plant were enumerated as described below. Uninoculated plants served as controls.

Enumeration of Bacteria Colonizing the Rice Roots and Stems

Bacteria present on both the surface and inside of the rice roots and stems were enumerated as described by Saxena *et al.* [21]. Briefly, for enumerating the surface colonizing bacteria, the roots and stems were vortexed separately for 1 min in sterile distilled water, and the resulting solution

was serially diluted and plated on LB agar plates containing appropriate antibiotics (see above) and $20 \mu\text{g ml}^{-1}$ of 5-bromo-4-chloro-3-indoxyl- β -D-glucuronide (X-gluc). To enumerate the endophytic populations of the bacteria, the roots and stems were surface-sterilized by immersion in 95% ethanol for 5 min, followed by 1 min treatment with 3% calcium hypochlorite containing 0.1% sodium dodecyl sulfate (SDS). After three washes with sterile distilled water, the plant material was macerated in normal saline, serially diluted, and placed on LB agar plates containing appropriate antibiotics and X-gluc as described above. Bacterial colonies showing blue color were counted.

GUS Staining of the Rice Seedlings

At least three plants from each of the three different replicated experiments were collected at 7 DAI and stained for GUS activity by incubating the seedlings in 50 mM potassium phosphate buffer, pH 7.0, containing $400 \mu\text{g ml}^{-1}$ of X-gluc as described by Jefferson *et al.* [15].

Growth Promotion of IR72 Seedlings by IRBG500 and Z67

To study the growth-promoting activity of *S. marcescens* IRBG500 and *H. seropedicae* Z67 in rice, the non-surface sterilized seeds were soaked overnight in sterile distilled water and then germinated on water agar plates. The seedlings were transferred to tubes containing FM 3 d after germination and were inoculated with respective bacteria, as described for the seedlings derived from surface-sterilized seeds. Bacteria were reisolated from the plant material 7 DAI and enumerated. Rice seedlings inoculated with bacteria and the uninoculated seedlings were harvested after 20 DAI, and their root and shoot length were recorded. The plant materials were then dried in an oven at 70°C until constant weight for dry weight measurements.

Data Analysis

Data were subjected to analysis of variance (ANOVA) and means were compared by Duncan's multiple range test (DMRT). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of pH

Surface and endophytic colonization of rice roots and stems by *S. marcescens* IRBG500 and *H. seropedicae* Z67 was evaluated with *gusA*-marked strains by reisolation and histochemical staining methods. To determine the optimal pH for further colonization studies, rice seedlings were inoculated with *S. marcescens* IRBG500 and *H. seropedicae* Z67 in FM adjusted to a pH 5 to 9 range. Bacterial colonization was not significantly different in the pH range

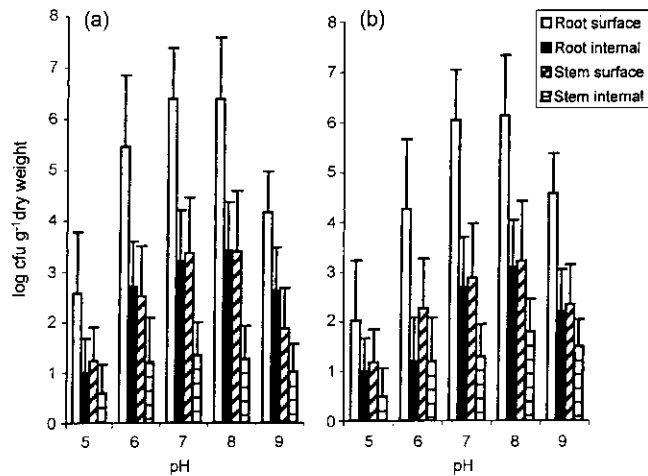


Fig. 1. Effect of pH on colonization of IR72 by *gusA*-marked strains of *S. marcescens* IRBG500 (a) and *H. seropedicae* Z67 (b).

of 5 to 9, although both bacteria exhibited a tendency to colonize better in the range of 7 to 8 (Fig. 1). Therefore, further experiments were done at pH 7.0. No significant variation in the pH value was observed in the medium at the end of the experiment.

Effect of Inorganic Nitrogen Sources on Colonization

The presence of NH_4^+ had the most profound effect by reducing the surface colonization of both roots and stems by more than 90%, and the endophytic colonization was below the detection limit of approximately 10^2 bacteria per

Table 1. Colonization of *S. marcescens* IRBG500-*gusA* and *H. seropedicae* Z67-*gusA* in IR72 grown in the presence of various supplements.

Treatment	Total no. bacteria (log cfu g ⁻¹ dry wt)			
	Root surface	Root internal tissues	Stem surface	Stem internal tissues
IRBG500-<i>gusA</i>:				
FM-N (control) ^a	6.38	3.32	3.08	1.59
+ NH_4^+ (10 mM)	1.47*	<1.00*	<1.00*	<1.00*
+ NO_3^- (10 mM)	5.87	2.89	2.76	1.25
+ Fe^{2+} (0.5 mM)	2.23*	1.0*	2.88	1.06
+ Ca^{2+} (5 mM)	5.95	1.52*	1.42*	<1.00*
Z67-<i>gusA</i>:				
FM-N (control) ^a	6.05	3.14	3.03	1.89
+ NH_4^+ (10 mM)	1.22*	<1.00*	<1.00*	<1.00*
+ NO_3^- (10 mM)	5.82	3.11	2.65	1.28
+ Fe^{2+} (0.5 mM)	6.27	2.92	3.28	1.78
+ Ca^{2+} (5 mM)	5.82	1.34*	1.52*	<1.00*

Results are the mean of three independent experiments.

^aFahraeus medium (FM) has 0.5 mM Ca^{2+} and 0.02 mM Fe^{2+} .

*Significantly different from control (FM-N) at 5% level by Duncan's multiple range test.

gram dry weight (Table 1). In contrast, the presence of NO_3^- did not show any adverse effect on colonization. Similar differential effects of nitrogen source in colonization of wheat roots by growth-promoting *Pseudomonas fluorescens* 2-79RL1 were earlier observed [20]. The endophytic colonization of sugarcane by *Acetobacter diazotrophicus* was also inhibited by high NH_4NO_3 fertilization of the medium [10], although it was not determined whether the inhibition was due either to ammonium or nitrate: The form of nitrogen supplied can affect the quality and quantity of the root exudates, thereby affecting the colonization ability of the bacteria [27]. It was also possible, however, that the bacteria were responding differently to the two sources of nitrogen, because NH_4^+ and NO_3^- are known to differentially regulate gene expression in bacteria [3]. The adverse effect of NH_4^+ did not seem to be a direct effect on the growth of the bacteria, since both the strains grew well in NH_4Cl as the N source (data not shown). The differential effect of the two nitrogen sources on the colonization of rice could be of significance. NO_3^- is the major N source for dryland rice grown under aerobic conditions, whereas NH_4^+ is the form available to rice under submerged growth conditions [6]. The present results are in accordance with earlier observations [10], and suggest that high nitrogen fertilization of soils in the form of urea or ammonium nitrate negatively affects association of diazotrophic endophytes with monocots. The presence of fixed nitrogen in the form of NH_4^+ in the growth medium also affected root development of rice. NH_4^+ -grown seedlings had lower root length and fewer lateral roots per plant than seedlings grown in N-free medium or with NO_3^- (Table 2). In spite of fewer lateral roots, the growth of the seedlings was better in the NH_4^+ -supplemented medium. The reduction of the number of lateral roots in the NH_4^+ -grown seedlings might have at least partially contributed to the reduced colonization because the sites of the emergence of lateral roots are the major entry zones for both these bacteria [13].

Effect of Ca^{2+} and Fe^{2+}

Apart from nitrogen, calcium ions play an important role in regulating infection and plant colonization by phytopathogenic bacteria [22] and fungi [7] as well as colonization of legumes by N_2 -fixing rhizobial symbionts [24]. The present

Table 2. Effect of fixed nitrogen on root length and lateral root development in rice variety IR72.

Treatment	Root length (mm)	No. lateral roots/plant
FM-N (control)	44	26
+ NH_4^+ (10 mM)	20*	8*
+ NO_3^- (10 mM)	39	22

The results are the mean of two independent experiments with 10 plants each.

*Significantly different from control (FM-N) at 5% level by Duncan's multiple range test.

study with *S. marcescens* IRBG500 and *H. seropedicae* Z67 showed that increasing the Ca^{2+} levels in the medium drastically inhibited the surface colonization of the stems and endophytic colonization in both the roots and stems of rice (Table 1). The presence of 1 mM Ca^{2+} was shown to be inhibitory to infection and disease production by *Erwinia carotovora* subsp. *carotovora* [22] and Ca^{2+} could also inhibit disease production by phytopathogenic fungi. It has been suggested that Ca^{2+} can chelate organic acids secreted by pathogenic fungi, thereby preventing access of cell-wall components to the hydrolytic enzymes of the fungi [7].

In addition to Ca^{2+} , the presence of Fe^{2+} also reduced plant infection and colonization by *E. chrysanthemi* by repressing expression of various isoforms of pectate lyase that are involved in plant infection [9]. Unlike with Ca^{2+} , increasing the concentration of Fe^{2+} showed a differential effect on the colonization of rice by IRBG500 and Z67 (Table 1). Both surface colonization and endophytic root colonization by *S. marcescens* IRBG500 were drastically inhibited by the presence of 0.5 mM Fe^{2+} in the medium. The colonization of the stem, however, was not affected. In contrast, colonization by *H. seropedicae* Z67 was not affected by the presence of Fe^{2+} in the medium.

Histochemical Localization of Colonization of Bacteria

β -glucuronidase (GUS) staining has been used to visualize rhizobial colonization in rice roots [21] and colonization of sugarcane by *A. diazotrophicus* [10]. In this study, we used the expression of GUS as a qualitative indicator to assess the extent of colonization of rice roots by *S. marcescens* IRBG500 and *H. seropedicae* Z67. The intensity of GUS staining observed on the inoculated seedlings corresponded with results obtained by enumerating of the bacterial populations associated with the plant. The most intense staining was detected on the seedlings grown in N-free medium which had the maximum colonization (Fig. 2B), whereas negligible staining was observed in NH_4^+ -grown seedlings which showed the least colonization (Fig. 2D). No GUS staining could be seen on the uninoculated control seedlings (Fig. 2A).

Growth Promotion of Rice by *S. marcescens* IRBG500 and *H. seropedicae* Z67

Coating bacteria on the seed could be an easy means to inoculate growth-promoting bacteria under field conditions. To determine whether IRBG500 and Z67 could increase rice growth, nonsurface-sterilized rice seeds were coated with these bacteria and their potential for rice growth promotion was monitored. The results showed that at 20 DAI both IRBG500 and Z67 were able to increase the root and shoot length as well as the dry weight of inoculated seedlings (Table 3). Bacterial enumeration showed 10^6 cfu g^{-1} dry wt surface-colonizing bacterial cells and 10^3 cfu g^{-1} dry wt endophytic bacterial cells of IRBG500 and Z67 in

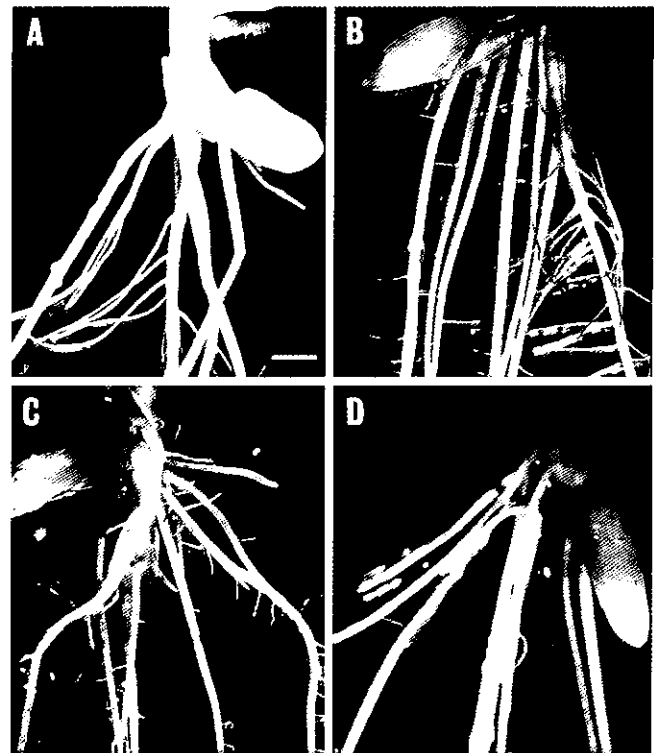


Fig. 2. Effect of fixed nitrogen on colonization of IR72 by *gusA*-marked strains of *H. seropedicae* Z67.

Uninoculated control plant showing no GUS activity (A), colonization of rice by *H. seropedicae* in media without N (B), with KNO_3 (C), and with NH_4Cl (D). Note a drastic reduction in the number of lateral roots in plants growing in the medium supplemented with NH_4Cl (D). The magnification is the same in all images. Bar=2 mm.

the inoculated seedlings. The results indicate that both IRBG500 and Z67 promote rice growth. Since we could not detect nitrogenase (acetylene reduction) activity in the inoculated seedlings (data not shown), the observed growth promotion of rice seemed to be due to a mechanism other than nitrogen fixation. Phytohormones such as indoleacetic acid (IAA) have been implicated in growth promotion elicited by bacteria [11], and further studies are in need to determine whether these two bacteria also secrete IAA or other phytohormones.

Table 3. Growth promotion of IR72 by seed-inoculated *S. marcescens* IRBG500-*gusA* and *H. seropedicae* Z67-*gusA*.

Inoculation	Root length (mm)	Root dry wt. (mg)	Shoot length (mm)	Shoot dry wt. (mg)
Nil (control)	41.0	3.65	68.0	4.41
+IRBG500- <i>gusA</i>	68.3*	4.80*	92.0*	5.92*
+Z67- <i>gusA</i>	55.5*	4.35*	98.5*	6.23*

Results are the mean of three different experiments with 10 observations each.

*Significantly different from the control at 5% level by Duncan's multiple range test.

In legume plants, nitrogen status was found to influence the expression of symbiosis-related plant genes that modulate infection by rhizobia [12]. Likewise, in bacteria, synthesis of exopolysaccharides, a major determinant of the infection by pathogenic and symbiotic bacteria [1, 5], was shown to be under the control of nitrogen regulation by the *ntrBC* system [25]. In addition, nitrogen, similar to Ca^{2+} and Fe^{2+} , was also found to inhibit the production of plant cell-wall degrading enzymes by bacteria thus curtailing their infective ability [9, 22]. These findings suggest that, in legume/rhizobia as well as plant/pathogen communications, nutrient status of both bacteria and host plant play a regulatory role in determining the ultimate fate of the interaction between the two partners. The present results show that the nutrient status of the medium also differentially affects the colonization of rice by endophytes. Further studies should determine whether there are changes in synthesis of polysaccharides and/or hydrolytic enzymes by IRBG500 and Z67 or in susceptibility/resistance of the rice plant to nutrient amendments. Such studies will help elucidate the molecular basis of rice-endophyte interactions.

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