Growth promotion of rice by inoculation of acid-tolerant, N₂-fixing bacteria isolated from acid sulfate paddy soil in South Kalimantan, Indonesia

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ABSTRACT In South Kalimantan, Indonesia, a large area of rice paddy field is suffered with acid sulfate soil and the productivity of rice is supposed to be low. While in some paddy fields we have found that the yield of rice is larger than 5 ton ha⁻¹ without any fertilizer. There assumed to be the contribution of nitrogen fixing bacteria for rice production by biological nitrogen fixation (BNF) under those low pH condition. We have screened bacteria from the rhizosphere and rhizoplane of local rice plant growing in natural field based on their growth on nitrogen free medium. We have obtained 26 isolates, and the majority of isolated bacteria belonged to the β -proteobacteria (34.6 %) and others are α -proteobacteria (19.2 %), γ -proteobacteria (0.38 %), Bacilli (19.2 %), and Actinobacteria (23.1 %) based on their 16S rDNA sequence. We have determined their nitrogenase activity and the existence of *nifH* gene by using PCR technique. Though all of the isolates can grow on nitrogen free medium, only five isolates belong to β -proteobacteria have high nitrogenase activity. The existence of *nifH* gene was also confirmed in theses five isolates by using PCR technique. We presume that one of the mechanisms of the adaptation of these bacteria under acid sulfate soil is changing the surrounding pH. In bacterial culture medium, the change of pH during the incubation of these isolates was deeply regulated by the existence of several organic acid anions. In rice culture with soil experiments, under initial soil pH 3.5 condition, though rice growth was inhibited with the addition of 500 μ M Al, inoculation with obtained isolate significantly alleviated Al stress. It is suggested that beneficial interactions of rice and free living nitrogen fixing bacteria might be operating to

reducing root Al stress in acid sulfate soil.

Key words: rice, nitrogen fixing bacteria, interaction, acid sulfate soil, isolation, organic acid, rhizosphere, rhizoplane, Al-resistance, pH increasing

Abbreviations: BNF, biological nitrogen fixation; N, nitrogen

INTRODUCTION

Beneficial interactions between plant and microbes in the rhizosphere are the determinants of plant growth and soil fertility (Jeffries et al. 2003). Plant-microbe interactions in the rhizosphere can be beneficial to the plant, the microbes or to neither of them. However, our focus should be to exploit the beneficial interaction of plants and microbes.

Nitrogen availability is the major limiting factor for the growth of many crops including rice, (Osaki et al. 1992; Trn Van et al. 1994; Trn Van et al. 2000). While in Banjarmasin of South Kalimantan, Indonesia, Purnomo Erry et al. (2004) reported that local farmers culture very high yield variety, called as Padi Panjang, which is native modern cultural variety selected from progenitor Padi Bayar by farmer, and can yield more than 4 t ha⁻¹, 7-8 t ha⁻¹ at highest in acid sulfate soil, without any application of nitrogen (N) fertilizer. This fact indicates a beneficial interaction exist in these rice growing system. In this system, the contribution of biological nitrogen fixation (BNF) might be operating as endophytes and/or rhizobacteria. In those paddy fields, the diazotrophs play a vertical role in the maintenance and building-up soil fertility, and then consequently increase N uptake, rice growth and yield (Kennedy et al. 2004; Song et al.

2005). On the other hand, microorganisms especially diazotrophs that are metabolically active in such an extremely acidic environment are important for their unique ecology and physiology and named as acidophilic microorganism (Bond et al. 2000; Burton et al. 2000; Johnson et al. 2001; Brofft et al. 2002; González-Toril et al. 2003). Though, acidophilic microorganisms have been intensively studied (Johnson, 1998; Johnson et al. 2003), but studies on acidophilic diazotrophs are limited (Stephan et al. 1991; Kuhner et al. 2000).

It is generally believed that acid sulfate soils are generally characterized as very strong acid (pH lower than 3–4) under peat soil in tropical coastal zone and in the areas originating from sediment or pyrogenic rocks because of drainage of water from acid sulfate soil (Sammut et al. 1996; Wilson et al. 1999; Smith et al. 2004). In acid sulfate paddy soil, though Padi Panjang rice seedlings suffer very acid condition and no fertilizer application, it can still yield high production, thus we speculated that functional bacteria such as nitrogen fixing bacteria may therefore play important role in high yield rice growth and nitrogen acquisition. To increase the soil pH may play a major role in the bioavailability of many pH-dependent nutrients, and decrease the effects of potentially toxic metals such as Al. Thus, inoculation experiment was conducted to mimic natural acid sulfate soil conditions to confirm whether there is beneficial interaction which can help to improve rice seedlings growth under very acid conditions.

In the present study, attempts were also made to identify potent plant growth promoting rhizobacteria for rice which may prove better inoculants for the rice. Thus, 26 rhizobacterial isolates were obtained from rice rhizosphere on the basis of N-free medium plating technique. A total of 5 isolates were selected on the basis of high ARA. Four out of five selected isolates were further tested for other characteristics under mixture carbon source incubation condition. Two isolates were further evaluated to examine the influence of these rhizobacteria on the growth (biomass) of rice under mimic acid sulfate soil condition. We also tried to understand the possible mechanism(s) involved in the

promotion of rice growth upon nitrogen fixing bacteria inoculation. Thus, isolation and incubation of diazotrophs have been conducted to check the nitrogen fixing and pH increasing ability under acid conditions.

MATERIALS AND METHODS

Experiment 1

Nitrogen fixing bacteria isolation and classification

Sampling sites description

Rice roots and soil samples were collected from rice paddy field at heading stage from two different local rice cultural varieties of Siam Unus (site 1; 3°10'13"N, 114°36'06"E), and Padi Panjang (site 2; 3°22'25"N, 114°32'18"E). Sites 1 and 2 were located at in Belandean District and Kuin District of South Kalimantan, Indonesia respectively.

Samples were collected from Siam Unus field in August 2002 and from Padi Panjang field in August 2004, respectively.

Preparation of soil and rhizosphere samples

The bulk soils (the region of soil without the influence of the root) and rhizosphere soils were sampled in plastic bags from the surface to a depth of 10 cm with sterile spatulas between rice rows and under the canopy of rice plants within a row, respectively. Obtained samples were immediately transferred to the laboratory and stored at 10 °C for successive analysis. The characteristics of soils are shown in Table 1.

Screening of bacteria

To isolate free living nitrogen fixing bacteria, a modified Winogradsky's nitrogen free mineral medium (Hino & Wilson, 1958) was prepared with the following composition (mM): KH_2PO_4 , 18.28; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.05; NaCl, 21.28; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.22, Na_2MoO_4 , 0.21, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.33. To 1000 ml of medium solution, 10 g of glucose and 100 mg powdery CaCO_3 were added into the medium. Depending on the purpose such as semi-solid or solid medium, 0.24 % gelrite (Wako Pure Chemical industries, Japan) and 1.5 % agar powders (Wako Pure Chemical industries, Japan) were added to the medium

Table 1. Characteristics of soils.

Location	pH (H ₂ O)	CEC (cmol kg ⁻¹ soil)	Organic C (%)	Organic N (%)	Total N (g kg ⁻¹ soil)	P Bray 1	Textural classification
Site 1	3.62	17	11	1.66	NT	0.14	NT
Site 2	4.02	37.5	4.86	NT	0.28	3.51	Silty clay loam

NT, not test

respectively. The pH was adjusted to 6.8.

For determination of nitrogenase activity of isolates, Rennie medium (RM) was prepared for acetylene reduction assay from solutions A and B (Elbeltagy et al. 2001). Solution A consisted of 0.8 g of K_2HPO_4 , 0.2 g of KH_2PO_4 , 0.1 g of NaCl, 28 mg of $Na_2FeEDTA$, 25 mg of $Na_2MoO_4 \cdot 2H_2O$, 100 mg of Bacto™ Yeast Extract (Difco Laboratories, subsidiary of Becton, Dickinson and Company), 3.0 g of mannitol, 5.0 g of sucrose, 0.5 ml of 60 % (v/v) sodium lactate, 2.0 g of sodium malate, 2.4 g of gelrite (for semisolid medium) and 900 ml of distilled water (the final pH of solution A was adjusted to 7.0 before autoclaving). Solution B consisted of 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of $CaCl_2 \cdot 2H_2O$, and 100 ml of distilled water. The solutions were autoclaved separately and mixed after cooling. Filter-sterilized biotin and *p*-aminobenzoic acid (100 µl each) were added at final concentrations of 5 and 10 µg/liter, respectively.

Isolating bacteria from soil, five-gram of fresh soil were transferred to glass bottles containing 45 ml of Winogradsky's mineral liquid medium, then shaken for 60 min at room temperature for free living bacterial suspension preparation (Othmana et al. 2004). For isolating bacteria from the surface of root, one-gram fresh roots was taken and washed with sterile water and dipped with parafilm at the both end were transferred to glass bottles containing 45 ml of Winogradsky's mineral liquid medium then shaken for 60 min at room temperature as rhizoplane bacterial suspension preparation (Othmana et al. 2004). Bacterial suspensions were sequentially diluted from 10^0 to 10^7 in 0.9 % (w/v) NaCl solution and then 100 µl of the diluted solution was inoculated into 7 ml of vial containing 3 ml of semi-solid Winogradsky's mineral medium with the prepared original dilutions. After static incubation for 7 days or until a significant increase in turbidity was observed at 30 °C, acetylene reducing activity (ARA) of culture vials was measured according to the method of Hegazi (1980) to monitor nitrogen fixing ability. Cultures producing more than 5 nmole C_2H_4 vial⁻¹ h⁻¹ were selected for bacteria isolation. Take 100 µl of 10^2 and 10^4 diluted selected cultures solution to streak on Winogradsky's mineral medium agar plates. Plates were incubated at 26 °C about 5 to 7 days. Isolated colonies were further purified by successive streaking on agar plates of Winogradsky's mineral medium (usually four to six times). Pure isolates were re-examined for ARA with Rennie medium and then stored cryogenically at -80 °C (Kennedy et al. 1991; Adam & Zdor, 2001).

DNA isolation, PCR amplification and sequencing of the 16S rDNA gene

For genomic DNA extraction, the isolates were cultured in a modified of soft RM which containing 0.2–0.3 % gelrite (pH 7.0) for 48 h at 30 °C, then harvested by centrifugation at $6000 \times g$ for 30 min at 4 °C and washed twice by adding sterile phosphate buffered saline (PBS) solution on the pellet and DNA extraction was performed by using ISOPLANT II (Nippon Gene, Japan) according to the instructions of manufacturers. Each 16S rDNA gene was amplified by PCR using universal primers of pr0R (5'-AGAGTTTGATCCTGGCTCAG-3') corresponding to positions 8 to 27 and 1492rev (5'-GGCTACCTTGTTACGACTT-3') corresponding to positions 1510 to 1492 of the *Escherichia coli* numbering system. The amplification of the 16S rDNA specific fragments was performed by using the following program: 5 min at 95 °C followed by 35 cycles of 1-min denaturation at 95 °C, 1-min annealing at 55 °C, and 2-min extension at 72 °C followed by 10 min at 72 °C and cooling at 4 °C by using TaKaRa PCR thermocycler. The PCR products were visualized by electrophoresis on 1.5 % agarose gel with SYBR-green I (Molecular Probes Inc., Japan). After checking the PCR product gives only one band, the PCR product was purified by using Exosap-IT (USB Corp., Cleveland, OH, USA). A modified Exosap-IT protocol is as follows: 0.2 µl of ExoSAP-IT and 1.8 µl sterile milliQ water were added directly to 5 µl PCR products and incubated at 37 °C for 30 minutes. After removing unwanted dNTPs and primers from PCR products, two hydrolytic enzymes of ExoSAP-IT, Exonuclease I and Shrimp Alkaline Phosphatase were inactivated simply by heating to 80 °C for 15 minutes.

Sequencing was performed with Genome Lab™ DTCS-Quick Start Sequencing Kit (Beckman Coulter Inc, Fullerton, CA, USA) and a CEQ™ 8000 Genetic analysis system sequencer (Beckman Coulter Inc, Fullerton, CA, USA). A modified protocol was as follows: the DNA sequencing reaction was prepared using 4 µl master-mix containing thermo sequenase DNA polymerase, pyrophosphatase, reaction buffer, dNTPs and dye terminator, 1.6 pmol primer and DNA templates added with 15 ng PCR products. These reagents were mixed and sterile milliQ water was added to 10 µl. Five primers including 27f, U529r, E786F, U926R and 1492r (shows in Table 2) were used to sequence whole strand of the 16S rDNA. For sequence primer 27f and 1492r, the PCR operation condition program was as follows: 1-sec at 96 °C followed by 30 cycles of 20-sec denaturation at 96 °C, 20-sec annealing at 50 °C, and 4-min extension at 60 °C

and cooling at 4 °C. For other three primers U529r, E786F and U926R, PCR program was the same as follows: 1-sec at 96 °C followed by 30 cycles of 20-sec denaturation at 96 °C, 20-sec annealing at 44 °C, and 4-min extension at 60 °C and cooling at 4 °C. Reactions were stopped by immediate cooling on ice and addition of 1 µl 3 M NaOAc, 1 µl 100 mM EDTA and 0.5 µl of UltraPure™ glycogen (Invitrogen, USA) to 10 µl of sequencing PCR products. Samples were precipitated with ethanol. The PCR product was added with 30 µl of 99.5 % EtOH, centrifuged at 14000g 4 °C, 15 minutes. Then discard the washing suspension solution through flash under 250 rpm. And then wash the sequence PCR products twice with 200 µl of 70 % EtOH. After purification, the samples were dried and dissolved in Sample Loading Solution (Beckman Coulter Inc., Fullerton, CA, USA). Finally, the sample plate with the purified sequence PCR product was put on a CEQ8000 Sequencing System (Beckman Coulter Inc., Fullerton, CA, USA) for sequence determination. Ribosomal Database Project-II (<http://rdp.cme.msu.edu/>) was used to compare the resulting sequences with those identified in Ribosomal Databases.

Table 2. Primers used in this study.

Name	Sequence (5'-3')
27F	AGAGTTTGATCCTGGCTCAG
U529R	ACCGCGGCKGCTGGC
E786F	GATTAGATACCCTGGTAG
U926R	CCGTCAATTCCTTTAGTTT
1492R	GGCTACCTTGTTACGACTT

Nested nifH PCR amplification

Fragments of *nifH* genes were amplified using nested PCR (Poly et al. 2001a; Diallo et al. 2004). Two pairs of primers were used: the first pair (FGPH19/PolR) were those defined by Poly et al. (2001a) as the most successful to amplify a 320 base pair *nifH* fragment from DNA isolated from a wide range of diazotrophic microorganisms. The second pair of primers was AQER/PolF. The first PCR was performed with a 20 µl reaction mixture containing 5 ng of purified DNA. The nested reaction was also performed with a 20 µl reaction mixture containing 1 µl of 100 times diluted the first PCR product as the template. After the initial denaturation consisting of 1 sec at 94 °C, amplification was carried out for both reactions for 30 cycles as follows: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C for the first PCR and 48 °C for the second PCR, and extension for 2 min at 72 °C.

A final 5 min extension step at 72 °C was performed and then cooling to maintain at 4 °C. The quality and quantity of the amplification products were analyzed on 1.5 % UltraPure agarose gels (Cambrex Bioscience Rockland Inc. Rockland. ME. USA).

Sequence alignment and phylogenetic analysis

The partial 16S rDNA sequences of selected representative clones obtained in this study were compared to the GenBank database by using the algorithm BLAST-N to identify the most similar 16S rDNA sequences (Da Silva et al. 2003). The strains were assigned to a genus based on the obtained 16S rDNA sequence similarity.

Experiment 2

Characteristics of N- fixing bacteria

Nitrogenase activity determination

For screening of nitrogen fixing bacteria, the Acetylene Reduction Assay (ARA) has been conducted as screening parameter. Briefly, the semisolid RM medium (pH 7.0) was inoculated with the isolates and incubated for 7 days at 30 °C without shaking. Pure acetylene gas (Hokkaido Acetylene Inc. Japan) was introduced to make a final concentration of 10 % acetylene in the air space of the vial. The produced ethylene (C₂H₄) concentrations in 0.5 ml headspace samples were determined using a gas chromatograph (GC-4000 Gas Chromatograph, GL science, Japan) equipped with a H₂-FID detector and Poropak-N column.

Bacteria growth under aerobic and acid condition with sugar and organic salt as carbon source

Time course experiments of bacteria growth were conducted to check pH change of the culture solution. A 300-ml inoculated culture of each isolate was incubated in 500 ml glass flask aerobically (100 rpm) at 30 °C in initial pH 4.0 of liquid Rennie medium supplying with sugar and organic salt (malate, lactic acid, sucrose and mannitol) mixture carbon sources and only sugar (sucrose and mannitol) carbon sources. The experiment was conducted with 3 replicates. Flasks containing culture medium without isolate inoculums were included as controls. The pH of the solution was measured at 0, 1, 2, 3, 5 days after inoculation. Ten ml of the cultured solution has been taken for the determination of bacteria growth, pH at every sampling time. The change in the concentration of the bacterial suspension during the time of cultivation was characterized by OD600 values obtained on spectrophotometer (Hitachi, Ltd. Tokyo, Japan). The pH

was measured by pH meter (F-51, HORIBA, CITY Japan).

Experiment 3

Construction of beneficial interaction between rice and nitrogen fixing bacteria

Acid sulfate soil preparation

The experimental design attempted to mimic acid sulfate soil, in brief, 20 g of air-dry cover soil was added into 20 ml deionized water in 50 ml falcon tubes then Soil pH was adjusted to 3.5 or 4.8 by adding 1 M H₂SO₄. Finally, Al was added as 1 M AlCl₃ to a final concentration 500 µM of Al. Without addition of Al treatments were as control.

Preparation of rice and bacteria

Rice (*Oryza sativa* L. cv. Padi Panjang and Nipponbare) seeds were surface sterilized in a 2.5 % solution of sodium hypochlorite (Sigma, USA) for 15 min followed by thorough washing in distilled water, and then germinated on filter paper for one week. One-week-old seedlings were cultured in standard plant culture solution (pH 5.0). Culture solution containing: Macronutrients 2.1 mM N (NH₄NO₃), 0.32 mM P (NaH₂PO₄ · 7 H₂O), 0.77 mM K (ratio 0.26 mM K₂SO₄ : 0.26 mM KCl), 1.2 mM Ca (CaCl₂ · 2 H₂O), 0.82 mM Mg (MgSO₄ · 7 H₂O); and micronutrients 36 µM Fe (FeSO₄ · 7 H₂O), 9.1 µM Mn (MnSO₄ · 5H₂O), 46 µM B (H₃BO₃), 3.1 µM Zn (ZnSO₄ · 7 H₂O), 0.16 µM Cu (CuSO₄ · 5 H₂O), 0.052 µM Mo ((NH₄)₆Mo₇O₂₄ · 4 H₂O) hydroponic nutrient culture solution. The solution was changed twice a week. Rice plants were cultivated and constantly pumped air into culture solution in a growth room for 2 weeks with a 16 h light: 8 h dark cycle at 28 °C in a growth room.

Cells of isolates H11 and Su10 were cultured in 500 ml flasks, containing 300 ml of sterile nutrient Luria-Bertani broth (LB). To obtain bacterial cultures in late log phase, flasks were incubated overnight on a rotary shaker (100 r.p.m.; 30 °C). Bacterial cells were harvested by centrifugation (7,000 g, 4 °C for 20 min). The bacterial pellet was then re-suspended once in sterile saline solution (9 g l⁻¹ NaCl) and centrifuged again (7,000 g, 4 °C for 20 min). Pellets cells were then re-suspended in sterile saline solution (9 g l⁻¹ NaCl) to 1/10 of original volume; the inoculation mixture thus contains approximately 8 × 10¹⁰ CFU ml⁻¹.

Incorporation of bacteria into soil

The bacterial suspension was added to pH 3.5 and 4.8 tubes containing 20 g air-dry soil, to make a population density of 10⁸ and 10⁶ cfu g⁻¹ air-dry soil. Inoculated soils were shaken by rotary shaker (100 r.p.m) to ensure that

an even distribution of the inoculated bacterium.

Rice growth with or without inoculation

Two 3-week-old rice seedlings were transplanted to bacteria incorporated soil. Rice plants were cultivated in a growth chamber for 3 or 5 weeks with a 16 h light: 8 h dark cycle at 28 °C in a growth room, respectively. Rice plant was watered every two days with deionized water. Plant nitrogen status was determined by leaf chlorophyll meter (SPAD-502, Minolta Camera Co. Ltd., Osaka, Japan). Two uppermost fully expanded leaves SPAD were used to judge nitrogen status (Johnkuty et al., 2000; Wang et al., 2006). After measuring shoot height, rice plants were divided into shoots and roots then dried in a forced-draft oven at 80 °C for 3 days.

RESULTS

Isolation of bacteria

Twenty six bacterial isolates were selectively isolated from the rhizosphere and rhizoplane of local rice plant by using nitrogen free medium. Modified Winogradsky's nitrogen free mineral medium containing 10 % glucose was used for growth of bacteria. There were 5 *α*-proteobacteria, 9 *β*-proteobacteria, 1 *γ*-proteobacterium, 5 bacilli and 6 actinobacteria respectively (Table 3). Of the 26 isolates, there are 5 isolates which have high nitrogenase activities including H3, H11, H14, H15 and Su10 (Table 4). Though other isolates also present nitrogenase activities, the values of their activities are relatively low compare with that of those 5 isolates.

The majority of the isolates belonged to the *β*-proteobacteria (34.6 %) and others are *α*-proteobacteria (19.2 %), *γ*-proteobacteria (0.38 %), Bacilli (19.2 %), and Actinobacteria (23.1 %). Of the 26 isolates, the existence of *nifH* gene was confirmed only from 5 isolates (Isolates H3, H11, H14, H15 and Su10) by using PCR with universal primers for *nifH* gene (Table 4) and these isolates belong to *Burkholderia* based on 16S rDNA sequence (Table 3).

Bacterial growth with sugar and/or organic compounds

The bacterial growth in mixed carbon sources, pH change and nitrogenase activity of the cultured medium were investigated by using high nitrogenase activity isolates H3, H11, H15, Su10. All of the tested isolates grew in pH 4.0 Rennie medium. The fastest growth was found in Su10 (Fig. 1). The pH of the medium increased rapidly during the substantial bacteria growth of *nifH* confirmed isolates (Fig. 2). As shown in Fig. 2, when H11, Su10, H15 and H3

Table 3. Isolates taxa associated with bacteria 16S rDNA sequence by using Ribosomal Database Project-II (RDP-II).

SN	Location	Isolation point	Phylum	Class	Order	Family	Genus
H20	1	Rhizosphere	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhizobiaceae</i>	<i>Rhizobium</i>
H8	1	Rhizosphere	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhizobiaceae</i>	<i>Rhizobium</i>
H17	1	Rhizosphere	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhizobiaceae</i>	<i>Rhizobium</i>
H31	1	Rhizosphere	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Bradyrhizobiaceae</i>	<i>Rhodopseudomonas</i>
203	2	Rhizosphere	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Caulobacterales</i>	<i>Caulobacteraceae</i>	<i>Brevundimonas</i>
H27	1	Rhizosphere	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Incertae sedis</i>	<i>Aquabacterium</i>
H3	1	Rhizosphere	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Burkholderiaceae</i>	<i>Burkholderia</i>
H14	1	Rhizosphere	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Burkholderiaceae</i>	<i>Burkholderia</i>
H11	1	Rhizoplane	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Burkholderiaceae</i>	<i>Burkholderia</i>
H15	1	Rhizoplane	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Burkholderiaceae</i>	<i>Burkholderia</i>
H19	1	Rhizoplane	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Burkholderiaceae</i>	<i>Burkholderia</i>
Su10	2	Rhizoplane	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Burkholderiaceae</i>	<i>Burkholderia</i>
H25	1	Rhizosphere	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Comamonadaceae</i>	<i>Acidovorax</i>
212	2	Rhizosphere	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Comamonadaceae</i>	<i>Delftia</i>
H10	1	Rhizosphere	<i>Proteobacteria</i>	<i>Gammaaproteobacteria</i>	<i>Xanthomonadales</i>	<i>Xanthomonadaceae</i>	<i>Fratureia</i>
H22	1	Rhizosphere	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
H37	1	Rhizosphere	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
H21	1	Rhizosphere	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
H2	1	Rhizosphere	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
H36	1	Rhizosphere	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Paenibacillaceae</i>	<i>Paenibacillus</i>
H33	1	Rhizosphere	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Actinomycetales</i>	<i>Arthrobacter</i>
H28	1	Rhizosphere	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Intrasporangiaceae</i>	<i>Intrasporangium</i>
H7	1	Rhizosphere	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Mycobacteriaceae</i>	<i>Mycobacterium</i>
H32	1	Rhizosphere	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Nocardiaceae</i>	<i>Rhodococcus</i>
H39	1	Rhizosphere	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Streptomycetaceae</i>	<i>Streptomyces</i>
H6	1	Rhizosphere	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Streptomycetaceae</i>	<i>Streptomyces</i>

SN, series isolate number

Table 4. Isolates nitrogenase activity and their closest associated bacteria accession number in Ribosomal Database Project-II (RDP-II).

SN	Nitrogenase activity (nmol C ₂ H ₄ /vial/h)	<i>nifH</i> amplification	Closest strain in RP-II data base	Closest strain ID	Similarity score
H2	0.02	-	<i>Bacillus niacini</i> (T)	IFO15566	0.836
H3	14.25	+	<i>Burkholderia</i> sp.	ON1	0.904
H6	0.00	-	<i>Streptomyces tumescens</i>	Km-2-1	0.936
H7	0.00	-	<i>Mycobacterium</i> sp.	HE5	0.942
H8	0.00	-	<i>Rhizobium</i> genosp	BDV5102	0.958
H10	0.00	-	<i>Fratureia aurantia</i>	IFO3245	0.861
H11	12.86	+	<i>Burkholderia sacchari</i>	AF263278	0.871
H14	12.73	+	<i>Burkholderia</i> sp.	STM1441	0.917
H15	14.97	+	<i>Burkholderia</i> sp.	ON1	0.902
H17	0.00	-	<i>Rhizobium</i> genosp	BDV5102	0.96
H19	0.00	-	<i>Burkholderia pyrrocinia</i>	R11805	0.973
H20	0.00	-	<i>Rhizobium</i> genosp	BDV5102	0.873
H21	0.00	-	<i>Bacillus</i> sp.	No.49	0.971
H22	0.00	-	<i>uncultured eubacterium</i>	AJ222833	0.885
H25	0.00	-	<i>Acidovorax</i> sp.	BSB421	0.995
H27	0.00	-	<i>uncultured bacterium</i>	AY212679	0.732
H28	0.22	-	<i>Intrasporangium calvum</i> ; type strain	DSM43043	0.856
H31	0.00	-	<i>Rhodopseudomonas palustris</i>	DSM126	0.977
H32	0.00	-	<i>Rhodococcus equi</i> (T)	ATCC7005T	0.971
H33	0.00	-	<i>Arthrobacter atrocyaneus</i> (T)	DSM20127	0.875
H36	0.00	-	<i>Bacillus</i> sp.	GL1	0.874
H37	0.00	-	<i>Bacillus fumarioli</i>	LMG18418	0.882
H39	0.00	-	<i>Streptacidiphilus luteoalbus</i>	IS069	0.906
203	0.00	-	<i>uncultured alpha proteobacterium</i>	S15A-MN37	0.855
212	0.00	-	<i>elftia</i> sp.	EK3	0.951
Su10	13.27	+	<i>Burkholderia vietnamiensis</i> (T)	TVV 70	0.989

SN, isolate series number; +, *nifH* gene PCR amplification; -, non *nifH* gene PCR amplification.

were cultivated in organic salt and sugar mixture media, the pH of the media increased from 4.0 to approximately 0.5 to 2 pH units during their growth. Significant pH change was found in isolate H11.

The bacteria isolates Su10 and H11 can also grow in the Rennie medium supplemented with sucrose and mannitol but without malate and lactic acid, at initialing pH 4.0 (Fig. 3). While the pH of media inoculated with isolate Su10 and H11 decreased approximately 1.2 and 0.6 pH units after incubation respectively (Fig. 4).

Nitrogenase activity

The nitrogenase activity (acetylene reduction activity) was measured on semi-solid Rennie medium with all the isolates. Only 7 of the 26 isolates tested have high reducing acetylene ability (Table 4). Some of them have very high nitrogenase activity, such as isolates H3, H11, H14, H15, and Su10, which all belonged to *Betaproteobacteria*. Most of them were isolated from rhizoplane except for H3 isolated from rhizosphere.

Growth promotion of rice by isolates H11 and Su10 under Al stress condition

Both Padi Panjang and Nipponbare seedling growth were inhibited with addition of 500 μ M Al at initial pH 3.5 after 5 weeks growing. Inoculation of Padi Panjang with both isolates H11 and Su10 alleviated this inhibition after 5 weeks growing compared with the un-inoculated control (Table 5). Promoted growth of Nipponbare by isolate H11 was also observed. There was, however, no significant difference in initial pH 4.8 with or without Al. Neither Su10 nor H11 affected rice leaf SPAD reading and plant height after 5 weeks growing (data not shown).

DISCUSSION

N_2 -fixing *Burkholderia* isolates were screened from the rhizosphere, rhizoplane of rice plants grown in tropic acid sulfate soil in Indonesia. The 16S rRNA gene sequence of all the isolates representing different genotypes, were compared with available 16S rRNA gene sequences database (Table 3). The similarity among the

Fig. 1. Free living nitrogen fixing bacteria were grown in initialing pH 4.0 Rennie liquid medium with 300 ml culture solution in 500 ml Erlenmeyer flask incubated in 30°C at 100 rpm. Samples were taken at 0, 1, 2, 3, 5 days respectively and data shown are mean values of three replicates assays. Bars indicate standard error (SE) and symbols without bars means that the SE is so small that it is covered by the symbols. NB, No bacteria; H3, isolate H3; H11, isolate H11; H15, isolate H15; Su10, isolate Su10.

Fig. 2. pH changes in Rennie liquid medium in bacteria the growth process at the initialing pH 4.0. Cultures were incubated at 30 °C in a rotatory shaker (100 rpm). Samples were taken at 0, 1, 2, 3, 5 days respectively and data shown are mean values of three replicates assays. Bars indicate standard error (SE) and symbols without bars means that the SE is so small that it is covered by the symbols. NB, No bacteria; H3, isolate H3; H11, isolate H11; H15, isolate H15; Su10, isolate Su10.

Fig. 3. Bacteria growth in the bacteria growing process after inoculated in 500 ml-flask containing 300 ml of Rennie medium supplemented with sucrose (5 g l⁻¹) and mannitol (3 g l⁻¹) but without malate and lactic acid, at initialing pH 4.0. Cultures were incubated at 30 °C in a rotatory shaker (100 rpm). Samples were taken at 0, 1, 2, 3, 5 days respectively and data shown are mean values of three replicates assays. Bars indicate standard error (SE) and symbols without bars means that the SE is so small that it is covered by the symbols. NB, No bacteria; H11, isolate H11; Su10, isolate Su10.

Fig. 4. pH changes in the bacteria growing process after inoculated in 500 ml-flask containing 300 ml of Rinnie medium supplemented with sucrose (5 g l⁻¹) and mannitol(3 g l⁻¹) but without malate and lactic acid, at initialing pH 4.0. Cultures were incubated at 30 °C in a rotatory shaker (100 rpm). Samples were taken at 0, 1, 2, 3, 5 days respectively and data shown are mean values of three replicates assays. Bars indicate standard error (SE) and symbols without bars means that the SE is so small that it is covered by the symbols. NB, No bacteria; H11, isolate H11; Su10, isolate Su10.

Table 5. Effects of inoculation of nitrogen fixing bacteria isolate H11 and Su10 on rice hydroponic cultured 3-week-old seedling dry weight (g plant⁻¹) after 5 weeks grown in artificial acid sulfate soil.

		Nipponbare		Padi Panjang	
		Mean	LSD	Mean	LSD
Initial pH 4.8	Al 0	0.798	n.s.	0.949	n.s.
	Al 0 H11	0.820	n.s.	1.088	n.s.
	Al 0 Su10	0.865	n.s.	0.981	n.s.
	Al 500	0.827	n.s.	0.818	n.s.
	Al 500 H11	1.016	n.s.	1.098	n.s.
	Al 500 Su10	0.994	n.s.	0.871	n.s.
Initial pH 3.5	Al 0	0.787	n.s.	0.923	n.s.
	Al 0 H11	0.838	n.s.	1.008	n.s.
	Al 0 Su10	0.845	n.s.	1.086	n.s.
	Al 500	0.662	n.s.	0.782	n.s.
	Al 500 H11	0.941	0.022***	0.958	0.320*
	Al 500 Su10	0.755	n.s.	1.005	0.006**

Least significant difference (LSD) at *P = 0.05, **P = 0.01 and ***P=0.001 were shown over control (without inoculation) and n.s.: difference in the same column was not statistically significant.

16S rRNA gene sequences of these strains ranged from 83.6 to 99.5 % (Table 4). Among all of the isolates, *nifH* PCR amplification was only successfully amplified in 5 isolates. All 5 isolates belong to *Burkholderia* sp. Isolate H11 closeted to *B. sacchari*, and isolate Su10 closeted to *B. vietnamiensis*. According to the *Burkholderia* N_2 -fixing species cluster by Reis et al. (2004), the N_2 -fixing species *B. vietnamiensis*, which belongs to the second major lineage of *Burkholderia*, clearly constituted a well-supported cluster separate from the cluster formed by the diazotrophic species *Burkholderia sacchari*. All of the *nifH* gene PCR positive isolates have high nitrogenase activities. Other isolates also present activity but very small comparing with that of 5 isolates. In an ecological survey of *Burkholderia* nitrogen-fixing bacteria, it widely distributed in sugarcane, maize, teosinte plants in Brazil, Mexico and South Africa (Reis et al. 2004). It can also distribute in Vietnamese rice ecosystem (Trần Van et al. 1994) and Indonesia rice ecosystem, according to our investigation.

The free-living nitrogen fixing bacteria are postulated to play an important role in sustainable rice crop production. In the present study, no nitrogen contribution was observed by using our candidates isolates (Table 5) because no different rice leaf SPAD has been found between the inoculation and non-inoculation treatments. The probable mechanism of growth promotion in acid sulfate soil may be by increasing the soil pH especially around the rhizosphere. In standard agriculture practice, lime in combination with a suitable organic material would probably be an excellent management approach to ameliorate acid soils for plant production (Shamshuddin et al. 1991; Shamshuddin et al. 2004; Muhrizal et al. 2006; Xu et al. 2006). Those organic materials might contribute to soil pH increasing through their direct ligand exchange between hydroxyl groups of soil aluminium and iron hydroxy oxides and organic compounds, and dissociation and association of organic compound of added plant organic matters, or ammonification of plant residue N, uptake of NO_3^- (Hinsinger et al. 2006; Xu et al. 2006). On the other hand, the presence of easily mineralizable organic material would indirectly increase soil pH by speeding up reduction of Fe(III) into Fe (II) and SO_4 into H_2S in acid sulfate soil under anoxic condition (Lin et al. 2003; Chu et al. 2006). The increased pH correlated directly with aqueous Fe(II) concentration during Fe(III) oxide reduction under submergence condition (Roden, 2006). But under field conditions, increase of soil pH at soil surface due to plant residue in organic matters addition largely contributed

pH stratification in topsoil profiles (Paul et al. 2001b). The acidity in subsoil especially in the rhizosphere of rice may persist. Furthermore, introducing of standard agricultural practice such as lime application is not always the best way for the farmers because of the cost for them. Alternative agricultural practice such as using microorganisms will help it.

Yan et al. (1996) demonstrated the increase of soil pH following the addition of malate and citrate was highly correlated with CO_2 evolution during the decomposition of these two anions. After addition of malate and citrate, a large part of organic acid salt would be consumed by microbes in the soil and oxidized completely to CO_2 with a corresponding consumption of H^+ (Kirk et al. 1998). The decarboxylation of organic anions during the decomposition requires one proton per carboxylic group decarboxylated (Mengel, 1994). Thus with the consumption of organic acid anion, the pH increased gradually in the soil. In rice plant species, organic acid anions are present in root cell mainly in the dissociated salt form and can be released substantially from roots, particularly malate, citrate and oxalate (Liu et al. 1990; Kirk et al. 1998; Kirk et al. 1999; Aulakh et al. 2001; Begun et al. 2006; Hoffland et al. 2006). Those organic acid anions may contribute benefits both in increasing soil pH and nitrogen fixation if the nitrogen fixing bacteria can utilize those carbon sources released from rice root.

Based on our observation that some nitrogen fixing bacteria isolates can utilize malate as carbon sources and increase medium pH, we can postulate that in the acid condition, these nitrogen fixing bacteria can increase rice rhizosphere pH (Fig. 2) by utilizing organic acid anions from rice roots as carbon source if the beneficial interaction between rice and nitrogen fixing bacteria in rice rhizosphere can be established. Such pH increasing that occur in the rhizosphere or severely speaking, rhizoplane as a consequence of root activity have thus a dramatic influence on the biogeochemistry of a whole range of elements, from major to minor nutrients and other trace elements, from toxic form to un-toxic form, such as Al. Although it is not known how long the effects of organic acid anions on pH increase will persist, such an effect creates a short-term window of opportunity for improvement of rice production on acid sulfate soils (Haynes & Mokolobate, 2001).

A number of anaerobic process including methanogenesis (Goodwin & Zeikus, 1987), denitrification (Shirey & Sexston, 1989) and sulfate reduction (Wieder et al. 1990) have been demonstrated to occur in acidic soils. However, the information of

whether nitrogen fixing bacteria are capable of aerobic growth at extreme acid conditions is still limited. In our study, isolates H3, H11, H15 and Su10 can grow at pH 4.0 (Fig. 1) by using organic acid salt and sugar mixture medium and isolates H11 and Su10 can even drive the pH to values below 4.0 (Fig. 4) by utilizing sugar mixture carbon sources. It is perhaps because these bacteria possess an inherent acid resistance (Belli et al. 1991). These acid-tolerant bacteria have been attributed in large part to the possession of a proton-extruding F-ATPase that is expressed at higher levels that functions well at pH values of 4.0 and below, allowing the bacteria to maintain adequate Δ pH when the external pH falls to 4.0 and lower. Among the tested isolates, isolate Su10 (Fig. 4) is capable of aerobic growth at very acid (pH < 3) condition suggesting this bacterium possesses not only an inherent acid resistance but also an excellent oxygen protection mechanisms of nitrogenase (Poole, 1994). This inherent acid-tolerance was seen not only in their ability to initiate and maintain growth at low pH levels, but also in the ability to fix nitrogen in organic acid salt and sugar mixture conditions as expected very close area of rhizoplane.

Al toxicity is a primary factor limiting agronomic production in acidic soils, which constitute more than 30 % of the world's arable land (Von Uexkull et al. 1995). The chemical behavior of Al in complex ecosystems or living organisms, its toxicity and reactivity or bioavailability, depends mainly on its chemical forms. In very acidic soils (pH < 5.0), Al speciates to a soluble octahedral hexahydrate form, commonly called Al³⁺, which is believed to be the primary phytotoxic Al species (Kochian, 1995). The initial and most dramatic symptom of Al toxicity is inhibition of root growth, which results in a reduced and damaged root system and can lead to mineral deficiencies and water stress, thus cause growth inhibition of the whole plant. In the present study, soil pH was conducted as two levels at pH 3.5 and pH 4.8, respectively. The significant growth inhibition was found under initial soil pH 3.5 with the addition of 500 μ M Al. This inhibition was alleviated by inoculated with isolate H11 after 5 weeks growing in Padi Panjang (Table 5). It is suggested that nitrogen fixing bacteria play a beneficial role in rice growth promotion.

Many Al-resistance mechanisms have been proposed (Juan et al. 2002): internal detoxification in upper plant parts, exclusion of aluminium which is including exudation of organic acid anions, phenolic compounds and rhizodeposition of Mucilage and border cells and alkalization of rhizosphere. There have been many

reports of a general correlation between Al resistance and transient increases in growth solution pH for several species, including wheat (Foy et al. 1967, 1974; Mugwira et al. 1976, 1978; Mugwira & Elgawahry, 1979; Foy & Fleming, 1982; Fleming, 1983; Dodge & Hiatt, 1992), barley (Foy et al. 1967), pea (Klimashevsky & Bernadskaya, 1973), rye (Mugwira et al. 1976, 1978), and triticale (Mugwira et al. 1976, 1978; Mugwira & Patel, 1977). To date, Al resistance of alkalization of rhizosphere is still keep unknown, probably because of lacking full understanding of soil microorganisms. Soil microorganisms play pivotal roles in various biogeochemical cycles and are responsible for the cycling of organic compounds. In acid sulfate paddy soil, rice can release substantial quantities of organic acid anions from their roots (Liu et al. 1990; Kirk et al. 1998; Kirk et al. 1999; Aulakh et al. 2001; Begun et al. 2006; Hoffland et al. 2006). A large part of organic compounds would be consumed by microbes in the rhizosphere and oxidized completely to CO₂ with corresponding consumption of proton, which may reduces Al stress. It is still not known whether a special alkalization (pH increasing) range on the rhizoplane surface could be developed under acid condition, further research to determine the pH of the area very close to the rhizoplane is required.

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Received 10th Feb. 2006Accepted 4th Dec. 2006