Neutral rhizoplane pH of local rice and some predominant tree species in South and Central Kalimantans: A possible strategy of plant adaptation to acidic-soil

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ABSTRACT To investigate the acid sulfate soil-adapting strategies of South Kalimantan local rice varieties, their rhizoplane pH was preliminarily examined using a glass-made micro-electrode. Although raw acid-sulfate soils in paddocks in South Kalimantan were generally pH 3.5-4.5 and sometimes pH 2.5, pH values of the rhizoplane of living roots directly measured by the microelectrode always converged to approximately 7.0 (variance 0.02-0.22, $n=10$). On the other hand, the pH of dead roots, which was measured after soil was thoroughly washed from the surface, was relatively low and often close to the soil pH itself. Similarly constant neutral rhizoplane pH was also observed in the major Central Kalimantan tree species, *Cratoxyllum arborescens* (upland) and *Combretocarpus rotundatus* (swampy forests). Whilst, two other predominant grasses, *Xyris complanata* and *Imperata cylindrica*, inhabiting acidic soils in upland and lowland, had relatively low rhizoplane pH (6.05 and 5.86, respectively), suggesting that these grasses have different strategies than the paddy rice varieties for adapting to acidic conditions. Rhizoplane bacteria are likely to be one of the factors for the maintenance of neutral rhizoplane pH. In fact, some *Sphingomonas* sp. raised medium pH, originally set at pH 4.0, to 5.5. These findings suggested the importance of cationic charge in the rhizoplane of local rice varieties for adapting to acid-sulfate soil, which is similar to strategy of some arboreal trees inhabiting acidic peat soil.

Key words: *Oryza sativa* L., acid-tolerant local rice, rhizoplane pH, adaptation to acidic soil.

INTRODUCTION

Main problems of acidic soil for plants are a strong leaching of substantial mineral elements and high concentrations of aluminum cations ($Al(OH)^{2+}$ or Al^{3+}). Pylite-containing clay in acid-sulfate soil releases a large amount of aluminum cations that are toxic to the root tissues and directly or indirectly disturb nutrient assimilation of the roots (Rengel, 1992). Nevertheless, some acid-sulfate tolerant plants, such as *Melastoma malabathricum*, *Juncus* sp. and *Melaleuca cajuputi*, can manage with excess aluminum cations and are able to regenerate on strongly acidic soil lands. Genus *Melastoma including M. malabathricum* L. and genus *Camellia* including *C. sinensis* (tea plant) are known to be Al-tolerant plants or Al-accumulators (Watanabe & Osaki, 2002a), while *Juncus* spp. and *M. cajuputi* are typical Al-excluders (Watanabe & Osaki, 2002b). Paddy rice plant is also an Al-excluder (Watanabe & Osaki, 2002b), having the ability to selectively intake nutritional cations. It is, however, not yet known exactly how acid-tolerant plants acquire nutritional elements, including N and P, from such acid-sulfate soils that induce serious cationic leaching and phosphate immobilization.

The rice production investigated so far in a paddy rice cultivating area in South Kalimantan, Indonesia, often reached to 3-4 t/ha, without any fertilization or lime-input (Hasegawa *et al*., 2001). To determine the reasons for the high productivity of the local rice varieties, three local paddocks were chosen as study plots and a) rice productivity, b) chemical properties of the soil, c) rhizoplane and soil microflora, and d) any of correlation amongst these factors were investigated (Hasegawa *et al*., 2002). Initially, their characteristic root systems spreading over shallow (2-10 cm) soil, whose soil acidity is relatively low in deeper layers (> 10 cm), were observed. Therefore, an investigation of root behavior in this nutrient-poor acidic soil and rhizoplane microfloral component characteristics was started. If rhizoplane microorganisms contribute to nutrient uptake from the strongly acidic soil, they are certainly involved in the local paddy plants' strategy for adapting to acidic paddocks. In this paper, the rhizoplane pH of the local rice varieties and behaviors of nitrogen-fixing bacteria in the rhizoplane are reported on. The rhizoplane pH of some predominant native plants adapted to acidic soils is compared and the important ecological role of rhizoplane diazotrophs in acid-tolerant plants is discussed.

MATERIALS AND METHODS

Plant Used in This Study

The plant specimens used in this study were as follows: rice plant (*Oryza sativa*) from Jerapatbaru and Gambut, South Kalimantan; *Xyris complanata* from Kalampangan, Central Kalimantan, *Imperata cylindrica* from Arboretum, Central Kalimantan, *Combretocarpus rotundatus* from Kapuas border, Central Kalimantan, and *Cratoxyllum arborescens* from Kapuas border and Lahei, Central Kalimantan (Figure 1).

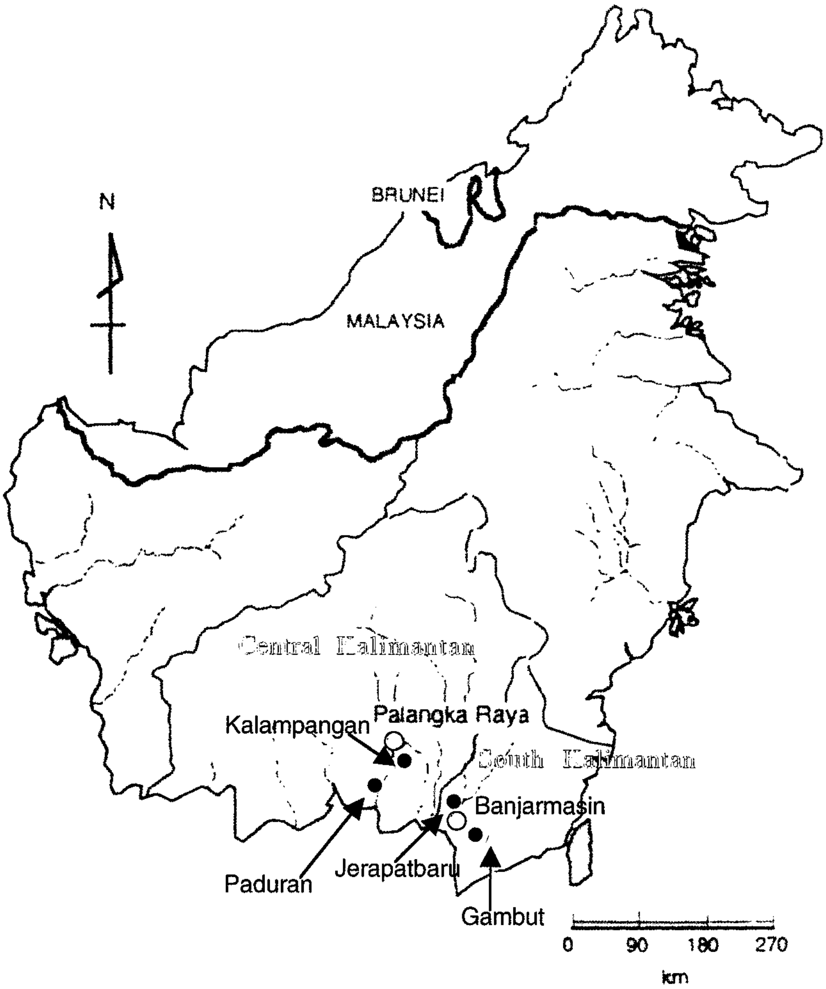

Fig. 1. Sampling plots in Central and South Kalimantans.
Tropical peat land is widely distributed throughout Kalampangan area, while the other three sites are acid-sulfate soil land. At Paduran, only IR64 was cultivated but nothing harvested, but Jelapatbaru and Gambut are paddy field areas with local rice (Siam Unus etc) production.

Measurement of Soil pH

Soil pH was measured as follows: wet soil cake pH was directly measured with a portable pH meter (Model 250A, ThermoOrion, USA) equipped with a needle tip micro-pH electrode (Orion 9863BN, ø 1.0 mm, 3 mm for minimum immersion depth, ThermoOrion) that had 5 mm of half of the stainless cover replaced with glass tip protection. For a more precise measurement of soil pH, approximately 1 g of soil was mixed with 2 ml deionized water in a small plastic bag with air removed, and left at least for 10 min. The soil suspension was directly measured with a compact pH meter (B-212 Twin pH, Horiba) .

Preliminary Experiments for Measuring Rhizoplane pH with Needle Tip Micro-pH Electrode

When the glass needle tip micro-pH electrode, with the stainless cover removed, was touched to soft or hard agarose gel, the pH meter indicated correct pH values for the object. Hence, this electrode was used for measuring rhizoplane pH in the field. To determine the influence of rhizospherous soil on rhizoplane pH measurements using a needle tip micro-pH electrode, 10 root samples were collected together from the acidic paddock, washed with clean water and cut from ca. 5 cm from the root tip. The root samples were put in polyethylene bags with zippers and were then soaked in ca. 3 ml of pure water for over 30 min without exposure to air. Five replications were prepared. Using a handy pH meter (Horiba D24S), pH values of the root-soaking water in each plastic bag was measured. At the same time, root samples prepared without rinsing out rhizospherous soil, but only physically removing the soil cake from the rhizoplane, were also collected and treated with pure water as described above ($n=5$).

In the local rice varieties, pH values of the root-soaking water converged at pH 6.0 (5.95 ± 0.11, with washing and 6.07 ± 0.11, without washing) regardless of the preliminary treatment. On the other hand, paddock soil taken from the points sampled the roots showed comparatively lower pH (5.21 ± 0.61). These results led to the conclusion that the washing process of the rhizospheric soil rarely affects the rhizoplane pH. To minimize the effect of root-attached soil on the rhizoplane pH, the roots were henceforth washed with clean water in a spray bottle to remove the soil.

Preliminary Measurement of Rhizoplane pH for Acid-tolerant Plants in Field.

For preliminary and simple rhizoplane pH measurement of the acid-tolerant plants inhabiting acid-sulfate soil, peat soil or sandy spodozol-like soil, the same portable pH meter with the needle tip micro-pH electrode as described above was used. The soil attached to the surface of fresh root was washed away with clean water using a spray bottle. Generally, ion-exchanged water is used, but in the field usually we preliminarily washed the soil away from the rhizoplane with drinking water, and finally sprayed it with pure water. In the case of only using drinking water for washing the root, no difference between the rhizoplane pH values after brief rinsing with pure water was observed. With a soft, absorbing polyurethane foam, free water on the root surface at 2-4 cm from the root tip was removed. So, the root was kept moist but without excess water on the surface. The root and the bare glass electrode were together enfolded gently with the polyurethane foam, and the pH value was recorded when the pH meter indicated a stable number for over 10 seconds. In the case of muddy paddock soils, the microelectrode was directly attached on a wet soil cake, and hold it until pH value became stable for 10 seconds.

Soft Gel Medium for Culturing Free-living Nitrogen-fixing Bacteria from the Rhizoplane of Acid-tolerant Plants

For observation and evaluation of rhizoplane microflora, we used a nitrogen-free soft gel medium solidified with 0.3% gellan gum (Hashidoko *et al*., 2002). In this experiment, we used Winogradsky's salt mixture originally developed for an *Azotobacter* medium (Tchan & New, 1984) adding 1% glucose as sole carbon source and adjusted its pH to 6.2 or 4.0 with 4*M* H_2SO_4 as the basal medium. The root fragment (1 cm long) was gently washed several times with 20-25 ml of sterile water, and finally vortexed for 30 sec in 10 ml of sterile water in an 18-cm test tube. The resulting washings regarded as rhizoplane bacterial cell suspension were used as the inocula. Since the gellan gum-base N-free medium is known as a convenient, primary culture medium for observing and evaluating behaviors of nitrogen-fixing bacteria inhabiting rhizoplane, 100 μl of the root washings was generally added to the liquefied soft gel medium and briefly vortexed 3-times as described previously (Hashidoko *et al*., 2002).

Bromothymol blue (BTB, Acros Industry), a pH indicator, when added in a final concentration of 20-40 mg/L, is capable of monitoring ammonia or organic acid accumulation by the inocula in the soft gelled medium in real time (yellow below pH 6.0, green in the range from pH 6.0-7.6, and blue-green to deep blue over 7.6). In some cases, we also used bromocresol purple (Wako Pure Chemical Industries Ltd.) instead of BTB, to reduce the toxicity of the pH indicator (yellow below pH 4.5, and purple over pH 4.5) (Roa *et al*., 2000).

Ability of Rhizoplane Microorganisms to Acidify or Maintain pH in Nitrogen-free Soft Gel Medium

The root washing inoculants on Winogradsky's medium (1% glucose as sole carbon source) solidified with 0.3% gellan gum (adjusted to be pH 6.2 or 4.0 by 2M H_2SO_4, containing 40 mg/L BTB) were incubated for 2-4 weeks to monitor pH change in the N-free soft gel medium. At the end of the incubation period, the resulting gel, sampled around the bacteria-colonized zone and/or whole gel medium vortexed to mix up uniformly, were directly measured by a table pH meter (pH Meter F22, Horiba) equipped with a glass electrode (type 6366, Horiba). Concurrently, the pH change was monitored in the liquid medium without the gel matrix, in which the same inoculant was added and standing-cultured in the dark. The medium pH was measured with the same table pH meter every 5 days (Figure 5).

RESULTS

Rhizoplane pH of Local Paddy Plants Grown in Acidic Paddocks

Rhizoplane pH values of several acid-tolerant tropical plants, including local rice varieties and forest trees, were measured using a glass microelectrode. All of the root samples from all of the species grown in different types of soil had a similar rhizoplane pH. In local rice grown in acid-sulfate paddocks, the living roots always indicated a rhizoplane pH of approximately 7.0 (Figure 2). The rhizoplane of the dead roots of a local rice specimen, from a hill that almost died after the harvest, had a more acidic pH, closer to the soil pH. This suggests that only living roots have an ability to maintain neutral rhizoplane pH. This tendency was also observed in *O. sativa* subsp. *japonica* (var. Koshihikari and Hoshitaro) grown in the experimental paddock of Hokkaido University Farm, of which mean soil pH was over 7.6 and the rhizoplane pH was also around 7.0 (Figure 2).

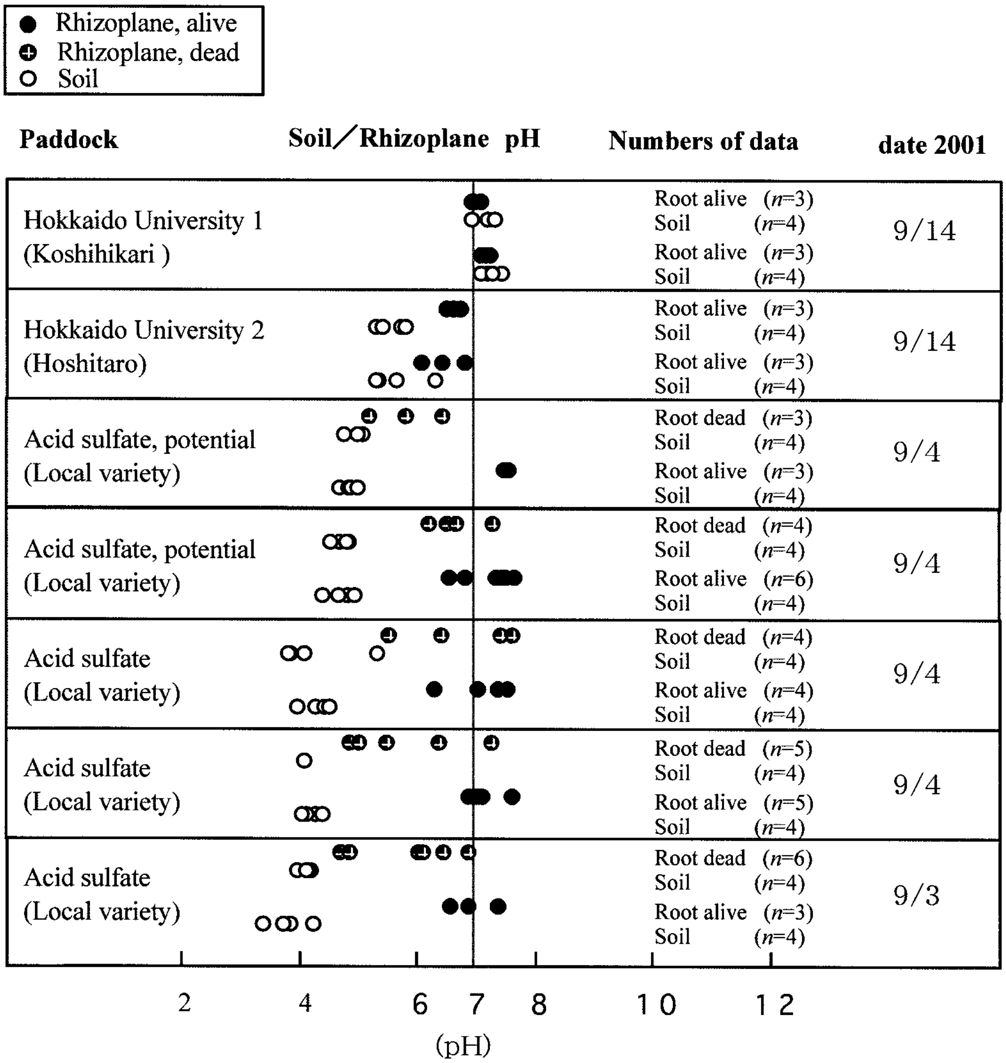

Fig. 2. Rhizoplane pH of paddy rice grown in acid-sulfate soil or another soil.
Soil pH was measured with the glass electrode to touch directly on the bulk soil. The dead roots had already lost their column structure and became flat. The rhizoplane soil was also washed away completely for measuring pH on the surface.

When rhizoplane pH was measured from Siam Unus on two hills (ten times-repetition for one hill), the mean pH values for the two replications were 6.93 (variance, 0.02) and 6.87 (variance, 0.22). The pH values of the paddock soil when suspended in H_2O were 4.70 and 4.75 (water in the paddock was pH 3.5-3.7). So, it demonstrated that our rhizoplane measurements were thus reproducible (Figure 3).

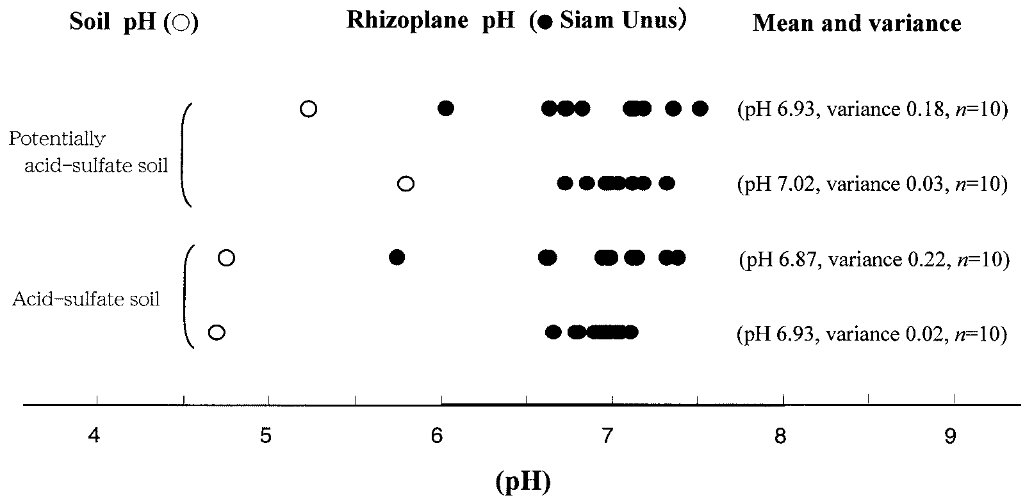

Fig. 3. Precise rhizoplane pH of paddy rice from one hill in paddocks in South Kalimantan.
Two hills from an acid-sulfate soil paddock and two more hills from a potentially acid-sulfate soil paddock (both at Gambut district) were sampled together with bulk soil. A part of the soil was washed away and rhizoplane pH of the resulting living roots were measured by the glass electrode with 10 replications for each hill. The soil pH was measured on soil suspension as described in Materials and Methods.

Rhizoplane pH of Predominant Grasses in an Open Area and Major Trees in a Natural Forest on Acidic Peat/Spodozol Soil

In the same manner, we also measured pH values of the rhizoplane of *Imperata cylindrica* (family Gramineae) and *Xyris complanata* (family Xyriceae), predominant vegetation in deforested lands in Kalimantan. Their rhizoplane pH values were 6.05 (variance, 0.08, n=6) and 5.86 (variance, 0.08, n=8), respectively. The mean pH values were obviously lower than those of local rice. In contrast, the rhizoplane pH of *Combretocarpus rotundatus* seedlings from a peat swamp and *Cratoxyllum arborescens* from a sandy upland area with shallow peat was more alkaline. Means of the rhizoplane pH for *Cr. arborescens* and *Co. rotundatus* were 7.02 (variance 0.08, n=7) and 7.05 (variance 0.30, n=7), respectively (Figure 4). Previously the rhizoplane pH of *Melaleuca cajuputi* grown in spodozol soil in Thailand was measured with the same equipment and manner. Its mycorrhizal roots were often below 4.0, although the soil pH itself was over 6.0 (Hashidoko & Osaki, unpublished data).

Maintenance of pH in the N-free Soft Gel Medium by Rhizoplane Microflora

Bacteria developed in the soft gel medium showed some patterns of colony emergence, according to their physiological and ecological characters, specifically: a) respiratory type, b) motility and oxygen adaptability, c) bacterial cells population, and d) interference by other competitive or accompanying bacteria (Hashidoko *et al*., 2002). In many cases, when root washings that had been prepared from plants grown in nutrient poor soils were inoculated and incubated at 20℃, pH value of the cultured medium (originally pH 6.2) became acidic within 2-3 days. However, some root washings from representative acid-tolerant plants, such as the rhizoplane microflora of a *M. malabathricum* and the South Kalimantan local rice varieties grown in strongly acidic soils (pH 2.6-3.5), did not make the medium acidic within 2-3 days.

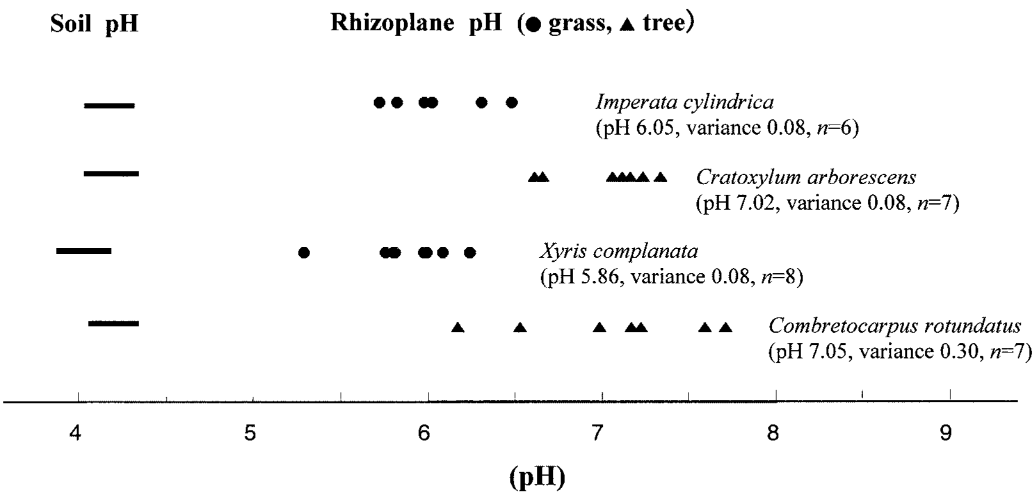

Fig. 4 Comparison of rhizoplane pH of predominant grasses and native trees grown in lowland and upland acidic soils.

Imperata cylindrica was collected from its pure community at 20 km north from Palangka Raya (Spodzol upland) and *Cratoxylum arborescens* near Kalampangan (dry peat land), both distributed throughout upland of Central Kalimantan. *Xyris complanata* and *Compretocarpus rotundatus* were sampled at a swampy peat land near Kalampangan. All of the data were collected during rainy seasons. The pH of flood water in the sampled area was used as the soil pH, except Spodzol, of which pH was measured after suspended in pure water.

Classification of Rhizoplane Microfloral Communities into Type A or B according to Their Ability to Maintain Culture Medium pH.

Rhizoplane microorganisms from many grasses collected at Kalampangan site, including *X. complanata*, caused the pH of the N-free liquid medium to change from pH 6.2 to acidic (pH 3.5-3.0) (type A). Moreover, those from most of the rice plants and stunted IR64 in acid-sulfate paddocks at Jelapatbaru and Paduran, Central Kalimantan, showed a similar tendency. In soft gel medium, medium acidification occurred within 24 h and reached to the maximal values after 3 days. Some samples of type A, representative of those from *Nephrolepis biserrata* (a fern, Davalliaceae), produced gas in the N-free soft gel medium.

In contrast, rhizoplane microorganisms from the acid-tolerant local rice varieties, Siam Unus and Siam Pandak at Gambut, and Jerapatbaru, South Kalimantan, respectively, maintained the initial pH value (6.2) of the cultured N-free soft-gel medium throughout the 4-week incubation (type B). Also, those from *M. malabathricum* sampled at Paduran, Central Kalimantan, and Jerapatbaru, South Kalimantan were also type B. The root washings from the *M. malabathricum* eventually increased the pH to alkaline to turn the medium color to greenish blue/deep blue after 2-3 months. These facts suggest that the plants so far investigated, whose rhizoplane pH was around neutral, generally had rhizospherous microflora of type B. Moreover, a *Ficus* sp. (family Moraceae) youngster naturally grown on peat land at Kalampangan, Central Kalimantan, also maintained the medium pH (Figure 5), although another specimen inhabiting from the same site possessed type A-microfloral compositions.

DISCUSSION

In this research, measurement of rhizoplane pH of acid-tolerant plants, by means of the needle tip micro-pH electrode, displayed well the differences between soil and rhizoplane of the micro-environment, helping to clarify the mechanisms of acid-tolerance. In local rice plants highly adapted to acid-sulfate soil, roots extending into acidic soil use cationic charges on the rhizosphere neutralizing the pH of rhizoplane and rhizosphere. It is known that root assimilating cations are capable of releasing H^+ to maintain the electroneutrality of the soil-root interface, particularly when the crop actively uptakes NH_4^+ (Gill & Reisenauer, 1993) or Ca and Mg cations (Jentschke *et al.*, 2001). Then, new queries emerge. How does the acid-tolerant plant manage self-releasing H^+ from the root, and how does the plant acquire such cationic elements from strongly

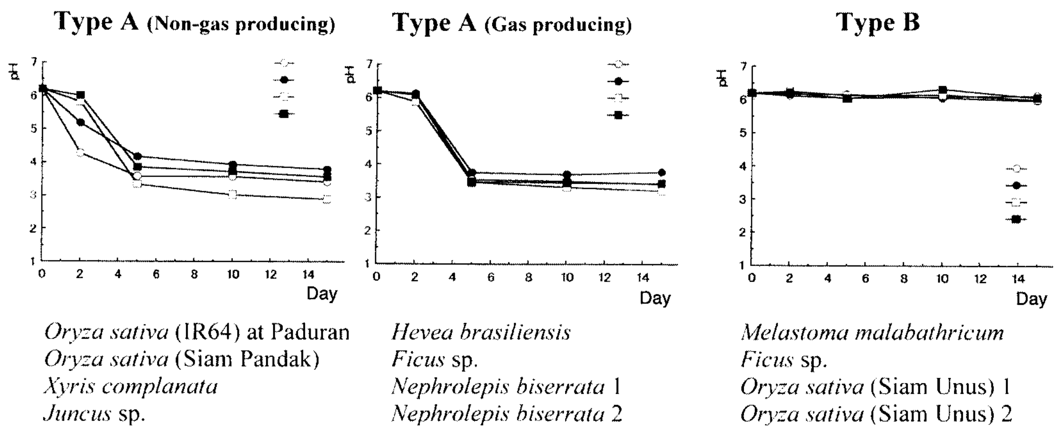

Fig. 5. Two types of rhizoplane microorganisms either acidifying or neutralizing the cultured N-free medium

Each inoculant, obtained from a washed root fragment, was cultured in 10 ml liquid medium of Winogradsky's mineral mixture with 1% glucose without shaking in a 18-cm-test tube (i.d. 15 mm), and each of medium pH was monitored to classify each rhizoplane microfloral community into types A and B. The samples tested here displayed a good accordance with the patterns of pH alteration in the BTB-containing Winogradsky's medium solidified with 0.3 % gellan gum.

acidic soil?

Not only the root system but also rhizospherous microorganisms, namely those in the rhizo-biocomplex, seem to regulate the rhizospherous micro-environment, including the rhizoplane pH. All of the phenomena that we observed in productivity and physiological responses of the local rice varieties to the soil condition (Hasegawa *et al*., 2002b) are likely to be strongly linked with the capability of rhizoplane microflora to regulate rhizospherous conditions. As Hasegawa *et al*. recently revealed, local rice varieties show relatively low response to fertilized nitrogen (Hasegawa *et al*., 2004). Moreover, nitrogen contents in the aboveground biomass of local rice, known as an ammonia-assimilating plant (Watanabe *et al*., 1998), were reasonably high, although potent mineralization activity of the acidic paddock soil was low through the rice cultivation (Purnomo *et al*., 2003).

We therefore investigated and screened ammonia-producing free-living nitrogen-fixing bacteria from the rhizoplane of the local rice varieties, based on the hypothesis that these ammonia-producing bacteria are key factor in the adaptation of the local varieties to acid-sulfate soil. As a result, many nitrogen-fixing bacteria from the rhizoplane of paddy plants were isolated, although the contribution of those nitrogen fixers to rice production is still doubtable (Cocking, 2003). More importantly, those microfloral members, particularly *Sphingomonas* sp. characteristically found in the rhizoplane of acid-tolerant plants grown in acid-sulfate soil, are able to keep their culture medium pH neutral. This is unusual for saprophytic and some representative diazotrophic bacteria.

The variance of rhizoplane pH of *Co. rotundatus* (n=8) was larger than others, probably due to the spatially diverse microenvironments of the root surface. In fact, *Co. rotundatus* possesses a relatively poor root system with much less branching for fibrous root development. The main root is covered with sponge-like soft bark, providing a niche for diverse epiphytic and endophytic microorganisms. Generally, different types of root architecture have different functions in nutrient uptake and the diverse rhizospherous environments on the root bark surface provide habitats to various rhizospherous microfloral communities. Such characteristic, poor root systems with sponge-like root are observed in some tree species that predominate in peat swamp forests. Local rice varieties also have remarkably thick roots, in which aerenchyma is remarkably well developed. These facts may clarify the issue mentioned above.

This method for measuring approximate rhizoplane pH may be applicable to other plants tolerant to different types of environmentally stressed soils. For more precise measurements of rhizoplane pH, some fluorescent pH indicators such as 2',7'-bis-(2-carboxyethyl)-5 (and-6)-carboxyfluorescein (BCECF) or 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) originally used for intracellular pH measurement for several cells (Molenaar *et al*., 1991; Aguedo *et al*., 2001) should be used, particularly for in situ monitoring of rhizoplane pH in intact roots. The pH values of the rhizoplane obtained in the simple and easy method presented in this paper might be different from the true microenvironmental conditions of the rhizoplane, as the root surface is now recognized as a diverse microfloral habitat (Ikenaga *et al*., 2000). We, however, believe that the tendencies of the rhizoplane pH as determined by the simple method in this research, partially answer how acid-tolerant plant can adapt to strongly acidic soil. Complete identification and determination of the ecological behaviors of the rhizoplane bacteria isolated from this systematic investigation on rhizoplane microflora will be reported elsewhere.

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