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Isolation of Diazotrophs from Different Soils of Tanjong Karang Rice Growing Area in Malaysia

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ABSTRACT

Isolation and biochemical characterization of diazotrophs were carried out on seven soil series/types of Tanjong Karang Rice Irrigation Project area. The soil population ranged from 4×10^4 to 2.2×10^6 cfu g^{-1} soil. Diazotrophic populations were significantly ($P<0.01$) influenced by soil types, plant age and rice varieties. Higher soil and rhizosphere populations were recorded in Organic Clay and Muck, Bakau, Sedu and Serong soils. The highest root (6.3×10^7) and shoot (2.5×10^7) populations were found in MR219 rice planted in Organic Clay and Muck and Sedu soil series, which had higher C, N and P contents. The highest acetylene reduction assay (ARA) value (1.26×10^{-6} µmoL C_2H_4 cfu^{-1} hr^1) was found in isolate Sb35. Several diazotrophic strains produced 32 to 69 mg L^{-1} of indoleacetic acid (IAA). The highest IAA was produced by the diazotrophic strain Sb41 (*Corynebacterium* sp). Eleven of the diazotrophic strains isolated from root and shoot of the rice varieties were capable of producing cellulose degrading enzyme. Tanjong Karang rice growing area harbor a diverse group of bacteria and most of the isolates belonged to the genera of *Rhizobium*, *Burkholderia* and *Corynebacterium*.

Key Words: Diazotrophs; Endophytes; Indoleacetic acid; Acetylene reduction assay; Cellulase activity

INTRODUCTION

About 2.4 billion people worldwide depend on rice as staple food (IRRI, 1996) and nitrogen (N) is one of most limiting nutrient for rice production. More than 50% of the applied chemical N is lost through various processes. Higher N application may result in NO_3^- pollution of ground water (Sherestha & Ladha, 1998). An increased denitrification process resulting in higher emission of N_2O to the atmosphere can also lead to global warming. Two potential approaches are being explored to solve this problem; one is to breed rice plants that can fix atmospheric N (Ladha & Reddy, 1995) and the other is to improve the uptake and utilization of native and applied N by rice plants.

Wetland rice ecosystem can harbor a diverse group of diazotrophs. Diazotrophic bacteria such as *Klebsiella oxytoca*, *Enterobacter cloacaeae* (Fujie *et al*., 1987), Alcaligenes (Tou & Zhou, 1989) and *Azospirillum* (Baldani & Dobereiner, 1980) have been isolated from the rhizosphere of wetland rice. *Burkholderia* sp. isolated from rice plants in Brazil and Vietnam was classified as diazotrophic endophytes (Neves & Rumjanek, 1998). *Azoarcus* sp. from Kallar grass abundantly colonizes and expresses nif genes and nitrogenase protein inside the original host as well as rice roots (Sevilla *et al*., 2001). Diazotrophs, which efficiently colonize the root endosphere has been shown to fix nitrogen (Hurek *et al.*, 1997). It has been reported that inoculated rice plants can get similar benefit (Chalk, 1991). The transfer of fixed N_2 varied from 1.5 to 21% (Sherestha & Ladha, 1996). *Rhizobium leguminosaram* bv. *Trifolli* can colonize rice roots endophytically in the field, where rice is grown in rotation with Egyptian berseem clover (*Trifolium alexandrium*) and can supplement 25-33% of the recommended rate of N fertilizer for rice (Yanni & El-Fattah, 1999). Diazotrophs associated in the rhizosphere can improve growth and development of rice plants. The production of hormonal substances by the diazotrophs can alter root morphology, increase extended surface area and enable plants to absorb more nutrients from the soils.

The growth and colonization of the bacteria on plant roots can be influenced by several soil chemical, physical and biological factors (Harries, 1998). The availability of C, N or P can limit bacterial growth in aquatic systems (Torre´ton *et al.,* 2000). In general neutral pH of soil solution provides the most suitable environment for nutrient availability and microbial growth. Soil type with variable chemical and biological properties, can also affect the association between plants and bacteria.

Limited information is available on strain diversity and effect of soil chemical properties on diazotrophic association in rice soil, which is important for regulation of

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the microbial population in rice production. Hence, the present study was undertaken to isolate indigenous diazotrophs from different rice varieties grown in different soil types in Tanjong Karang Rice irrigation area and to determine some of their beneficial traits for rice cultivation.

MATERIALS AND METHODS

Sampling of soil and rice plant. Samples were taken from seven soil types (Jawa, Sedu, bakau, Bernam, Serong, Organic Clay and Muck & Brown Clay) in Tanjong Karang Rice Irrigation Project area. Roots and shoots of three rice varieties (MR219, MR220 & MR232) grown in these soils were collected for the estimation of diazotrophic populations. The soil samples were randomly collected between the hills and rice plants. Composite soil and plant samples were brought to the laboratory in an ice box and kept at 4°C temperature before analyses.

Estimation of population of diazotrophs from soil and rice rhizosphere. A series of 10-fold dilutions were prepared up to 10^{-8} of rhizosphere and non-rhizosphere soil and the diazotrophs populations were determined using most-probable number (MPN) method in nitrogen free (Nfb) semi-solid medium. Composition of the Nfb semisolid medium (Prasad *et al*., 2001) contained: 5 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$. 7 H_2O, 0.1 g NaCl, 0.02 g $CaCl_2$ and 0.5% bromothymol blue in 0.2 N KOH (2 mL), 1.64% Fe-EDTA solution (4 mL), 2 g agar and 0.02 g yeast extract (pH 7.2). Yeast extract provided a trace amount of nitrogen for the isolation of most diazotrophs from the rhizosphere of rice (Watanabe & Barraquio, 1979).

Estimation of population and isolation of endophytic diazotrophs from rice root and stem. The root sample (2-5 g) was washed and surface sterilized with 70% ethanol for 5 min and then with 3% Clorox for 30 sec. The stems were surface sterilized by dipping in 95% ethanol. After surface sterilization that root and stem were washed several times with sterile distilled water. The root and stem samples were checked for the efficacy of surface sterilization by rolling them on 0.1% trypic soy agar (TSA) plates. The roots and stem were meshed by using a sterilized mortar and pestle. A 10-fold series of dilution was prepared up to 10^{-7} and the diazotrophic populations were determined using MPN method in Nfb semi-solid medium.

Soil analyses. Soil was analyzed for total nitrogen following Kjeldhal method, soil organic carbon (OC) according to Walkley and Black (1934) method, soil available P determined by Molybdenum Blue method (Bray & Kurtz, 1945) and exchangeable K, Ca, Mg and Na was determined by Leaching method (Anonymous, 1980).

Determination of indoleacetic acid (IAA) production. Isolates were inoculated in Jensen's broth with addition of tryptophan (2 mg mL^{-1}) and incubated at 28 ± 2°C for 48 h. The culture was centrifuged at 7000 rpm for 7 min and 1 mL of the supernatant was mixed with 2 mL of Salkowsky's reagent. The IAA concentration was determined using spectrophotometer at 535 nm.

Estimation of acetylene reduction assay (ARA). A 1.0 mL of the culture was transferred into an air-tight 30 mL bottles containing 10 mL of Nfb semi-solid medium. After the pellicle formation (72 h), the bottles were injected with 5% (v/v) acetylene gas with simultaneous removal of the same volume of air. The bottles were incubated at 30ºC for 24 h. 1 mL of gas was withdrawn and transferred to 7 mL Vacutainer™ tubes. The ethylene gas produced was assayed using G-300 gas chromatograph (American™) equipped with a FID and 1-m Porapak N column.

Determination of cellulase activity. The Cellulase activity was determined according to James *et al.* (2002). Cellulose degradation was carried out on nutrient agar plates with 0.1% crboxymethyl cellulose (CMC). Plates were spot inoculated with 10 µL liquid culture of the strains. After incubation the colonies were washed off with sterile water and discarded. The plates were stained with 0.1% Congo red solution for 30 min and rinsed with 1 M NaCl. The halo zones indicated positive reaction for cellulose activity.

Strain identification and diversity of the isolates. Diazotrophic strains were grown on Biolog Universal Growth Agar and identified using Biolog Database Software. Strain diversity studied using rep PCR genomic finger-prints. The bacterial genomic DNA was extracted from pure culture of bacteria using GF-1 bacterial DNA extraction kit. Rep PCR done using primer: REP IR: 5′ - IIIICgICgICATCIggC-3′ and REP 2I: 5′ - ICgITTATCIggCCTAC-3′ according to Versalovic *et al.* (1991). One percent Agarose gel, 10 × TAE buffer and 6 × loading buffer were used to separate the rep-PCR generated fragments. After running Gel electrophoresis the PCR product was placed in ethidium bromide (10 mg mL^{-1}) to make the band visible. The computer software, NTSYS-pc program was used to analyses of rep-PCR generated finger prints and dendogram. The un-weighted pair group method average (UPGMA) was applied to analyses for genetic relatedness among the 27 indigenous isolated diazotrophs.

RESULTS

Physico-chemical properties of the experimental soil. Soil samples were randomly collected form seven soil series or types in Tanjong Karang Rice Irrigation Project area. Soil pH ranges from 4.4 to 5.2 in the sampling area (Table I). Significantly highest soil N (0.66%) and soil organic carbon (9.11%) was observed in Organic Clay and Muck soil, followed by Sedu soil series. There was no significant difference in organic carbon content between Bernam and Jawa soil series. The available soil P among the soil series varied from 3.38 to 78.03 ppm. The highest exchangeable P was found in Sedu series followed by Organic Clay and Muck soil. There were no significant

Table I. Chemical properties of different soils in Tanjong Karang Rice Irrigation Project

Soil Series/types	Soil pH	N (%)	OC (%)	P (ppm)	K ($cmol^{(+)}$ kg^{-1})	Mg ($cmol^{(+)}$ kg^{-1})	Ca ($cmol^{(+)}$ kg^{-1})	Na ($cmol^{(+)}$ kg^{-1})
Brown Clay	4.63^{d}	0.33^{d}	5.13^{d}	3.94^{c}	2.95^{b}	1.76^{b}	32.40^{a}	0.24^{d}
Jawa	4.71^{c}	0.44^{c}	6.67^{c}	3.76^{c}	2.46^{c}	1.11^{b}	38.33^{a}	0.18^{e}
Org. Clay & Muck	4.5^{d}	0.66^{a}	9.11^{a}	56.05^{b}	1.67^{c}	1.79^{b}	41.76^{a}	0.28^{c}
Bakau	5.18^{a}	0.27^{e}	3.08^{e}	8.21^{c}	3.20^{a}	2.75^{a}	33.75^{a}	0.58^{a}
Sedu	4.40^{e}	0.55^{b}	7.89^{b}	78.03^{a}	1.64^{c}	1.79^{b}	25.98^{a}	0.32^{c}
Serong	4.90^{b}	0.38^{d}	2.94^{e}	3.48^{c}	2.55^{c}	2.77^{a}	40.16^{a}	0.41^{b}
Bernum	4.87^{b}	0.24^{e}	5.43^{c}	3.38^{c}	2.21^{c}	2.53^{a}	47.35^{a}	0.48^{b}

Means in column followed by different letters differ significantly at 1% level by Tukey's Studentized Range test

Table II. Diazotrophs population in soil, rhizosphere, root and shoot of different rice varieties grown on Tanjong Karang rice area

Soil series/ types	Rice Variety	Plant growth stage	Diazotrophs Population (cfu g^{-1})			
			Soil	Rhizosphere	Root	Shoot
Brown Clay	MR219	2 days after seeding	$4.3\times10^{4\,b}$	4.0×10^{3b}	-	-
Jawa	MR219	7 days after seeding	$2.9\times10^{4\,b}$	4.4×10^{3b}	$5.7\times10^{4\,c}$	$5.9\times10^{3\,c}$
Organic Clay and Muck	MR219	Maximum tillering	$2.2\times10^{6\,a}$	2.2×10^{7a}	$6.3\times10^{7\,a}$	1.6×10^{6b}
Bakau	MR232	Maximum tillering	$2.2\times10^{6\,a}$	4.2×10^{7a}	$8.4\times10^{6\,b}$	$3.2\times10^{6\,b}$
Sedu	MR219	Maximum tillering	1.78×10^{6a}	$1.4\times x10^{7a}$	$7.2\times10^{6\,b}$	$2.5\times10^{7\,a}$
Serong	MR219	Maximum tillering	$2\times10^{6\,a}$	2.2×10^{7a}	$2.2\times10^{6\,b}$	$2.2\times10^{6\,b}$
Bernam	MR220	Ripening	$3.8\times10^{5\,b}$	4.2×10^{3b}	-	-

Means in column followed by different letters differ significantly at 1% level by Tukey's Studentized Range test

differences were found in soil extractable P content among Brown Clay, Jawa, Bakau, Serong and Bernum soil series. The soil exchangeable K varied from 1.64 to 3.20 $cmol^{(+)}$ kg^{-1} soil. The highest amount of exchangeable K, Mg, Ca and Na were found in Bakau soil series.

Population of diazotrophs. There were significant differences in the population of diazotrophs found in different rice varieties, plant age and soil types (Table II). The diazotroph population was higher in the rhizosphere than non-rhizosphere soil. Rice cultivar MR219 showed higher endophytic populations compared to MR220 and MR232 varieties. Plants sampled at maximum tillering stage showed the highest soil and rhizosphere populations compared to seedling and ripening stages. The young seedlings of two and seven days old in Brown clay and Jawa series showed lower soil and rhizosphere populations. Lower populations of diazotrophs were also recorded at harvesting stage in Bernam soil series. The soil diazotrophic populations of the seven soil types ranged from 2.3×10^4 to 2.2×10^6 cfu g^{-1} soils. Higher populations of root endophytes (6.3×10^7) in Organic Clay and Muck soil and the shoot endophytes (2.5×10^7) in Sedu soil series were recorded.

Acetylene reduction assay (ARA), cellulase activity and indoleacetic acid production (IAA). Nineteen bacterial strains were evaluated for N_2 fixation activity (Table III). The ARA values ranges from 6.1×10^{-11} to 1.2×10^{-6} µmol C_2H_4 cfu^{-1} hr^{-1}. The highest ARA was found in isolate Sb35 (*Corynebacterium* sp.). Several isolates were high in ARA, as observed for Sb6 and Sb16. Jensen-CMC plate was assayed to determine cellulase activity. The plates spotted with diazotrophic broth showed a clear halo zone after staining with 0.1% Congo red indicating cellulase activity (Table III). Eleven of the root and shoot isolates were positive for cellulase activity. Isolated diazotrophs were able to produce higher amounts of IAA (Fig. 1). In the presence of tryptophan the bacterial isolates produced higher IAA, which varied from 32 to 69 mg L^{-1}. The highest IAA was recorded in strain Sb41 (*Corynebacterium* sp.) followed by Sb32 strain, while the lowest IAA was produced by Sb33 strain.

Table III. Nitrogen fixation of isolated diazotrophs and some of the biochemical properties

Isolates	ARA (µmol $C_2H_4^{-1}cfu^{-1h}$)	Cellulose degradation	Gram reaction	KOH reaction
Sb2	$1.89x10^{-8}$	-	+	+
Sb3	$2.25x10^{-10}$	-	-	-
Sb4	Nd	+	-	-
Sb6	$1.33x10^{-7}$	+	+	+
Sb7	Nd	+	-	-
Sb10	$2.74x10^{-10}$	-	-	-
Sb13	$3.14x10^{-9}$	-	-	-
Sb16	$1.39x10^{-7}$	+	-	-
Sb18	$2.71x10^{-10}$	-	+	+
Sb20	$3.89x10^{-9}$	-	+	+
Sb26	$2.98x10^{-10}$	+	+	+
Sb28	$2.1 x 10^{-9}$	+	+	+
Sb32	$5.35x10^{-10}$	+	+	+
Sb33	$1.8x10^{-10}$	+	+	+
Sb34	$4.9x10^{-7}$	+	+	+
Sb35	$1.26x10^{-6}$	-	+	+
Sb37	$2.47x10^{-10}$	+	-	-
Sb38	$6.1x10^{-11}$	+	-	-
Sb41	$9.1x10^{-10}$	+	+	+

Note: Nd represents not done, (-) ive for absence, (+) ive for presence

Biolog identification and strain diversity. The diazotrophic strains from the Tanjong Karang Rice Irrigation Project area were identified as Rhizobium, Burkholderia and *Corynebacterium* spp. by using Biolog identification method. The REP-PCR finger-prints using Jaccard's coefficient data analyses found 27 isolates formed

two majore groups at genetic distance = 0.1. Cluster-1 contained 17 isolates and cluster-2 contained 10 isolates (Fig. 2). Cluster-1 comprised six subgroups (Sb1 & Sb3, Sb4 & Sb33, Sb9 & Sb23, Sb17 & JRO10, Sb20 & Sb26, Sb15 & Sb19), while cluster-2 had only one subgroup (Sb41 & Sb19).

DISCUSSION

Among the soil properties pH, organic matter content and nutrient availability, were strong determinants of microbial community structure in soil (Grayston *et al.,* 2004). The soils of this project area were inherently rich in organic matter and developed over brackish water deposit (Parmananthan, 2000). These soils had shallow or deep organic phase, which were high in nitrogen and organic carbon contents. It was recorded that organic matter from organic rich layers provided carbon and energy sources for microbial growth (Coolen *et al.*, 2002). The available nutrient elements were also comparatively high in all soil samples, as they were collected from fertilized plots. Higher OC and N content were found in Sedu and Organic Clay and Muck soils; the former was previously mapped under Organic Clay and Muck soil (Parmananthan, 2000). The highest root and shoot endophytes were found in these two soils. The soil and rhizosphere populations were higher in Organic Clay and Muck, Sedu, Serong and Bakau soils. These soils contained higher amounts of OC, N and P, which may enhance (Alden *et al.*, 2001), or the plant growth stage influenced rhizobacterial population positively. Previous study showed that cultivars and plant growth stages were strong determinants of microbial community development (Macdonald *et al*., 2004; Herschkovitz *et al.*, 2005). In the present study, the soil carbon and nitrogen content of Serong and Bakau soil series were lower than Sedu and Organic Clay and Muck soils but higher soil and rhizosphere diazotrophs population recorded. This indicated a possible effect of plant age (active tillering stage) in enhancing population growth besides the inherent soil nutrient status. Comparatively lower diazotrophs population were found in Jawa, Bernam and Brown Clay soil series, which could probably be due to early plant growth stage and soil being newly ploughed at the time of sampling in Jawa and Brown soil clay. A low soil moisture content and death of roots may induce low populations in Bernam soil series. Bueno dos Reis *et al*. (2000) found similar reduction in numbers of *A. diazotrophicus* in sugarcane at harvesting stage. In general, the differences in the diazotroph populations were due to the differences in the soil chemical properties and plant age.

All the isolates grew well in the Burks N-free media, ATTC (Eskew *et al.*, 1977) media and Jensen media. After 24 h of inoculation period isolates formed pellicles in N free semisolid malate media, which indicated their abilities to fix N_2 under aerobic and microaerophilic conditions (Azlin *et al.,* 2003).

Fig. 1. IAA production of the isolated diazotrophs, Means in column followed by different letters differ significantly at 1% level by Tukey's Studentized Range test

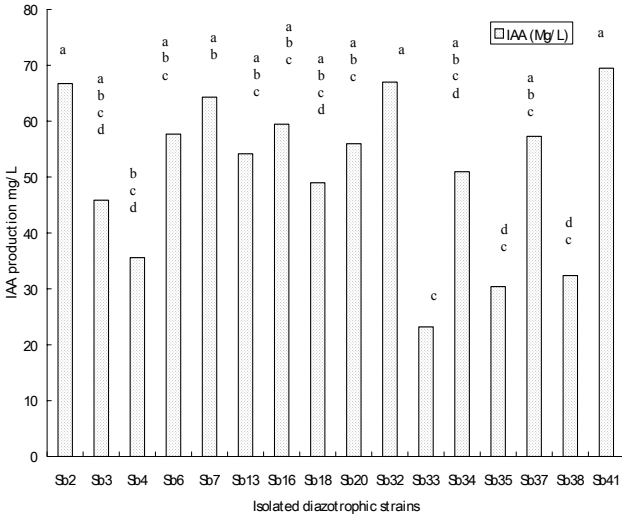
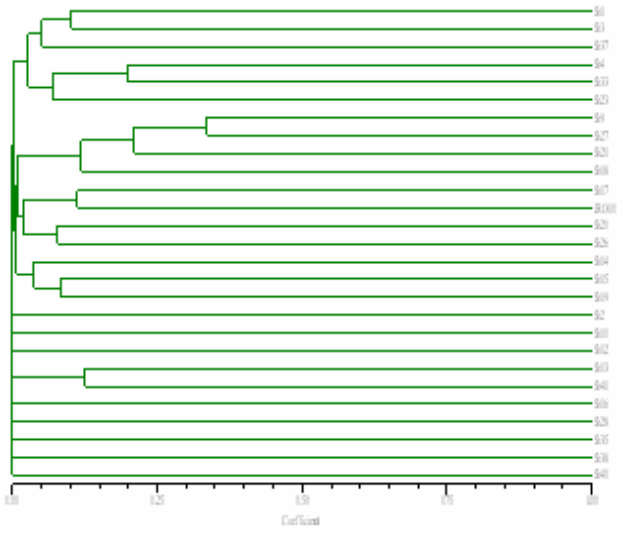


Fig. 2. The dendogram of cluster analyses of isolated diazotrophs based on REP-PCR marker (Versalovic *et al.*, 1997), Dendogram constructed by Jaccard similarity coefficient matrix by UPGMA method



A number of the diazotrophs isolates were capable of producing IAA, which are known to have pronounced effects on plant growth and development (Glick, 2005). The microbially produced IAA increased seed germination and seedling growth (Biswas *et al.*, 2000). The effects of IAA on seedling growth were concentration dependent; the low concentration may stimulate growth, while high concentrations may be inhibitory (Arshad & Frankenberger, 1991). The isolates producing cellulose degrading enzyme may be competent to penetrate the root cells and grow endophytically. Several studies showed production of cell

wall degrading enzymes such as cellulase, phosphatase or pectinase in PGPR was important in facilitating entry of the bacteria into the intercellular spaces of plant root hairs (Mateos *et al.*, 2001).

Biolog identification, used to identify strain diversity, depends upon the ability of individual species to catabolize specific substrates. It is an assay of carbon substrate utilization patterns by combining a broad range of substrates (95 in a 96- well microtiter plate) with a detection method based upon the reduction of tetrazolium violet in each well. In the present study, among the10 selected isolates *Rhizobium*, *Corynebacterium* and *Burkholderia* spp. were identified from experimental area. The *Corynebacterium and Burkholderia* genera have been proven to have greater affinity with the water environment of the rice irrigation channel and rice plots (Reche & Fiuza, 2005). Burkholderia sp. was isolated from the rice plants in Brazil and Vietnam and classified as diazotrophic endophytes (Neves & Rumjanek, 1998). In the present study Burkholderia sp. was also endophytic. Rhizobium radiobacter has been shown to be abundant in marine sediments with organic rich layers (SÜB *et al.*, 2006). Presence of this species in Sedu soil could be due to the high black organic clay layer as soil was developed over non-sulfidic marine alluvium with black organic clay (Parmananthan, 2000).

The study of cluster analysis based on REP-PCR marker (Versalovic *et al.*, 1997) is a simple technique, which uses a single set of primers closely related as well as divergent groups of strains can be classified. Using this technique we found that Tanjong Karang Rice Irrigation project area (18591 ha) harbor a diverse group of diazotrophs. Although it is a small project area, there existed many diazotrophic species, which were variable in biochemical properties such as N_2 fixation, cellulose degradation and hormone production. Some of the isolates were endophytes. The above findings shows the genetic distance of these isolates were vast even when isolated from only one project area.

In conclusion, Tanjong Karang Rice Irrigation project area is rich in diverse group of bacteria. The diazotrophic populations were significantly influenced by soil types, plant age and varieties. According to Biolog identification, *Rhizobium*, *Burkholderia* and *Corynebacterium* were the prominent diazotrophs in this area. Several strains of diazotrophs from different soil types and rice varieties of this area were capable of fixing N_2, producing cellulase enzyme and IAA.

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