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Growth Enhancement and Root Colonization of Rice Seedlings by *Rhizobium* and *Corynebacterium* spp.

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ABSTRACT

Diazotrophs form natural association with rice plant. An *in vitro* experiment was conducted to study the colonization and association of Sb16 and Sb26 diazotrophic strain (previously isolated from rice rhizosphere) on two rice genotypes namely Mayang Segumpal and MR219. Root colonization was observed under scanning and transmission electron microscope. After 5 days of inoculation diazotrophs colonized on the surfaces and internals of lateral roots, root hairs and epidermal cells of the rice roots. Sb16 (*Rhizobium* sp.) formed aggregated cells and produced mucilaginous materials that may be involved in their attachment on roots. Rice seedlings inoculated with diazotrophs produced significantly higher biomass compared to control and 35 kg ha^{-1} of nitrogen treatments. The diazotrophs association increased rice seedlings root and shoot biomass. Mayang Segumpal rice colonized by *Rhizobium* sp. (SB16) content higher tissue nitrogen (4.47%) and increased plant biomass by 36% over the non-inoculated control and 22% over 35 kg ha^{-1} of N fertilizer. While the MR219 rice inoculated with *Corynebacterium* sp. contained 4.30% tissue N and increased 32% of plant biomass over non-inoculated control and 21% over 35 kg N ha^{-1} The study showed that inoculation with diazotrophic strains (Sb16 & Sb26) improve plant growth including tissue nitrogen content of rice and differences in the association between diazotrophs and rice genotypes.

Key Words: Corynebacterium*;* Diazotrophs; Nitrogen*;* Rhizobium*;* Rice; Root colonization

INTRODUCTION

Nitrogen is the primary nutrient element in rice production. The efficiency of added urea-N is very low and only 30-40% used by the plants (Choudhury & Khanif, 2001). One of the approaches to reduce N losses and improve the uptake and utilization of native and applied nitrogen by rice plants is the biological nitrogen fixation (Ladha & Reddy, 1995). Diazotrophs are nitrogen fixing free living bacteria that utilize rhizosphere carbon compounds for growth and development and subsequently fix nitrogen for the plant. Besides consequences of nitrogen fixation, diazotrophs exhibit plant growth enhancement activities such as production of phytohormones, antifungal or antibacterial agents, siderophore and induction of systemic acquired host resistance and increased availability of mineral nutrients to plants (Sessitsch *et al*., 2002).

Diazotrophs that efficiently colonize in the rice root interior might have higher potential to fix nitrogen (Hurek *et al*., 1997). It has been reported that endorhizosphere contributions of plant nitrogen are more extensive than rhizosphere contributions due to the lack of competition from other rhizosphere organisms and the availability of carbon sources with small fluctuations in pO_2 (Boddey *et al.*, 1995). In 1989 first evidence of intracellular colonization of rice seedlings by *Alcaligenes faecalis* was reported by You and Zhou (1989). Several endophytic diazotrophs have been isolated from different cultivated and wild rice species, which includes *Klebsiella, Enterobacter, Alcaligenes*, *Azospirillium, Corynebacterium* and *Burkholderia* genus. The diazotrophic endophytes actively colonize in healthy roots. Endophytic diazotrophic strain BH72, *Herbasprillium seropedicae*, *Gluconacetobacter diazotrophicus, Rhizobium leguminosarum* and *Klebsiella pneumoniae* has been reported to colonize in the root arenchymatic tissues, cortex regions, stele, xylem and shoots of grasses and cereals (Yanni *et al*., 1997; James & Olivares, 1998; Reinhold-Hurek & Hurek, 1998). *Azorhizobium caulinodans* enter into the root system of cereals by intercellular invasion between epidermal cells and the xylem. The xylem colonization might provide a non-nodular niche for endosymbiotic nitrogen fixation in rice, wheat, maize, sorghum and other non-legume crops (Cocking, 2003). Vascular tissue is an ideal niche for endophytic diazotrophic colonization as there is low pO_2 and allocation of photosynthate. The inoculation of diazotrophs with various non-legumes especially cereals found intercellular establishment of those bacteria in the

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root system and were capable of fixed nitrogen endophytically and providing combined growth enhancement effect to the crop. Application of *Rhizobium leguminosaram* bv. *Trifolli* has been shown to successfully colonize rice roots and supplied 25-33% of the recommended rate of N fertilizer (Yanni *et al*., 1997). The benefits of diazotrophs may result from better uptake of soil nutrients rather than from nitrogen fixation (Cocking, 2003). Some of the diazotrophs such as *Azospirillum brasilense* cells aggregate and flocculate, which may enhance successful inoculum survival in the soil (Sadasivan & Neyra, 1985).

The diazotrophs colonize only surface of the root remain vulnerable to competition with other rhizospheric microorganism. Endophytic diazotrophic population may ensure better beneficial effect. For sustainable and environmental friendly crop production need enhancement of biological nitrogen fixation by endophytic diazotrophs especially for the main cereals such as rice, wheat and maize crop. Hence present study undertaken with the aim to observe the root colonization by *Rhizobium* sp. (Sb16) and *Corynebacterium* sp. (Sb26) in different cultivars of rice seedlings and the consequence of inoculation on biomass production and nitrogen fixation in the host plant under *in vitro* condition.

MATERIALS AND METHODS

Bacterial isolates. Diazotrophic strains *Rhizobium* sp. (Sb16) and *Corynebacterium* sp. (Sb26) were isolated from Tanjong Karang rice irrigation project area in December 2006.

Seed surface sterilization. The method of seed surface sterilization was adopted from Amin *et al.* (2004). Rice seeds were agitated in 70% ethanol for 5 sec. The ethanol was discarded and the seeds washed in sodium hypochlorite solution comprising 3% Chlorox ^{TM} (2.6% NaOCl), with a few drops of Tween 20. The seeds were rinsed with sterile water followed by 2% sodium thiosulphate solution to neutralize chloramine residue. The efficacy of sterilization was checked by germinating seeds on the NA plates.

Inoculums preparation. *Rhizobium* and *Corynebacterium* strains were grown in ATCC broth for 48 h. The bacterial cells were harvested by centrifugation at 13500 rev min^{-1} for 10 min in eppendorf tube and washed with 0.85% sterilized phosphate buffer saline. Approximately 10^9 mL^{-1} live bacterial cells were used to inoculate plants in each planting unit. The population was confirmed by cell enumeration in drop plate method on NA.

***In vitro* growth of rice seedlings.** Mayang Segumpal and MR219 rice seedlings were grown in plastic container (15 cm × 10 cm) containing 500 g of sterilized washed sand. Twelve seedlings were planted in each container. Plant nutrient solution, free of carbon and nitrogen modified from Egener *et al.* (1999) was applied. Each seedling was subsequently inoculated with 1×10^9 cfu mL^{-1} of the respective bacterial isolates. Plants were grown for 45 days in growth chamber with 12 h light/dark cycle and temperature of 28°C.

Determination of rhizosphere population. Plants were harvested and roots were gently washed with sterile water and placed in conical flask containing 99 mL of distilled water. The content in flasks were shaken for 10 min and a series of 10 fold dilutions were prepared and bacterial populations were determined as described previously.

Root interior population. The roots (1.0 g) were washed and surface sterilized with 70% ethanol for 5 min and then treated with 3% Clorox for 30 sec. The roots were checked for the efficacy of surface sterilization by rolling them on NA plates. Using a sterilized mortar and pestle the roots were meshed (Gyneshwar *et al.,* 2001). A 10 fold series of dilution were prepared up to 10^{-10} and the diazotrophic populations were determined following drop plate count method.

Scanning electron microscope and transmission electron micrioscope observation. Rice seedlings were grown in *in vitro* condition. Five days old seedlings were inoculated (10^9 cfu mL^{-1}) and 3 days after inoculation root sample were washed with sterile water and cut into 1 cm for Scanning Electron Microscope (SEM) and 1 mm pieces for Transmission Electron Microscope (TEM) observation. Root samples were pre-fixed with 4% glutaraldehyde overnight and washed with 0.1 M sodium cacodylate buffer for 3 changes at 30 min each. Osmium tetraoxide buffer (1%) was used for post fixation. After a series of dehydration in acetone (35, 50, 75, 95 & 100%) the samples were dried in a critical point dryer and mounted on aluminum stubs, sputter coated in gold and viewed under Scanning Electron Microscope (JEOL JSM-6400 attached with OXFORD INCA ENERGY 200 EDX). For Transmission electron microscope observation the samples were infiltrated with acetone and resin mixture and embedded. After polymerized steps samples were sectioned, coated with gold and observed under TEM (PHILIPS HMG 400).

Plant biomass and total nitrogen determination. After harvest plant sample were dried in oven at 80°C temperature and dry biomass was recorded. The plant tissue nitrogen was determined by Kjeldahl method.

Statistical analyses. The experiment was laid out in a complete randomized design with four replicates. Data were analyzed using the SAS (9.1 version) statistical software. Treatments means were separated using Tukeys test.

RESULTS

Root colonization. The applied diazotrophs proliferated and colonized in the roots. Scanning electron micrographs showed that 5 days after inoculation bacterial isolates were able to colonize on the surfaces of the primary and lateral roots, root hair zone, lateral root junction, in crevices and the root tips (Fig. 1). Cell aggregates were also observed on the root tip,

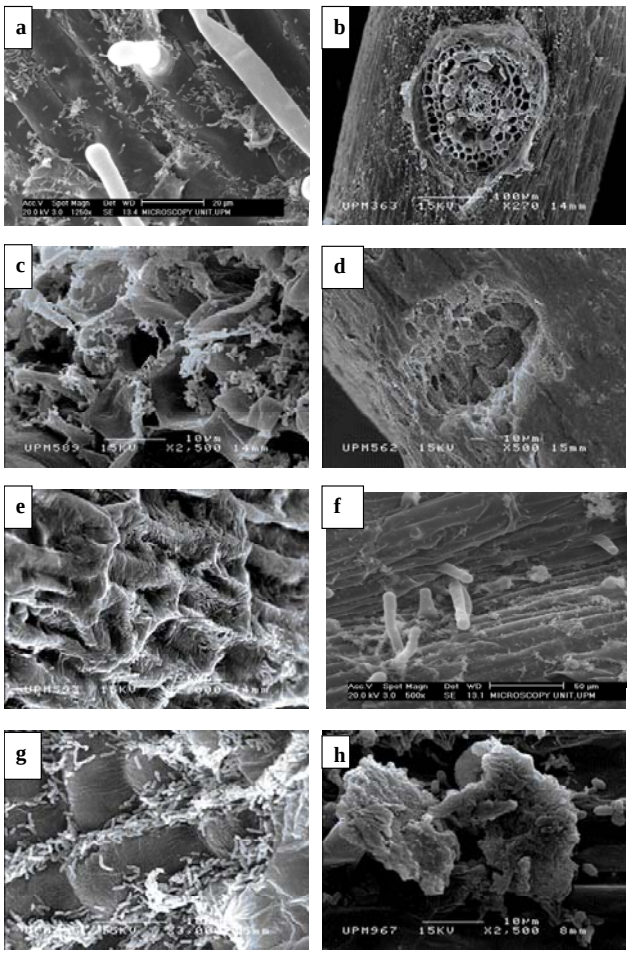
near the elongation and differentiation zones (Fig. 1f). Among the two diazotrophs, *Rhizobium* sp. was observed to form clusters on the root surfaces, which was covered with gummy material (Fig. 1h). The transverse sections of the root viewed under TEM found that diazotrophs lived in the intercellular spaces of cortical parenchyma, within epidermis, inner cortex and xylem vessels (Fig. 2). The rice plants grown in *in vitro* condition in sand culture were harvested after 45 days of inoculation and bacterial population of root interior and in rhizosphere were determined. The higher endophytic population (9.8×10^7) was found by *Rhizobium* Sp. in Mayang Segumpal rice (Table I).

Effect of inoculation on plant growth and tissue N content. The diazotrophs association increased rice seedlings root and shoot biomass (Fig. 3). Differences occurred in the association between diazotrophs and rice genotypes. The association between *Rhizobium* sp. and Mayang Segumpal produced higher shoot biomass compared to *Corynebacterium* sp. The association with *Corynebacterium* sp. and MR219 rice produced higher shoot biomass. Mayang Segumpal rice inoculated with *Rhizobium* sp. (Sb16) content higher tissue nitrogen and increased 22% biomass production over (35 kg ha^{-1}) nitrogen doses. Rice variety MR219 inoculated with *Corynebacterium* sp. (Sb26) increased 21% biomass yield and contained 4.30% of tissue nitrogen (Table I).

DISCUSSION

The *Rhizobium* and *Corynebacterium* species were locally isolated diazotrophic strains from rice rhizosphere in Malaysia. Both of the diazotrophs were able to colonize endophytically. Three days after inoculation the view from scanning electron microscopy showed bacterial colonization on the surface of the primary and secondary root, zone of elongation, root hair, lateral root junction, in crevices and in the root tips. The view of transmission electron microscopy and the longitudinal view of SEM showed an extensive colonization of the diazotrophs from intra and intercellular spaces and extending into cortex and vascular system in the lateral roots. Similar findings were recorded by de Bruijn *et al.* (1995). The cortex region of roots and the arenchymatic tissues of rice were the colonization sites for endophytic diazotropphs as these might provide the suitable conditions for N_2-fixation with low concentrations of oxygen and supplement of available nutrients. Diazotrophs may enter into the cell through lateral root junction and by crevices. Previous study in rice root tips showed the zone of elongation and differentiation and the junction between the primary and the lateral roots were the primary sites for entry of diazotrophs (Hurek *et al*., 1994; Liu *et al*., 2006). Lateral roots originate from the pericycle and grow through the cortex of the main root so entrance through lateral roots led to the bacteria into the inner cortex. Emergence points of lateral roots might be other primary sites of colonization. Previous studies reported that the entry through the root

Fig. 1. Diazotrophs colonization, (a) Root hair zone, (b & c) Junction of lateral root and lateral root meristem, (d) Crevices, (e) Inside crevices, (f) Zone of elongation, (g) In between cell wall of root tip, (h) Bacterial cell aggregation on the root hair zone covered by mucilage material



zone of elongation can help diazotrophs to invade inter and intracellular and penetrate central tissues in the stele (James, 2000; Liu *et al*., 2006).

Scanning electron micrograph showed that both the diazotrophs entered into the cell through crack. The diazotrophs concentrated at the lateral root junction and the crack formed at the lateral root junction is probably another entry sites into the roots. Crack entry infections by diazotrophs allow entering host plants intercellular between adjacent cells. After lateral root crack colonization, bacteria may move into intercellular spaces within the cortical cell layer of roots. The crack entry infection involves in Rhizobia in non-legumes between the adjacent cells and not by the formation of infection threads at the root tips of root hairs (Webster *et al*., 1998).

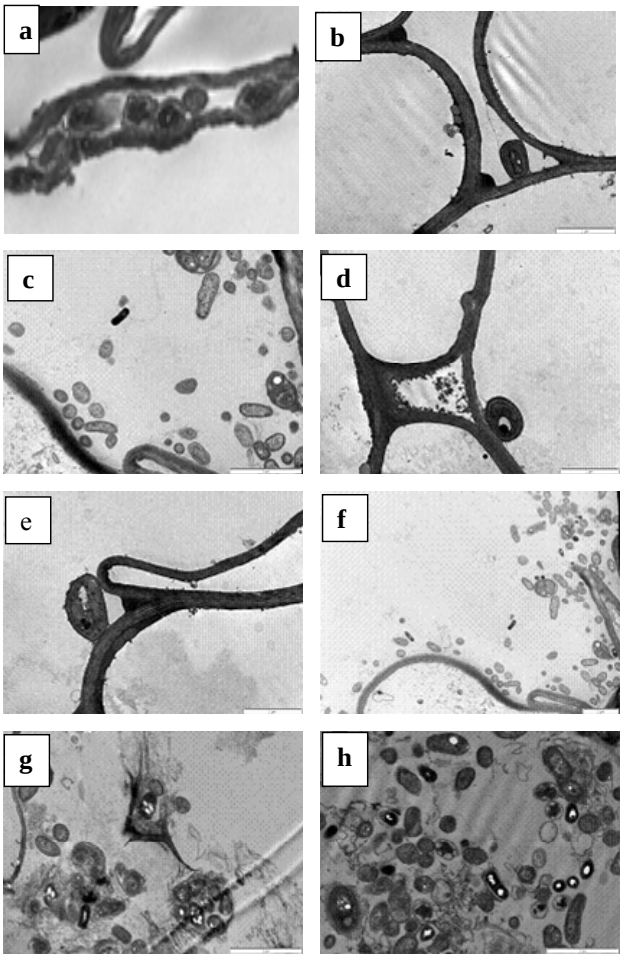
Another most probable mechanism of entry of bacteria into the cell is degrading of cell wall or tissue. In the previous study, it was found that both of the diazotrophs were capable

Table I. Effect of diazotrophs on biomass production in rice genotypes

	Root interior population (cfu ml^{-1})		Rhizosphere population (cfu ml^{-1})		N Content (%)		Plant dry weight (mg)		Dry Matter increased over control (%)		Dry Matter increased over 35 kg N (%)	
	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2
B1	9.8×10^7	1.4×10^7	3.1×10^9	1.5×10^9	4.47 a	3.88 b	49a	51a	36	25.49	22	9
B2	1.6×10^7	5.6×10^7	8.0×10^7	1.8×10^{10}	3.34 b	4.30 a	46a	58a	30.43	32.48	15.21	20.68
Nf	--	--	--	--	2.97 b	3.11 b	39b	46b	--	--	--	--
N0	--	--	--	--	2.40 c	2.75 c	32b	38b	--	--	--	--

V_1 = Mayang Segumpal, V_2 = MR219, B_1= *Rhizobium* sp, B_2 = *Corynebacterium* sp. N_f= 35 kg of N, N_0= without N
In each column, values with different letters show significant difference ($P \leq 0.05$) as determined by Tukeys test

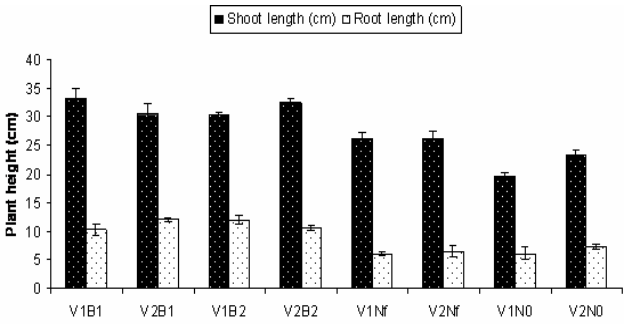
Fig. 2. Diazotrophs colonization (Transverse section), (a & b) In between cell wall, (c, d & e) Inter cellular spaces and within epidermis, (f) Inner cortex, (g & h) near vascular bundles. The bacteria surrounded by a matrix of microfibrillar material within the cell, Scale bars: (a) 1 µm, (b-h) 2 µm



of producing cellulolytic enzyme, which may initiate the invasion process (Mateos *et al*., 2001). Hurek *et al.* (1993) found Azoarcus sp. posses enzymes that degrade cellulose and facilitate the infection process by localized digestion of plant cell walls. The production of extracellular polysaccharide and cell aggregation might help in colonization process. In the present study Rhizobia produced extracellular polysaccharide and cluster of cell aggregation

Fig. 3. Effect of diazotrophs on biomass production in rice genotypes

V_1= Mayang Segumpal, V_2= MR219, B_1= *Rhizobium* sp, B_2= *Corynebacterium* sp. N_f= 35 kg of Nitrogen, N_0= without Nitrogen



was found in the root hair and elongation zone. This might have positive effect on plant colonization. During colonization process large cell aggregation was also found in *Bacillus megaterium* C4 and *Azospirillum brasilense* (Bahat-Samet *et al*., 2004; Liu *et al*., 2006). Bacterial surface components, such as extracellular polysaccharids and proteins were involved in plant colonization and extrapolysaccharide used as a sole carbon source for its own development (Burdman *et al*., 2001). Bacteria with flagella might play role in root surface attachment. *Rhizobium* and *Corynebacterium* spp. have flagella like structure, which may help to bind the bacteria on to the root surface. Burdman *et al.* (2001) also reported the pili of *Azoarcus* sp. involved in the cell attachment with plant surface.

Diazotrophs association and growth enhancement. The rhizospheric and endophytic bacterial population depends on the bacterial species, inoculum density, host plant genotypes, plant growth stage and other soil physico-chemical and biological properties. In the rhizosphere competition between the microorganisms exists to survive in the root exudates. Comparatively, the root interior environment favoured endophytes to survive with less competition although Rosenblueth and Martínez-Romero (2006) found some of the strains were equally competitive for colonizing in the rhizosphere and inside root tissues. The association and colonization depends on rice cultivars. In the present study, higher rhizosphere population was found in MR219 rice with *Corynebacterium* sp. and higher endophytic population found in *Rhizobium* sp. in Mayang Segumpal rice. Our result showed rice cultivars play an active role in the colonization of both rhizosphereic and

endophytic with the same bacteria. Similar finding was obtained by Rosenblueth and Martínez-Romero (2006). In axenic condition, probably the root exudates carbon compounds determined colonization. For endophytic colonization probably the rice apoplast provide suitable microenvironment for growth and proliferation of the diazotrophs, which may vary between rice varieties. The variety-specific differences of rice-associated nitrogenase expression in gnotobiotic culture with *Herbaspirillum seropedicae* was also reported by Gyneshwar *et al.* (2002). In the present study, higher tissue nitrogen content and plant biomass obtained with *Rhizobium* sp. in Mayang Segumpal rice and *Corynebacterium* sp. in MR219 association. Similar results of ^{15}N isotope and N-balance studies revealed that rice varieties genotypically differed in growth promotion and N fixation activities with the respect of host-bacteria interactions (Sherestha & Ladha, 1996). In the present study, inoculated rice plant dry biomass, root-shoot length and tissue nitrogen content significantly increased over non-inoculated plant. Rice seedlings root and shoot biomass and growth enhancement by the diazotrophs was also recorded by Biswas *et al.* (2000) and Govindarajan *et al.* (2008). The beneficial plant growth promoting association with rice root may not only N_2 fixation but the association leading to a greater root proliferation that may led to efficient nutrient acquisition.

The diazotrophic isolates were able to colonized endophytically in the root tissue and produce higher plant biomass and tissue N content. There were differences in the association between diazotrophs colonization and growth enhancement with the rice varieties. The root exudates carbon compounds and the plant tissue architecture may influenced the diazotrophs colonization and growth enhancement activity. In this study, *Corynebacterium* and *Rhizobium* spp. Colonized both surface and endophytically and the association significantly increased seedling root and shoot biomass, which can supplement about 35 kg ha^{-1} of nitrogen requirement in MR219 and Mayang Segumpal rice seedlings.

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