Australian Journal of Crop Science AJCS
AJCS 5(10):1210-1217 (2011) ISSN:1835-2707

Effect of root exuded specific sugars on biological nitrogen fixation and growth promotion in rice (*Oryza sativa*).

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Abstract

Biological Nitrogen Fixation (BNF) is an energy involving process. A ^{15}N tracer study was conducted under growth chamber and glasshouse conditions to determine the effect of glucose, galactose and arabinose (common sugars found in root environments) on BNF by two diazotrophs, *Rhizobium* sp. Sb16 and *Corynebacterium* sp. Sb26, previously isolated from rice genotypes (Mayang Segumpal and MR219). Diazotrophs have preferences for specific sugar utilization and plant association. Sb16 showed high preference for galactose, and Sb26 preferred arabinose. Application of 10 mM sugar in the experimental pot (5 kg soil), either galactose or arabinose, to the respective rice genotype enhanced diazotroph population growth, N_2 fixation activity and simultaneously plant growth. Mayang inoculated with Sb16 applied with galactose increased plant N concentration 4.2 ± 0.07 %, whereby, 42 ± 1.06 % of the N was derived from the atmosphere. About 40 ± 1.29 % of the N concentration of MR219 inoculated with Sb26 and arabinose was obtained from BNF. The association between Mayang with Sb16 increased 195 ± 40 % of plant biomass as compared to control, and 36 ± 19.8 % over 60 kg ha^{-1} of N-fertilizer. On the other hand, the association of MR219 with Sb26 resulted in 108 ± 37.07 % biomass increment as compared to control, and 89 ± 22.34 % over fertilized-N in different sugar treatments. The association between the plant-diazotrophs along with sugar significantly increased photosynthetic activity. The study indicated that growth and N_2 fixation activity of rice can be increased by increasing the availability of specific sugars in the rhizosphere.

Key words: Arabinose; Diazotrophs; Glucose; Galactose; Indoleacetic acid; Photosynthesis.

Abbreviation:

NFb	Nitrogen free media
Ndfa	Nitrogen derived from atmosphere
^{15}N a.e.	^{15}N atom excess
A_{max}	Net photosynthetic rates at light saturation points
ARA	Acetylene Reduction Assay
Cfu	Colony forming unit
IAA	Indoleacetic acid
PGPR	Plant Growth Promoting Rhizobacteria
SAS	Statistical Analyses System
OD	Optical Density

Introduction

Root exudates are the main attractant for rhizobacteria. The carbon compounds released from roots provide energy sources for diazotrophs to fix nitrogen from atmosphere. Nitrogen is crucial for crop production and it is known that rice plants need one kilogram nitrogen to produce 15-20 kg grain (Janssen et al., 1990). The applied fertilizer nitrogen can be lost from rice ecosystem in various ways. If half of the nitrogen fertilizer could be replaced with biologically fixed nitrogen, 7.6 billion tons of fossil fuel can be saved annually (Reddy et al., 1997). This would not only decrease the use of chemical fertilizer-N but also reduce environmental problems to a considerable extent. Nitrogen fixation is an energy dependent process. Free-living and associative diazotrophs depend on soil organic carbon or simple low molecular weight carbon compounds from root exudate as their energy source (Bürgmann et al., 2005). Simple sugars such as glucose, fructose, sucrose, mannose, arabinose and xylose are the main root exuding compounds of Mayang Segumpal, MR219 and Mahsuri rice (Naher et al., 2008). It was found that sugar concentrations in the root exuded differ with rice genotypes (Hertenberger et al., 2002; Naher et al., 2008). In Morelos A-88 rice, main sugar derivatives were found as arabinose, mannose, galactose, xylose and glucuronic acid (Bacilio-Jimenez et al., 2003). Diazotroph utilize rhizosphere carbon substrates as their energy and it was found that about 64-86% of the carbon released into the rhizosphere is respired by microorganisms (Hutsch et al., 2002). Biological nitrogen fixation is a spontaneous process when adequate carbon sources are available (Kennedy et al., 2004). Nitrogen fixation by *Azotobacter* species was fully dependent on adequate supply of reduced carbon compounds such as sugars and addition of rice straw as carbon source can also improve nitrogen fixation (Kanungo et al., 1997). The ability of nitrogen fixation by *Azospirillum* sp. was improved with addition of malate as carbon source (Wood et al., 2001). Several diazotrophs have preferences of sugars for their activity, such as *Acetobacter* which prefers a sucrose rich medium (Kennedy et al., 2004). Glucose and most amino acids do not support growth and activity of *A. brasilense* SP7 (Hartmann et al., 1988). Identification of specific carbon substrates for growth and nitrogen fixation of diazotrophs in rice rhizosphere may lead to greater understanding in maintaining the population density and N use efficiency in a soil-plant system. Diazotrophs are plant growth promoting rhizobacteria (PGPR), that the most of them endophytically colonize in plant roots and stems (Naher et al., 2009a). Besides contributing to BNF, diazotrophs enhance crop growth in various processes. These microbes may alter root development by producing auxin, ethylene, cytokinins and volatile compounds such as acetone, or modulate plant ethylene levels (Glick, 2005). The overall impact on root morphogenesis is the increase of nutrient uptake due to wider root surface area which enhanced biomass production and yield. Several studies observed growth improvement and nitrogen fixation in rice by free-living and associative diazotrophs. Govindarajan et al. (2007) reported an increase of 38% shoot and 83% root biomass in rice 60 d after inoculation with diazotrophs. Inoculation with *Rhizobium* sp. replaced 25-33% of the recommended rate of nitrogen fertilizer and increased yield of rice by 3.8 t ha^{-1} (Yanni et al., 2001). Inoculation with *Herbaspirillum* sp. contributed around 31-54% of N in rice plants through BNF (James, 2000) and *Azorhizobium* sp. has been shown to save 50% of nitrogen fertilizer (Saleh et al., 2001). A yield increase of 54% in rice by *Burkholderia* sp. was reported by Guimarães et al. (2002). Besides improving the nitrogen uptake, diazotroph inoculation also increased leaf photosynthesis in rice plant and it was observed that inoculation with *Rhizobium* sp. increased single leaf photosynthesis in rice by 11-13% (Cong et al., 2008; Peng et al., 2002). The activity of diazotrophs could be influenced by root exuding compounds produced by the rice genotypes. An increased or altered rhizodeposition may stimulate expected population of diazotrophs, which may ensure a desired community structures in the rhizosphere (Griffiths et al., 1999). Identification of efficient diazotrophs for specific rice genotypes and the appropriate carbon sources to maximize and maintain the population level and activity for maximum yield benefit is crucial. Hence, the present study was conducted to evaluate the effect of selected rhizospheric carbon sources on diazotrophic nitrogen fixation activity and plant growth promotion.

Results

a) *Growth chamber study*

Effect of sugars and diazotroph population on plant-N concentration of rice genotypes

Application of external carbon sources increased diazotrophs populations and plant tissue-N concentration of both rice genotypes (Table 1). The association of Sb16 with Mayang Segumpal and Sb26 with MR219 increased plant-N concentration compared to control and 60 kg ha^{-1} of equivalent nitrogen fertilizer treatments. Application of galactose significantly increased Sb16 population and plant tissue-N concentration of Mayang Segumpal rice. On the other hand, the Sb26 population significantly increased with arabinose application in MR219 rice that resultant higher tissue nitrogen concentration in this rice.

b) Glasshouse study

Plant-N concentration and estimation of fixed N (mg) in rice genotypes

Sugar application along with diazotrophs inoculation increased total plant-N concentration of both rice genotypes (Table 2). In general, higher plant-N concentration was found in plant inoculated with both of the diazotrophs compared to non-inoculated and 60 kg ha^{-1} fertilizer-N treatments. The same trend of result was also obtained in growth chamber study. The ^{15}N analyses revealed that both diazotrophs were able to fix N from atmosphere and diazotroph inoculated treatments showed significantly higher amount of biologically fixed N_2 compared to control treatment. The highest atom excess (a. e.) was found in the non-inoculated than the inoculated plant. The lowest atom excess was found in Sb16 inoculated Mayang Segumpal rice with galactose applied treatment and Sb26 inoculated MR219 rice with arabinose amended treatment. Diazotrophs inoculation and sugar application influenced total N fixed in plant. Significantly higher total nitrogen fixed was observed in inoculated and sugar (arabinose and galactose) supplemented treatments in both rice genotypes. The estimation of percent N derived from the indirect ^{15}N isotope labeling technique, showed that applied diazotrophs contributed about 22 to 42% of the total N through N_2 fixation (Fig. 1). Mayang Segumpal inoculated with Sb16 and applied with galactose showed the highest 42 ± 1.06 %, while in MR219 rice, 40 ± 1.29 % was obtained in Sb26 inoculation supplied with arabinose.

Leaf Area Index and photosynthesis of rice genotypes

Leaf area index varied from 2 to 7 cm^2. The highest leaf area of Mayang Segumpal and MR219 rice were found in inoculated, galactose and arabinose supplemented treatments (Table 3). There were significant differences found in leaf photosynthesis rate among the treatments. The application of diazotrophs and sugars significantly increased leaf photosynthesis in both rice genotypes. In general, Mayang Segumpal showed higher photosynthetic activity compared to MR219 rice. Mayang Segumpal inoculated with Sb16 and supplemented with galactose showed the highest leaf photosynthesis, while, the same rice genotype, inoculated with Sb26 showed higher photosynthesis activity with arabinose application compared to Sb16 inoculation. There was a significant ($P \leq 0.05$) relationship found between photosynthesis and plant-N contents where the leaf photosynthesis increased with plant-N concentration (Fig. 2).

Biomass increment of rice genotypes

Application of diazotroph along with sugar significantly increased plant biomass yield of both rice genotypes. Differences in biomass yield observed between diazotrophs and sugar treatments. Application of galactose significantly increased plant biomass (7.41 ± 0.19 g $plant^{-1}$) of Sb16 inoculated Mayang Segumpal rice, while arabinose increased MR219 biomass (6.99 ± 0.52 g $plant^{-1}$) with Sb26 inoculation (Fig.3). Mayang Segumpal produced high biomass increment when inoculated with Sb16. This rice genotype showed 195 ± 40 % of biomass yield increment over non-inoculated plant and 36 ± 19.87 % over fertilizer-N treatment with different sugar application. While, the same rice, inoculated with Sb26, yielded 154 ± 31.05 % of biomass increment over control treatment (Table 4). The highest biomass yield increment was obtained in galactose and Sb16 inoculum application over the control treatment. In MR219, Sb16 inoculation

Table 1. Effect of sugar application on diazotrophs population after 60 days of inoculation into two rice genotypes at *in vitro* condition

Rice genotypes	Sugar	Rhizosphere population		Plant-N concentrations (%)			
		Sb16	Sb26	N_0	N_f	Sb16	Sb26
				Mean	Mean	Mean	Mean
Mayang Segumpal	Without sugar	2.6 x 10^6c	3.7 x 10^6c	1.8	3.1f	4.0 abc	3.4def
	Glucose	1.4 x 10^6c	6.1 x 10^7b	2.0g	3.2 def	4.2ab	3.7bcde
	Arabinose	3.6 x 10^6c	5.7 x 10^7b	2.1g	3.4 def	4.2ab	4.2ab
	Galactose	2.6 x 10^8 a	5.8 x 10^7b	2.1g	3.4 def	4.4a	3.8 abcd
MR219	Without sugar	2.0 x 10^6c	4.8 x 10^7b	1.7g	3.3ef	3.9 abcd	4.0abc
	Glucose	3.1 x 10^6c	5.8 x 10^7b	2.0g	3.4 def	4.2ab	4.2ab
	Arabinose	2.4 x 10^6c	2.7 x10^8 a	2.2g	3.5cedf	4.2ab	4.4a
	Galactose	3.5 x 10^6c	4.8 x 10^7b	2.1g	3.7 bcde	4.1ab	4.2ab

N_0 = without nitrogen, N_f = 60 kg ha^{-1} of equivalent N, Sb16 = *Rhizobium* sp. Sb26 = *Corynebacterium* sp., SD = Standard deviation. N_0, N_f, Sb16 and Sb26 at different sugars were significant at $p \leq 0.05$. Means in column with same letters are not statistically significant.

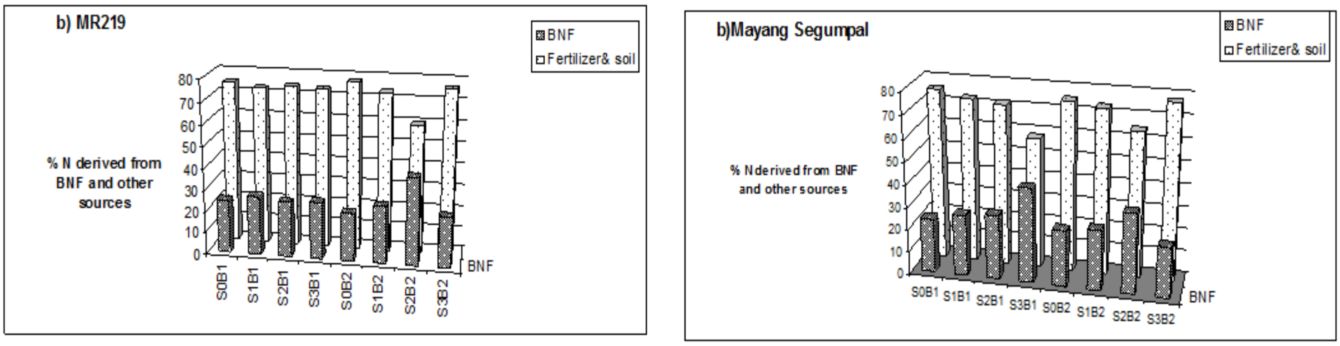

Fig 1. Percent nitrogen derived from atmosphere and other sources: (a) Mayang Segumpal rice (b) MR219 rice. S_0 = without sugar, S_1 = glucose, S_2 = arabinose, S_3 = galactose, B_1 = Sb16 and B_2 = Sb26, BNF = Biological Nitrogen Fixation.

produced 101 ± 29.85 % of biomass yield over non-inoculated and 82 ± 20.15 % over fertilizer-N in different sugar treatments. Inoculation with Sb26 produced 108 ± 37.03 % of biomass yield over control and 89 ± 22.39 % over fertilizer-N. The highest biomass increment was obtained in Sb26 inoculation applied with arabinose compared to non-inoculated control rice.

Residual sugar

At harvest none of the sugar residue was detected in both glasshouse and growth chamber study.

Discussion

Rhizosphere is the major sink of photo-assimilated carbon and these readily available carbon substrates required for asymbiotic microorganisms. It was found that among the root exuded components; only sugar-containing substrates were able to induce nitrogen fixation and artificially application of single sugars rapidly induced nitrogen fixation in diazotrophs (Bürgmann et al., 2005). Previous study showed the concentration of glucose, galactose and arabinose were considerably low in both rice root exudates, but in growth chamber studies these sugar utilization by the diazotrophs and its population growth were higher (Naher et al., 2008). Result from growth chamber study also proved that the external application of glucose, arabinose and galactose significantly increased diazotrophs populations in the rhizosphere and the increased populations were subsequently related to the higher tissue nitrogen concentration of inoculated rice genotypes. The incidence of sugar application and increased tissue nitrogen concentration also reflected in glasshouse study. The low ^{15}N atomic excess (a. e.) in the diazotrophic treatments compared to control (glasshouse study) indicated the efficiency of diazotrophs for biological nitrogen fixation. Thus it is proved that diazotrophs were able to fix N_2 and application of sugars significantly increased the nitrogen fixation activity. The diazotrophic strain Sb16 and Sb26 showed high affinity to galactose and arabinose, respectively. In growth chamber study, application of galactose in Mayang Segumpal, with Sb16 inoculation significantly increased population and tissue nitrogen concentration. On the other hand, MR219 inoculated with Sb26 produced highest tissue N in arabinose treatment. This may be due to their affinity to these specific carbon substrates. Previous study also revealed that several diazotrophs had affinity for particular sugars as carbon substrate for their metabolic activity and that affinity to certain sugars is due to the ChvE protein (Dommelen et al. 1997). Diazotrophs contain a ChvE homologous protein showed high affinity for D-galactose, L-arabinose, and D-fructose (Shimoda et al., 1993; Dommelen et al., 1997). Differences in nitrogen fixation occurred between rice genotypes, diazotrophs and sugars. It has been found that Sb16 (*Rhizobium* sp.) preferred galactose and both of rice varieties exerted less amount of galactose compared to other root exudates sugars (Naher et al., 2008; Naher et al., 2009). External application of galactose increased nitrogen fixation activity in Mayang Segumpal rice. While in MR219, the high N was fixed by the Sb26 strain in arabinose treatment. This may be due to the specific diazotroph association with plants and their preferences for sugar uptake. The results of sugar utilization study revealed that *Corynebacterium* sp. (Sb26) prefers arabinose than glucose (Naher et al., 2008). Previous study by Govindarajan et al. (2007) also showed preferences of diazotrophs for N_2 fixation in different sugars. They showed that isolate MGK3 was capable of N_2 fixation with the presence of fructose, mannitol, malate, azelaic, sucrose, and glucose, but *Burkholderia vietnamiensis* showed negative activity when glucose was applied as carbon source. Some of the diazotrophs and their N_2 fixation activities are

Table 2. Effect of diazotroph, fertilizer-N and sugar on plant-N concentrations, N atom excess (a.e.) and total fixed N in two rice genotypes (60 d of growth) at glasshouse

Sugar	Plant-N concentrations (%)				^{15}N (a.e.) (%)			fixed N (mg)	
	N_0	N_f	Sb16	Sb26	N_0	Sb16	Sb26	Sb16	Sb26
	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD
Without sugar	2.2 ± 0.03	3.1 ± 0.07	3.9 ± 0.16	4.0 ± 0.06	0.80 ± 0.02	0.60 ± 0.02	0.60 ± 0.02	0.93 ± 0.11	1.00 ± 0.10
Glucose	2.8 ± 0.14	3.7 ± 0.21	4.2 ± 0.04	4.1 ± 0.02	0.69 ± 0.03	0.50 ± 0.01	0.50 ± 0.01	1.15 ± 0.11	1.11± 0.10
Arabinose	2.6 ± 0.06	3.3 ± 0.05	4.2 ± 0.05	4.2 ± 0.04	0.66 ± 0.01	0.47 ± 0.02	0.43 ± 0.02	1.20 ± 0.14	1.57 ± 0.05
Galactose	2.4 ± 0.06	3.6 ± 0.13	4.3 ± 0.05	4.2 ± 0.12	0.66 ± 0.01	0.38 ± 0.01	0.51 ± 0.01	1.83 ± 0.02	0.95 ± 0.04
Without sugar	2.3 ± 0.18	2.7 ±0.27	3.6 ± 0.14	3.8 ± 0.33	0.76 ± 0.03	0.57 ± 0.02	0.59 ± 0.02	0.89 ± 0.13	0.84 ± 0.09
Glucose	2.7 ± 0.18	3.4 ± 0.03	4.1 ± 0.06	4.2 ± 0.03	0.68 ± 0.02	0.50 ± 0.02	0.50 ± 0.02	1.11 ± 0.11	1.12 ± 0.16
Arabinose	2.5 ± 0.18	3.3 ± 0.19	4.1 ± 0.07	4.3 ± 0.08	0.67 ± 0.04	0.50 ± 0.01	0.40 ± 0.02	1.05 ± 0.22	1.75 ± 0.10
Galactose	2.6 ± 0.02	3.1 ± 0.17	4.2 ± 0.07	4.1 ± 0.06	0.67 ± 0.02	0.49 ± 0.01	0.51 ± 0.01	1.10 ± 0.05	0.96 ± 0.12
Variety			***			NS		**	
Sugar			***			***		***	
Diazotroph			***			***		NS	
Variety x sugar			NS			***		**	
Variety x diazotroph			***			**		***	
Sugar x diazotroph			***			***		***	
Variety x sugar x diazotroph			*			**		***	

N_0 = without nitrogen, N_f = 60 kg ha^{-1} of equivalent N, Sb16 = *Rhizobium* sp. Sb26 = *Corynebacterium* sp., Treatment means were from 3 replications. N_0, N_f, Sb16 and Sb26 at different sugars were significant at $p \leq 0.05$. SD = Standard deviation. Significance levels are * = 0.05, ** = 0.01, and *** = 0.001 respectively. NS = not significant.

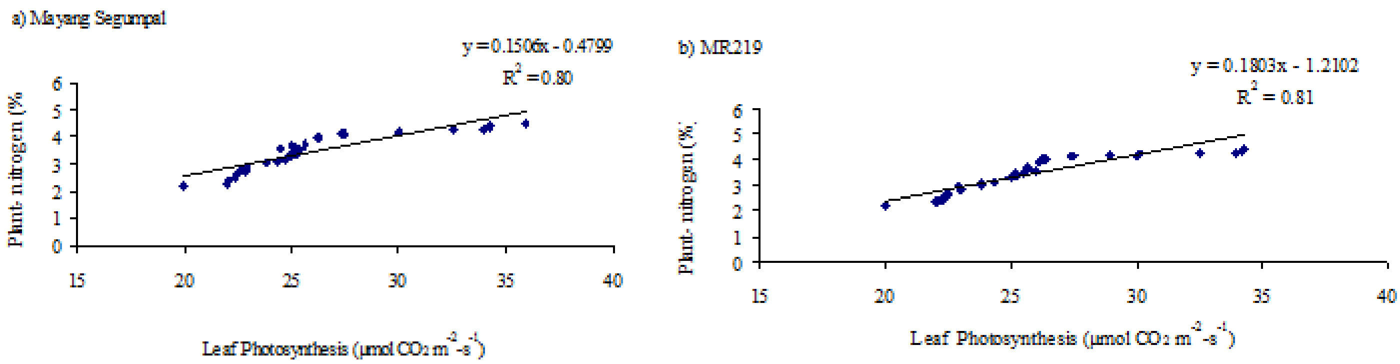

Fig 2. Relationship between leaf photosynthesis and plant-N concentration: a) Mayang Segumpal b) MR219 rice.

restricted by the plant associations such as *Acetobacter* that need high sugar concentration in plant for their activity (Kennedy et al., 2004). Besides sugar application, the proportion of nitrogen derived from atmosphere and the amount of N_2 fixed in plant tissues varied with diazotrophs. Diazotrophic strain Sb16 fixed the highest N_2 (42 %) and Sb26 strain (41 %) under glasshouse condition. About 59% of N derived from atmosphere in rice ecosystem has been reported by Mirza et al. (2000). Govindarajan et al. (2007) reported 40.4% of N_2 fixed by *Burkholderia vietnamiensis* under glasshouse condition. However, in the present study there were no significant difference in N-increment found between diazotrophs but significant differences were observed withvarious sugar applications. The external application of sugars stimulated diazotroph activities. Previous study showed that leaf N plays a central role in leaf photosynthesis and is highly correlated with leaf photosynthesis capacity (Midgley et al., 1999). The study showed a significant correlation between leaf N concentration and leaf photosynthesis in the both rice genotypes. A higher photosynthesis rate was obtained in diazotroph inoculated treatments compared to control and nitrogen treatments. Previous study also showed that inoculation with *Rhizobium* increases the leaf photosynthesis in rice (Peng et al., 2002). The photosynthesis rate correlated with high tissue N concentration of inoculated treatments. Similar findings were also reported earlier which showed that higher N content led to increased photosynthetic rate (Evans, 1989). The high leaf photosynthesis as well as nitrogen concentration was significantly associated with sugar application. As the process of N_2 fixation required high amounts of energy, the sugar application could have improved the energy balance in the diazotrophs for N_2 fixation as well as photosynthesis activities. Inadequate supply of energy may reduce the N_2 fixation process for symbiotic and non symbiotic associations. Pausch et al. (1996) found that reduction of N_2 fixation capacity of nodulated soybean was related to the inadequate photosynthate translocation to the nodule which indicates the diazotroph's energy requirement and carbon balance for nitrogen assimilation. The present study also showed the ability of the locally isolated diazotrophs to supply N for the rice plant requirement leading to significantly higher plant biomass increment over non-inoculated and fertilizer-N treatments. These two diazotrophs were capable of producing significant amount of indoleacetic acid (Naher et al., 2008). Inoculated plant produced higher leaf area and higher photosynthetic activity over the controls. Application of sugars especially galactose and arabinose, increased diazotroph activities which may enhanced the root surface area for increased nutrients uptake that subsequently produced higher leaf area index and plant biomass. Several

Table 3. Effect of diazotroph, fertilizer-N and sugar on leaf area index and photosynthesis in two rice genotypes (60 d of growth) at glasshouse

Rice genotypes	Sugar	Leaf photosynthesis rate (μmol CO_2 $m^{-2}s^{-1}$)				Leaf Area Index (cm^2)			
		N_0	N_f	Sb16	Sb26	N_0	N_f	Sb16	Sb26
		Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD
Mayang Segumpal	Without sugar	21.3 ±1.16	24.3 ± 0.46	25.9 ± 0.64	26.7 ± 0.69	2 ± 0.06	3 ± 0.18	4 ± 0.11	4 ± 0.16
	Glucose	22.7 ± 0.24	25.2 ± 0.43	30.9 ± 1.43	28.3 ± 1.50	3 ± 0.06	4 ± 0.12	4 ± 0.16	4 ± 0.21
	Arabinose	22.5 ± 0.23	25.0 ±0.14	30.8 ± 1.46	34.8 ± 0.98	2 ± 0.06	4 ± 0.22	6 ± 0.27	6 ± 0.28
	Galactose	22.1 ± 0.17	25.4 ± 0.26	34.1 ± 0.17	30.6 ±3.41	3 ± 0.06	5 ± 0.16	7 ± 0.22	6 ± 0.22
MR219	Without	20.7 ± 1.36	22.5 ± 0.42	25.6 ± 0.06	25.8 ± 0.53	3 ± 0.11	4 ± 0.21	5 ± 0.16	4 ± 0.16
	Glucose	22.6 ± 0.34	25.4 ± 0.46	27.0 ± 0.68	31.3 ± 2.26	3 ± 0.38	4 ± 0.27	4 ± 0.22	5 ± 0.16
	Arabinose	22.4 ± 0.12	24.9 ± 0.50	27.6 ± 1.26	32.8 ± 2.40	4 ± 0.49	4 ± 0.16	6 ± 0.43	6 ± 0.11
	Galactose	22.4 ± 0.12	24.2 ± 1.2	30.0 ± 2.54	26.7 ± 0.70	4 ±0.37	4 ± 0.11	5 ± 0.21	6 ± 0.22
Variety				**				***	
Sugar				***				***	
Diazotroph				***				***	
Variety x sugar				***				***	
Variety x diazotroph				***				***	
Sugar x diazotroph				***				***	
Variety x sugar x diazotroph				***				***	

N_0 = without nitrogen, N_f = 60 kg ha^{-1} of equivalent N, Sb16 = *Rhizobium* sp., Sb26 = *Corynebacterium* sp. Treatment means were from 3 replications. N_0, N_f, Sb16 and Sb26 were significant at $p \leq 0.05$. SD = Standard deviation. Significance levels are * = 0.05, ** = 0.01, and *** = 0.001, respectively. NS = not significant.

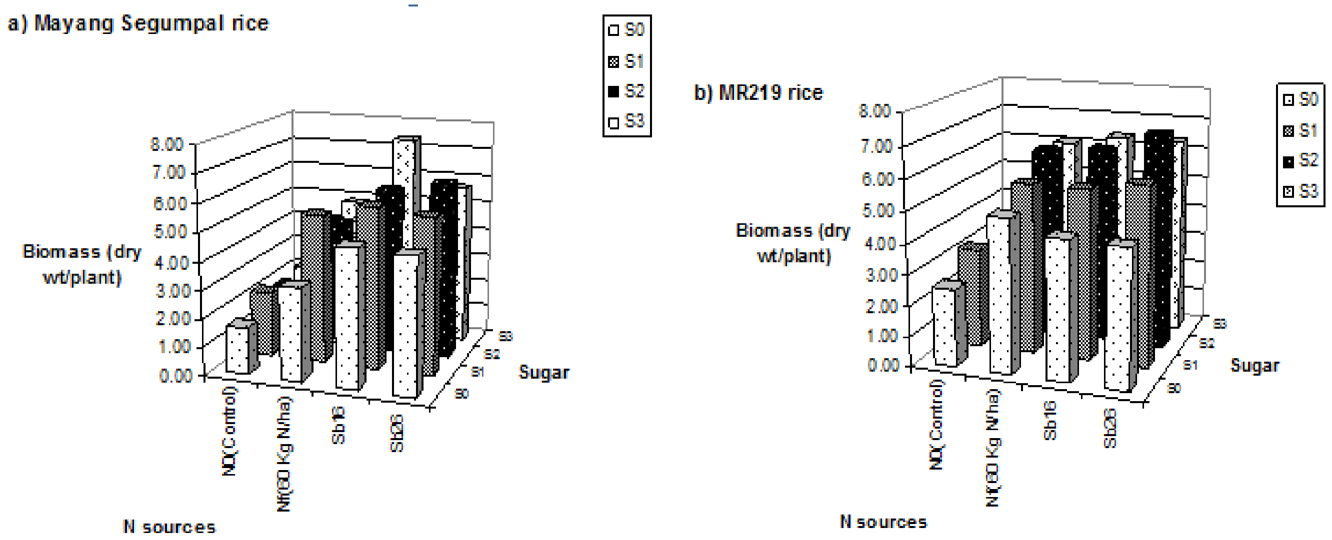

Fig 3. Effect of inoculation and sugar application on biomass production of rice genotypes: a) Mayang Segumpal b) MR219 rice. N_0 = without nitrogen, Nf = 60 kg ha^{-1} of equivalent N, Sb16 = *Rhizobium* sp. Sb26 = *Corynebacterium* sp., S_0 = without sugar, S_1 = glucose, S_2 = arabinose, S_3 = galactose. N0, Nf, Sb16 and Sb26 at different sugars were significant at $p \leq 0.05$.

evidences of rice yield increment by diazotrophs inoculation were reported earlier. Govindarajan et al. (2007) reported that application of inoculum (mixed MGK3 and *Burkholderia vietnamiensis)* increased shoot biomass by 12 to 69% in different rice varieties after 60 days of inoculation. Kecskés et al. (2008) and Peng et al. (2002) observed a significant biomass yield in rice inoculated with *Rhizobium* and *Azospirillum* cells respectively. Besides N fixation activity and growth promotion in rice are probably due to the combined effect of biological nitrogen fixation and phytohormone production by the diazotrophs.

Materials and methods

The experiments were conducted simultaneously in both growth chamber and glasshouse conditions. Diazotrophs population growth and tissue nitrogen concentration were determined in growth chamber study. At glasshouse, photosynthesis, nitrogen fixation and plant biomass were recorded.

Selection of rice genotypes, root exuded sugars and diazotrophic strains

Two rice genotypes (Mayang Segumpal and MR219), two locally isolated diazotrophic strains (*Rhizobium* sp., Sb16 and *Corynebacterium* sp., Sb26) and three sugars commonly exuded from rice root (glucose, arabinose and galactose 2 mM kg soil^{-1} each) were used in this study. Previously, the diazotrophic strains were isolated from Mayang Segumpal

(Malaysian local accession) and MR219 (a high yielding rice variety and derived from *indica* × *japonica* rice) rice root and its nitrogen fixing potentiality determined accordingly. Acetylene Reduction Assay (ARA) for *Rhizobium* sp., Sb16 and *Corynebacterium* sp., Sb26 were recorded as 1.4×10^{-7} and 2.9×10^{-10} µmol $C_2H_4^{-1}cfu^{-1}h^{-1}$, respectively (Naher et al., 2009b). Both diazotrophs were epiphytic PGPR. The root exuded sugars of these two rice and diazotrophs population growth with selected sugars were determined.

Preparation of soil and planting

The experimental soil (0-15 cm depth) was collected from Tanjong Karang rice irrigation project area, Malaysia. The soil belongs to Bernam soil series, clay loam in texture containing; 0.14 % of total N, and 3.33 % of organic carbon. The soil was rich in P, K, Ca, Mg Zn and with a soil pH of 5.01, which was suitable for rice cultivation. The soil was ground and passed through 2 mm mesh. Exactly 2.5 kg of air dried soil was weighed and packed into plastic bag. The soil was sterilized by gamma ray (40 kGy). The soil with plastic bag was placed in the plastic pot (25 cm × 17.5 cm). Before putting the soil the bottom of the pot was sealed to prevent leaching of nutrients and ^{15}N fertilizer. The potted soil was soaked with distilled water for 2 days. Exactly 0.1 g of ^{15}N urea with atomic excess (a. e. 10.18) was diluted with distilled water and poured uniformly in each pot (IAEA, 2001). Blanket doses of P_2O_5, and K_2O from sodium phosphate (monobasic), and potassium chloride at the rates of 60 and 40 kg ha^{-1} equivalent, respectively, were applied to each pot. Mayang Segumpal and MR219 rice seeds were surface sterilized according to Amin et al (2004). Seeds were grown in axenic conditions on sterile plastic tray lined with filter paper. The sterile distilled water was applied daily to keep the filter paper moist. After 7 days of growth the seedlings were uprooted and transplanted into the respective pots for both growth chamber and glass house studies. The pots were irrigated and kept in moist condition. In growth chamber temperature was maintained at 28±1^{0}C with a 12 hour light and dark cycle. Plants were grown for 60 days.

Preparation of inocula and inoculation

Diazotrophic strains were grown in ATCC (American True Type Culture Collection) broth for 48 h. The samples were transfered to a microcentrifuge tube and bacterial cells were harvested by centrifugation at 13500 rev min^{-1} for 10 min and washed with 0.85% sterilized phosphate buffer saline (PBS). Optical density (OD_{600}) of washed cells were observed and adjusted accordingly. Approximately 5×10^7 ml^{-1} live washed bacterial cells were suspended in 100 ml of sterile distilled water and applied immediately to rice seedlings. The non-inoculated pots received the same amount of killed (autoclaved for 30 min at 121^{0}C), washed with PBS and suspended cells with sterile distilled water. Bacterial population applied, was confirmed by using drop plate method on NFb (nitrogen free) media.

Application of nitrogen fertilizer to the non-inoculated rice genotypes

At the same time as the diazotroph inoculation, 60 kg ha^{-1} of equivalent N from urea was applied in to the nitrogen fertilized treatment pots. The urea was dissolved in distilled water and applied uniformly in each pot.

Application of sugars

Exactly 10 mM of individual glucose, arabinose and galactose were applied in each pot (5 kg soil) as external carbon sources to enhance diazotrophs population. A preliminary experiment with various sugar concentrations were done to determine the diazotrophs survival and suitability of plant growth. From preliminary study sugar dose was selected. Glucose, arabinose, galactose each were suspended separately in 20 ml of sterile distilled water and pH was adjusted to 7.5 (Bürgmann et al., 2005). During inoculation, individual sugar solution was applied to each planting unit according to the treatments. The sugars were applied only once at the time of inoculum application.

Determination of diazotroph population in the rhizosphere

After 60 days of growing period (at harvest), approximately 1.0 g of plant roots were gently washed with sterile water and placed in 250 ml of Erlenmeyer flasks containing 99 ml distilled water. The contents were shaken for 15 minutes and a series of 10-fold dilutions were prepared. Aliquot of 0.1 ml from each dilution was dropped onto NFb agar (nitrogen free media) plates and populations were determined following the procedure of Somasegaran and Hoben (1985).

Determination of residual sugar

During harvest about 2 ml of soil solution was taken out from each planting unit and filtered through 2 µm filter paper. The filtrate solution was stored at -20 ^{0}C for residual sugar analyses. Exact 20 µl of samples were injected in high performance liquid chromatography (HPLC, Jasco Borwin software). The amount of residual glucose, galactose and arabinose, were determined using Apex column (60^{0}C temp.) and refractive index (R.I.) detector. Acetonitrile (75%) was used as mobile phase with a flow rate of 1.8 ml min^{-1}.

Biological nitrogen fixation, tissue-N concentration, photosynthesis, leaf area index and plant biomass determination

At harvest the total tissue-N concentration was determined by semi-micro Kjeldahl method (Bremner, 1996). N_2 fixation rate was estimated by ^{15}N isotope dilution method according to Boddey (1987). The percent of atom excess ^{15}N (a. e.) in each plant part was estimated using the following formula (Warembough, 1993).

$$\%^{15}N(a.e.)in\ \ each plant part\ = \%N abundance - 0.3663\ \%^{15}N\ (natural\ \ abundance)$$

It is assumed that 0.3663% ^{15}N is present in the atmosphere. The proportion of N derived from the atmosphere was calculated as follows:

$$\%\ Ndfa\ = \left[1 - \frac{\%^{15}N\ \ a.e.\ in\ the\ \ fixing\ \ system}{\%^{15}Na.e.\ in\ the\ non\ \ fixing\ \ system} \right] \times 100$$

The total N_2 fixation in plant tissue was calculated as:

$$N_2\ fixed\ \ (mg) = \frac{\%\ \ Ndfa \times total\ \ N\ \ content}{100}$$

Table 4. Influence of diazotroph inoculation and sugar application on biomass yield increment of two rice genotypes at 60 d of growth

Rice genotypes	Sugar	Sb16		Sb26	
		Biomass increment over control	Biomass increment over fertilized-N	Biomass increment over control	Biomass increment over fertilized-N
		%			
Mayang Segumpal	Without sugar	147	8	140	51
	Glucose	192	48	186	45
	Arabinose	195	37	115	29
	Galactose	245	52	167	17
MR219	Without sugar	72	62	77	68
	Glucose	79	69	81	71
	Arabinose	133	93	156	111
	Galactose	119	105	119	105

Single-leaf net photosynthesis rate (A_{max}) was determined (60 days of growth) from the Youngest Expanded Leaf (YEL) using LI-6200 Portable photosynthesis system, LI-COR Inc. Measurements were done under full sunlight and constant CO_2 of 380 µmol CO_2 mol^{-1} in a closed chamber. The Leaf Area Index (LAI) was measured using leaf area meter, model, LI-3100 Area meter; LI-COR. Inc. LAI was calculated using

the following formula: $LAI = \frac{Mean\ leaf\ area\ of\ whole\ plant}{Surface\ area\ of\ pot\ (cm^2)}$

The harvested plant samples were thoroughly washed and dried in an oven at 70 ^{0}C temperature until constant weight was achieved.

Statistical analyses

The experiment was conducted in factorial using completely randomized design with 3 replications. Data were analyzed using SAS statistical program version 9.1. The treatments means were separated using Tukey's Studentized Range test at the 5% level of probability.

Conclusion

The study proved that the diazotrophic strain Sb16 and Sb26 were capable of forming natural associations with Mayang Segumpal and MR219 rice genotypes and have preferences for sugar utilization and N_2 fixation. The association of Mayang Segumpal with Sb16 fixed high N_2 and produced higher plant biomass with galactose application, while MR219 showed better association with Sb26 in the presence of arabinose. In general, the study showed that diazotrophs have preferences for certain sugars which accelerate their growth and activity. Application of preferential carbon source established better diazotroph association with specific plant genotypes and consequently enhanced growth promotion, photosynthesis as well as N_2 fixation in rice.

Acknowledgements

The authors are grateful to the Third World Organization for Women in Science (TWOWS) and Universiti Putra Malaysia, Ministry of Science, Technology & Innovation Malaysia for providing financial support for the project. Appreciations are due to the technical staff of Malaysian Nuclear Agency for ^{15}N isotopic study.

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