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Frequent isolation of sphingomonads from local rice varieties and other weeds grown on acid sulfate soil in South Kalimantan, Indonesia

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ABSTRACT We preliminarily investigated the correlation between productivity and the diversity of free–living nitrogen–fixing bacteria on the rhizoplane of local rice varieties, including Siam Unus, Siam Adil and Siam Pandak, all tolerable to acid–sulfate soil in South Kalimantan, Indonesia. The rhizobacteria of some unidentified weeds of native–grown in the acidic paddocks were also searched. Subsequently, sphingomonads, including *Sphingomonas* spp. and *Sphingobium* spp., are found as the most dominant rhizobacteria in local rice and also in weeds that are adapted to the strongly acidic wet soil. Other unique rhizobacteria are *Alcaligenes* spp. that powerfully neutralized their cultured media. To understand how the local variety of paddy rice can tolerate acid–sulfate soils with very low soil pH (2.5–3.5), studies of these acid–tolerant, root–associating, and often nitrogen –fixing, rhizospherous bacteria are likely to be important key factors.

Key words: acid–sulfate soil, *Oryza sativa* L., rhizobacterium, rhizosphere, *Sphingomonas*.

Since the tropical peat swampy forests distributed throughout the lowland areas of Central Kalimantan were reclaimed for farming, the emergence of acid–sulfate soil on farmland has become a major problem. It is said that drastic decreases in crop production within several years in reclaimed farmlands, forced many farmers to abandon their farmlands and resort to illegal logging. After the illegal logging, the remaining forest trees are burned to clean–up and open–up new farmlands, which invariably turn into acid–sulfate soil. To avoid deforestation in Kalimantan, it is most necessary to break this vicious cycle. So, the establishment of agricultural methods for sustainable crop production and field management on land with acid–sulfate soil is one solution to this pressing problem. Hence, we focused on low pH–tolerable wild plants and crops to understand the function of their root systems, which are involved in the acid–adapting strategy of the plants.

The main problem of acidic soil for plants is the toxicity of aluminum cations (Al^{3+}). Acid–sulfate soils with a very low pH (2.5–3.5) release large amounts of Al^{3+} that directly or indirectly disturbs nutrient assimilation from the roots. Some acid–sulfate tolerable plants, such as *Melastoma malabathricum, Juncus* sp. and *Melaleuca cajuputi,* however, can cope with excessive Al^{3+} and are able to regenerate on such strongly acidic soil lands. As the representative acid–tolerant plants, genera *Melastoma* and *Camellia,* including *M. malabathricum* and the tea plant (*Camellia sinensis*), respectively, are known to be Al–accumulators, while *Juncus* spp. and *M. cajuputi* are representative Al–excluders.

Local paddy rice (*Oryza sativa*) is comparatively tolerant of acid–sulfate soils, and is an Al–excluder (Watanabe & Osaki, 2002). Since acid–sulfate soils with very low pH (2.5–3.5) have usually suffered serious leaching, acid–tolerable plants must employ certain strategies to acquire nutrients, including N and P. The local rice production, so far as we investigated, reached 3 –4 t ha^{-1}, without any fertilizer or lime–inputs (Hasegawa *et al.,* 2004a). Since we regarded the high yield performance of rice in the acid–sulfate paddock as a good model for low–input sustainable agriculture (LISA), three paddies were chosen as plots to monitor the chemical properties of the soil, the rhizoplane microflora and rice productivity.

Since paddy rice is known as an ammonia–assimilating plant that prefers NH_4^+ rather than NO_3^- for its nitrogen source, the presence of free–living nitrogen–fixing bacteria on the rhizoplane of the local rice varieties is thought to be beneficial for survival in acid–sulfate soil land. Releasing ammonia into the rhizosphere may also play a role in the neutralization of rhizospheric soil by reducing aluminum toxicity. So our idea was to investigate free–living nitrogen–fixing bacteria inhabiting

the rhizosphere of local paddy plants. So far during our preliminary investigation, free–living nitrogen–fixing, and/or neutral pH–maitaining bacteria were frequently isolated from the rhizoplane of the local rice. On a parallel with rhizobacterial examination, Hasegawa *et al.* have investigated rice producivity in acidic paddocks, and have revealed that rice productivity is positively correlated to soil acidity in the local paddocks in the Gambut area (2002a). In this paper, we report on the investigation of rhizospherous microflora of the local rice varieties, and frequent isolation of sphingomonads from the rhizosphere to discuss further their ecological roles.

MATERIALS AND METHODS

Screening method for rhizoplane microorganisms

We used a gellan gum–based soft gel medium (Doebereiner, 1995) for observation and evaluation of the microflora of the rhizoplane nitrogen–fixing bacteria (Hashidoko *et al.,* 2002). Initially, we solidified this gel matrix (0.3%) together with a nitrogen–free medium (Winogradsky's salt medium) containing 1% glucose as the sole carbon source (Tchan & New, 1984). A root fragment (1 cm long) was washed several times with 20–25 ml sterile water, and finally vortexed for 30 sec. in 10 ml of sterile water in an 18–cm test tube. The resulting washings contained rhizoplane bacteria, and as such were used as inocula. For inoculation, generally 100 µl of washings were added to the liquefied soft gel medium and briefly vortexed 3 times.

For convenience, the root samples collected in the local paddy field were washed with spraying sterilized water in a hand spray bottle, and each resulting root fragment was inoculated into a soft gel–containing screw –capped 10–cm–long glass tube. After 1– day–incubation, the root fragment was removed under aseptic conditions. Rhizoplane bacteria trapped in the soft gel medium were further spread onto a modified Winogradsky's agar medium (Hashidoko *et al.,* 2002).

Identification of Rhizobacteria

Some bacteria were purified on a modified Winogradsky's medium (Winogradsky's mineral mixture, 0.5% mannitol or sucrose, 0.005% yeast extract and 2% agarose), and their phenotypic and physiological characters were investigated. For identification, 16S rRNA gene sequencing determination was conducted. The total DNA used for the template for PCR amplification of the 16S rRNA gene regions was prepared by Isoplant II (Wako Pure Chemical Industries, Osaka, Japan). For the reaction, the PCR kit, HotStarTaq (Quiagen, USA), was used according to its instruction protocol. The first amplification for a 16S rRNA gene region with universal forward (5'–AGARTTTGATCCTGGCTCAG–3', 27f) and reverse (5'–AAAGGAGGTGATCCAGCC–3', 1525r) primers (Hiraishi, 1992) was done as 30 cycles of 94 ºC for 1 min, 53 ºC for 1 min and 72 ºC for 1 min. The PCR product was diluted 10 times with pure water, and used directly as the template for the second amplification with forward (5'–CTACGGGAGGCAGCAGT–3', 357f) and reverse (5'–ACGAGCTGACGACA–3', 1080r) primers under the same reaction conditions as above (Weisburg *et al.,* 1991). The resulting PCR product was sequenced using an ABI PRISM® 310 Genetic Analyzer with BigDye Terminator Cycle Sequencing, FS (Applied Biosystems, USA). The sequence homology was then investigated on the database program, BLASTN, provided by DDBJ on its web site.

Survival and recovery test for soil bacteria and rhizobacteria

In this experiment, two strains of sphingomonads EC–K013 and EC–K005 isolated from the rhizoplane of *M. malabathricum* and from the rhizosphere of acid–tolerant local rice variety, Siam Unus, respectively, were used. Both of these strains were pre–incubated on MW–agar plates for 5 days. As a reference bacterium, approximately the same population size of *Burkholderia cepacia* EC–K014 was also inoculated to the identical materials under the same conditions.

For the recovery test, semi–dried acidic paddock soil that was collected from Gambut, South Kalimantan (in August 2000), and local rice (Siam Unus) root residues that had been soaked in MeOH for several months and then removed the solvent to be dried were used for the matrix. To 1 g of the paddock soil, 0.2 g of dry rice root residues, or their mixture, and 1 ml of deionized water was added and then twice autoclaved at 120ºC for 30 min with 2–day–interval. Control was set as only 1 ml of sterilized water. After each of the bacterial strains (1×10^6 cells suspended in 0.2 ml water) was inoculated to matrix –containing 10–ml glass vials, these were incubated at 28ºC for 3 weeks in the dark.

After the 3–week–incubation, sterilized water was added to all of the bacteria–inoculated glass vials to be final volume of 5 ml. Samples were then vortexed for 10 sec and were allowed to settle for 10 min. The resulting supernatants (200 µl) were respectively spread onto potato–dextrose agar plates to incubate at 23ºC in the dark for 2 days. Colonies which emerged on the plate

were then counted and recorded. All treatments were performed in 2 replicates, including the spreading process onto the agar medium.

RESULTS AND DISCUSSION

Characterization of rhizoplane bacteria from local paddy rice

Among the rhizospherous microorganisms cultured in the soft gel medium, those from local rice varieties inhabiting acid–sulfate soil paddocks in Southern Kalimantan showed a high diversity of the rhizobacteria that were able to maintain pH in the culture medium at neutral region. In the isolation of microfloral composers, the 16S rRNA gene sequences of all the bacterial isolates were investigated by PCR followed by sequence determination. A homology search (with the BLAST system at NRIGA) led to the local variety isolates being close to *Sphingomonas rose, S. adhaesiva, S. parapaucimobilis,* or *S. melonis,* but most of them are identifiable only in the genus level. *Sphingobium yanoikuyae* and *S. yabuuchiae* were identifiable in the species level (Table 1). On the other hand, *Alcaligenes* spp. were characteristically isolated from the cultured medium containing B–type microflora, capable of adjusting N–free media to be neutral to alkalin regions (Hashidoko *et al.,* 2005).

Among these isolates, approximately half of them were sphingomonads, a group of Sphingomonadaceae,

Table 1. Isolated bacteria from plants acid-sulfate soil paddock (pH 3.0～3.3)

Source	Identification	Source	Identification
Siam Pandak–1	*Sphingomonas* sp.	a dicot	*Sphingobium yanoikuyae*
	Methylobacterium sp.		*Methylobacterium* sp.
	Unidentified	Siam Unus–7	*Sphingobium yanoikuyae*
Siam Pandak–2	*Sphingomonas* sp.	a Pontederiaceae	*Asticcacaulis excentricus*
	Sphingomonas sp.		*Commamonas* sp.
Azolla	*Sphingomonas* sp.	a monocot	*Sphingobium yanoikuyae*
	Sphingobium yanoikuyae		*Commamonas* sp.
	Sphingobium yanoikuyae		*Methylobacterium* sp.
	Unidentified	Siam Unus–8	*Sphingomonas* sp.
Siam Pandak–3	*Sphingomonas* sp.	*Juncus* sp.	*Alcaligenes faecalis*
Siam Pandak–4	*Sphingomonas* sp.		*Alcaligenes faecalis*
	Sphingomonas sp.	Siam Adil	*Sphingobium yanoikuyae*
	Sphingomonas sp.		*Sphingobium yanoikuyae*
Siam Unus–1	*Ralstonia* sp.		*Methylobacterium* sp.
Siam Unus–2	Acidobacteriaceae		
	Acidobacteriaceae		
	Sphingomonas sp.		
Siam Unus–3	*Sphingomonas* sp.		
	Caulobacter endosymbiont		
	Caulobacter endosymbiont		
Melastoma	*Burkholderia* sp.		
	Sphingomonas rosa		
Siam Unus–4	*Burkholderia* sp.		
Siam Unus–5	*Alcaligenes faecalis*		
	Sphingomonas sangus		
Siam Unus–6	*Sphingomonas* sp.		
	Alcaligenes faecalis		
A dicot weed	*Sphingomonas yabuuchiae*		
	Alcaligenes faecalis		
	Methylobacterium sp.		

genetically close to genus *Sphingomonas*. A highly frequent appearance of these sphingomonads in the acidic soil–tolerant local rice and a high productivity of local rice in non–fertilized acidic soil (Hasegawa *et al.,* 2004a) strongly suggests that the rice root–associating sphingomonads have characteristic functions in the rhizosphere. Although there is only one reliable report about nitrogen fixation by *Sphingomonas* sp. (Adhikari *et al.,* 2001) among rhizospherous sphingomonads from rice plants, a direct evidence for nitrogen fixation in some acid–tolerant *Sphingomonas* spp. isolated from rhizoplane of *Xyris complanata* has also been obtained by means of acetylene reduction assay (Ogita, 2006). Since *X. complanata* preferring water–logged, acidic soil land had a similar habitat with the local Kalimantan rice varieties, and also many of rice–root associating sphingomonads are oligotrophic and able to grow in N–free media, not few isolates probably possess nitrogen–fixing abilities.

Relatively high affinity of a *Sphingomonas* sp. inhabiting rhizosphere of a local rice variety Siam Adil to rice root residues

In the recovery test for soil bacteria and rhizobacteria, all of the three strains (*Sphingomonas* sp. EC–K005, S. *rosa* EC–K013 and *B. cepacia* EC–K014) showed a 50–90 % bacterial cell recovery from the water–only. From the acidic paddock soil, only *B. cepacia* EC–K014 showed over 50% recovery, but neither *Sphingomonas* sp. EC–K005 from *M. malabathricum* nor *S. rosa* EC–K013 from local rice emerged on the plates. This result suggested that both of the *Sphingomonas* bacteria are either acid–susceptible or highly absorbed to the soil particles. On the other hand, over 50% bacterial cells of *S. rosa* EC–K013 were recovered from supernatant of the rice root residues–containing water, whereas *Sphingomonas* sp. EC–K005 did not. Since 10% of S. rosa EC–K013 was recovered from the root residue–containing acidic soil, bacterial cells attaching on the root residues are separable and become planktonic cells into the supernatant (Table 2). Taken together, the latter sphingomonad from rice root may show a high affinity to the surface of rice rhizoplane.

CONCLUSION

In acidic soil regions, particularly those in Indonesia, sphingomonads are most likely to be predominant rhizospherous bacteria, and our preliminary investigation (Hashidoko *et al.,* 2006) suggests that many of such shpingomonads are functional rhizobacteria in acidic soil ecosystem. It is not yet clear whether or how such local rice–associating sphingomonads that function in the rhizosphere for host survival and production in adverse acid–sulfate soil. Our DNA array research suggested a bio–rational agricultural production in the traditional rice farming system that is unique among tropical and semi–tropical rice farming regions (Hashidoko *et al.,* 2006). Many phenomena that we have observed in productivity and physiological behaviors of local rice varieties are likely to be highly linked with their functional rhizospherous microflora, which regulate rhizospherous conditions.

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Table 2. Tolerance of two *Sphingomonas* spp and a *Burkholderia* in acidic soil conditions

strain / conditions	*Sphingomonas* sp. EC-K005 from rice root	*Sphingomonas rosa* EC-K013 from *M. malabathricum*	*Burkholderia cepacia* EC-K014 from *M. malabathricum*
Paddock soil	−	−	++
Paddock soil + Root residues	−	+	NT
Root residues*	−	++	NT
Ion-exchanged water	++	++	NT

++; 90～50% recovery, +; 10 % or less, −: none, NT : not tested..

grown on acid sulfate soil in South Kalimantan. Japanese *Journal of Crop Science* **73:** 222–223.

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