EVALUATION OF ORGANIC ACID PRODUCTION POTENTIAL OF PHOSPHATE SOLUBILIZING FUNGI ISOLATED FROM SOILS IN OKINAWA, JAPAN

ISLAM, M. K.1,2,4 – SANO, A.1,2 – MAJUMDER, M. S. I.1,2 – SAKAGAMI, J-I.1,5 – GIMA, S.3 – HOSSAIN, M. A.1,2*

1The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan
2Faculty of Agriculture, University of the Ryukyus, Okinawa 903-0213, Japan
3IRC, University of the Ryukyus, Okinawa 903-0213, Japan
4Department of Soil Science, Patuakhali Science and Technology University, Patuakhali, Bangladesh
5Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan

*Corresponding author
e-mail: amzad@agr.u-ryukyu.ac.jp; phone: +81-98-895-8824; fax: +81-98-895-8741

(Received 30th Jun 2019; accepted 25th Oct 2019)

Abstract. Deficiency of available phosphorous (P) in soil is one of the major factors that limit plant growth and yield. Microorganisms play an important role to improving available P status in soil by solubilization. Although phosphate solubilizing mechanism is not clearly understood, organic acid production seems to be the main mechanism of P solubilizing. Therefore, present study evaluated the organic acid production potentials of 16 P solubilizing fungal strains (2 Aspergillus floscosus, 3 Aspergillus niger, 2 Aspergillus niveus, 2 Penicillium oxalicum, 5 Penicillium spp. and 2 Talaromyces pinophilus isolates) isolated from soils in Okinawa, Japan to select outstanding strains that could facilitate the P solubilization process. Results revealed that both type and quantity of microbial organic acids production depend on the P sources and fungal strains. The highest quantity of organic acids was found when Ca\(_2\)(PO\(_4\))\(_2\) was used as substrate followed by FePO\(_4\) and AlPO\(_4\). Based on the organic acids production potential, A. niger (SI-12URAgr) considered as outstanding P solubilizing fungi regardless of substrates followed P. oxalicum (SI-6URAgr, SI-16URAgr) and A. niger (SI-10URAgr). These strains could have great potential as promising bioresource for efficient P utilization in agricultural production.

Keywords: phosphorous, organic acids, Aspergillus niger, Penicillium oxalicum, agricultural production

Introduction

Phosphorous is the second major nutrient after nitrogen that limits plant growth and yield (Gyaneshwar et al., 2002). This nutrient exists in nature in a variety of organic and inorganic forms. The majority of soils contain insoluble inorganic phosphates, which are of no use to plants unless they are solubilized (Singh et al., 2011). Acidic environment can enhance the solubility of P minerals significantly (Zhen, 2016). This is a feasible pathway to improve the P release from phosphate minerals. Although phosphate solubilizing mechanism is still not fully understood, the production of organic acids seems to be the main mechanism of P solubilizing (Alam et al., 2002; Siddique and Robinson, 2003). Organic acids also have multiple industrial applications as food additives, pharmaceutical and cosmetic excipients (Sauer et al., 2008). They are fully
degradable molecules and can be used as chemical intermediates or as for the production of biodegradable polymers replacing synthetic chemicals (Sauer et al., 2008).

Many phosphate solubilizing microbes (PSM), including bacteria and fungi have the ability to produce organic acids (Kavanagh, 2011) and they contributes to dissolving insoluble P through the process of acidification, chelation and exchange reaction, thus promotes plant growth (Gerresten, 1948; Singh et al., 2011). Compared to bacteria, phosphate solubilizing fungi (PSF) have ten times higher ability to secrete organic acid (Kavanagh, 2011). Among these, Aspergillus spp., Penicilium spp., Talaromyces spp. and Eupenicilium spp. are considered “key organisms” in the P cycle (Jose et al., 2010).

The ability of organic acids production by fungi is basically determined by genes, but it can also be affected by environmental condition (Zhen, 2016). For example, type of phosphate compounds could affect both phosphate solubilization and organic acid production. Previously we isolated phosphate solubilizing fungi from subtropical soils in Okinawa, Japan and studied their potentiality to solubilize different insoluble phosphate compounds. However, organic acid production ability of the fungal strains for different P sources were not documented. Therefore the study evaluated the organic acid production potential of 16 phosphate solubilizing fungal strains isolated from soils in subtropical Okinawa, Japan to select outstanding strains that could facilitate the P solubilization process.

**Materials and Methods**

**Description of the Soil sampling area**

The sampling area located at 26.5000°N and 128.0000°E. Its climate is subtropical, temperatures range from 10 to 32°C. Low temperature (10 to 26°C) exists in winter season and higher temperature (27 to 32°C) exists in summer with a humidity level near 100%. The major soil types are dark-red, red and grey soils in this area.

**Isolation of phosphate solubilizing fungi**

This study was carried out in the Mycology Laboratory, Faculty of Agriculture, University of the Ryukyus, Okinawa, during 2017–2018 under a class II biohazard cabinet (BHC-1306IIA/3B, AIRTECH, Tokyo, Japan) followed to the biosafety classification by National Institute of Infectious Disease of Japan, because of possibilities of including toxic fungal species treated as BSL2 during the isolation. Zero to fifty cm depth soil samples were collected from ten different locations of each soil type in Okinawa using sterile auger. One-hundred-gram soil was taken from each sampling point and it makes a total of 500 g composite sample (five points from each location make one composite sample). The samples were transferred to laboratory in sterile sealed polythene bag under aseptic condition and isolation was done by serial dilution method (Rao, 1982).

**Identification of phosphate solubilizing fungi**

**Morphological identification**

The genera of phosphate solubilizing fungal isolates were identified based on the taxonomic keys based on morphologies (Watanabe, 2010). The keys were the colour and tint in colony overs and revers, presence of aerial hyphae, colony surface texture, colony margin and pattern of pigment exudations. Wet mounts prepared from micro culture were mounted in lacto phenol and lacto phenol cotton blue. Microscopic examination and
photomicrography were performed with an OLYMPUS BX50 microscopy equipped with image Analysis system (Olympus Corporation, Tokyo, Japan).

**Molecular identification**

DNA was extracted from one piece of fungal mycelia from a culture incubated at 25°C for 48 h on Sabouraud medium containing 2% glucose and 1% peptone using a DEXPAT kit (TaKaRa, Japan) to identify the isolates at genetic level (Yamaguchi et al., 2014). Beta-tubulin gene sequences amplified with primers bt2a and bt2b and calmodulin genes amplified with primers CMD5 and CMD6 were determined (Samson et al., 2014). Sequences were analysed by the NCBI BLAST tool to classify and identify closely related fungal sequences. We identified the isolates to the certain species if the BLAST results showed similarity values of 98% or higher. Nucleotide sequences were deposited into DNA data bank of Japan under accession number (Table 1).

**Table 1. List of fungal strains with gene bank accession number isolated from different soils in Okinawa, Japan used in this study**

| Isolates | Strain in gene bank | Soil types | Sampling places | Organisms | Accession number |
|----------|---------------------|------------|----------------|-----------|-----------------|
| 1        | SI-1UR Agr          | Dark red soil | Nishihara, Okinawa | Penicillium sp. | LC425316         |
| 2        | SI-2UR Agr          | Dark red soil | Nishihara, Okinawa | Aspergillus floccosus | LC425317         |
| 3        | SI-3UR Agr          | Dark red soil | Nishihara, Okinawa | Aspergillus niveus | LC425318, LC425334 |
| 4        | SI-4UR Agr          | Grey soil   | Nishihara, Okinawa | Talaromyces pinophilus | LC425319, LC425335 |
| 5        | SI-5UR Agr          | Grey soil   | Nishihara, Okinawa | Aspergillus niveus | LC425320, LC425336 |
| 6        | SI-6UR Agr          | Grey soil   | Nishihara, Okinawa | Penicillium oxalicum | LC425321         |
| 7        | SI-7UR Agr          | Red soils   | Kunigami, Okinawa | Penicillium sp. | LC425322         |
| 8        | SI-8UR Agr          | Red soils   | Kunigami, Okinawa | Penicillium sp. | LC425323         |
| 9        | SI-9UR Agr          | Red soils   | Kunigami, Okinawa | Penicillium sp. | LC425324         |
| 10       | SI-10UR Agr         | Red soils   | Kunigami, Okinawa | Aspergillus niger | LC425325, LC425337 |
| 11       | SI-11UR Agr         | Red soils   | Yanbaru forest, Okinawa | Aspergillus niger | LC425326, LC425338 |
| 12       | SI-12UR Agr         | Red soils   | Yanbaru forest, Okinawa | Aspergillus niger | LC425327, LC425339 |
| 13       | SI-13UR Agr         | Dark red soil | Nishihara, Okinawa | Penicillium sp. | LC425328, LC425340 |
| 14       | SI-14UR Agr         | Dark red soil | Nishihara, Okinawa | Aspergillus floccosus | LC425329         |
| 15       | SI-15UR Agr         | Grey soil   | Nishihara, Okinawa | Talaromyces pinophilus | LC425330         |
| 16       | SI-16UR Agr         | Dark red soil | Nishihara, Okinawa | Penicillium oxalicum | LC425331         |

**Medium preparation for organic acid production study**

Pikovskaya’s (PKV) broth medium consisted of 10.0 g glucose, 5.0 g Ca₃(PO₄)₂, 0.5 g (NH₄)₂SO₄, 0.1 g MgSO₄·7H₂O, 0.02 g NaCl, 0.02 g KCl, 0.003 g FeSO₄·7H₂O, 0.003 g MnSO₄·H₂O, 0.5 g yeast extract and 1000 mL distilled water (Pikovskaya, 1948). In this medium Ca₃(PO₄)₂ was used as source of insoluble phosphate that was replaced by insoluble FePO₄ and AlPO₄. The medium was autoclaved at 121°C for 15 minutes.
Chloramphenicol (Wako Pure Chemical Corporation, Osaka, Japan) was also used to avoid bacterial growth.

**Culturing and preparation of spore suspension**

For conducting organic acid production experiment, fungal cultures were made from the re-slanting of pure culture slants that preserved at 4°C. Sporulated culture slants were selected for preparation of spore suspension. A total volume of 5 ml sterile water with tween 80 (Wako Pure Chemical Corporation, Osaka, Japan) was added in culture slants and the fungal colony surface was lightly scraped by a sterile inoculation loop (Thermo Scientific™, Nunc™ Disposable Loops and Needles, Thermo Scientific™ 251586, Fisher Scientific, Tokyo, Japan). Then cultures were passing through a syringe with a 4×4 cm sheet of a sterile absorbant cotton (Kyualet, Kawamoto Sangyo, Osaka, Japan). Spore count was done by a hemocytometer and the suspension was adjusted to approximately 10⁶ spores mL⁻¹.

**Incubation**

The experiments were carried out using Erlenmeyer flask containing 40 ml Pikoveskaya’s (PKV) broth medium supplemented with 0.5% tricalcium phosphate [Ca₃(PO₄)₂], aluminium phosphate (AlPO₄) and iron phosphate (FePO₄). After sterilization, the medium of each flask was inoculated with the 5% (v/v) spore suspension of a particular fungal strain containing 10⁶ spore mL⁻¹. Sterile distilled water inoculated flaks was treated as control (Fig. 1.).

![Figure 1. Fermented Pikovskaya broth culture for organic acid determination by HPLC inoculated with 16 phosphate solubilizing fungal strains](image-url)

Three replicates were maintained for each test isolate. Incubation was done at 25°C in an incubator shaker at 120 rpm up to 7 days. The samples were autoclaved and centrifuged at 5000 rpm for 25 minutes to remove any suspended solids and mycelial parts. The culture supernatants were filtered through 0.22 μm pore size syringe filter unit (Merck KGaA, Darmstadt, Germany).

**Detection and quantification of organic acids**

Detection and quantification of organic acids were done by High Performance Liquid chromatography (Prominence HPLC system, Shimadzu-CBM-20A, Japan) equipped with diode array detector (SPD-M20A), refractive index detector (RID-10A), column ICE-ION-300 (300 mmX7.8 mm), auto sampler (LC-20AD) and fraction collector (FRC-10A). The injection volume, temperature and flow rate was 50 μl, 50°C and 0.5 ml/min, respectively. Sulfuric acid of 0.01N was used as solvent of mobile phase. Peaks were identified against a set of standards from known organic acids (oxalic, citric, tartaric, malic, lactic, formic and acetic acid).
Statistical analysis

All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program (version 2016). The mean values were compared by Dancan’s Multiple Range Test and significant differences were detected at $p<0.05$ level.

Results

We detected and quantified seven different organic acids from medium containing insoluble tricalcium phosphate (TCP), aluminium phosphate (Al-P) and iron phosphate (Fe-P). Acids were oxalic, citric, tartaric, malic, lactic, formic and acetic acid. Fungal strains showed significant variation to organic acid production based on phosphate substrates. Detail results presented below under specific headlines.

Organic acid production by fungal strains in TCP $\left[{\text{Ca}}_3(\text{PO}_4)_2\right]$ supplemented medium

All the strains produced oxalic acids and lactic acids. The amount ranged from 2.3-342.0 and 26.3-320.7 µg/ml, respectively. Except SI-3URAg and SI-5URAg, other strains produced malic acid ranged from 22.7-139.7 µg/ml, whereas both citric acid and tartaric acid were released by the strains SI-6URAg, SI-7URAg, SI-8URAg, SI-9URAg, SI-10URAg, SI-11URAg, SI-12URAg, SI-14URAg and SI-16URAg. The produced citric acid ranged from 3.0-566.7 and tartaric acid was 1.7-27.3 µg/ml. SI-5URAg produced citric acid and SI-2URAg, SI-3URAg and SI-13URAg produced tartaric acids. Most of the strains produced formic acid except SI-4URAg, SI-7URAg, SI-8URAg, SI-9URAg, SI-15URAg, and most of the strains produced acetic acid except SI-2URAg, SI-3URAg, SI-5URAg and SI-8URAg. It was ranged from 16.7-1102.7 µg/ml and 9.7-2812.3 µg/ml, respectively. The highest amount of oxalic (342.0 µg/ml), citric (566.7 µg/ml), tartaric (173.3 µg/ml), malic (139.7 µg/ml), lactic (320.7 µg/ml), formic (1102.7 µg/ml) and acetic (2812.3 µg/ml) acids were produced from TCP containing broth by the strain SI-11URAg, SI-7URAg, SI-16URAg, SI-2URAg, SI-12URAg, SI-13URAg and SI-12URAg, respectively (Table 2).

Organic acid production by fungal strains in Al-P $\left[\text{AlPO}_4\right]$ supplimented medium

In aluminium phosphate (Al-P) supplemented medium, most of the strains produced oxalic acid, tartaric acid, malic acid and lactic acid ranged from 3.0-461.3 µg/ml, 3.0-461.3 µg/ml, 16.0-198.0 µg/ml and 5.7-119.0 µg/ml, respectively. The strain SI-2URAg and SI-8URAg could not produce oxalic acid and SI-2URAg, SI-4URAg and SI-15URAg could not produce tartaric acids. Only SI-5URAg could not produce both tartaric and malic acids. The citric acid was produced by most of the fungal strain ranged from 3.7-367.7 µg/ml except the strains SI-2URAg, SI-3URAg, SI-4URAg, SI-5URAg, SI-14URAg and SI-15URAg. Both formic and acetic acids were not detected from the culture filtrate of SI-2URAg, SI-4URAg, SI-8URAg and SI-9URAg. SI-7URAg, SI-15URAg could not produce formic acid. The highest amount of oxalic (461.3 µg/ml), citric (367.7 µg/ml), tartaric (61.0 µg/ml), malic (198.0 µg/ml), lactic (119.0 µg/ml), formic (1313.7 µg/ml) and acetic (1556.0 µg/ml) acids were produced from Al-P containing broth by the strain SI-12URAg, SI-9URAg, SI-12URAg, SI-7URAg, SI-11URAg, SI-13 URAg and SI-6URAg, respectively (Table 3).
Table 2. Types and quantities of produced organic acids in the Pikoveskaya’s medium supplemented with insoluble Ca₃(PO₄)₂ by 16 phosphate solubilizing fungal strains

| Strains      | Type of fungi | Organic acid (µg/ml) |
|--------------|---------------|----------------------|
|              | Oxalic        | Citric               | Tartaric | Malic | Lactic | Formic | Acetic |
| SI-1URAg     | Penicillium sp. | 3.0±0.1³                 | N.D.     | N.D.  | 34.7±2.1⁶d | 48.7±9.3⁶c| 567.0±33.9³| 1148.0±11.0³ |
| SI-2URAg     | A. floccosus  | 46.3±4.7²d               | N.D.     | 6.0±1.0⁶b | 139.7±7.5³ | 170.7±13.5³ | 183.3±18.2³ | N.D.               |
| SI-3URAg     | A. niveus     | 23.0±2.6³                   | N.D.     | 7.3±0.6⁶d | N.D.    | 77.7±6.0³c | 162.3±29.7³ | N.D.               |
| SI-4URAg     | T. pinophilus | 3.7±1.2³                  | N.D.     | N.D.  | 43.0±3.0⁶d | 26.3±2.1³  | N.D.     | 29.0±2.0³          |
| SI-5URAg     | A. niveus     | 4.0±1.0³                  | 426.3±22.7³ | N.D.  | 90.3±13.0³c | 16.7±4.0³  | N.D.     |                   |
| SI-6URAg     | P. oxalicum   | 77.0±10.8³               | 52.0±2.6³  | 13.7±2.5³ | 22.7±3.1³ | 74.3±6.1³  | 749.7±42.1³ | 1503.0±42.0³      |
| SI-7URAg     | Penicillium sp. | 2.7±0.6³                 | 566.7±32.1³ | 17.3±3.1³ | 114.3±16.3³ | 50.7±7.8³c | N.D.     | 36.3±8.0³          |
| SI-8URAg     | Penicillium sp. | 5.3±1.5³                 | 3.0±1.0³  | 18.7±3.5³ | 42.0±5.6³  | 41.7±4.0³  | N.D.     | N.D.               |
| SI-9URAg     | Penicillium sp. | 5.0±1.0³                 | 53.0±8.2³ | 5.3±1.5³ | 31.0±6.6³  | 37.7±5.7³  | N.D.     | 9.7±0.7³           |
| SI-10URAg    | A. niger      | 69.0±4.0³f               | 10.3±1.5³ | 6.0±1.0³ | 79.7±6.0³ | 154.3±5.0³ | 172.7±31.6³ | 28.7±4.2³         |
| SI-11URAg    | A. niger      | 342.0±20.4³              | 8.0±1.0³  | 16.3±2.5³ | 82.7±6.7³ | 272.3±26.6³ | 311.0±22.3³ | 628.0±16.1³      |
| SI-12URAg    | A. niger      | 49.0±10.5³e               | 13.7±2.5³ | 16.0±4.4³ | 78.7±9.5³ | 320.7±13.8³ | 340.3±52.6³ | 2812.3±76.6³     |
| SI-13URAg    | Penicillium sp. | 5.7±0.6³                 | N.D.     | 1.7±0.6³ | 54.0±5.6³ | 84.7±7.4³ | 1102.7±80.3³ | 15.3±2.1³       |
| SI-14URAg    | A. floccosus  | 52.3±5.9⁶d               | 10.3±2.1³ | 26.7±2.5³ | 35.7±6.4³ | 94.0±4.6³  | 54.3±12.7³ | 20.0±4.6³         |
| SI-15URAg    | T. pinophilus | 2.3±0.6³                 | N.D.     | N.D.  | 43.7±4.0² | 44.3±5.9³  | N.D.     | 31.0±4.6³         |
| SI-16URAg    | P. oxalicum   | 12.3±1.5³c               | 100.7±6.5³ | 27.3±3.1³ | 39.7±4.0³ | 68.3±6.0³c | 696.3±54.8³ | 1402.0±46.0³      |

Values given are the mean of three replicates ± standard deviation of the mean. Values with common letters in each column do not differ statistically according to Duncan’s Multiple Range Test (DMRT) at p<0.05
N.D.: Not detected
Organic acid calculated as micrograms per milliliter

Organic acid production by fungal strains in Fe-P (FePO₄) supplemented medium

In iron phosphate (Fe-P) supplemented medium, all strains showed the production of tartaric acid (11.3-408.3 µg/ml), malic acid (12.0-383.0 µg/ml), lactic acid (2.7-88 µg/ml) and formic acid (8.3-1082.0 µg/ml), whereas SI-5URAg did not produce lactic acid. The oxalic acid was produced (1.5-811.0 µg/ml) by the strain SI-4URAg, SI-9URAg, SI-11URAg and SI-12URAg. Strains SI-3URAg, SI-5URAg, SI-7URAg, SI-10URAg, SI-11URAg, SI-12URAg SI-15URAg and SI-16URAg produced both citric and acetic acids, whereas SI-2URAg, SI-13URAg produced citric acid, SI-9URAg and SI-14URAg produced acetic acid. The highest amount of oxalic (811.0 µg/ml), citric (955.7 µg/ml), tartaric (408.3 µg/ml), malic (383.3.0 µg/ml), lactic (88.0 µg/ml), formic (1082.7 µg/ml) and acetic (342.3 µg/ml) acids were produced from Fe-P containing medium by the strain SI-10URAg, SI-12URAg, SI-13URAg, SI-10URAg, SI-4URAg, SI-13 and SI-12URAg, respectively (Table 4).

Comparison of quantities of organic acids produced by 16 fungal strains in different P substrates

In this study, the strongest organic acid production ability of fungal strains was found in medium containing tri-calcium phosphate (TCP) followed by iron phosphate (Fe-P) and aluminium phosphate (Al-P). The produced organic acid ranged between

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(6):15191-15201.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1706_1519115201
© 2019, ALÔKI Kft, Budapest, Hungary
102.0-3630.0 µg/ml, 22.3-2486.9 µg/ml and 118.7-1803.3 µg/ml in the medium supplemented with TCP, Al-P and Fe-P, respectively. Among the fungal strains, the highest amount of organic acids was produced by Aspergillus niger strain SI-12URAgr (3630.7 µg/ml) in the medium supplemented with TCP followed by Penicillium oxalicum strain SI-6URAgr (2492.3 µg/ml) and SI-16URAgr (2346.7 µg/ml) in Al-P medium and Aspergillus niger strain SI-10URAgr (1803.0 µg/ml) in Fe-P medium. These strains were considered as outstanding because this quantity of organic acids was higher than sum of the mean and standard deviation of the total quantities of organic acids produced by 16 fungal strains in this study (Table 5). HPLC chromatograms of outstanding fungal strains shown in (Fig. 2).

**Table 3.** Types and quantities of produced organic acids in the Pikoveskaya’s medium supplemented with insoluble AlP by 16 phosphate solubilizing fungal strains

| Strains    | Type of fungi | Oxalic | Citric | Tartaric | Malic | Lactic | Formic | Acetic |
|------------|---------------|--------|--------|----------|-------|--------|--------|--------|
| SI-1URAgr  | Penicillium sp. | 18.7±3.5<sup>ab</sup> | 3.7±0.6<sup>ab</sup> | 44.0±3.6<sup>ab</sup> | 36.3±2.1<sup>a</sup> | 11.3±1.5<sup>a</sup> | 443.3±14.2<sup>a</sup> | 787.0±27.1<sup>a</sup> |
| SI-2URAgr  | A. floccosus    | N.D.   | N.D.   | 10.3±1.5<sup>a</sup> | N.D.  | 12.0±2.0<sup>a</sup> | N.D.   | N.D.   |
| SI-3URAgr  | A. niveus       | 3.0±1.0<sup>ab</sup> | 6.0±1.0<sup>bc</sup> | 16.0±2.6<sup>a</sup> | 21.7±0.6<sup>de</sup> | 23.0±3.0<sup>bc</sup> | 34.0±3.6<sup>ab</sup> |
| SI-4URAgr  | T. pinophilus   | 13.3±1.5<sup>abc</sup> | N.D.   | 46.0±4.0<sup>cd</sup> | 16.0±2.6<sup>bc</sup> | N.D.   | N.D.   |
| SI-5URAgr  | A. niveus       | 13.7±3.5<sup>abc</sup> | N. D. | N. D.    | 25.0±4.0<sup>bc</sup> | 21.0±1.0<sup>bc</sup> | 74.0±12.2<sup>bc</sup> |
| SI-6URAgr  | P. oxalicum     | 7.0±1.0<sup>de</sup> | 11.0±1.0<sup>de</sup> | 6.5±0.5<sup>e</sup>  | 39.0±3.0<sup>de</sup> | 50.7±5.9<sup>bc</sup> | 816.7±10.5<sup>bc</sup> | 1556.0±27.2<sup>bc</sup> |
| SI-7URAgr  | Penicillium sp. | 12.0±1.0<sup>ef</sup> | 356.7±16.8<sup>g</sup> | 8.3±0.6<sup>bc</sup> | 198.0±8.5<sup>ef</sup> | 5.7±1.2<sup>de</sup> | N.D.   | 32.7±4.5<sup>bc</sup> |
| SI-8URAgr  | Penicillium sp. | N.D.   | 7.3±1.2<sup>ef</sup> | 7.5±0.5<sup>bc</sup> | 54.3±11.6<sup>de</sup> | 5.7±0.6<sup>de</sup> | N.D.   |
| SI-9URAgr  | Penicillium sp. | N.D.   | 367.7±25.8<sup>de</sup> | 4.7±0.6<sup>de</sup> | 151.3±11.4<sup>de</sup> | 30.0±3.0<sup>de</sup> | N.D.   |
| SI-10URAgr | A. niger        | 20.0±1.0<sup>bc</sup> | 13.3±2.1<sup>de</sup> | 20.3±2.1<sup>de</sup> | 27.0±2.6<sup>de</sup> | 6.0±1.0<sup>bc</sup> | 315.7±6.7<sup>de</sup> | 96.7±6.8<sup>bc</sup> |
| SI-11URAgr | A. niger        | 22.3±3.2<sup>bc</sup> | 31.0±3.6<sup>de</sup> | 7.0±1.0<sup>bc</sup> | 45.0±5.3<sup>de</sup> | 119.0±5.6<sup>de</sup> | 268.0±22.9<sup>de</sup> | 12.0±1.0<sup>bc</sup> |
| SI-12URAgr | A. niger        | 461.3±13.9<sup>g</sup> | 22.7±4.0<sup>de</sup> | 61.0±6.6<sup>de</sup> | 24.0±3.6<sup>de</sup> | 108.7±5.7<sup>de</sup> | 299.3±12.2<sup>de</sup> | 578.0±40<sup>de</sup> |
| SI-13URAgr | Penicillium sp. | 23.7±2.5<sup>bc</sup> | 18.0±2.6<sup>de</sup> | 16.0±2.6<sup>de</sup> | 40.0±2.0<sup>de</sup> | 41.7±6.5<sup>de</sup> | 1313.7±33.6<sup>de</sup> | 567.0±19.7<sup>de</sup> |
| SI-14URAgr | A. floccosus    | 20.0±3.0<sup>de</sup> | N.D.   | 24.7±2.1<sup>ef</sup> | 36.3±2.5<sup>de</sup> | 31.0±3.6<sup>de</sup> | 136.7±3.1<sup>de</sup> | 37.0±4.6<sup>bc</sup> |
| SI-15URAgr | T. pinophilus   | 11.0±1.0<sup>ef</sup> | N.D.   | 48.0±7.0<sup>de</sup> | 26.3±3.2<sup>de</sup> | N.D.   | 21.7±2.9<sup>de</sup> |
| SI-16URAgr | P. oxalicum     | 16.3±1.5<sup>abc</sup> | 70.0±6.6<sup>bc</sup> | 19.7±1.5<sup>de</sup> | 37.3±2.1<sup>de</sup> | 42.3±7.0<sup>de</sup> | 643.7±10.0<sup>de</sup> | 1196.7±36.9<sup>bc</sup> |

Values given are the mean of three replicates ± standard deviation of the mean. Values with common letters in each column do not differ statistically according to Duncan’s Multiple Range Test (DMRT) at p<0.05.
N.D.: Not detected.
Organic acid calculated as micrograms per milliliter.

**Discussion**

The sixteen P solubilizing fungal strains used in this study were isolated from different soils in Okinawa, Japan under subtropical environment. The isolates were identified as the genera of Aspergillus, Penicillium and Talaromyces (Islam et al., 2019). Both type and the quantity of organic acids produced by fungal strains varied with the nature of phosphate substrates and fungal strains. In this study, acetic, lactic and formic acids were the major acids in TCP medium, oxalic, citric, malic, tartaric and acetic acids in Fe-P medium, and formic, lactic, malic and citric acids in Al-P medium. Fungal strains produced the highest amount of organic acids in TCP supplemented medium...
followed by Fe-P and Al-P. It might be the result of interaction between fungal strains and the P sources. Zang et al. (2018) and Scervino et al. (2013) reported that the quantity of organic acid produce by fungi differed with the nature of phosphate substrates. Another point is that organic acid production by microorganism depends on their genetic variation and each strain has specific ability of producing organic acid during the P solubilization (Protiva et al., 2009).

Table 4. Types and quantities of produced organic acids in the Pikoveskaya’s medium supplemented with insoluble FePO₄ by 16 phosphate solubilizing fungal strains

| Strains | Type of fungi | Organic acid (µg/ml) |
|---------|---------------|---------------------|
| SI-1URAg | Penicillium sp. | 214.7 ± 10.5<sup>a</sup> |
| SI-2URAg | A. floccosus | 2.7 ± 0.6<sup>a</sup> |
| SI-3URAg | A. niveus | 15 ± 1.0<sup>a</sup> |
| SI-4URAg | T. pinophilus | N.D. |
| SI-5URAg | A. niveus | 1.7 ± 0.6<sup>a</sup> |
| SI-6URAg | P. oxalicum | 14.0 ± 1.0<sup>a</sup> |
| SI-7URAg | Penicillium sp. | 3.0 ± 0.6<sup>a</sup> |
| SI-8URAg | Penicillium sp. | 2.0 ± 0.0<sup>a</sup> |
| SI-9URAg | Penicillium sp. | N.D. |
| SI-10URAg | A. niger | 811.0 ± 66.8<sup>a</sup> |
| SI-11URAg | A. niger | 466 ± 12.3<sup>a</sup> |
| SI-12URAg | A. niger | 955.7 ± 9.5<sup>a</sup> |
| SI-13URAg | Penicillium sp. | 104.7 ± 4.5<sup>a</sup> |
| SI-14URAg | A. floccosus | 459 ± 14<sup>a</sup> |
| SI-15URAg | T. pinophilus | 2.7 ± 0.6<sup>a</sup> |
| SI-16URAg | P. oxalicum | 270.7 ± 6.5<sup>a</sup> |

Values given are the mean of three replicates ± standard deviation of the mean. Values with common letters in each column do not differ statistically according to Duncan’s Multiple Range Test (DMRT) at p<0.05

N.D.: Not detected
Organic acid calculated as micrograms per milliliter

Present study showed that A. niger, strain SI-12URAg have the strongest organic acid production ability regardless of P substrates. Isolates P. oxalicum (SI-6URAg and SI-16URAg) produced higher amount of organic acids in the medium supplemented with TCP and Al-P. Whereas, A. niger SI-10URAg and A. floccosus SI-14URAg were capable to produce organic acids in the medium supplemented with Fe-P. In our previous study A. niger (strain SI-10URAg, SI-11URAg and SI-12URAg) considered as the outstanding P solubilizing fungi due to their high capabilities to solubilize three insoluble phosphate compounds by decreasing pH of the culture medium (Islam et al., 2019). It suggested that higher amount of microbial organic acids were produced during P solubilization that accelerate the solubilization process by providing protons and complexing anions, or ligand exchange reactions or complexion of metal ions release to solution (Zang et al., 2018). The solubilization of P mostly depended on the amount of organic acids production by fungi (Bo et al., 2011). They also reported that tricarboxylic acids such as citric acid, oxalic acid, malic acid, formic acids and other lower molecular...
weight organic acids are the main contributors to solubilization of phosphate and decrease pH in the medium.

Among the filamentous fungi, *Aspergillus* are prominent for higher concentrations of a variety of organic acid production (Liaud, 2014). The fungi of genus *Aspergillus* are widely used for the industrial production of bio-based products, including enzymes and organic acids (Yang et al., 2017). In fact, *A. niger* has been regarded as the workhorse microorganism for the industrial production of organic acids (Show et al., 2015). These acids contributed to P solubilization (Silva et al., 2014; Li et al., 2016). Besides *A. niger* species, *P. oxalicum* also showed an excellent organic acid production ability in the medium supplemented with TCP and Al-P. This fungal species is important in food and drug production (https://en.wikipedia.org/wiki/Penicillium_oxalicum). Some members of the genus produce penicillin (https://en.wikipedia.org/wiki/Penicillium). The molecule penicillin is used as an antibiotic.

### Table 5. Comparison of organic acid production form different P sources (TCP, Al-P and Fe-P) by 16 phosphate solubilizing fungal strains

| Strains     | Type of fungi        | Organic acid (µg/ml) from |
|-------------|----------------------|--------------------------|
|             | TCP                  | Al-P                     | Fe-P                     |
| SI-1URAg    | *Penicillium* sp.    | 1801.3                   | 1344.3                   | 596.3                   |
| SI-2URAg    | *A. floccosus*       | 546.0                    | 22.3                     | 620.7                   |
| SI-3URAg    | *A. niveus*          | 270.3                    | 103.7                    | 549.3                   |
| SI-4URAg    | *T. pinophilus*      | 102.0                    | 75.3                     | 208.0                   |
| SI-5URAg    | *A. niveus*          | 537.3                    | 133.7                    | 1439.0*                 |
| SI-6URAg    | *P. oxalicum*        | 2492.3*                  | 2486.9*                  | 614.0                   |
| SI-7URAg    | *Penicillium* sp.    | 788.0                    | 613.3                    | 578.0                   |
| SI-8URAg    | *Penicillium* sp.    | 110.7                    | 74.8                     | 152.7                   |
| SI-9URAg    | *Penicillium* sp.    | 141.7                    | 553.7                    | 118.7                   |
| SI-10URAg   | *A. niger*           | 520.7                    | 499.0                    | 1803.0*                 |
| SI-11URAg   | *A. niger*           | 1660.3                   | 504.3                    | 986.0                   |
| SI-12URAg   | *A. niger*           | 3630.7*                  | 1555.0*                  | 1655.3*                 |
| SI-13URAg   | *Penicillium* sp.    | 1264.0                   | 2020                    | 1720.7                  |
| SI-14URAg   | *A. floccosus*       | 293.3                    | 285.7                    | 1506.3*                 |
| SI-15URAg   | *T. pinophilus*      | 121.3                    | 107.0                    | 186.3                   |
| SI-16URAg   | *P. oxalicum*        | 2346.7*                  | 2026.0*                  | 571.7                   |
| Mean ± S    | 1039.1±1061.9*       | 775.3±828.5              | 831.6±599.6              |

TCP: tricalcium phosphate; Al-P: aluminium phosphate and Fe-P: iron phosphate.

An asterisk (*) indicated outstanding values of produced organic acid. It was higher than sum of the mean and standard deviation of organic acid produced by 16 fungal strains. It also indicated the best substrate for organic acid production.

### Conclusions

From the above discussion it can be concluded that both type and quantity of microbial organic acid production depended on the P sources and fungal species/strains. All the fungi produced more organic acids in TCP medium compared to FePO₄ and AIPO₄ supplemented medium, which contributed to P solubilization. Among the isolates, *A. niger* (SI-12URAg) considered as outstanding P solubilizer based on...
organic acids production potential regardless of substrates followed *P. oxalicum* (SI-6URAg, SI-16URAg) and *A. niger* (SI-10URAg). These strains could have great potential as promising bioresource for efficient P utilization in agricultural production. Future experiment is necessary to evaluate the performance of the outstanding strains on growth and yield of plant in the soils contain insoluble phosphates.

**Figure 2.** Chromatograms of organic acids analyzed by HPLC. The acids were produced by outstanding P solubilizing fungal strains [*A. niger* (SI-12URAg), *P. oxalicum* (SI-6URAg, SI-16URAg) and *A. niger* (SI-10URAg)]

**Acknowledgement.** This manuscript has been considered as the requirement of doctoral degree for the first author.

**REFERENCES**

[1] Alam, S., Khalil, S., Ayub, N., Rashid, M. (2002): In vitro solubilization of inorganic phosphate by phosphate solubilizing microorganisms (PSM) from maize rhizosphere. – Int J Agric Biol 4: 454-458.

[2] Bo, C., Yan, W., Pengming, L., Biao, L., Meiying, G. (2011): Isolation and phosphate solubilizing ability of a fungus, *Penicillium* sp. from soil of alum mine. – JBM 51: 5-14.

[3] Gyaneshwar, P., Kumar, G. N., Parekh, L. J., Poole, P. S. (2002): Role of microorganisms in improving P nutrient of plants. – Plant Soil 245: 83-93.

[4] https://en.wikipedia.org/wiki/Penicillium.

[5] https://en.wikipedia.org/wiki/Penicillium_oxalicum.

[6] Islam, M. K., Sano, A., Majumder, M. S. I., Hossain, M. A., Sakagami, J.-I. (2019): Isolation and molecular characterization of phosphate solubilizing filamentous fungi from subtropical soils in Okinawa. – Applied Ecology and Environmental Research 17(4): 9145-9157.
[7] Jain, R., Saxena, J., Sharma, V. (2017): The ability of two fungi to dissolve hardly soluble phosphates in solution. – Mycology 8(2): 104-110.

[8] Jose, M. S., Milton, P. M., Ivana, D. M., Marina, R., Nubia, S. M., Alicia, G. (2010): Soil fungal isolates produce different organic acid patterns involved in phosphate salts solubilization. – Biol Fertil Soils 46: 755-763.

[9] Kavanagh, K. (2011): Fungal fermentation systems and products in Fungi: biology and applications. – Wiley.

[10] Liaud, N. (2014): Exploring fungal biodiversity: organic acid production by 66 strains of filamentous fungi. – Fungal Biology and Biotechnology 1: 1.

[11] Pikovskaya, R. I. (1948): Mobilization of Phosphorus in Soil Connection with the Vital Activity of Some Microbial Species. – Microbiology 17: 362-370.

[12] Pratibha, V., Arvind, G. (2009): Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate solubilizing fluorescent Pseudomonas. – BMC Microbiology 9: 174.

[13] Rao, N. S. S. (1982): Phosphate Solubilization by Soil Microorganisms. – In: Rao, N. S. S. (ed.) Advances in Agricultural Microbiology. Butterworth-Heinemann, Oxford.

[14] Samson, R. A., Visagie, C. M., Houbraeken, J., Hong, S. B., Hubka, V., Klaassen, C. H. W., Perrone, G., Seifert, K. A., Suska, A., Tanny, J. B., Kocsube, S., Szigeti, G., Yaguchi, T., Frisvad, J. C. (2014): Phylogeny, identification and nomenclature of the genus Aspergillus. – Studies in Mycology 78: 141-173.

[15] Sauer, M., Porro, D., Mattanovich, D., Branduardi, P. (2008): Microbial production of organic acids: expanding the markets. – Trends Biotechnol 26: 100-80.

[16] Scervino, J. M., Mesa, M. P., Mónica, I. D., Recchi, M., Moreno, S., Godeas, A. (2013): Soil fungal isolates produce different organic acid patterns involved in phosphate salts solubilization. – Biol Fertil Soils 49(6): 779-779.

[17] Show, P. L., Oladele, K. O., Siew, Q. Y., Zakry, F. A. A., Lan, J. C. W., Ling, T. C. (2015): Overview of citric acid production from Aspergillus niger. – Front. Life Sci. 8(3): 271-283.

[18] Silva, U. D. C., Mendes, G. D. O., Silva, N. M. R. M., Duarty, J. L., Silva, I. R. (2014): Fluoride Tolerant Mutants of Aspergillus niger Show Enhanced Phosphate Solubilization Capacity. – PLOS ONE 9: 10.

[19] Singh, M. S., Yadav, L. S., Singh, S. K., Singh, P., Singh, P. N., Ravindra, R. (2011): Phosphate solubilizing ability of two Arctic Aspergillus niger strains. – Polar Research 30: 72-83.

[20] Watanabe, T. (2010): Pictorial atlas of soil and seed fungi morphologies of cultured fungi and Key to species. – 3rd. CRC press, Florida.

[21] Yamaguchi, S., Sano, A., Hiruw, A. M., Murata, M., Kaneshima, T., Murata, Y., Takahashi, H., Takahashi, S., Takahashi, Y., Chibana, H., Touyama, H., Nguyen, H. T. T., Nakazato, Y., Uhera, Y., Hirakawa, M., Imura, Y., Tereshima, Y., Kawamoto, Y., Takahashi, K., Sugiyama, K., Hiruma, M., Murakami, M., Hosokawa, A., Uezata, H. (2014): Isolation of dermatophytes and domestic fowl (Gallus domesticus). – Mycopathologia 178: 135-143.

[22] Yang, L., Lubeck, M., Lubeck, P. S. (2017): Aspergillus as a versatile cell factory for organic acid production. – Fungal Biol. Rev. 31(1): 33-49.

[23] Zhang, Y., Chen, F.-S., Wu, X-Q., Luan, F-G., Zang, L-P., Fang, X-M., Wan, S-Z., Hu, X-F., Ye, J-R. (2018): Isolation and characterization of two phosphate solubilizing fungi from rhizosphere soils of moso bamboo and their functional capacities when exposed to different phosphorus sources and pH environment. – PLOS ONE 13: 7.

[24] Zhen, L., Tongshuo, B., Letian, D., Fuwei, W., Jinjin, T., Shiting, M., Yunxiao, H., Shimei, W., Shujin, H. (2016): A study of organic acid production in contrasts between two phosphate solubilizing fungi: Penicillium oxalicum and Aspergillus niger. – Sci Rep. 6: 25313.