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Nitrogen-fixing activity in various parts of sago palm (*Metroxylon sagu*) and characterization of aerobic nitrogen-fixing bacteria colonizing the sago palm

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Abstract: Various parts (root, rachis, petiole, leaflet, bark, and pith) of the sago palm were collected in the Phillipine islands of Leyte, Cebu, and Aklan, and their nitrogen-fixing ability was measured with an acetylene reduction assay (ARA). Almost all the samples, except for the leaflets, showed positive ARA activity ranging from 1.1 to 961 nmol $g^{-1}day^{-1}$. Among the samples, roots collected during July, 2003 showed the highest activity, followed by the pith. ARA activity in the petiole, rachis, and pith samples was quite variable. Some parts showed high activity, while others showed very low activity, suggesting a heterogeneous distribution of the nitrogen-fixing potential. Nitrogen-fixing bacteria (NFB) were then isolated by aerobic cultures using a N-free Rennie semi-solid medium from the samples showing high ARA activity. A homology search of 16S rDNA sequences revealed that the nitrogen fixers belonged to various genera, such as *Klebsiella pnuemoniae*, *Pantoea agglomerans*, *Enterobacter cloacae*, *Burkholderia* sp., and *Bacillus megaterium*. Some of the Gram-negative strains were confirmed by physiological tests using API20E and API20NE, while the physiological identification and 16S rDNA sequence did not match in a few strains. The relationship between the ARA of the isolates and their carbon sources showed that all the isolates preferred simple sugar compounds, such as glucose, sucrose, and mannitol, but showed very low activity in pectin, starch, and hemi-cellulose media. This study demonstrated the extensive colonization of sago palms by various types of nitrogen-fixing bacteria for the first time.
Keywords: *Bacillus* spp., biological N_2-fixation, *Burkholderia* spp., *Enterobacter* spp., *Klebsiella* spp., *Pantoea* spp.

サゴヤシ各部位の窒素固定活性とサゴヤシに生育する好気性窒素固定細菌の特徴

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要約　サゴヤシの様々な部位（根, 葉軸, 葉柄, 小葉, バーク, 髄）がフィリピンのレイテ島，セブ島，アクラン島から採取され，アセチレン還元法により窒素固定活性が評価された．小葉を除くすべての部位で窒素固定活性が確認され, その活性は1.1～961 nmol g^{-1} day^{-1}であった. 2003年7月に採取された

根がもっとも高い活性を示し,ついで髄が高い値を示した．葉軸，葉柄，髄では非常に活性が変動し，窒素固定活性が不均一であることが示唆された．ついで,アセチレン還元活性が高かったサンプルから,窒素を含まない軟寒天培地を用いて好気的に窒素固定細菌が分離された．16S rDNA塩基配列の相同性検索から,窒素固定細菌は*Klebsiella pnuemoniae, K. oxytoca, Pantoea agglomerans, Enterobacter cloacae, Burkholderia* sp., *Bacillus megaterium*といった様々な属に属することがわかった. グラム陰性の分離菌についてはAPI20E とAPI20NE を用いた生理的な同定により再確認された菌株がある一方, 生理的同定結果と塩基配列の相同性結果が矛盾した菌株もあった. 分離菌の様々な炭素源下での窒素固定活性を見たところ, すべての分離菌はグルコース, スクロース, マンニトールで高い活性を示し, ペクチン, デンプン, ヘミセルロースといった高分子化合物を利用した窒素固定活性は極めて低かった. 本研究結果は, サゴヤシに様々な窒素固定細菌が定着していることを初めて示した.

キーワード　*Bacillus* spp., 生物的窒素固定, *Burkholderia* spp., *Enterobacter* spp., *Klebsiella* spp., *Pantoea* spp.

Introduction

Biologically fixed nitrogen is a free and renewable resource that constitutes an integral part of sustainable agro-ecosystems (Jensen and Nielsen 2003). Biological nitrogen fixation (BNF) is known to occur to a varying degree in various environments, including soils, fresh and salt waters, and sediments, and on or within the roots, stems, and leaves of certain plants (Hubbell and Kidder 2003). The potential for nitrogen fixation exists in any environment capable of supporting the growth of microorganisms. The global terrestrial amount of BNF is between 100 and 290 million tons per year (Cleveland et al. 1999), 40-48 million tons per year of which is fixed by agricultural crops in fields (Jensen and Nielsen 2003). About 80 million tons of global fertilizer nitrogen is used, and the amount has been static in North America and Europe over the last decade (Jenkinson 2001). The evidence of significant BNF in economically important *Gramineous* species, particularly sugarcane (Urquiaga et al. 1992), rice (Shrestha and Ladha 1996), and forage grass, such as Kallar grass (Malik et al. 1997), has generated tremendous interest in N_2 fixation by non-legumes.

Cavalcante and Dobereiner (1988) and Gills et al. (1989) isolated new acid-tolerant nitrogen-fixing bacteria (NFB), *Gluconacetobacter diazotrophicus*, associated with sugarcane. Baldani et al. (1986) reported on *Herbaspirillum seropedicae* and *Pseudomonas rubrisubalbicans*, now called *Herbaspirillum* (Baldani et al. 1996), from the surface-sterilized roots and stems of corn, rice, and sorghum. Reinhold-Hurek and Hurek (1998) found *Azoarcus indigens* and *A. communis* in the rhizosphere of Kallar grass. McInroy and Kloepper (1995b) reported on *Burkholderia* spp., *Enterobacter* spp., and *Bacillus* spp. from the roots and stems of sweet corn and cotton. Moreover, *Klebsiella pneumoniae* and other *Klebsiella* species were found in the roots and stems of *Zea mays* and *Zea luxurians* (Palus et al. 1996), *Burkholderia* spp. and *Herbaspirillum* spp., in the roots, stems, and leaves of banana and pineapple (Weber et al. 1999), and *Stenotrophomonas maltophilia*, in the rhizosphere of dune grass (Dalton et al. 2004). Some novel NFB have also been found, e.g. *Azospirillum doebereinerae* (Eckert et al. 2001) in the rhizosphere of C4-grass *Miscanthus* and *Gluconacetobacter johannae* and *G. azotocaptans* (Ramirez et al. 2001) in the rhizosphere of coffee plant. Other nitrogen fixers, such as *B. fusiformis* and *Pseudomonas fluorescens*, were isolated from the rhizosphere of such plants as sesame, maize, wheat, soybean, lettuce, pepper, and rice grown in Chungbuk Province, Korea (Myoungsu et al. 2005). Most of the studies on free-living NFB have suggested that the colonization of these bacteria largely occurs in the roots and rhizosphere of plants, although there have been some reports of colonization

in the stems and leaves. Attempts to isolate NFB from palm trees in the Amazon region showed an abundance of such bacteria. Palm trees of the varieties Dende (*Elaeis guineensis*) and Pupunha (*Bactris gasipaes*) were colonized by *A. brasilense*, *A. amazonense*, and *H. seropedicae*, which are well-known potential nitrogen fixers. These bacteria were present in the roots, stems, leaves, and endosperm of the fruit (Reis et al. 2000).

Among the palm trees, the sago palm is a promising agricultural plant for its starch production. It is one of the oldest tropical plants exploited by man and has been utilized in southeast Asia, especially in Papua New Guinea, Indonesia, Malaysia, and Pacific Oceania. In the last 25 years, interest in this crop has increased considerably. According to Stanton (1993), one of the first to advocate sago palm research, the advantages of the crop are that it is (1) economically acceptable; (2) relatively sustainable; (3) environmentally friendly; (4) uniquely versatile; (5) vigorous; and (6) socially stable in an agro-forestry system. The sago palm has its own importance in terms of its starch yield and wide range of uses as a staple food, an ingredient in noodles and confectionery, and a substrate for alcohol fuel. Even though rice is now a staple food for most people in Indonesia, many people still eat sago starch as a staple and prepare traditional food from sago (Bintoro 2002). Today, sago is still an important crop playing a vital role as a subsistence food in some areas of Papua New Guinea and Irian Jaya (Oates 2002). The sago palm grows well in humid tropical lowlands up to an altitude of 700 m (Oates 2002) and is also found in coastal and hilly regions up to its altitude limit of 1,150 to 1,250 m (Oates 1999). It is sensitive to soil moisture and prefers to have its roots in damp mud (Oates 1999). Water shortages are detrimental to its growth, and rainfall should be evenly distributed above 2,000 mm per year without any pronounced dry periods (Oates 1999). The sago plant prefers mineral soils but grows more slowly in notoriously poor and usually unfertilized peat soils (Jong and Flach 1995), suggesting that it may possess some strategies for nutrient acquisition, especially nitrogen. To test this hypothesis, we concentrated on microbial activities and anticipated that there might be free-living nitrogen-fixing bacteria colonizing the sago palm. The objective of this work was to evaluate the nitrogen-fixing activity of various parts of the sago palm and study the existence of NFB in/on the sago palm and identify those bacteria.

Materials and methods

Sample collection and acetylene reduction assay (ARA) of plant materials

Various parts (root, petiole, leaflet, bark, and pith) of the sago palm were collected from several regions of the Philippines: Dulag (N10º 58' 94", E124º 59' 98"), Alangalan (N11º 11' 43", E124º 52' 97"), and Hilusig (N10º 38' 80", E124º 56' 72"), Leyte; Labangon Tulik (N9º 52' 20", E123º 35' 22"), Langsted (N9º 53' 73", E123º 36' 20"), and Campo Guadalupe (N10º 06' 53", E123º 38' 98"), Cebu; and Balate (N11º 32' 43", E122º 21' 76"), Lezo (N11º 40' 94", E122º 18' 65"), Malinao (M11º 39' 21", E122º 18' 09"), and Banga (N11º 38' 02", E122º 19' 75"), Panay. Roots were washed with tap water to remove all the attached soil particles and rinsed with sterile distilled water. Petioles were also washed with tap water and sterile distilled water. Pith was obtained from mature palms containing starch. The washed roots and petioles were cut into 1 to 2 cm segments with scissors, and 3 g (fresh basis) of the samples was transferred into a vial. Three g (fresh basis) of pith sample was also put into a vial. The vials were capped with rubber stoppers, and acetylene gas was injected into the headspace of the vial at a final concentration of 10 %. Experiments were done in one replicate, duplicates, or triplicates, depending on the availability of samples. The amount of ethylene in the headspace was quantified 4 to 7 days after the injection of acetylene by injecting 0.5 ml of the headspace into a gas chromatograph (GC-14B; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and

a column packed with a Shincarbon S (3 mm x 2 m, 60/80 mesh, Shimadzu GLC Center, Tokyo, Japan).

Isolation and identification of bacteria

A Rennie medium (Rennie 1981) was used for the isolation of nitrogen-fixing bacteria. It consists of solution A (0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.1 g NaCl, 28 mg $Na_2FeEDTA$, 0.1 g NaCl, 25 mg Na_2MoO_4 $2H_2O$, 100 mg yeast extract, 5 g mannitol, 5 g glucose, 0.5 ml sodium lactate (60 % v/v), 12 g of agar for the solid media and 0.3 g for the semi-solid media, and 900 mL distilled water) and solution B (0.2 g $MgSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2$, and 100 mL distilled water). Solutions A and B were separately autoclaved at 121ºC for 20 min and mixed after cooling to 50 to 60 ºC, and filter-sterilized 5 μg biotin and 10 μg p-aminobenzoic acid were then added.

The sago palm parts (root, petiole, rachis, leaflet, and pith) possessing high ARA activity were selected and cut into small pieces and macerated on a sterilized mortar and pestle. The macerated samples were serially diluted, and portions of the dilutions were spread onto a Rennie medium for the isolation of NFB. After 2 to 3 days of incubation at 27ºC, individual colonies with various morphologies were picked and sub-cultured several times on a fresh Rennie medium to obtain pure cultures. The nitrogen-fixing activity of the isolates was then confirmed using an acetylene reduction assay. Individual isolates were inoculated into a 30ml of an air-tight vial containing 3 ml of a semi-solid Rennie medium. After inoculation, 10 % of the headspace was replaced with acetylene, and the vials were incubated for 24 h at 27ºC. The production of ethylene was measured using the same method mentioned above. The isolates possessing high ARA activity were selected and characterized on the basis of the sequence analysis of 16S rDNA. DNA was extracted from purified bacterial strains with the conventional method (Miyashita 1992), and the PCR primers (27f) 5`-AGAGTTTGATCCTGGCTCAG-3`and (1378r) 5`-TACAAGGCCCGGGAACG-3` were used to amplify the segment of the bacterial 16S rDNA from nucleotides 27 to 1378 (*Escherichia coli* numbering) (Lane, 1991). PCR was carried out using a basic reaction mixture containing an Ex *Taq* buffer (Takara, Otsu, Japan), 2.5 mM of each deoxyribonucleotide triphosphate, 2.5 U of Ex *Taq* (Takara), 0.2 mM of each oligonucleotide primer, and 1 μl template (purified DNA) in a total volume of 25 μl. The thermocycling conditions consisted of an initial denaturing step at 94ºC for 3 min, 30 amplification cycles of 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 3 min, and a final extension step of 72ºC for 7 min with the GeneAmp PCR System (Takara, PCR Thermal Cycler, PERSONAL). The PCR products were confirmed in electrophoresis using a 0.7% agarose gel with 0.5 μg ml^{-1} with ethidium bromide. Then, direct sequencing of the PCR products derived from various isolates was done by Hitachi Science Systems, Ltd. (Tokyo, Japan). The phylogenetic position of the sequences obtained was examined using the DDBJ homology search system BLAST. Some of the strains were characterized by physiological tests using the bacterial identification kits API20E for enteric bacteria and API20NE for non-enteric Gram-negative bacteria (BioMerieux Inc., Hazelwood, MO). The method was followed according to the company's instructions.

Nitrogen-fixing activity under various carbon substrates

Various kinds of carbohydrates, such as sucrose, glucose, mannitol, starch, lactate, pectin, and hemi-cellulose, were used to estimate the suitable carbon sources for high nitrogen-fixing ability. Experiments were conducted with a semi-solid nitrogen-free Rennie medium using various individual carbohydrates. In this experiment, a 30ml sterilized rubber capped vial, into which 3 ml of media was dispensed, was used. Individual pure bacterial colonies were then inoculated into 3 ml sterile distilled water, and a loopful of bacterial suspension was inoculated into each medium containing a single

carbohydrate source. The vials were capped with rubber stoppers, and acetylene gas was injected into the headspace of the vial at a final concentration of 10 % and incubated at 28°C for 48 hours without shaking. The amount of ethylene in the headspace was quantified as described above.

Results

Acetylene reduction assay (ARA) activity of plant materials

All of the thin (less than 2 mm in diameter) and thick (more than 2 mm) root samples collected from the various sites in the Philippines in March 2003 showed positive ARA activity. On the basis of the measured activity, the two thin-root samples were determined to possess higher nitrogen-fixing ability than thick roots, whereas the thin roots collected from Campo Guadalupe, Cebu, showed lower activity (Table 1). Of the samples collected in July 2003, the root and petiole samples showed high ARA activity, but the leaflet sample did not. The highest activity was measured in the root (961 nmol g^{-1} d^{-1}) and petiole (65.9 nmol g^{-1} d^{-1}) samples, but there was low activity in the upward root sample (10.2 nmol g^{-1} d^{-1}) (Table 1). In July 2004, in addition to the root samples, samples of other parts, such as the petiole, pith, and bark, were collected. All the selected samples showed positive ARA activity with high variation, ranging from 1.1 to 144 nmol g^{-1} d^{-1}. The lowest activity was from a pith sample collected from Banga, and the highest, from a pith sample collected from Hilsug, Leyte. However, the pith samples collected from Lezo, Panay, showed moderate activity of 109 nmol g^{-1} d^{-1}. Similarly, in the petiole samples, high variation in ARA activity was also observed, ranging from 24.5 to 65.9 nmol g^{-1} d^{-1}. In the case of the root samples, the activity varied from 4.5 to 961 nmol g^{-1} d^{-1} (Table 1).

Isolation and identification of nitrogen-fixing bacteria

The isolation of bacteria from the roots and petiole samples normally allowed the recovery of some nitrogen-fixing bacteria on a Rennie medium. Although many other bacteria grew on "N-free" media, this ability was not taken as an indicator of nitrogen fixation because roughly half of the bacteria that grew on the Rennie medium did not show positive ARA activity. No ARA positive strains were isolated from the bark and pith samples, and, in spite of that, they showed positive ARA activity.

According to 16S rDNA sequencing and a homology search by BLAST, most of the isolates with high ARA activity belonged to the *Enterobacteriaceae* family: *Pantoea agglomerans, Klebsiella oxytoca, K. pneumoniae*, and *Enterobacter cloacae* (Table 1). On the other hand, *Bacillus megaterium* isolates with positive ARA activity were successfully isolated from the root, petiole, and rachis samples. Of all species isolated, *K. pnuemoniae* strain RD3, isolated from the root sample collected in July 2004, showed the highest ARA activity of 37.1 nmol culture^{-1} h^{-1}. *K. oxytoca* strain RH1, isolated from a root sample, demonstrated 20.4 nmol culture^{-1} h^{-1}. Likewise, *Pantoea agglomerans* strains RA2 from a root sample and Mr2 from a petiole sample showed ARA values of 35.1 and 26.2 nmol culture^{-1} h^{-1}, respectively. *Enterobacter cloacae* strain RA1, isolated from a root sample, showed ARA values of 22.3 nmol culture^{-1} h^{-1}. Of all the isolates, the *B. megaterium* isolate from the petioles, rachis, and roots showed comparatively low ARA values that varied from 1.6 to 8.2 nmol culture^{-1} h^{-1}, although its corresponding source showed quite a high ARA value in the root (2310 nmol $g^{-1}d^{-1}$) and petiole (160 nmol $g^{-1}d^{-1}$) samples, while the rachis sample possessed a low activity of 1.6 nmol $g^{-1}d^{-1}$. The nitrogen fixers, *P. agglomerans* RA2 and *E. cloacae* RA1, were isolated from the same root sample and showed an ARA activity of 25.6 nmol g^{-1} d^{-1}. Similarly, *B. megaterium* Mr3 and *P. agglomerans* Mr2 were recovered from the petiole sample and demonstrated to have an ARA activity of 160 nmol g^{-1} d^{-1} (Table 1). *K. oxytoca* RH1 and *K. pnuemoniae* RH2 colonized the same root sample and showed an ARA activity of 32.1 nmol g^{-1} d^{-1}.

Another major identification tool used in this study was the physiological test for Gram-negative bacteria. API20E or API20NE (Table 1) showed almost similar identification to those shown by 16S rDNA sequencing. In the case of isolates RH2 and LR1, the closest species as identified by 16S rDNA were *K. pnuemoniae* and *Burkholderia tropicalis*, respectively. However, the physiological test (API 20NE) suggested *Sternotrophomonas maltophilia* for isolate RH2 and *Pasteurella haemolytica* for isolate LRI, which indicates similar physiological properties in the two species.

Enzymes for the substrate, such as L-lysine, L-ornithine, L-tryptophane, gelatin, and urea, were absent in most of the isolates, whereas the tests were positive for L-arginine and galactopyranoside (Table 2). Biochemical tests, such as the sodium thiosulphate and indole tests, were negative in almost all the isolates, while the citrate and Voges-Proskauer tests were positive. The oxidation/fermentation test of most carbohydrates was positive for almost all the strains, except for LR2.

Nitrogen-fixing activity under various carbon substrates

NFB isolated in this study showed a preference for carbon as a sole source of energy to fix high amounts of nitrogen. In all the isolates, a high nitrogen-fixing ability was observed with simple carbohydrates, such as glucose, sucrose, and mannitol (Table 3). In a glucose medium, high activity was observed in *K. pnuemoniae* RH2, *P. agglomerans* RA2, and *E. cloacae* RA1 with ARA values of 57.1, 40.5, and 42.5 nmol culture^{-1} h^{-1}, respectively. In a sucrose medium, *P. agglomerans* RA2 and *E. cloacae* RA1 showed ARA activities of 28.9 and 20.5 nmol culture^{-1} h^{-1}, respectively. Other carbon sources, such as starch, pectin, and hemi-cellulose, demonstrated low nitrogen-fixing ability (Table 3).

Discussion

In this study, nitrogen-fixing activity was detected in almost all the parts of the sago palm collected from the Philippine Islands of Leyte, Cebu, and Panay except for the leaflet samples, suggesting that nitrogen fixation may occur in the sago palm. Inubushi et al. (2005) suggested, using a stable isotope technique, that biological nitrogen fixation occurs in the sago palm. Nitrogen fixation in the sago palm will be supported by the successful isolation of nitrogen-fixing bacteria from the parts with higher ARA activity. In the present study, rates as high as 961 nmol g^{-1} h^{-1} were detected in the root sample, and two nitrogen-fixing strains were isolated. Both the API test and 16S rDNA sequencing suggested that one of the strains belongs to *Klebsiella pneumoniae*, while the other was identified as *Bacillus megaterium* by sequencing. The highest activity in the root sample might be due either to the high number of nitrogen-fixing bacteria or the favorable conditions for NFB. Kirchhof et al. (1997) observed a higher number of NFB associated with graminaceous plants under infertile conditions.

In terms of identification, the phylogenetic position is more reliable, while physiological tests make it possible to estimate the function or behavior of the microbes in the natural environment. Thus, the nearest species names based on the 16S rDNA sequencing of the isolates were used to describe the phylogenic position of NFB in this study. To our knowledge, this study constitutes the first report on the isolation and identification of NFB from the various parts of the sago palm.

The high variation in ARA activity, which ranged from 1.1 to 961 nmol g^{-1} d^{-1} (Table 1), suggested that there might be a heterogeneous distribution of NFB in the sago palm or various conditions for the expression of nitrogen-fixing ability. Microbial interactions, which generally occur in natural habitats between individual microbes, might be one of the factors involved in such a variation in nitrogen-fixing ability. These ecological phenomena might cause the conditions to become harmful or beneficial. No ARA positive strains were isolated from the bark and pith

samples in this study, which might be due to the heterogeneity of NFB, the preferences of NFB for the substrates in the natural sago palm habitat, or the need for beneficial interaction with non-NFB for the expression of nitrogen fixation. However, the results of this study illustrate an almost ubiquitous association between nitrogen-fixing bacteria and the sago palm. Whatever the specific relationships involved, plant colonization by bacteria constitutes a vast and, as yet, little mapped ecological niche (Sturz et al. 1997).

Oxygen tension is known as one of the environmental factors affecting nitrogen fixation (Haatehl et al. 1983). Haatehl et al. (1983) found that anaerobic conditions were required for the maximal expression of nitrogenase activity of *Klebsiella* spp. and *Enterobacter* spp. isolated from plants belonging to the Graminae family, such as creeping bent grass, red canary grass, and couch grass. In a natural microenvironment, bacterial respiration may decrease the oxygen level, thereby activating an oxygen-sensitive nitrogenase enzyme in NFB, which, in turn, enhances the nitrogen-fixing ability. In this study, NFB was individually inoculated into a Rennie medium and, therefore, the nitrogen-fixing activity might have increased in the presence of other non-NFB. The exchange of headspace gas with dinitrogen to decrease the oxygen level was not conducted in this study, although a semi-solid medium was used to generate heterogeneous microhabitats in a single vial. This study was the first step to reveal the diversity and characterization of nitrogen-fixing bacteria colonizing the sago palm, and, therefore, an aerobic isolation strategy was adopted. Anaerobic isolation is now in progress.

Although most of the diazotrophs described in the literature utilize simple carbon substrates, little is known of their nitrogenase activity in more complex types of carbon substrates. As efforts are being made to learn more about the potential carbon source for a nitrogen fixer in the natural environment, an understanding of their ability to utilize complex substrates and the mechanism whereby they fix nitrogen is essential.

The isolation of nitrogen-fixing *Klebsiella* from rice leaves (Ladha et al. 1983) and *K. pneumoniae* from maize stems (Palus et al. 1996) and the roots, stems, and leaves of banana (Martinez et al. 2003) has been reported. *K. oxytoca* has been isolated as an endophytic diazotroph from Japanese sweet potato stems (Asis and Adachi 2003). Other nitrogen-fixing *Klebsiella* spp. have been isolated from the stems of sweet potato cultivated in Brazil (Paula et al. 1993), surface-sterilized stems and roots of sweet corn and cotton (McInroy and Kloepper 1995a), and stems of *Zea mays* and *Z. luxurians* (Palus et al. 1996). These studies and our report of the isolation of *K. oxytoca* and *K. pnuemoniae* from roots suggested that *Klebsiella* species might colonize a variety of plants.

E. cloacae and *P. agglomerans* strains were isolated from the roots and petioles of the sago palm. These two genera are closely related and have been frequently reported as nitrogen fixers. *P. agglomerans* was isolated from sweet potato stems endophytically and was also detected in maize, winter wheat, and seeds of a deepwater rice variety as endophytic bacteria (Verma et al. 2001) as well as in the stems and roots of corn and cotton plants (McInroy and Kloepper 1995a). *E. cloacae* have been isolated from the roots of coco palm in Brazil (Fernandes 2001) and from the rhizosphere of rice (Mehnaz et al. 2001). Other studies have reported its isolation from the rhizosphere of wetland rice (Fujie et al. 1987) and the stems of potato (Reiter et al. 2002) and the roots, leaves, and stems of banana (Martinez et al. 2003).

Bacilli, especially nitrogen-fixing strains, were found in association with grass roots (Line and Loutit 1971). *Bacillus polymyxa* is a well-documented colonizer of the wheat rhizosphere (Chanway et al. 1988; Heulin et al. 1994) as well as of rice roots (Khammas et al. 1992). *B. circulans* was found to be a dominant N_2-fixing bacterium in maize rhizosphere in various regions of France (Berge et al. 1991). According to Bergey's manual (Holt et al. 1994), *B. megaterium* is not described as a nitrogen fixer, but

Rozycki et al. (1999) reported *B. megaterium* from oak root with an ARA activity of 13 nmol culture^{-1} h^{-1}. Recently, *B. megaterium*, with an ARA activity of 4.57 nmol culture^{-1} h $^{-1}$, has been isolated from rice plant rhizosphere in a temperate region of northeast China (Myoung et al. 2005). The present work is the first report of *B. megaterium* as a frequently isolated N_2-fixer from the roots, petioles, and rachis of the sago palm.

The occurrence of *Burkholderia* spp. in the sago palm is intriguing because diazotrophic members of this genus have recently been found to have substantial capability to colonize diverse host plants for N_2-fixation (Estrada-de los Santos et al. 2001). In this study, one strain *Burkholderia* spp. LR1 was isolated from the root, although it showed quite low activity.

The isolates *E. cloacae, P. agglomerans, K. pneumoniae, K. oxytoca,* and *B. megaterium* have been recovered from the roots of sago palm (Table 1); this may be solid evidence that the root is the most favorable part for NFB, as root exudation may provide sufficient and suitable carbon sources for nitrogen-fixing ability. Nitrogen fixation is often enhanced in the rhizosphere due to asymbiotic microorganisms utilizing root exudates as an energy source (Burgmann et al. 2005). However, on the root surface, NFB is in heavy competition with other microorganisms for root exudates as a nutritional source (Kirchhof et al. 1997). Therefore, further research is still necessary to investigate the colonization of NFB in the sago root.

BNF by nitrogen fixers is a process in which soil nitrogen is limited and adequate carbon sources are available (Kennedy et al. 2004). Our understanding of the ability of colonization of these bacteria in the sago palm is only beginning to expand; therefore, further research is essential, particularly, to estimate the amount of biologically fixed nitrogen that may contribute substantially to the nitrogen nutrition of the plant as well as the contribution of anaerobic NFB and a microbial consortium so as to establish the phenomenal success of the nitrogen-fixing ability in the sago palm.

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Running title

Free-living nitrogen-fixing bacteria are present in the sago palm tree

Table 1. Nitrogen fixing activity (nM ethylene produced g $^{-1}$ day $^{-1}$) in different parts of sago palm collected form the Philippines and affiliation of the bacterial strains isolated from the different parts of sago palm

Date of sampling	Parts	Locations	ARA activity Mean	(Individual values)	Strain name	ARA activity In Rennie	16S rDNA sequence Closest species	% similarity	API Test Closest species	% Homology
March 2003	Thick root	Labangon Tulik, Cebu	249	(248, 250)	LR1	0.1± 0.0	*Burkholderia sp.*	98	*Pasteurella haemolytica*	61
	Thin root		209							
	Thick root	Langsted, Cebu	89.2	(131, 89.2)						
	Thin root		495							
	Thick root	Campo Guadaklupe, Cebu	35.8	(47.4, 24.2)						
	Thin root		4.5							
	Thick root	Dulag, Leyte	36.2	(30.2, 35.1, 43.4)						
	Thin root		102	(57.3,146)						
July, 2003	Root	Dulag, Leyte	961	(266, 164, 1330, 183,1520, 2310)	RD1	2.6 ± 0.0	*Bacillus megaterium*	99		
					RD2	8.2 ± 0.7	*B. megaterium*	99		
					RD3	37.1 ± 0.3	*K. pnuemoniae*	97	*K. pneumoniae*	62
					RD4	12.4 ± 0.3	*K. pnuemoniae*	95	*K. pneumoniae*	62
	Upward root		10.2	(8.7, 17.1, 4.87)						
	Petiole		65.9	(57.6, 88.8)						
	Leaflet		0	(0, 0)						
July, 2004	Root	Hilusig, Leyte	25.5	(32.1, 20.4, 23.9)	RH1	20.4 ± 0.1	*Klebsiella oxytoca*	99	*K. pneumoniae*	99
					RH2	28.9 ± 0.6	*K. pnuemoniae*	99	*Stenotrophomonas maltophilia*	56
	Root	Alangalan, Leyte	13.3	(25.6, 13.7, 0.7)	RA1	22.3 ± 0.1	*Enterobacter cloacae*	95	*E. cloacae*	92
					RA2	35.7 ± 0.8	*Pantoea agglomerans*	95	*E. cloacae*	92
	Root	Balate, Panay	2.8	(5, 0.6)						
	Root	Lezo, Panaly	84	(109, 29.1, 114)						
	Root	Malinao, Panay	33.1	(40.2, 30.1, 29.0)						
	Rachis	Banga, Panay	1.6	(2.1, 2.2, 1, 1.1)	Mr4	1.6 ± 0.0	*B. megaterium*	96		
	Petiole	Lezo, Panay	24.5	(63.7, 1.3, 26.1, 6.2)						
	Petiole	Malinao, Panay	28.2	(2.6, 159.6, 1.6, 1.7, 1.9,1.7)	Mr1	1.9 ± 0.4	*B. megaterium*	99		
					Mr2	26.2 ± 0.0	*P. agglomerans*	99	*E. cloacae*	92
					Mr3	2.6 ± 0.0	*B. megaterium*	99		
	Pith	Banga, Panay	1.1	(0.8, 1.4)						
	Pith	Hilusig, Leyte	144	(152, 136)						
	Pith	Lezo, Panay	109	(137, 81.1)						
	Bark	Tayhawan,Leyte	42.3	(65.43, 19.09)						

Table 2. Physiological and biochemical test as demonstrated by API 20E and API 20NE tests

Diagnostic tests	Strain name							
	RA1	RA2	Mr2	RH1	RH2	RD3	RD4	LR1
Presence of enzyme								
2-nitrophenyl-β-D- galactopyranoside	+	+	+	+	+	+	+	+
L-arginine	-	-	-	-	+	+	+	-
L-lysine	-	-	-	-	+	+	+	-
L-ornithine	-	-	-	-	+	+	+	-
L-trytophane	-	-	-	-	+	+	+	-
Gelatin	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-
Biochemical test								
Trisodium citrate	+	+	+	+	+	+	+	NT
Sodium thiosulphate	-	-	-	-	-	-	-	NT
Indole	-	-	-	-	-	-	-	NT
Vogues-Proskauer	+	+	+	+	+	+	+	NT
Fermentation/Oxidation								
Glucose	+	+	+	+	+	+	+	+
Mannitol	-	+	-	+	+	+	+	-
Inositol	+	-	+	-	+	+	+	-
D-sorbitol	+	+	+	+	+	+	+	-
L-rhamnose	+	+	+	+	+	+	+	-
D-sucrose	+	+	+	+	+	+	+	-
D-melibiose	+	+	+	+	+	+	+	-
Amygdalin	+	+	+	+	+	+	+	-
L-arabinose	+	+	+	+	+	+	+	-
Other tests in API 20NE								
Potassium nitrate	NT	NT	NT	NT	NT	NT	NT	+
Esculin ferric citrate	NT	NT	NT	NT	NT	NT	NT	-
D-mannose	NT	NT	NT	NT	NT	NT	NT	-
N-acetylglucosamine	NT	NT	NT	NT	NT	NT	NT	-
D-maltose	NT	NT	NT	NT	NT	NT	NT	-
Potassium gluconate	NT	NT	NT	NT	NT	NT	NT	-
Capric acid	NT	NT	NT	NT	NT	NT	NT	-
Adipic acid	NT	NT	NT	NT	NT	NT	NT	-
Malic acid	NT	NT	NT	NT	NT	NT	NT	-
Phenylacetic acid	NT	NT	NT	NT	NT	NT	NT	-

NT:Not tested

Table 3. Nitrogen-fixing activity of the sago palm isolates in media with various carbon sources

Strain name	Bacterial species by 16S rDNA	ARA activity (nmol ethylene produced culture^{-1} h^{-1})							
		Rennie	Glucose	Sucrose	Mannitol	Lactate	Starch	Pectin	Hemi-cellulose
RA1(Root)	*Enterobacter cloacae*	22.3 ± 0.1	42.5 ± 0.8	20.5 ± 0.6	2.2 ± 0.0	6.6 ± 0.2	1.0 ± 0.0	21 ± 0.1	0
RA2(Root)	*Pantoea agglomerans*	35.7 ± 0.8	40.5 ± 1.7	28.9 ± 0.0	0.0	2.9 ± 0.0	0.9 ± 0.1	6.5 ± 0.2	0.6 ± 0.0
RH1(Root)	*Klebsiella oxytoca*	20.4 ± 0.1	0.8 ± 0.0	2.2 ± 0.0	1.1 ± 0.0	2.2 ± 0.0	1.0 ± 0.1	1.9 ± 0.0	0
RH2(Root)	*K. pnuemoniae*	28.9 ± 0.6	57.1 ± 0.9	2.8 ± 0.1	0.0	2.7 ± 0.1	1.5 ± 0.0	3.3 ± 0.1	0.4 ± 0.0
RD1(Root)	*Bacillus megaterium*	2.6 ± 0.0	9.4 ± 0.2	7.9 ± 0.1	0.0	0.0	0.2 ± 0.0	0.0	0.0
RD2(Root)	*B. megaterium*	8.2 ± 0.7	1.7 ± 0.0	0.3 ± 0.0	0.0	0.0	0.3 ± 0.0	0.0	0.0
RD3(Root)	*K. pnuemoniae*	37.0 ± 0.3	0.2 ± 0.0	0.2 ± 0.0	0.0	0.3 ± 0.0	0.2 ± 0.1	0.0	0.0
RD4(Root)	*K. pnuemoniae*	12.4 ± 0.3	25.0 ± 0.8	7.2 ± 0.0	0.0	0.0	0.2 ± 0.0	0.0	0.0
LR1(Root)	*Burkholderia* sp.	0.1 ± 0.0	0.1 ± 0.0	0.0	0.0	0.1 ± 0.0	0.0	0.1 ± 0.0	0.1 ± 0.0
Mr1(Petiole)	*B. megaterium*	1.9 ± 0.4	0.4 ± 0.3	2.2 ± 0.0	0.5 ± 0.0	2.0 ± 0.0	0.8 ± 0.3	2.1 ± 0.0	0.0
Mr2(Petiole)	*P. agglomerans*	26.2 ± 0.0	32.5 ± 1.7	30.7 ± 0.7	0.0	3.5 ± 0.1	1.2 ± 0.3	4.7 ± 0.1	0.0
Mr3(Petiole)	*B. megaterium*	2.6 ± 0.0	0.0	2.2 ± 0.0	7.1 ± 0.1	2.0 ± 0.0	2.4 ± 0.4	5.4 ± 0.1	0.0
Mr4(Rachis)	*B. megaterium*	1.6 ± 0.0	0.0	1.9 ± 0.0	0.0	2.1 ± 0.0	3.5 ± 0.1	2.0 ± 0.0	0.0

Each ARA value represents mean of triplicates ± SD