Introduction

Nitrogen (N), phosphorus (P), and potassium (K) are among several inorganic elements that are essential nutrients for plant growth. Phosphorus is used for the formation of nucleic acids, phospholipids, and ATP in plant cells. Inorganic P in soil is classified into insoluble adsorbed P such as AlPO₄, Fe(H₂PO₄)₂·2H₂O and Ca₃(PO₄)₂; available adsorbed P such as Ca(H₂PO₄)₂ and CaHPO₄; and water-soluble P (WSP) such as H₂PO₄⁻ and H₃PO₄ (Altomare et al., 1999; Gyaneshwar et al., 2002; Sanyal and De Datta, 1991).

Plants absorb WSP and available P from the soil through their roots (Altomare et al., 1999).

Chemical fertilizers have been developed for efficient agricultural systems. They have high concentrations of WSP and available P, thereby providing a direct fertilizer effect for plant growth. Agricultural yields were greatly increased by the use of chemical P fertilizers in the 20th century (Cordell and White, 2011; Natasha, 2009).

In recent years, environmental pollution arising from the excessive use of chemical fertilizers has become a serious global problem (Sharpley, 1999). Inorganic P from chemical fertilizers may be released into the environment (Hart et al., 2004) and may enter aquatic environments, which leads to eutrophication and “red tide” (Smith, 2003). In addition, chemical P fertilizers are produced from phosphate rocks, but the global reserves of phosphate rocks are gradually decreasing. It
is for these reasons that agricultural systems using organic materials have recently received much attention (Crowder et al., 2010; Mäder et al., 2002; Trewavas, 2001).

Up to 80% of P in the soil is present in organic forms, and phytates are a major constituent of the organic P fraction (Richardson et al., 2005; Turner et al., 2002). WSP is produced from phytates by the action of phytases from plant roots and soil microorganisms (Irving, 1980; Richardson and Hadobas, 1997; Richardson et al., 2001). Part of the produced WSP is adsorbed and precipitated on active soil surfaces and metal ions (Doolittle et al., 2010; Oberson et al., 2001). Recently, several phytase-secreting microorganisms, such as Aspergillus niger, Peniophora lycii, and Pseudomonas sp., have been isolated and identified for improving mineralization of phosphorus in soils (George et al., 2007; Richardson et al., 2001). The activation of phytase-secreting soil microorganisms plays an important role in the mineralization of phytate.

In this study, a method to evaluate the mineralization of phytate in the soil environment was developed based on the production of WSP, and the relationship between the phytate mineralization activity and bacterial biomass in the agricultural soils was investigated. In addition, phytate-degrading bacteria with the potential to improve the mineralization of phytate in the soil environment were isolated and identified.

Materials and Methods

Soil sampling and phosphorus fractions in the soil samples. Soil samples were collected from agricultural fields around Kusatsu City, Shiga Prefecture, Japan. Soil samples (sandy loam in texture) were collected from a depth of 5–10 cm without the surface layer. To analyze the different forms of P, soils were extracted following the procedures of Bowman and Moir (1993) as follows: 1.0 g soil sample was mixed with a 25 ml of extraction solution (0.25 M NaOH and 0.5 M sodium EDTA) followed by heating at 60°C for 2 h, centrifuging at 6,000×g for 15 min, and filtering the suspension through a filter paper (No. 5B, Advantec, Tokyo, Japan).

To analyze the inorganic P, the filtrate was digested with H₂SO₄ (1.8 M) and filtered again, followed by the molybdenum blue method (Murphy and Riley, 1962). The total P was analyzed by digesting the filtrate with K₂S₂O₄ at 150°C for 30 min followed by the molybdenum blue method. Organic P was calculated by subtracting the inorganic P fraction from the total P. The moisture content in each sample was analyzed by oven drying method (105°C for 24 h) to convert the fractions of P on a dry weight basis.

Evaluation of phytate mineralization. Phytate (sodium salt) solution (pH 7.0) containing 3.9 mg P was added to 1.0 g soil sample, which was then incubated for 3 days at room temperature at 60% water retention capacity. A blank experiment (without phytate) was carried out simultaneously by the same method. WSP was extracted from the incubated 1.0 g soil sample with 20 ml H₂O, and analyzed by the molybdenum blue method. The increment in WSP in phytate-supplemented soil after 3 days compared to that in the blank was defined as the phytate mineralization activity in that soil (Fig. 1). The mineralization activity was expressed in percentage assigning 0 for no mineralization and 100 for complete mineralization of the added 3.9 mg phytate P.

Bacterial biomass in the soil sample. Bacterial biomass in the soil was measured by a slow stirring method (Aoshima et al., 2006) by analyzing environmental DNA (eDNA). To extract the eDNA from soils, a 1.0 g soil sample was mixed with 8.0 ml of DNA extraction buffer (100 mM tris(hydroxymethyl)aminomethane, 100 mM sodium EDTA, 100 mM sodium dihydrogenorthophosphate, 1.5 mM sodium chloride and 1% (w/v) hexadecyltrimethylammonium bromide) and 1.0 ml of 20% (w/v) sodium dodecyl sulfate solution, and digested with a propeller for 20 min. The suspension was centrifuged at 5,000×g for 10 min, and then about 700 μl of supernatant was transferred into a 1.5 ml micro tube and slowly supplemented with 700 μl of chloroform-isooamylic alcohol and centrifuged at 18,000×g for 10 min followed by the further addition of 300 μl of isopropanol and purified by centrifugation at 18,000×g for 20 min. The pellet of crude nucleic acid was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) after drying. The extracted eDNA was quantified based on the nanodrop spectrophotometer reading and the intensity of the DNA bands on the agarose gel electrophoresis using Kodak 1D 3.6 Image Analysis Software (Kodak, New Haven, CT). The bacterial biomass in the soils was estimated by using the equation Y =1.70×10⁵X (R²=0.96) (Aoshima et al., 2006), where Y and X are the bacterial biomass g⁻¹ dry soil and the amount of eDNA, respectively.

Isolation and identification of phytate-degrading bac-
Phytate-degrading bacteria and their contribution to phytate mineralization

To isolate the phytate-degrading bacteria from the soils, a 10 times diluted soil suspension was added to the M9 phytate liquid medium (0.4% phytate, 0.1% NH₄Cl, 0.012% MgSO₄·4H₂O, and 0.00147% CaCl₂·2H₂O, buffered with 0.1% HEPES in water) and incubated at 30°C for 48 h by shaking on a rotary shaker at 120 rpm. The cultures showing an optical density at 600 nm (OD₆₀₀) of more than 0.1 were selected for further screening by plating the cultures on M9 phytate solid medium (containing 1.5% agar) to isolate a single colony. Each of the selected colonies was transferred and multiplied in the M9 phytate liquid medium, and the strains showing an OD₆₀₀ of more than 0.5 were selected for further characterization.

 Cultures of the selected strains were analyzed for the WSP content, and those showing a higher amount (1,000 mg ml⁻¹) were selected for taxonomic classification. For taxonomic classification, 16S rRNA gene sequences were analyzed following the procedures of Koma et al. (2003) and Sanpa et al. (2006). The 16S rRNA gene primers 20F (5'-TGTAATCGTCGGCCAGTAGAGTTTGATCCTGGCTC-3') and 1510R (5'-CAGGAAACAGCTATGACCAGGCTACCTTGTTACGACT-3') were used for PCR to amplify the 16S rRNA gene. The PCR reaction was carried out for 30 cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 1 min). The amplified DNA was purified using a PCR product purification kit (Gel/PCR DNA Isolation System, Viogene, Sunnyvale, CA). The 16S rRNA gene sequences were determined and the data were compared with the reference strains available in the GenBank database using BLAST search (Altschul et al., 1997) available at http://www.dna.affrc.go.jp.

Enhancement of phytate mineralization in agricultural soil by inoculation with phytate-degrading bacteria.

To enhance the mineralization of phytate in the agricultural soils, a phytate-degrading bacterium was cultured with LB medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl) at 30°C for 24 h. Then, the cells were washed with sterilized water one time and resuspended in sterilized water. The bacterial suspension was inoculated into the soil samples at 4 x 10⁵, 4 x 10⁶, 4 x 10⁷, and 4 x 10⁸ cells g⁻¹ soil including a non-inoculated control. Bacillus subtilis MT-2 was used as a control (Kubo et al., 1996). The phytate mineralization activity was measured as described in Fig. 1.
Nucleotide sequence accession number. All 16S rRNA gene sequences have been deposited in the DNA Data Bank of Japan under accession numbers AB819473–AB819480.

Results and Discussion

Phosphorus status in the soil

Fractions of different forms of P in 10 agricultural soils are presented in Fig. 2. The concentrations of total P ranged between 0.50 and 7.22 mg g\(^{-1}\) dry soil (average: 1.90 mg g\(^{-1}\) dry soil), and organic P between 0.40 and 4.25 mg g\(^{-1}\) dry soil (average: 1.10 mg g\(^{-1}\) dry soil). Of the total P in the soils, the fraction of organic P ranged between 48.5 to 80.0% (average: 57.8%).

The transformation of organic P to WSP is an important reaction in agricultural soil. Analysis of the transformation of organic P to WSP, therefore, helps for better understanding of agricultural soil conditions. Organic P constitutes the largest proportion of total P in most soils, of which phytates and their derivatives comprise about 80% of the total organic P (Anderson, 1980).

Evaluation of phytate mineralization in soil environment

The phytate mineralization activity differed among the soils ranging from 3.0–41.0% (average: 24.1%) (Fig. 3), but no mineralization activity was observed in the sterilized soils, indicating that the phytate mineralization is carried out solely by the microorganisms. While mineralization activity was compared with the amounts of different forms of P in the soils (Fig. 2), it was related to neither the organic P (\(R^2=0.055\)) nor the inorganic P (\(R^2=0.046\)) (data not shown). The low mineralization activity in most of the soils and their un-

![Fig. 2. Concentration of different forms of P in agricultural soils.](image)

In each bar, organic and inorganic P fractions are represented by white and black spaces. Bars are standard deviations (\(n=3\)).

![Fig. 3. Phytate mineralization activity in agricultural soil samples.](image)

The sterilized control is indicated by "Cont.". Numbers from 1 to 10 correspond to the soils of the same number in Fig. 2. Bars are standard deviations (\(n=3\)).

![Fig. 4. Phytate mineralization activity in the ascending order (above) and corresponding bacterial biomass (below) in soil samples collected from 60 agricultural fields (from 11 to 70).](image)

Phytate mineralization activity denotes the % P mineralized from the added phytate P after 3 days of incubation. Bacterial biomass was estimated by the eDNA method (Aoshima et al., 2006).
relatedness to the P content in soils might be due to the lack of phytate-degrading microorganisms. The conversion of organic P to WSP could be an effective way for sustainable P management. Since the potential mineralization of WSP from the added phytate was 3.9 mg g\(^{-1}\) soil, the average mineralization value of 0.94 mg g\(^{-1}\) soil in this study corresponded to 24.1% only. The gap of 75.9% might be due to the adsorption of mineralized phosphate (as shown in Fig. 1) or due to incomplete mineralization of phytate in the soil. Therefore, for the proper P nutrition of crop plants, continuous mineralization of the organic P fraction is important.

**Relationship between phytate mineralization activity and bacterial biomass**

The range of phytate mineralization activity in 60 agricultural soils was from 0 to 61.7% (average: 18.8%), and that of bacterial biomass from \(3.01 \times 10^8\) to \(14.28 \times 10^8\) cells g\(^{-1}\) soil (average: \(6.8 \times 10^8\) cells g\(^{-1}\) dry soil) (Fig. 4), which is almost ten times less than that of reported by Aoshima et al. (2006), who found \(5.95 \times 10^9\) bacterial cells g\(^{-1}\) soil in agricultural fields and non-agricultural forests of Japan. In addition, we found a weak correlation existed between phytate mineralization activity and bacterial biomass \((R^2=0.11)\), suggesting a need to enhance the bacterial biomass, particularly, phytate-degrading bacterial populations in the soils to improve the mineralization activity.

**Enhancement of phytate mineralization with phytate-degrading bacteria**

A total of 72 bacterial strains showing a high OD\(_{600}\) (>0.1) in a liquid culture containing phytate were isolated (data not shown). Of the selected isolates, 36 strains showed OD\(_{600}\) >0.5 and produced ≥242.8 \(\mu\)g WSP ml\(^{-1}\) culture, and 8 of them were able to release more than 1,000 \(\mu\)g WSP ml\(^{-1}\) (Table 1).

Based on the 16S rRNA gene sequences, out of the 8 strains showing higher P mineralization rates, JT29, JT32, JT33, JT34, JT35, and JT36 showed the highest homology with the *Pseudomonas rhodesiae* NO5 (100%), and those of JT30 and JT31 showed 99.9% and 99.6% homology with *Pseudomonas* sp. JCM 17186 and *Flavobacterium johnsoniae* strain LB-D, respectively (Table 2).

To enhance the mineralization of phytate, *P. rhodesiae* JT29 and *F. johnsoniae* JT31 (4 \(\times\) 10\(^5\) to 4 \(\times\) 10\(^8\) cells g\(^{-1}\) soil) were inoculated into the soil samples. The soil used for the inoculation was soil sample num-

<table>
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<tr>
<th>Strain name</th>
<th>WSP content in the culture ((\mu)g ml(^{-1}))</th>
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<tr>
<td>JT 1</td>
<td>242.8</td>
<td>JT 15</td>
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<tr>
<td>JT 2</td>
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<td>JT 3</td>
<td>411.5</td>
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<td>JT 4</td>
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<td>JT 5</td>
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<td>JT 6</td>
<td>472.2</td>
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<td>JT 7</td>
<td>485.7</td>
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<td>JT 32</td>
<td>1,079</td>
<td>JT 36</td>
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Table 1. WSP content in the bacterial cultures in M9 phytate liquid medium.
In this soil, the indigenous bacterial biomass was $2.72 \times 10^8$ cells g$^{-1}$ soil, and phytate mineralization activity was lowest (3%) among the 10 soils. Results show that inoculation of these strains at $4 \times 10^7$ and $4 \times 10^8$ cells g$^{-1}$ soil greatly increased the phytate mineralization activity (Fig. 5). Inoculation with *F. johnsoniae* JT31 at $4 \times 10^8$ cells g$^{-1}$ soil showed the highest phytate mineralization activity (53.5% of added phytate P). On the other hand, the control strain (*B. subtilis* MT2) did not activate the mineralization even at the inoculation rate of $8 \times 10^8$ cells g$^{-1}$ soil.

Previous studies have reported these bacteria as plant growth promoters. Kang et al. (2007) observed the dominant presence of *Pseudomonas rhodesiae* in the pepper (*Capsicum annuum* L.) stems and found a beneficial effect on plant growth. In a study by Tani et al. (2011) several species of *Pseudomonas* including *P. rhodesiae* were isolated from them a culture of hydroponically grown moss and found capable of promoting moss growth by producing plant growth substances. In this study, we found soil bacteria *P. rhodesiae* JT29 and *F. johnsoniae* JT31 were effective to degrade phytate in the soils. In addition, a high $R^2$ value of 0.84 between the inoculation rate and phytate mineralization activity of these two inoculants (see Fig. 5) suggests that the use of phytate-degrading bacteria such...
as *P. rhodesiae* JT29 and *F. johnsoniae* JT31 could improve the mineralization of organic P in soils poor in P circulation.

A quantitative method for assessing N mineralization activity has recently been constructed (Matsuno et al., 2013). In N mineralization, the activities of ammonium-oxidizing bacteria and nitrite-oxidizing bacteria are important. In this study phytate mineralization was enhanced by the phytate-degrading bacteria. It is likely that different bacterial genera are involved in the phytate mineralization in soil compared to those involved in N mineralization.

Phytases (myo-inositol hexakisphosphate phosphohydrolases) catalyze the hydrolysis of phytates. Phytases include cystein phytase, histidine acid phytase, purple acid phosphatase and β-propeller phytases (BPP) (Mullaney and Ullah, 2003; Rao et al., 2009). BPPs are produced by various soil bacteria (Jorquera et al., 2011; Lim et al., 2007). Higher phytase activity by the strains *P. rhodesiae* JT29 and *F. johnsoniae* JT31 may contribute to mineralization of phytate in soil. To better understand the mineralization of phytate, phytase activity in the soil environment should be further investigated.

**References**


Murphy, J. and Riley, J. P. (1962) A modified single solution...


