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Citation	Tropics, 16(1), 1-7 https://doi.org/10.3759/tropics.16.1
Issue Date	2007-01-31
Doc URL	https://hdl.handle.net/2115/35165
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Type	journal article
File Information	16_1.pdf



Screening of phosphate-solubilizing microorganisms in rhizosphere and rhizoplane of adverse soil-adapting plants in Southern Thailand

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Abstract Various adverse soils; peat, podzolic sandy, acid sulfate and salted-affected acid sulfate soils were widely distributed throughout Southern Thailand. These soils were infertile and strongly acidic. Phosphorus fertility of these soils was low due to the low content of available phosphorus (0.31–3.70 mg kg⁻¹) especially in podzolic sandy soil. Acid sulfate soil and salt-affected acid sulfate soil contain relatively high level of exchangeable Al (13.3 and 9.87 cmolc kg⁻¹, respectively). However, some native plants; *Melaleuca cajuputi*, *Melastoma malabathricum*, *Scleria sumatrensis* and *Oryza sativa* grow well in these adverse soils. Since the adverse soils distributed there are phosphorus-unavailable soils. Hence, base on an idea that phosphate-solubilizing microorganism is a key factor for plant growth, such functional rhizospheric microorganisms were screened using modified Pikovskaya's media containing 5 mg l⁻¹ P in different phosphate forms, Al-PO₄, Fe-PO₄ and Na-phytate, and adjusted pH 4.0. The number of microorganisms in the rhizosphere tended to be lower than in the rhizoplane. Twelve isolates showed remarkable growth performance on these solid media. Among these selected, 5 strains were obtained from acid sulfate soil, suggesting acid sulfate soil-adapting plant is a good source of the phosphate-solubilizing microorganism.

Key Words: adverse soil, low phosphorus soil, phosphate-solubilizing microorganism, rhizoplane and rhizosphere

INTRODUCTION

Various adverse soils, such as peat, sandy podzolic, acid sulfate and salt-affected acid sulfate soils are widely distributed throughout Southern Thailand. All adverse soils are acidic and have low fertility, and therefore, when

field crops are cultivated or introduced into these soils and serious nutritional problems often occur. Among soil productivity constraints, phosphorus plays a crucial role in crop productivity because these soils have low phosphorus availability (Osaki et al. 1998; Onthong et al. 1999). However, some plant species, such as *Panicum maximum*, *Pueraria phaseoloides*, *Melaleuca cajuputi*, *Panicum repens* and *Melastoma malabathricum*, all of which can adapt to such problematic soils, grow well without any fertilization (Osaki et al. 1997; Watanabe et al. 1997).

It is well known that a considerable variety of microorganisms are associated with the plant rhizosphere (Rodriguez and Fraga 1999). For example Oishi et al. (1999) found that a population size of bacteria in the root of reed-grass (*Lepiroonia articulate*) grown in acid sulfate soil was the same order as that of canola or wheat cultivated in neutral soil (Hong and Sato, 1994; Germida et al. 1998). This study suggested that the association between microorganism and plant rhizosphere provide the host plant a beneficial effect on plant growth, probably by means of the solubilization of soil-bound inorganic phosphates, particularly in acid sulfate soil. Moreover, in peat soil which contains relatively large amounts of organic phosphates, rhizospheric microorganisms are expected to hydrolyze such organic phosphates, including phytate.

As particular inhabitants in rhizosphere, some microorganisms play a critical role in mediating the availability of soil phosphorus to the host plants. Such functional microorganisms may directly act to phosphate solubilization and mineralization. An extensive range of microorganisms that are able to solubilize various forms of soil-bound phosphorus have been reported (Rodriguez and Fraga, 1999; Whitelaw, 2000), and among them, the most predominant and representative ones are *Bacillus* sp. and *Pseudomonas* sp., soil bacteria, and *Penicillium* sp. and *Aspergillus* sp. saprophytic fungus. On the basis of previous screening assays, it has been

shown that phosphate-solubilizing microorganisms often constitute maximal 30% of the culturable population size from the rhizosphere soils (Kucey, 1983). Therefore, it is suggested that phosphate solubilization by rhizosphere microorganisms is a major mechanism for phosphorus supply, and in fact there are a number of reports on these phosphate-solubilizing microorganisms. Nevertheless, less information is available on phosphate solubilizing by microorganisms in the rhizosphere or rhizoplane of plants, particularly those highly adapted to acidic and low phosphorus soils. The objectives of this work are, hence, to enumerate and investigate microorganisms having a capability to utilize practically unavailable phosphorus under low pH and low phosphorus conditions, from the rhizosphere and rhizoplane of the adverse soil-adapting plants.

Materials and Methods

Sample preparation

Roots of the adverse soil-adapting plants grown in low pH fields, including peat soil, sandy podzolic soil, acid sulfate soil and salt-affected acid sulfate soil were collected in Songkhla, Pattalung and Narathiwat province, Southern Thailand. The dominant species included *Melaleuca cajuputi* (Family Myrtaceae), *Melastoma malabathricum* (Family Melastomataceae), *Scleria sumatrensis* (Family Cyperaceae) and *Oryza sativa* cv. Kaodokmali 105 and Supunburi 2 (Family Poaceae) (See in Table 1). The rhizosphere soils were carefully sampled at the laboratory and then the living root was washed with

sterile distilled water, cut to a length of 0.5 cm using sterilized scissors, and the subjected to the isolation of rhizoplane microorganisms.

Bulky soil samples were collected at a depth of 0–15 cm from each field and air-dried. The resulting soils were ground, passed through a 2 mm sieve, and subjected to chemical analysis.

Analysis of soil chemical properties

Chemical properties of soil measured and methods used in this study include: soil pH (1:5, soil: distilled water), content of available P (Bray 2 method), exchangeable K, Ca, Mg and Na (extracted solution with 1 M ammonium acetate at pH 7, atomic absorption), content of organic matter (Walkley and Black method) (Certini et al. 2002) and total N (Kjeldahl method) (Bremner and Mulvaney, 1982) for triplication.

Enumeration of rhizosphere and rhizoplane microorganisms

One gram of soil or root samples was used for serial dilutions in 99 ml sterile distilled water. The enumeration medium was a modified Pikovskaya's agar containing per liter of distilled water: glucose 10 g, $(\text{NH}_4)_2\text{SO}_4$ 0.5 g, NaCl 0.2 g, KCl 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.005 g, P 5 mg l^{-1} and agar 15 g. Five mg l^{-1} of phosphorus were added to the medium and the pH was adjusted to 4.0 with 2 M sulfuric acid. In the cases of insoluble P (AlPO_4 and FePO_4), appropriate amount of the chemical powders were further grinded with a mortar and a pestle,

Table 1. Sampling site, soil type, host plant and phosphate-solubilization in the medium

Isolate no.	Site	Host	Sampling	Soil type	P_2O_5 from AlPO_4 (ppm)
AM 101	Ban Muang, Pattalung	<i>Melaleuca cajuputi</i>	Rhizoplane	Acid sulfate	0.04 ± 0.01
AM 102	Ban Muang, Pattalung	<i>Melaleuca cajuputi</i>	Rhizoplane	Acid sulfate	trace
AS 103	Ban Muang, Pattalung	<i>Scleria sumatrensis</i>	Rhizoplane	Acid sulfate	0.02 ± 0.00
AS 104	Ban Muang, Pattalung	<i>Scleria sumatrensis</i>	Rhizoplane	Acid sulfate	trace
AS 206	Bangtang, Pattalung	<i>Scleria sumatrensis</i>	Rhizosphere	Acid sulfate	0.02 ± 0.00
AS 218	Bangtang, Pattalung	<i>Scleria sumatrensis</i>	Rhizosphere	Acid sulfate	0.03 ± 0.00
AS 219	Bangtang, Pattalung	<i>Scleria sumatrensis</i>	Rhizoplane	Acid sulfate	0.04 ± 0.01
SM 112	Thananghom, Songkhla	<i>Melaleuca cajuputi</i>	Rhizoplane	Salt-affected acid sulfate	0.02 ± 0.00
SM 113	Thananghom, Songkhla	<i>Melaleuca cajuputi</i>	Rhizoplane	Salt-affected acid sulfate	0.03 ± 0.01
AR 101	Hat Yai, Songkhla	<i>Oryza sativa</i>	Rhizoplane	Acid sulfate	trace
AR 102	Hat Yai, Songkhla	<i>Oryza sativa</i>	Rhizoplane	Acid sulfate	trace
PM 103	Kokgradookmoo, Narathiwat	<i>Melaleuca cajuputi</i>	Rhizosphere	Peat	trace

* Although we tested not only AlPO_4 , but also FePO_4 , Al-phytate, Fe-phytate and Na-phytate, available P in the medium from these unavailable forms, except AlPO_4 , were all trace amount. All of the isolates acidified the medium pH from 4.0 into 3.63–2.54 after 12-day-incubation at r. t.

and the resulting micronized mineral was then added to the test medium. Drop plate method (Zuberer, 1994) was conducted using a modified Pikovskaya's agar containing KH_2PO_4 . The microorganisms were incubated at 37 °C for 5 days at which point colony forming unit were counted. The value (cfu g^{-1} of dry soil or dry root) of viable microorganisms for each plant samples was recorded. All of the test was done as 6 replications.

Isolation of phosphate-solubilizing microorganisms

One gram of soil or root samples was used for serial dilutions in 99 ml of sterile distilled water. As the isolation medium, a modified Pikovskaya's agar medium was used. Drop plate method (Zuberer, 1994) was conducted using modified Pikovskaya's agar containing Al-PO_4 , Fe-PO_4 , or Na-phytate. The microorganisms were incubated at 37 °C for 5 days. The isolates representative of the predominant and morphologically distinct colonies on the plate were selected and purified repeatedly. These isolates were cultured in modified Pikovskaya's broth containing Al-PO_4 , Fe-PO_4 , or Na-phytate.

Tentative identification of selected isolates

Microbial colonies of selected isolates grown on modified Pikovskaya's agar containing phosphorus in form of K_2HPO_4 were scraped with a loop and used for DNA extraction by Isoplant II kit (Wako Pure Chemical Industries, Japan). Total DNA was used as the template for PCR amplification with either Gene Taq (Nippon Gene, Japan) or HotStarTaq DNA polymerase (QIAGEN, USA). The first amplification for the 16S ribosomal RNA (rRNA) gene region with universal forward (27f) and reverse (1525r) primer was performed with 30 cycle of

denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 75 °C for 1 min. PCR products were sequenced by an ABI PRISM 310 Genetic Analyzer with a BigDye Terminator version (3.0). Cycle Sequencing Ready Reaction kit (Applied Biosystems). A reverse (1080r) primer was utilized for PCR direct sequencing. The sequence homology of the bases determined was then searched on the BLASTN database program (Hoo et al. 2004). For prokaryotic microorganisms, PCR and sequencing were done with the same method, but the region between NS1 and NS7R was amplified by PCR, using NS1 and NS7R primers. The PCR product was sequenced using sequencing primers, M13F, M13R, basid2, and basid3 to determine 1.75 kb. The results of tentative identification are shown in Table 2. Three isolates, eventually all identified as *Ustilago* spp., had the same characters as yeasts to show budding in younger stage and characteristic cell division from long hyphae into ovary-shaped short cells in matured stage.

RESULTS

Soil properties analysis

The pH of the peat soil used in this study was 3.75, and the fertility of all soils was lower than averaged mineral soils. As shown in Table 3, the EC of the salt-affected acid sulfate soil was higher than those of the other soils due to high content of exchangeable Mg and Na, which were 0.5 and 16.5 cmolc kg^{-1} , respectively. The content of available phosphorus in each adverse soil was also low level (0.31–3.7 mg kg^{-1}). The podzolic sandy soil had the lowest available P among all the test soils, while a high level of available S (10.6 mg kg^{-1}) and the highest level

Table 2. Identification of phosphate-solubilizing isolates

Isolate	Sequenced base size	Homology (%)	Tentative identification (family or closer genus)
AM 101	605	97	an Azotobacteriaceae bacterium (<i>Acidocella</i> sp.)
AM 102	628	99	<i>Burkholderia tropicalis</i> (Burkholderiaceae, bacterium)
AS 103	619	99	<i>Burkholderia tropicalis</i> (Burkholderiaceae, bacterium)
AS 104	515	93	an Acetobacteraceae bacterium (<i>Acidiphilium</i> sp.)
AS 206	632	97	an Acetobacteraceae bacterium (<i>Acidocella</i> sp.)
AS 218	506	96	an Acetobacteraceae bacterium (<i>Acidocella</i> sp.)
AS 219	566	97	an Acetobacteraceae bacterium (<i>Acidovorax</i> sp.)
SM 112	660	97	an Acetobacteraceae bacterium (<i>Acidocella</i> sp.)
SM 113	498	95	a Burkholderiaceae bacterium (<i>Burkholderia</i> sp.)
PM 103	1750	99	<i>Ustilago</i> sp. (Ustilaginaceae, yeast)

When 99% or more DNA alignment was obtained for the 16S rRNA gene regions of the test bacterium by BLAST DNA homology research (DDBJ), we finally concluded that the most aligned bacterial species in the DNA database was identical of the test strain in the species level (AM 102 and AS 103). The eukaryotic microorganism, PM 103, also showed 99% homology, but due to a poor database for fungi and yeast, we identified it as the genus level (*Ustilago* sp.). The others aligned less than 98% in BLAST homology search was identified only in the genus level.

Table 3. Chemical properties of peat, podzolic sandy, acid sulfate and salt-affected acid sulfate soils (value are mean of 3 replication $\pm$ SE)

Soil properties	Soil type			
	Peat	Podzolic sandy	Acid sulfate	Salt-affected acid sulfate
pH (soil : water 1:5)	3.75 $\pm$ 0.05	4.77 $\pm$ 0.12	3.87 $\pm$ 0.46	4.69 $\pm$ 0.03
EC (dS cm ⁻¹)	0.46 $\pm$ 0.07	0.07 $\pm$ 0.00	1.17 $\pm$ 0.18	5.48 $\pm$ 0.44
Avai. P (mg kg ⁻¹)	3.21 $\pm$ 0.58	0.31 $\pm$ 0.03	3.60 $\pm$ 2.22	3.70 $\pm$ 1.04
Avai. S (mg kg ⁻¹)	3.78 $\pm$ 0.61	0.62 $\pm$ 0.03	10.66 $\pm$ 0.50	8.96 $\pm$ 0.79
Exch. Ca (cmol _c kg ⁻¹)	2.64 $\pm$ 0.54	0.04 $\pm$ 0.00	0.93 $\pm$ 0.12	1.67 $\pm$ 0.08
Exch. Mg (cmol _c kg ⁻¹)	1.48 $\pm$ 0.24	0.04 $\pm$ 0.02	4.44 $\pm$ 0.48	10.5 $\pm$ 0.92
Exch. Na (cmol _c kg ⁻¹)	0.08 $\pm$ 0.03	0.16 $\pm$ 0.03	4.60 $\pm$ 1.21	16.5 $\pm$ 0.99
Exch. K (cmol _c kg ⁻¹)	0.04 $\pm$ 0.03	0.16 $\pm$ 0.03	0.31 $\pm$ 0.14	0.42 $\pm$ 0.26
Exch. Al (cmol _c kg ⁻¹)	2.08 $\pm$ 0.31	0.97 $\pm$ 0.28	13.3 $\pm$ 1.96	9.87 $\pm$ 0.58
OM (g kg ⁻¹)	322 $\pm$ 3.36	3.81 $\pm$ 0.81	70.5 $\pm$ 6.72	48.8 $\pm$ 3.25
Total N (g kg ⁻¹)	10.6 $\pm$ 2.52	0.36 $\pm$ 0.03	4.13 $\pm$ 0.43	8.13 $\pm$ 0.07

Table 4. Enumeration of microorganisms in the rhizoplane and rhizosphere of tropical adapted plants in adverse soils (value are mean of 6 replication $\pm$ SE)

Plant specie	Soil type	Rhizosphere microorganism (cfu g ⁻¹ dry soil)	Rhizoplane microorganism (cfu g ⁻¹ dry root)
<i>Melaleuca cajuputi</i>	Peat	1.02 $\pm$ 0.23 x 10 ⁵	2.73 $\pm$ 0.43 x 10 ⁶
	Acid sulfate	9.04 $\pm$ 0.47 x 10 ⁵	8.52 $\pm$ 0.59 x 10 ⁶
	Podzolic sandy	2.30 $\pm$ 0.81 x 10 ⁴	4.87 $\pm$ 0.72 x 10 ⁵
	Salt-affected acid sulfate	1.24 $\pm$ 0.42 x 10 ⁵	2.29 $\pm$ 0.89 x 10 ⁶
<i>Melastoma malabathricum</i>	Acid sulfate	2.00 $\pm$ 0.32 x 10 ⁴	3.28 $\pm$ 1.01 x 10 ⁵
	Podzolic sandy	1.35 $\pm$ 0.48 x 10 ³	4.52 $\pm$ 0.98 x 10 ⁵
<i>Scleria sumatrensis</i>	Peat	4.31 $\pm$ 0.24 x 10 ⁴	9.53 $\pm$ 1.35 x 10 ⁵
	Acid sulfate	8.79 $\pm$ 0.94 x 10 ⁵	9.37 $\pm$ 0.94 x 10 ⁶
<i>Oryza sativa</i>	Acid sulfate	1.50 $\pm$ 0.48 x 10 ⁷	2.70 $\pm$ 0.19 x 10 ⁷

of exchangeable Al (13.3 cmol_c kg⁻¹) were found in salt-affected acid sulfate soil. For soil organic matter, the lowest amount of organic matter was obtained (3.8 g kg⁻¹) in the podzolic sandy soil, whereas peat soil contained a high amount of organic matter (322 g kg⁻¹), which was 4–9-fold higher than those found in salt-affected acid sulfate soil and acid sulfate soil and 85 fold higher than sandy podzolic soil. Total nitrogen content was similar level in both peat soil and acid sulfate soil (10.6 and 8.1%, respectively).

Enumeration of microorganisms in the rhizosphere and rhizoplane

The number of microorganisms in rhizoplane was higher than rhizosphere in all plant species and all soil types. In the rhizosphere and rhizoplane of *O. sativa*, the population size of microorganisms was highest (1.50 x 10⁷

cfu g⁻¹ soil and 2.70 x 10⁷ cfu g⁻¹ root, respectively), among sampled plants; *M. cajuputi*, *M. malabathricum* and *S. sumatrensis*. In contrast, the number of microorganisms in podzolic sandy soil was the lowest in both rhizosphere and rhizoplane (1.35 x 10³ cfu g⁻¹ soil and 4.52 x 10⁵ cfu g⁻¹ root, respectively) (Table 4).

Isolation of phosphate-solubilizing microorganisms

Excellent phosphate-solubilizing microorganisms were mainly isolated from the rhizoplane, and some from the rhizosphere (Table 1). Although podzolic sandy soil contained only a trace amount of phosphorous due to severe leaching, we could not isolate phosphate-solubilizing microorganisms from this soil in this method. *M. malabathricum* adapted to low pH soil, but we also isolated none of phosphate-solubilizing microorganisms from this plant. *M. cajuputi* is an adapting plant to various

adverse soils, and this plant possessed several phosphate-solubilizing microorganisms, mainly on the rhizoplane.

DISCUSSION

Soil properties

The adverse soils, peat, sandy podzolic, acid sulfate and salt-affected acid sulfate soils widely distributed in the south of Thailand, were acidic and had low phosphorus fertility (Table 3). Acidification of these soils is constituted from several factors, including climatic conditions in terms of high temperature and intensive rainfall. These conditions tend to accelerate decomposition of the rock, leach nutrients and finally weather the soils. The contents of available phosphorus in the soil were low due to most of P exists as unavailable soil-bound or organic form. Moreover, aluminum phosphate and iron phosphate were the main forms of inorganic phosphorus in the soils (Onthong et al. 1999). The amounts of available phosphorus of peat, acid sulfate and salt-affected acid sulfate soils were higher than that of podzolic sandy soil due to the contribution of organic matter. However, the highly weathered soils on the tropics, soils with low phosphorus availability are generally found in this region. Therefore, phosphorus deficiency is the major constraint on crop production in the tropical zone, as reported by many researchers (Sahrawat et al. 1995; Yan et al. 1995; Kang et al. 1995). Moreover, the amount of exchangeable sodium, potassium, calcium and magnesium were low, implying that these adverse soils have been developed under highly leaching conditions to result in poor chemical properties. They are commonly acidic with the low cation exchange capacity and low content of exchangeable base and organic matter, but exchangeable aluminum is exceptionally high (Kheoruenromne, 1990).

Enumeration and isolation of phosphate-solubilizing microorganisms

The involvement of microorganisms in phosphate solubilization was known in the early 1930s (Gyaneshwar et al. 2002). Since then, using phosphate-solubilizing microorganisms as root-specific biofertilizer has been proposed as a method to enhance the efficiency of applied phosphorus fertilizers and/or phosphorus containing organic compounds leading to enhancement of the land productivity. For screening of phosphate-solubilizing microorganisms, the solubilization of calcium phosphate complexes such as dicalcium phosphate, tricalcium phosphate, and hydroxyapatite, is commonly used. However, these microorganisms would be effective

in calcareous soil in which calcium phosphate complexes predominate, while not in other soils, such as acidic soil in which phosphate complex with Fe and/or Al ions. The modified Pikovskaya's medium containing Al-P or Fe-P was therefore used in this study, and as a result, we obtained 12 isolates that have the capability of solubilizing unavailable phosphorus of Al-PO_4 , Fe-PO_4 at pH 4.0.

These selected positive strains with phosphate-solubilizing ability were mainly isolated from the rhizoplane, and only some were from the rhizosphere of plants adapted to acidic and low available phosphorus soils (Table 4). This is probably because phosphate-solubilizing microorganisms are highly dependent to rich carbon sources provided from plant root, because they require carbon source for active production of organic acids that are utilized for solubilizing soil-bound phosphate. In fact, Nautiyal (1997) demonstrated that, in the microscaled rhizosphere, the more apart from the root surface, the less root exudates are contained in the soil. On the other hand, rhizobacteria from *M. cajuputi* grown in acidic peat soil, Na-phytate-utilizable bacteria are candidate for phosphorus-suppliers.

Although both *M. malabathricum* and *M. cajuputi* are well adapted to acidic soils, any phosphate-solubilizing microorganisms have not yet been isolated from *M. malabathricum*. In contrast, several selected phosphate-solubilizing microorganisms were isolated from rhizosphere and rhizoplane of *M. cajuputi*. These results implied that plant species were greatly influenced by the microfloral community in their rhizosphere and rhizoplane (Johri et al. 1999), according to their acid-adapting strategies, in which root exudates are highly related and involved.

In general, arbuscular mycorrhizal fungi often contribute to phosphorus availability of this acid-tolerance plant; however, the phosphate-solubilizing, mycorrhizal fungi cannot function or even survive themselves in some adverse soils, such as strongly acidic soil or swampy soil. Hence, root-associating bacteria that often form specific microfloral communities tolerant to such adverse soil environment are regarded as phosphate-solubilizers of *M. malabathricum* and some other adverse soil-adapting plants that actively provided root exudates from the living root and /or root residues (Kennedy, 2005). Hence, it is speculated that characteristics of such host-specific rhizobacterial communities are highly associated with adaptabilities of host plants to the soil conditions.

In fact, Osaki et al. (1998) concluded that *M. malabathricum* and *M. cajuputi* have different strategies from each other, particularly for dealing with aluminum.

Since *M. malabathricum* accumulates a huge amount of aluminum in leaves designated as an aluminum-accumulator plant (Osaki et al. 1998), the root actively assimilates aluminum cations. While, *M. cajuputi* does not accumulate aluminum in shoots to behave as an aluminum excluder plant (Osaki et al. 1998), so the root surfaces of the plant are defense zones against aluminum cation in acidic soils. Moreover, *M. cajuputi* exudes lower amounts of organic acids than other tropical adapted plants (Onthong, 2000). Although they are both representative acid-tolerant plants, their differences in the strategy against aluminum at root surface may result in different contribution degree of phosphate-solubilizing rhizobacteria. For the next step, a pot culture experiment should be conducted to obtain clear evidence whether phosphate-solubilizing bacteria are beneficial against P-deficiency in acidic soil conditions.

ACKNOWLEDGEMENTS We wish to thank Miss Amornrat Chumtong and Miss Wanit Rotniam, Prince of Songkla University for their assistance in the laboratory work. This study was partly supported by Grant-in-Aid for Scientific Research (No. 16208032 to Y.H.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan

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Received 15th July 2005

Accepted 2nd Sep. 2005