The potential of plant growth-promoting microbes from South Kalimantan acid sulfate soil in enhancing the growth of rice plants

E Yuniarti¹, Surono¹, Nurjaya¹ and D N Susilowati²

¹Indonesian Soil Research Institute, Bogor, Indonesia
²Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Bogor, Indonesia

E-mail: erny_yuniarti@yahoo.com

Abstract. Rhizobacteria and dark septa endophytic (DSE) fungi isolated from indigenous plant of acid sulfate soil (ASS) in South Kalimantan has potency as an adaptive plant growth promoter. The study aimed to test the effectiveness of the rhizobacteria and DSE in improving swamp rice growth in ASS. Inpara 2 rice variety was used in greenhouse experiment using randomized complete design. Inoculants and compost were applied to the potential ASS-filled pot. The observed parameters were plant height, tillers number, rice straw and root weight, as well as root length. The plant height at 14 and 21 days after transplanting (DAT) was significantly higher in three rhizobacteria treatments over three DSEs and uninoculated treatment. All rhizobacteria significantly increased tiller number, rice straw and root weight, and length compared to control. The DSE GS1-2 significantly improved tiller number, as well as, root weight, and length over control. Rhizobacterium KM19.2 revealed highest effect on rice straw fresh and dry weight, root fresh weight and length. Rhizobacterium PD5.3.1 produced highest plant height at 14, 21, and 56 DAT as well as, tiller number and root dry weight. Therefore, the rhizobacteria and DSE GS1-2 could be used as a promising bio-fertilizer for improving swamp rice growth in ASS.

1. Introduction
The acid sulfate soil (ASS) is a soil containing sulfidic (potential ASS) or sulfuric (actual ASS) materials. It occupies coastal areas or inland through lakes, rivers, or wetlands [1,2]. Potential acid sulfate has pyrite material within 0 to 100 cm from the soil surface (pH >3.5 which is higher following soil depth whereas actual acid sulfate soil has a sulfuric horizon (pH <3.5) due to pyrite oxidation [3]. In Indonesia, acid sulfate soil area reaches 3.5 million ha distributed in the three largest islands, i.e., Sumatra, Kalimantan, and Papua [4]. ASS is fragile and classified as one of marginal soils to be targeted as planting area expansion for increasing rice production in Indonesia [5].

A potential ASS is not entirely included as potential ASS area but there are some points undergoing sulphur oxidation causing problem of acid sulfate that affected soil productivity for crop production. It consists of high soil acidity, the solubility of toxic metal (e.g., Al³⁺, Fe²⁺, Mn²⁺), low P, low base cations (Ca, Mg, K) low base saturation, and high leaching occurrence triggered by sulfuric acid formation from pyrite oxidation [6]. Very low pH and high toxic Al³⁺ concentration are unsuitable for plant growth and development. High toxic Al³⁺ inhibits roots elongation and restricts absorption of nutrient minerals [1,7]. Plant growth-promoting microbes have shown their ability to
improve plant growth and tolerance to soil environment stress such as acidity and alkalinity [8]. However, limited research provided the effectiveness data of plant growth-promoting microbes in improving swamp rice growth in ASS. Shamsuddin et al. [1], Panhwar et al. [9] and Panhwar et al. [10] reported that organic acids produced by PGPB can alleviate toxicity of Al$^{3+}$ by chelation, as well as, exopolysaccharides increased pH of soil solution by chelation of H$^+$. Dark septate endophytic are roots-colonizing Ascomycetes fungi of a wide range plant species. They are characterized by the presence of brown to black mycelium and septate and pigmented hyphae, capable of colonizing the intracellular and intercellular region of root tissues where it can form densely septate intracellular structures called as microsclerotia [11]. DSE fungi have a role related to plant host resistance in environmental stress such as salinity and drought conditions. The association of plant with microorganisms, such as dark septate endophytic fungi, has mitigated the detrimental effects of abiotic and biotic stress on the host plant [12]. The effectiveness of DSE in improving plant growth in ASS hitherto has not yet been documented.

In the preliminary work, we had isolated plant growth-promoting microbes, i.e., rhizobacteria and DSE fungi from indigenous plants such as karamunting (Melastoma sp), purun (Eleocharis dulcis), kalakai (Stochaenapaluistris sp.), gelam (Melaleuca leucadendra) and indigenous swamp rice (Oryzae sativa) growing in acid sulfate soil ecosystem in Central Kalimantan. They were rhizobacteria and DSE fungi which were in vitro test showing the properties as a plant growth promoter and adapter to ASS condition such as acidity and Fe$^{2+}$ toxicity. The plant growth promotion potency of ASS-adapting microbes still needs to be proved. Therefore, the study aimed to test the effectiveness of the selected microbes in promoting swamp rice of Inpara 2 growth in potential acid sulfate soil.

2. Materials and methods

2.1. Microbial sources

The bacteria used in this study were 3 rhizobacteria (KM19.2, PR24.1, PD5.31) and three DSE (GS1.21, LK.11. K3a, KDS 1.3) isolated from from rhizosphere and roots of indigenous/pioneer plants such as karamunting (Melastoma sp), purun (Eleocharis dulcis), kalakai (Stochaenapaluistris sp.), gelam (Melaleuca leucadendra) and indigenous swamp rice (Oryzae sativa) growing in acid sulfate soil ecosystem in South Kalimantan. They were in vitro test showing the properties such as capable of growing in nitrogen-free medium, solubilizing insoluble P, as well as, producing indole acetic acid phytohormone, organic acids. Furthermore, the microbes were able to grow in low pH and high Fe i.e., 4 to 5.5 and 100 ppm, respectively for rhizobacteria, while for DSE were able to grow at pH and Fe values of 3 to 5.5 and 500 to 1,000 ppm, respectively for DSE (table 1).

Long term preservation of rhizobacterial cultures by lyophilization intended to maintain their genetic stability and viability. Before their use, lyophilized rhizobacteria were rejuvenated in TSB media. The glycerol solution-preserved DSEs were rejuvenated in Corn Meal Agar.

2.2. Location of soil sampling

Bulk soil sampling location was District of Tanjung Harapan, Alalak, Barito Kuala, South Kalimantan Alalak, Barito Kuala (3° 10’ 08”S and 144° 36’ 13”E). The soil was potential acid soil of type B (an area that can only be overflowed by large tides) of a newly opened land ecosystem. The grass, bush, weed growing in the sampling site was cleared away. The soil was drilled with a Belgium drill and the soil at each drilling step subjected to a hydrogen peroxide test to check the depth of the pyrite layer. At a depth of 0 to 30 cm, the pyrite layer is suitable for taking bulk soil samples at 0 to 20 cm tillage layer of the soil. Bulk soil sampling avoided the site with shallow pyrite levels and kept away from touching the pyrite layer. Taken bulk soil was free from decomposed pile plant residues or animal dirt, had a homogeneous weed growth, and was in a water-logged condition. Bulk soil samples at the depth of 0-20 cm were taken with a hoe. The plant root residues were removed from the surface of the bulk soil sample in a submerged condition to evade soil oxidation. Each bulk soil was put in a plastic bag lined
with the same size of a thick sack. Then, the bulk soil sample filled-plastic bag and its sack immediately tightly tied.

Table 1. Plant growth promotion properties of the microbes.

| No. | Microbial Code | Fix N | P-sol | IAA (mg L⁻¹) | Growth on media pH | Growth on Fe (mg L⁻¹) | Acetate acid (mg L⁻¹) | Lactate acid (mg L⁻¹) | Citrate acid (mg L⁻¹) | Malate acid (mg L⁻¹) | Oxalate acid (mg L⁻¹) |
|-----|----------------|-------|-------|--------------|---------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| 1   | KDS1.3         | NT    | NT    | NT           | 3 - 5               | 500-1000              | 0.207                | 0.259                | 0.037                | 0.670                | 0.075                |
| 2   | LK2K3a         | NT    | NT    | NT           | 3 - 5               | 500-1000              | 0.662                | 0.759                | 0.042                | 1.224                | 0.064                |
| 3   | GS1.2          | NT    | NT    | NT           | 3 - 5               | 500-1000              | 0.472                | 0.847                | 0.041                | ND                   | ND                   |
| 4   | KM19.2 Bacillus subtilis PD5.3.1 | +     | +     | 8,12        | 4 -5.5              | 100                  | 0.475                | 1.038                | 0.042                | 0.292                | ND                   |
| 5   | Bacillus calusii | +     | +     | 18,14       | 5 - 5.5             | 100                  | 0.280                | 0.322                | 0.028                | 0.418                | ND                   |
| 6   | PR24.11.2      | +     | +     | 13,95       | 5-5.5               | 100                  | 0.467                | 1.038                | 0.050                | 0.535                | ND                   |

Notes: NT = had not been tested; ND = Not detected.

2.3. Soil analysis
Before the greenhouse experiment, chemical analysis of the bulk soil sample was carried out at Chemical Laboratory of ISRI, including texture, pH, C-organic, N-Kjeldahl, a 25% HCl extracted P and K, available P (Bray 1/Olsen), and K (Morgan), cation exchange value (Ca, Mg, K, Na), NH₄OAc 1 N pH 7 extracted CEC, base saturation, Al³⁺, H⁺, Fe, S, and pyrite (13) were carried out at Chemical Laboratory of ISRI.

2.4. Greenhouse experiment
A pot experiment was conducted in a greenhouse of ISRI, IAARD, Ministry of Agriculture, Indonesia. Two rice seedlings were transplanted in each pot containing 5 kg of topsoil (0-20 cm) which was maintained in water-logged condition. The soil in the pot was amended with 2 ton ha⁻¹ compost and 1% (v/w) microbial inoculant. The treated soils in the pots were mixed thoroughly seven days before transplanting compost. The experiment had six treatments one control. They were repeated in three replications and was laid out in complete randomized design. The six treatments consisted of inoculants of KM19.2, PR24.1, PD5.3, GS1.21, LK.11. K3a, KDS1.3. The control was the soil without inoculation. A Swamp rice variety of Inpara2 is used as an indicator plant.

Seedling preparation steps as follows: firstly, seeds were soaked in hot water (60°C) for 20 minutes to open the seed pores. Afterward, the seeds were subjected to surface sterilization by immersing them in a 5% NaOCl solution for five minutes. Sterilized seeds were washed with sterile water three times which in each washing step, the water-soaked seeds were shaken for 10 minutes to clean the residual NaOCl on the seeds surface. Sterilized seeds were sown in autoclaved seedling media, a mixture of compost and acid sulfate soil (1:1). Sown seeds were cultivated in a growth chamber for 14 days.

Microbial inoculants were prepared as follows: each rhizobacterium was inoculated to NB (Nutrient Broth) media while each DSE was inoculated to PDB (Potato Dextrose Broth). Inoculated media were incubated on an orbital shaker machine at 28°C for 24 hours for rhizobacteria and 5 days for DSE. Rhizobacteria inoculation to NB medium was carried out 24 hours before a 5-day incubation period of DSE to both those inoculants available at the same time for the greenhouse experiment. Both culture rhizobacteria and DSE respectively were centrifuged at 5000 rpm and 4°C for five minutes. Each pellet was reconstituted with 0.85% saline solution and was centrifuged at the same condition as...
a first centrifugation process. The Pellet was subjected to two more washing processes to obtain
media-free inoculants. Pellet was reconstituted in a 0.85% saline solution up to a density of 1x10^7 cells
per ml (rhizobacteria) and 1x10^5 propagules per ml (DSE).

After seven days of soil incubation time, two 14-day old rice seedlings were transplanted in each
pot. During the experiment, tap water was added to each planting medium to give the submerged
condition.

The observed plant parameters included plant height at 14, 21, and 56 days after transplanting
(DAT) and the number of tillers, rice straw fresh weight, root fresh weight, and length at 56 DAT.
Plant height was measured from the stem base up to the tip of the leaves.

2.5. Data analysis
Data were analyzed by ANOVA using SPSS ver. 21. Differences among treatments were tested using
Duncan’ Multiple Range Test (DMRT).

3. Results and discussion

3.1. Properties of potential acid sulfate soil used in greenhouse experiment
The physico-chemical characteristic of potential acid sulfate soil before the greenhouse experiment
is presented in table 2. The topsoil had a silty clay texture and very low soil pH, high exchangeable Al
and extractable Fe, i.e., 13.51 cmolc kg^-1 and 1,600 mg kg^-1, the concentration of toxic level for rice
growth. Total N, P and K content were low. Base saturation, exchangeable K, Ca, Mg were low. The
potential ASS under this study required fertilization of N, P, K, Ca, and Mg.

Total C was high with value 3.29%. The high organic matter would reduce the availability of Al
and Fe through chelation. Chelated Al and Fe are non-toxic to rice plant in field [9]. Compost was
used in this study as sole source of fertilizer for rice seedling growth. Incorporation of organic matter
into the sulfuric soil significantly increased soil pH which the change in pH were correlated with Eh.
Application of OM to the “neutralized sulfuric soil” was only partially effective in preventing
acidification. The decomposition of OM by aerobic bacteria results in oxygen depletion which then
favors metabolic conversion of sulfates to sulfide by anaerobic bacteria [14]. Organic matter depend
on its quality could neutralize Al toxicity via chelation [15].

3.2. Effectiveness of microbes to improve rice seedling growth
The results of rice seedling growth improvement by rhizobacteria and DSE in the greenhouse are
presented in tables 3 and 4. Significant difference was observed for the growth parameters in rice
seedling among rhizobacteria, DSE treatments and control (uninoculated). The plant height at 14
and 21 DAT was significantly higher in three rhizobacteria treatments over three DSEs and uninoculated
Treatment. One DSE treatment, namely LKII.2K3a could significantly improve plant height at 21 DAT
compared to uninoculated. At 56 DAT, only rhizobacterium PD5.3.1 which was able to improve plant
height compared to those of uninoculated treatments.

All three rhizobacteria significantly increased rice straw fresh and dry weight compared to control
and DSEs whereas the rhizobacteria and DSE GS1-21 significantly improved number of tillers, root
fresh and dry weight, as well as root length compared to control. DSE KDS1.3 only revealed higher
root length compared than those of to control and DSE LK11K3a. Rhizobacterium KM19.2 produced
highest rice straw fresh (21.92 g) and dry weight (5.80 g), as well as, root fresh weight (27.39 g) and
length (29.97 cm) of rice plants. Rhizobacterium PD 5.3.1 produced highest number of tillers (11),
root dry weight (of Inpara 2 rice plant variety about 3.53 g), plant height at 14, 21, and 56 DAT, i.e.
35.1, 35.9, and 42.5 cm respectively.

Very acidic soil, aluminum, and ferrous toxicity, as well as nutrient scarcity are limiting factors in
acid sulfate soil used in this study. According to Slaski [7] that the toxicity of Al^3+ particularly is
evinced by inhibition in roots elongation, as a result of disturbance of cell division in root apical
meristem. Al toxicity causes foliar symptom which is similar to those caused by calcium and
phosphorus deficiency [7]. Furthermore, Yang et al. [16] stated that soluble aluminum form (Al³⁺) at level of 10-20 mg/kg or more can be toxic on plants. This aluminum can cause oxidative stress as result of an increase of oxygen species (ROS) production which influence the unsaturated fatty acid membrane and indirectly affecting plant metabolism. Aluminum also can replace to interfere expansion process of plant cells.

**Table 2.** Physico-chemical characteristics of potential acid sulfate soil used in the experiment.

| Soil Parameters  | Value       | category |
|------------------|-------------|----------|
| pH (1:5)         | 3.90        | Very acid|
| H₂O              | 3.40        |          |
| KCl              |             |          |
| Textural Grade (pipet) |   | Silty clay|
| Sand (%)         | 0           |          |
| Silt (%)         | 42          |          |
| Clay (%)         | 58          |          |
| Total C (%)      | 3.29        | High     |
| Total N (%)      | 0.18        | Low      |
| C/N              | 18.00       | High     |
| Extractant (HCl 25%) |          |          |
| Total P₂O₅ (mg.100 g⁻¹) | 8   | Low      |
| Total K₂O (mg.100 g⁻¹) | 6   | Very low |
| P-Bray 1 (ppm)   | 8.3         | Moderate |
| Cation exchange value (NH₄-Acetate 1N, pH 7) | | |
| Ca (cmol kg⁻¹)   | 0.57        | Very low |
| Mg (cmol kg⁻¹)   | 0.65        | Low      |
| K (cmol kg⁻¹)    | 0.06        | Very low |
| Number of cations (cmol kg⁻¹) | 1.53 |          |
| CEC (cmol kg⁻¹)  | 20.17       | Moderate |
| Base saturation (cmol kg⁻¹) | 8.00 | Very low |
| Exchangeable (KCN 1 N) | | |
| Al³⁺ (cmol kg⁻¹) | 13.51       | High     |
| H⁺ (cmol kg⁻¹)   | 1.74        | Low      |
| Fe, Total HNO₃ (%) | 1.60        | High     |

Iron ions (Fe³⁺) under submerged soil with the anaerobic condition and low soil pH (high negative potential) are reduced to FeAl²⁺ which a more soluble ferrous form. Excess Fe²⁺ is absorbed by roots and transported via the xylem to leaf causing Fe²⁺ accumulation in plant tissues. Accumulated Al²⁺ in plant tissue cause excess ROS production by Fenton reaction. ROS disrupt cell structure, DNA, and protein, as well as physiological process. Toxicity of Fe³⁺ in plant tissue eventually cause bronzing symptoms in leaf followed by the yield loss or crop failure. While Fe³⁺ around roots is oxidized to Fe³⁺ by oxygen transported from shoots to roots leading to the plaque formation on the root surface [17]. The Fe plaque coated root surface undergo constraint of other mineral nutrients uptake [18]. However, in figure 1 seemed that root growth of rice plants in all treatment showed no symptoms of toxic Fe exposure, i.e., thickening, browning, rough, and short root. These symptoms could inhibit nutrient absorption, reduce root oxidation ability on Fe³⁺, and inhibit the root ability to prevent the entry of
Fe$^{2+}$ [19]. Some factors were influencing the normal root growth of rice plants. Firstly, rice plant
Inpara 2 which is the rice variety for swampland so that it was able to cope with the productivity
problems of acid sulphate soil in term to toxicity of Al or Fe. Secondly, organic matter factor which
helped to neutralize toxic Al or Fe.

Then, microbe inoculation especially the rhizobacteria contributed to alleviate toxic metal and to enhance root growth, and eventually shoot growth of rice plant Inpara 2 variety in potential acid sulfate soil.

Table 3. Effect of microbes on plant height.

| No. | Microbes       | Average of Rice Plant Height (cm) |
|-----|----------------|----------------------------------|
|     |                | Day 14  | Day 21  | Day 56  |
| 1   | GS 1.2         | 28.106  | 30.2 c  | 41.2 b  |
| 2   | LK II.2. K3a   | 30.11 b | 32.22 b | 39.3 c  |
| 3   | KDS 1.3        | 29.5 b  | 30.5 c  | 41.0 b  |
| 4   | PR 24.1        | 34.4 a  | 35.4 a  | 40.7 bc |
| 5   | KM19.2         | 34.6 a  | 35.3 a  | 40.3 bc |
| 6   | PD 5.3.1       | 35.1 a  | 35.9 a  | 42.5 a  |
| 7   | Uninoculated (Control) | 29.3 bc | 30.0 c  | 40.3 bc |

Note: means in a column followed by the same letter are not significantly different at 5% level by DMRT.

Table 4. Effect of plant growth-promoting microbes on plant growth parameters of 56-day old rice plants.

| No | Microbes       | Number of Tillers | Rice Straw Fresh Weight (g) | Rice Straw Dry Weight (g) | Root Fresh Weight (g) | Root Dry Weight (g) | Root Length (cm) |
|----|----------------|-------------------|-----------------------------|---------------------------|-----------------------|---------------------|------------------|
| 1  | GS 1.2-21      | 7.00 c            | 13.44 b                     | 3.17 d                    | 25.78 b               | 2.62 d              | 29.67 ab         |
| 2  | LK 11. K3a     | 6.00 e            | 10.69 c                     | 2.60 e                    | 13.80 e               | 1.72 f              | 26.87 cd         |
| 3  | KDS 1.3        | 6.33 de           | 13.86 b                     | 3.25 d                    | 15.54 d               | 1.83 e              | 29.93 a          |
| 4  | PR 2.4.1       | 10.67 a           | 21.64 a                     | 5.65 b                    | 26.89 ab              | 3.24 b              | 27.30 ab         |
| 5  | KM 19.2        | 10.00 b           | 21.92 a                     | 5.80 a                    | 27.39 a               | 2.96 c              | 29.97 a          |
| 6  | PD 5.3.1       | 11.00 a           | 21.10 a                     | 5.65 b                    | 25.92 b               | 3.53 a              | 28.57 ab         |
| 7  | Uninoculated (Control) | 6.33 de       | 12.85 b                     | 3.57 c                    | 17.83 c               | 1.89 e              | 26.37 d          |

Note: means in a column followed by the same letter are not significantly different at 5% level by DMRT.

However, in figure 1 seemed that root growth of rice plants in all treatment showed no symptoms of toxic Fe exposed such as thickening, browning, rough, and short root so that they could inhibit nutrient absorption, reduce root oxidation ability on Fe$^{3+}$, and inhibit the root ability to prevent the entry of Fe$^{2+}$ [19].

The rhizobacteria (PR 24.1, KM 19.2, PD 5.3.1) were used in this experiment. In the preliminary work they showed as a growth promoter in vitro such as N fixer, phosphate solubilizer, IAA, and organic acids producer (acetate, lactate, citrate, malic acid). While the DSE (GS 1.2, LK II.2.K3a, KDS 1.3) revealed plant growth characteristics via the production of organic acids, i.e. acetate, lactate, citrate, malic (only for KDS1.3 and LK2K3a), and oxalate (only for KDS1.3 and LK2K3a). In a research results study (10) reported that PGPB (Burkholderia thailandensis, B. seminalis, and Sphingomonas putiuasa) produced organic acids (oxalic, citric, malic acid) which were able to reduce Al toxicity by chelating the Al ions. They also produced polysaccharides which might adsorb H$^+$ ions
increasing soil pH from 4 to 6. At this pH level, Al ions will be precipitated to not toxic form. The release phytohormone further enhanced rice growth and increased yield.

The rice plant Inpara 2 variety, existing soil organic matter, and added compost in this study had potency as a mitigator of abiotic stress of the acid sulfate soil (low pH, toxicity of Al and Fe and nutrient source). Overall, enhancement of Inpara 2 rice growth in the acid sulfate soil as evidence that the rhizobacteria and DSE are adaptive plant growth-promoting microbe.

![Figure 1](image_url)  
**Figure 1.** Root performance of 56-day old rice plants growing in potential acid sulfate soil filled-pot.

4. Conclusions  
Rhizobacteria PR24.1, KM19.2, PD 5.3.1, and DSE GS1.21 could enhance the growth of Inpara 2 paddy seedling variety in acid sulfate soil. The organic acids produced by rhizobacteria and DSE GS1-2 fungus might help to alleviate the toxicity of Al and Fe by chelation reaction. The IAA phytohormone produced by rhizobacteria promoted swamp rice growth in acid sulfate soil. However, their mechanisms to alleviate soil acidity, low nutrient content, and Al and Fe toxicity of ASS need more investigation to obtain a better strategy to formulate them as an adaptive bio-fertilizer for acid sulfate soil.

Acknowledgments  
This research was funded by the project of DIPA of Indonesian Soil Research Institute, Agricultural Research and Technology Dissemination) managed by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Year 2019. All authors contributed equally to the data generation and writing of this paper.

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