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Diazotroph Endophytic Bacteria in Cultivated and Wild Rice in Thailand

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ABSTRACT: The population size of endophytic nitrogen fixing or diazotrophic bacteria was determined for three varieties of cultivated rice and four populations of wild rice. The dynamics of the bacterial populations from both types of rice was estimated at different stages of plant growth. The number of diazotrophic bacteria in roots, stems and leaves of all varieties of cultivated rice increased with ageing of the plants to a maximum at the heading stage (60 days after transplanting). However, nitrogenase activity could not be detected in bacterial isolates from cultivated rice. The highest bacterial population (5.25×10^6 per gram fresh material) was found in the roots of *Oryza rufipogon,* and this population showed the highest nitrogenase activity. In wild rice genotypes the populations of endophytic diazotrophs were stable, with the higher population in *O. rufipogon* as compared to *O. rufipogon* (18883) and *O. nivara* (18852). Cultures of diazotrophic bacteria were isolated and characterized as species of the genera *Azospirillum*, *Herbaspirillum*, *Beijerinckia* and *Pseudomonas*. All isolates were Gram negative and motile, and produced both pectinase and cellulase. Optimum growth and nitrogen fixation activity for *Azospirillum* sp. was recorded at 30-35 °C and pH 6.0-7.0, for *Beijerinckia* sp. at 25-30 °C and pH 6.0-7.5, and for *Herbarspirillum* sp. at 30-35 °C and pH 6.0-7.5.

KEYWORDS: endophytic, diazotroph, cultivated rice, wild rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is important in the diet of the world population. Increases in the demand for rice, as a result of an increase in population, creates the need to improve rice productivity. Crop productivity is based on numerous factors including weather, soil type, moisture and nutrients. One of the most important factors for high yields of rice production is the availability of nitrogen in the form of industrial fertilizer. However, the use of high levels of nitrogen fertilizer in crop production has several drawbacks and for sustainable rice production one important aim is to replace industrial fertilizer with biologically fixed nitrogen.

Endophytic diazotrophic bacteria are microorganisms that live in plant tissues[1] and they may be responsible for the supply of biologically fixed nitrogen to their host plant[2]. They are distributed in the tissues of roots, stems and leaves, as seen in the endophytic infection of sugarcane by *Acetobacter diazotrophicus* and *Herbaspirillum* spp.[3]. Several endophytic diazotrophs, such as *Azospirillum* sp.[4], *Klebsiella* sp. and *Enterobacter* sp.[5], have been isolated from the rhizosphere of wetland rice. In the Philippines, rice plants have been shown to harbor a wide spectrum of endophytic diazotrophs in their tissues, and exhibit, to some degree, a varietal discrimination in forming associations with these organisms[6,7]. The endophytic diazotrophic bacteria of wetland rice varieties differed at different plant growth stages. Their population increased with plant age and were maximized at heading stage[6,8].

It has been speculated that cultivated rice originated from species of wild rice over 1000 years ago[9,10]. Wild rice is likely to harbor unique populations of nitrogen fixing bacteria that differ from those in modern varieties of cultivated rice. In the present paper we report on the enumeration and isolation of endophytic diazotrophic bacteria from various stages of growth in three varieties of cultivated rice, and determine the population and nitrogenase activity of diazotrophic bacteria in wild rice.

MATERIALS AND METHODS

Culture Media

Three semisolid N-free media were employed to count diazotrophic bacteria using the most probable numbers (MPN) method[11]. These were modified combined carbon medium[12], modified malate medium[8] and modified LGIP medium[13]. Tryptic soy broth (TSB

0.1%) was used for MPN-based enumeration of total counts of aerobic heterotroph bacteria.

Modified N-free medium contained the following components (g l^{-1}): glucose 2.5; mannitol 3.0; sucrose 2.5; sodium malate 2.0; K_2HPO_4 0.2; KH_2PO_4 0.6; $Na_2MoO_4.2H_2O$ 0.002; sodium lactate 60% (v/v) 5.0 ml; $MgSO_4.7H_2O$ 0.2; $CaCl_2.2H_2O$ 0.02; 0.5% bromothymol blue (in 0.2 M KOH); agar 1.8 g for semisolid medium; pH 6.5-7.0.

A half-strength of DYGS (½ DYGS) medium was used for the study of optimal temperature and pH for growth of bacterial population in wild rice. The medium contained per 1000 ml: dextrose 1.0 g; malate 1.0 g; peptone 1.5 g; yeast extract 2.0 g; $MgSO_4.7H_2O$ 0.5 g; L-glutamic acid 1.5 g; pH 6.0-6.5.

Rice Genotype

Three varieties of cultivated rice (khao dawk mali 105, purple glutinous rice kum doi saket and bue po lo) and four populations of wild rice (*Oryza granulata*, *O. rufipogon, O. rufipogon* 18883 and *O. nivara* 18852) were studied. Cultivated rice was grown at the experimental station of Multiple Cropping Center (MCC), Faculty of Agriculture, Chiang Mai University.

The wild rice, *O. rufipogon* was collected from Lumphun Province, *O. rufipogon* (18883) and *Oryza nivara* (18852) were collected from Patumtanee Province, and *O. granulata* was collected from Chiang Mai Province.

The cultivated rice was used to study the dynamics of endophyte populations. This rice was sampled at 15 day intervals starting from 30 days after transplanting to the field.

Enumeration of Diazotroph Endophytic Bacteria

Five plants of each rice genotype were carefully removed from the field, washed to remove all soil and separated into leaves, stems, and roots. The separated samples were washed with tap water, rinsed with sterile water and cut into 2-3 cm long sections. All tissues were surface sterilized with 70% alcohol for 1 min, 2% NaOCl for 2 min and 70% alcohol for 30 s, followed by four washes with sterile distilled water, and then drained on sterile absorbent paper[7,14,15]. The tissues were aseptically cut into small pieces and macerated in sterile water. Serial dilutions were prepared and 0.1 ml portions were inoculated into vials containing 3 ml of sterile semisolid nitrogen free medium. After incubation at 30 °C for 7 days, those vials showing a pellicle near the surface of the media were considered positive, and used to estimate the amount of diazotrophic bacteria present in the sample by the MPN technique.

The cultures from the positive vials were subjected to further purification by streaking them onto sterile semisolid N-free medium and transferring to a fresh medium for final purification. The nitrogenase activities of pure cultures were estimated by acetylene reduction assay[16,17].

Determination of Stability of Endophytic Bacteria Population

Three wild rice types of different genotypes were collected from the sites. The endophytic populations from various tissues of those plants were enumerated immediately after collection. Some of the plants were transplanted into pots in the greenhouse, and after 1 month the endophytic bacteria population was determined by the MPN technique. Stability of population numbers was compared in both ages of plants.

Determination of Nitrogenase Activity

The positive vials from the enumeration of endophytic bacteria were used for acetylene reduction assay (ARA). Atmosphere containing 10 % acetylene (v/v), which was achieved by removing air and replacing with equal volume of acetylene, were added to the vials. At 24 hour intervals 1 ml of the gaseous phase was injected into a gas chromatograph (GC) equipped with flame ionization detector (FID) and a Porapak N column, in order to assay ethylene concentration. The method of gas chromatography was described by Lee and Yoshida[18].

Characterization of Diazotrophic Bacteria

Morphological and biochemical characteristics were determined for Gram reaction, oxidase reaction, catalase reaction, acidification of glucose under aerobic and anaerobic conditions, and growth in nitrogen free medium. In addition, motility, pectinase and cellulase were assayed[14,19,20].

The Optimum Growth Temperature and pH

Fifteen endophytic diazotrophic bacteria isolated from wild rice were tested for optimal growth by measuring the optical density at 600 nm in liquid half-strength DYGS medium. An inoculum of 50 ml of cell culture, pre-grown in ½ DYGS medium (48 h, room temperature ~30 °C), was added to each 25 ml medium in 100 ml Duran bottle and incubated for two days with shaking at 150 rpm. The optimum temperature for growth was tested at 20, 25, 30, 35, 40 and 45 °C. The optimum pH was determined at 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0.

Results

Enumeration and Isolation of Diazotroph Endophytic Bacteria

Pellicle formation by nitrogen fixing bacteria were

Table 1. The most probable numbers (MPN) per g fresh weight of heterotrophic bacteria and diazotrophic endophytic bacteria in various tissues of cultivated rice.

Varieties	Tissues	Population of heterotrophic bacteria – no. of days after transplanting[a]				Population of diazotrophic endophytic bacteria – no. of days after transplanting[b]			
		30	45	60	75	30	45	60	75
Khao Dawk Mali 105	Leaves	1.1×10^5	5.0×10^4	1.1×10^5	1.1×10^5	3.0×10^2	4.0×10^4	2.1×10^5	2.0×10^4
	Stems	5.0×10^4	5.0×10^4	1.1×10^5	2.0×10^4	4.0×10^4	2.1×10^4	2.1×10^5	2.0×10^4
	Roots	2.0×10^4	5.0×10^4	1.1×10^5	5.0×10^4	4.0×10^4	3.0×10^4	2.3×10^5	9.0×10^3
Kum Doi Saket	Leaves	2.1×10^4	2.1×10^5	5.0×10^5	5.0×10^4	1.5×10^4	7.0×10^3	4.0×10^4	9.0×10^3
	Stems	7.0×10^3	5.0×10^5	5.0×10^5	2.1×10^4	7.0×10^3	7.0×10^4	1.1×10^6	9.0×10^3
	Roots	7.0×10^4	9.0×10^4	5.0×10^5	4.0×10^3	2.3×10^4	7.0×10^4	5.0×10^5	9.0×10^3
Bue Po Lo	Leaves	1.1×10^4	1.5×10^4	2.0×10^4	7.0×10^3	3.0×10^2	4.0×10^3	2.0×10^4	2.0×10^3
	Stems	5.0×10^4	5.0×10^4	1.1×10^5	2.0×10^4	4.0×10^4	2.1×10^4	2.1×10^5	2.0×10^4
	Roots	2.0×10^4	5.0×10^4	1.1×10^5	5.0×10^4	4.0×10^4	3.0×10^4	2.3×10^5	9.0×10^3

[a]MPN per g fresh weight in 0.1% TSB.
[b]The average diazotrophic MPN per g fresh weight from 3 semisolid N-free media.

observed in all semisolid N-free media inoculated with root, stem and leaf samples from the 3 varieties of cultivated rice. Nitrogen fixing bacteria were observed in different media used. The number varied from 10^{-2} to 10^{-5} per gram of fresh material.

The number of total heterotrophic and diazotrophic bacteria from various tissues of the three rice varieties fluctuated between 4.0×10^3 to 5.0×10^5 and 3.0×10^2 to 1.1×10^6, respectively. Nitrogen fixing endophyte populations were detected in plant tissues at 30 days after transplanting, but were highest at the heading stage (60 days), in roots, stems and leaves of all cultivated rice varieties (Table 1).

The numbers of bacteria varied between 3.0×10^2-1.1×10^6 MPN per g fresh weight in the three tissues types. Highest counts were observed in the stem, and varied between 7.0×10^3–1.1×10^6 MPN per g fresh weight in kum doi saket rice variety. In root tissues, the population ranged between 4.0×10^3-5.0×10^5 MPN per g fresh weight (Table 1). The leaf tissues of bue po lo and khao dawk mali 105 rice varieties had the lowest population of nitrogen fixing bacteria, 3.0×10^2 (at 30 days after transplant) MPN per g fresh weight.

The modified N-free semisolid medium was employed for MPN technique. In MPN tubes, all cultures positive for acetylene reduction exhibited typical microaerophilic growth. A pellicle developed several millimeters below the surface of the medium and then moved to the surface during further growth. Result of MPN counts and acetylene reduction activities at 10^{-2} dilutions are given in Table 2. Nitrogen fixing endophytic population was highest in the roots of wild rice. The highest endophytic bacteria population (5.25×10^6 MPN per gram fresh weight) was found in *O. rufipogon* roots, and the lowest population (1.41×10^3 MPN per g fresh weight) was found in leaf tissues of *O. granulata*.

The nitrogen fixing ability of the endophytic bacteria

Table 2. The number of diazotroph endophytic bacteria isolated from cultivated and wild rice in each nitrogenase activity rate.

Rice variety	Nitrogenase activity rate[a]		
	Low	Intermediate	High
Cultivated rice			
O. sativa (Khao Dawk Mali 105)	7	0	0
O. sativa (Kum Doi Saket)	7	0	0
O. sativa (Bue Po Lo)	5	0	0
Wild rice			
O. granulata	1	5	0
O. rufipogon	8	17	5
O. rufipogon (18883)	5	28	4
O. rufipogon (18852)	4	16	2

[a]Measuring by ARA (nmol of C_2H_4/tube/24 h) based on an average of three replicates, with five tubes in each replicate. Low = 0-50.0, Intermediate = 50.1-100, High >100.

from various tissues of cultivated and wild rice was examined based on the ability of cultures to reduce acetylene to ethylene. Acetylene reduction activities of MPN tubes at 10^{-2} dilutions were compared. There was a wide range of variation in nitrogenase activity among the 114 isolates tasted, with differences between rice varieties. Eleven endophytes isolated from wild rice gave the highest nitrogenase activity rate, while all endophytes isolated from cultivated rice gave low nitrogenase activity (Table 2). The rates of acetylene reduction also varied with different plant tissues and species. The roots of wild rice, *O. granulata*, *O. rufipogon* and *O. nivara,* exhibited high nitrogenase activity as estimated by ARA. Endophytic bacteria in the roots of *O. rufipogon* showed the highest activity of acetylene reduction, 1.35715 nmol of C_2H_4/tube/24 h (Table 3).

The endophytic diazotrophic bacteria populations observed in roots before, and one month after transplanting, seemed higher in *O. rufipogon* than in *O. rufipogon* (18883) and *O. nivara* (18852) (Fig 1a). The lowest numbers of endophytic diazotrophic bacteria was observed in leaves of the three rice varieties tested.

Table 3. Population and acetylene reduction activity (ARA) of endophytic N_2 fixing bacteria from various tissues of mature wild rice determined 30 days after transplanting.

Rice species	Tissues	MPN per g fresh weight	ARA[a] (nmol of C_2H_4/tube/24 h)[b]
O. granulata	Leaves	1.41×10^3	0.00195
	Stems	2.09×10^4	0.00201
	Roots	7.36×10^5	0.51710
O. rufipogon	Leaves	3.39×10^3	0.01466
	Stems	4.36×10^5	0.13133
	Roots	$\mathbf{5.25\times10^6}$	**1.35715**
O. rufipogon (18883)	Leaves	3.39×10^3	0.00351
	Stems	4.36×10^5	0.43255
	Roots	6.88×10^5	1.21083
O. nivara (18852)	Leaves	2.51×10^3	0.00216
	Stems	5.25×10^4	0.14327
	Roots	6.31×10^5	0.89753

[a]ARA at 10^{-2} dilution tubes.
[b]based on an average of three replicates, with five tubes in each replicate.

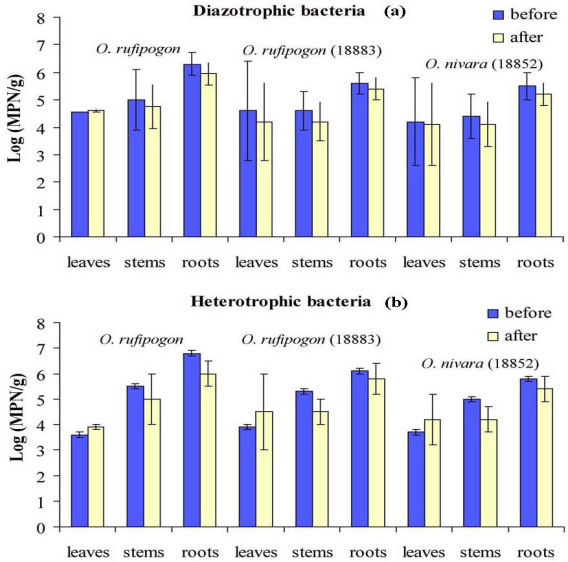

Fig 1. Population of endophytic diazotrophic bacteria (a) and heterotrophic bacteria (b) from various tissues of wild rice before and after 1 month transplanting.

A comparison of endophytic diazotrophic bacteria and heterotrophic bacteria showed that there was no significant difference in the number of bacteria within three tissue types (Fig 1). The number of bacteria also did not change after the plants were transplanted and placed in the greenhouse.

Endophytic Diazotrophic Bacteria from Cultivated and Wild Rice

A total of 114 endophytic bacteria isolates were obtained from various tissues of rice, comprising of 19 isolates from cultivated rice, and 95 isolates from wild rice. These cultures were purified and grouped

Table 4. Grouping and characteristics of diazotrophic bacteria isolated from various tissues of cultivated and wild rice.

Group	Source*	Characteristic	Genus
Group I	AS1,AR1,BS1,BR1,CS1,CR1,DS1,DR1,EL5,EL6,EL7,ES1,ES2, ES5,ES6,ES7,ES10,ER1,ER2,ER3,ER4,ER5,ER9,ER10,ER11, ER12,ER13,FL1,FL2,FS1,FS2,FS3,FS4,FS11,FS12,FR1,FR2, FR3,FR4,FR12,FR13,FR14,FR15,GL1,GL2,GS1,GS2,GS3, GS8,GS9,GS10,GR1,GR2	Gram negative,rod or vibrioid,motile,oxidase & catalase positive, large white colony and slimy on N-free medium,pink colony on BSM	*Azospirillum* sp.
Group II	AS2,AR2,BS2,BR2,CS2,DS2,DR2,ES4,FS10,FR5,FR6,GR8	Gram negative,short curved rod,motile, oxidase & catalase positive, small white colony and slimy on N-free medium,brown colony on BSM	*Herbaspirillum* sp.
Group III	AS3,AR3,BS3,BR3,DS3,ES3,ES8,ES9,ER6,ER7,ER8,FS5,FS6, FS7,FS8,FS9,FR7,FR8,FR9,FR10,FR11,FR16,FR17,FR18,FR19, FR20,GS4,GS5,GS6,GS7,GR3,GR4,GR5,GR6,GR7	Gram negative,short curved rod,motile, oxidase & catalase positive, copious tenacious and elastic slime and giant colony on N-free medium	*Beijerinckia* sp.
Group IV	AL1,BL1,CL1,CR2,DL1,EL1,EL2,EL3,EL4,FL3, FL4,FL5,GL3,GL4	Gram negative,straight rod,oxidase & catalase positive, cannot grow on N-free semi-medium, small white colony on combined carbon medium (CCM)	*Pseudomonas* sp.

*first letter = rice variety,second letter = tissue type

Cultivated rice:
A: *Oryza sativa* (Khao Dawk Mali 105)
B: *O. sativa* (Kum Doi Saket)
C: *O. sativa* (Bue Po Lo)

Wild rice:
D: *O. granulata*
E: *O. rufipogon*
F: *O. rufipogon* (18883)
G: *O. nivara* (18852)

Tissue types:
L: leaves
S: stems
R: roots

according to their morphological and physiological characteristics (Table 4). Four types of bacteria were obtained. Group I isolates were motile, vibrioid to S-shaped, Gram negative rods showing acetylene reduction and growth as a subsurface pellicle in N-free medium, as is typical for *Azospirillum*. Group II isolates showed morphological and physical characteristic resembling bacteria in the genus *Herbaspirillum*. They were slightly curved rods, producing a brown colony when grown on BSM, and were catalase and oxidase positive. The bacteria in group III showed morphological and biochemical characteristics of *Beijerinckia*. This group was Gram negative, motile, short curved rods, forming copious tenacious and elastic slime, and giant colonies on N-free medium. The group IV bacteria, which formed a subsurface pellicle in N-free semisolid agar medium with laboratory air in the headspace, failed to reduce acetylene. Thus, they were regarded as effective N scavengers and not diazotrophs. These cells were Gram negative, straight rods (0.5 × 2-3 mm), motile, catalase and oxidase positive, showing oxidative but not fermentative use of glucose. Results suggested that endophytic bacteria in this group belonged to the genus *Pseudomonas*. The diazotrophic endophytes were all Gram negative, motile and showed pectinase and cellulase activities.

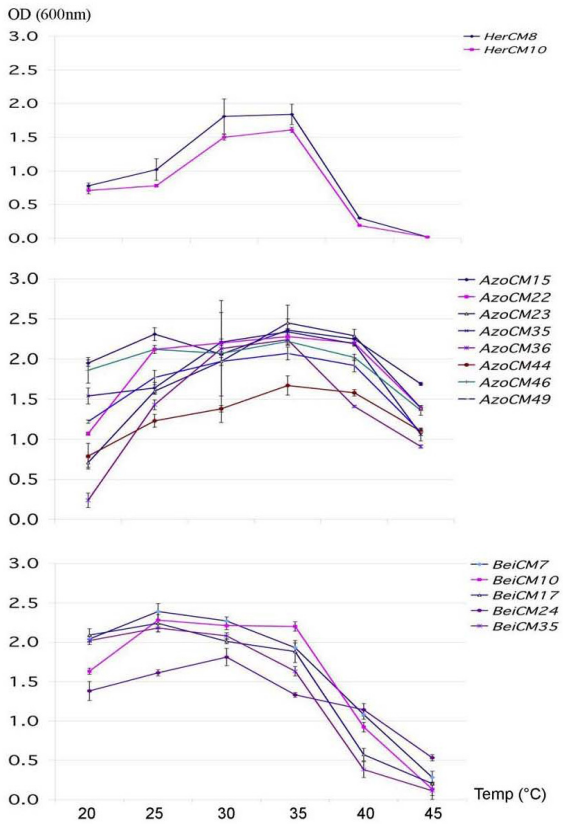

Fig 2. The growth of diazotrophic endophytic bacteria in ½ DYGS medium at various temperatures.

Fifteen endophytic diazotrophic bacteria with high nitrogenase activity (*Herbaspirillum* sp., *Azospirillum* sp. and *Beijerinckia* sp.) isolated from wild rice were selected for studying the optimum temperature and pH for growth (Figs 2 and 3). The optimal temperature and pH for growth of *Herbaspirillum* sp. were 30-35 °C and 6.0-7.0, respectively. The optimal temperature for *Azospirillum* sp. growth varied from 25-40 °C, with very poor growth at 20 and 45 °C, and the optimal pH was 6.0-7.0. For *Beijerinckia* sp. the optimum temperature and pH for growth were 20-35 °C and 6-9, respectively.

Discussion

Each of three media used for isolation and enumeration of endophytic bacteria from cultivated and wild rice varieties was theoretically interchangeable but did not consistently yield the highest number of nitrogen fixing bacteria. Each of them gave the highest relative counts. This result suggests that different groups of diazotrophic bacteria preferred different media. The pure bacteria that were isolated from one particular medium seldom grew on any the other media (data not shown), suggesting that the diversity of diazotrophic endophytes in rice tissues is large, as has been reported by Ueda *et al*.[21] for diazotrophs in the rhizosphere of wetland rice.

The highest numbers of diazotrophic endophytes from cultivated rice observed in this study are similar to those of *Herbaspirillum seropediae* in sorghum, sugarcane and forage grasses (about 10^5-10^7 per g dry

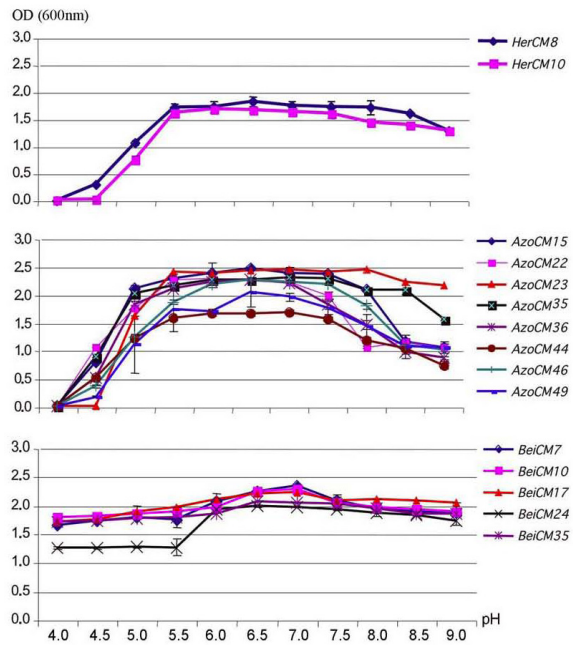

Fig 3. The growth of diazotrophic endophytic bacteria in ½ DYGS medium at various pH.

root)[22], *Azoarcus* in Kallar grass (7.3×10^7 per g dry root)[23] and *Acetobacter diazotrophicus* in sugarcane (10^6-10^7 per g dry root)[24]. The populations were sometimes found to be higher in the stems than in the roots. It is possible that the stem is a more suitable niche for nitrogen fixing endophytes than the root, because it has a seemingly less crowded microbial environment, and products from photosynthesis being transported downward through the phloem may reach the stem before the root. However, the endophytic diazotroph population did not differ between stems and roots of the cultivated rice. The occurrence of high populations of endophytic diazotrophs in rice tissues at the heading stage may be explained by the need of nitrogen during the accumulation of food in the grain. Although, there was high population of diazotrophs in rice tissues, there was less nitrogenase activity of bacteria isolated from cultivated rice. This result suggests that nitrogenase enzyme might not be synthesized in diazotrophs growing with sufficient or excess supply of fixed nitrogen in their environment[25].

A comparison of endophytic bacteria at 30 days after transplanting found that there was less endophytic bacteria in stems and roots of cultivated rice than in wild rice. Engelhard *et al.*[26] also observed that the number of diazotrophic endophytes in cultivated rice (*O. sativa*) was significantly lower than in wild rice (*O. officinalis* and *O. minuta*). These may be because cultivated rice adapts to response for N-fertilizer better than the ability of diazotrophs to fix N_2, and most stage of wild rice are vegetative growth. In the case of wild rice, the diazotrophic endophytic bacteria were found in root more than other tissues. This may be the result of higher inoculum of N_2 fixing bacteria in soil than in atmosphere. Several studies have indicated that indigenous and introduced endophytic bacteria populations were higher in roots and decreased gradually up to the stems and leaves[27,28,29,30,31,32].

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