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

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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 floccosus*, 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 $Ca_3(PO_4)_2$ 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, agricultaral 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 now 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

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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

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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 Sabrouraud 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	
					Beta tubulin gene	Calmodulin gene
1	SI-1URAgr	Dark red soil	Nishihara, Okinawa	*Penicillium sp.*	LC425316	Not done
2	SI-2URAgr	Dark red soil	Nishihara, Okinawa	*Aspergillus floccosus*	LC425317	Not done
3	SI-3URAgr	Dark red soil	Nishihara, Okinawa	*Aspergillus niveus*	LC425318	LC425334
4	SI-4URAgr	Grey soil	Nishihara, Okinawa	*Talaromyces pinophilus*	LC425319	LC425335
5	SI-5URAgr	Grey soil	Nishihara, Okinawa	*Aspergillus niveus*	LC425320	LC425336
6	SI-6URAgr	Grey soil	Nishihara, Okinawa	*Penicillium oxalicum*	LC425321	Not done
7	SI-7URAgr	Red soils	Kunigami, Okinawa	*Penicillium sp.*	LC425322	Not done
8	SI-8URAgr	Red soils	Kunigami, Okinawa	*Penicillium sp.*	LC425323	Not done
9	SI-9URAgr	Red soils	Kunigami, Okinawa	*Penicillium sp.*	LC425324	Not done
10	SI-10URAgr	Red soils	Kunigami, Okinawa	*Aspergillus niger*	LC425325	LC425337
11	SI-11URAgr	Red soils	Yanbaru forest, Okinawa	*Aspergillus niger*	LC425326	LC425338
12	SI-12URAgr	Red soils	Yanbaru forest, Okinawa	*Aspergillus niger*	LC425327	LC425339
13	SI-13URAgr	Dark red soil	Nishihara, Okinawa	*Penicillium sp.*	LC425328	LC425340
14	SI-14URAgr	Dark red soil	Nishihara, Okinawa	*Aspergillus floccosus*	LC425329	Not done
15	SI-15URAgr	Grey soil	Nishihara, Okinawa	*Talaromyces pinophilus*	LC425330	Not done
16	SI-16URAgr	Dark red soil	Nishihara, Okinawa	*Penicillium oxalicum*	LC425331	Not done

Medium preparation for organic acid production study

Pikoveskaya's (PKV) broth medium consisted of 10.0 g glucose, 5.0 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$,0.1 g $MgSO_4 \cdot 7H_2O$, 0.02 g NaCl, 0.02 g KCl, 0.003 g $FeSO_4 \cdot 7H_2O$, 0.003 g $MnSO_4 \cdot H_2O$ 0.5 g yeast extract and 1000 mL distilled water (Pikovskaya, 1948). In this medium $Ca_3(PO_4)_2$ was used as source of insoluble phosphate that was replaced by insoluble $FePO_4$ and $AlPO_4$. The medium was autoclaved at 121^0C for 15 minutes.

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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 4^0C. 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^{TM}, Nunc^{TM} Disposable Loops and Needles, Thermo Scientific^{TM} 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 approximately10^6 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 [$Ca_3(PO_4)_2$], aluminium phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). After sterilization, the medium of each flask was inoculated with the 5% (v/v) spore suspension of a particular fungal strain containing 10^6 spore mL^{-1}. Sterile distilled water inoculated flaks was treated as control (*Fig. 1.*).

Figure 1. *Farmented Pikovskaya broth culture for organic acid determination by HPLC inoculated with 16 phosphate solubilizing fungal strains*

Three replicates were maintained for each test isolate. Incubation was done at 25^0C 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^{o}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).

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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 [$Ca_3(PO_4)_2$] 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-3URAgr and SI-5URAgr, 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-6URAgr, SI-7URAgr, SI-8URAgr, SI-9URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-14URAgr and SI-16URAgr. The produced citric acid ranged from 3.0-566.7 and tartaric acid was 1.7-27.3 μg/ml. SI-5URAgr produced citric acid and SI-2URAgr, SI-3URAgr and SI-13URAgr produced tartaric acids. Most of the strains produced formic acid except SI-4URAgr, SI-7URAgr, SI-8URAgr, SI-9URAgr, SI-15URAgr, and most of the strains produced acetic acid except SI-2URAgr, SI-3URAgr, SI-5URAgr and SI-8URAgr. 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-11URAgr, SI-7URAgr, SI-16URAgr, SI-2URAgr, SI-12URAgr, SI-13URAgr and SI-12URAgr, respectively (*Table 2*).

Organic acid production by fungal strains in Al-P ($AlPO_4$) supplimented medium

In aluminium phosphate (Al-P) supplimented 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-2URAgr and SI-8URAgr could not produce oxalic acid and SI-2URAgr, SI-4URAgr and SI-15URAgr could not produce tartaric acids. Only SI-5URAgr 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-2URAgr, SI-3URAgr, SI-4URAgr, SI-5URAgr, SI-14URAgr and SI-15URAgr. Both formic and acetic acids were not detected from the culture filtrate of SI-2URAgr, SI-4URAgr, SI-8URAgr and SI-9URAgr. SI-7URAgr. SI-15URAgr 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-12URAgr, SI-9URAgr, SI-12URAgr, SI-7URAgr, SI-11URAgr, SI-13 URAgr and SI-6URAgr, respectively (*Table 3*).

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Table 2. *Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with insoluble $Ca_3(PO_4)_2$ by 16 phosphate solubilizing fungal strains*

Strains	Type of fungi	Organic acid (µg/ml)						
		Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
SI-1URAgr	*Penicillium sp.*	3.0±1.0^{ef}	N.D.	N.D.	34.7±2.1^{def}	48.7±9.3^{efgh}	567.0±33.9^{c}	1148.0±111.0^{c}
SI-2URAgr	*A. floccosus*	46.3±4.7^{d}	N.D.	6.0±1.0^{de}	139.7±7.5^{a}	170.7±13.5^{c}	183.3±18.2^{e}	N.D.
SI-3URAgr	*A. niveus*	23.0±2.6^{e}	N.D.	7.3±0.6^{cd}	N.D.	77.7±6.0^{de}	162.3±29.7^{e}	N.D.
SI-4URAgr	*T. pinophilus*	3.7±1.2^{ef}	N.D.	N.D.	43.0±3.0^{def}	26.3±2.1^{h}	N.D.	29.0±2.0^{e}
SI-5URAgr	*A. niveus*	4.0±1.0^{ef}	426.3±22.7^{b}	N.D.	N.D.	90.3±13.0^{d}	16.7±4.0^{f}	N.D.
SI-6URAgr	*P. oxalicum*	77.0±10.8^{b}	52.0±2.6^{d}	13.7±2.5^{bc}	22.7±3.1^{f}	74.3±6.1^{def}	749.7±42.1^{b}	1503.0±42.0^{b}
SI-7URAgr	*Penicillium sp.*	2.7±0.6^{ef}	566.7±32.1^{a}	17.3±3.1^{b}	114.3±16.3^{b}	50.7±7.8^{efgh}	N.D.	36.3±8.0^{e}
SI-8URAgr	*Penicillium sp.*	5.3±1.5^{ef}	3.0±1.0^{e}	18.7±3.5^{b}	42.0±5.6^{def}	41.7±4.0^{gh}	N.D.	N.D.
SI-9URAgr	*Penicillium sp.*	5.0±1.0^{ef}	53.0±8.2^{d}	5.3±1.5^{de}	31.0±6.6^{ef}	37.7±5.7^{gh}	N.D.	9.7±0.7^{e}
SI-10URAgr	*A. niger*	69.0±4.0^{bc}	10.3±1.5^{e}	6.0±1.0^{de}	79.7±6.0^{c}	154.3±5.0^{c}	172.7±31.6^{e}	28.7±4.2^{e}
SI-11URAgr	*A. niger*	342.0±20.4^{a}	8.0±1.0^{e}	16.3±2.5^{b}	82.7±6.7^{c}	272.3±26.6^{b}	311.0±22.3^{d}	628.0±16.1^{d}
SI-12URAgr	*A. niger*	49.0±10.5^{cd}	13.7±2.5^{e}	16.0±4.4^{b}	78.7±9.5^{c}	320.7±13.8^{a}	340.3±52.6^{d}	2812.3±76.6^{a}
SI-13URAgr	*Penicillium sp.*	5.7±0.6^{ef}	N.D.	1.7±0.6^{de}	54.0±5.6^{d}	84.7±7.4^{d}	1102.7±80.3^{a}	15.3±2.1^{e}
SI-14URAgr	*A. floccosus*	52.3±5.9^{cd}	10.3±2.1^{e}	26.7±2.5^{a}	35.7±6.4^{def}	94.0±4.6^{d}	54.3±12.7^{f}	20.0±4.6e
SI-15URAgr	*T. pinophilus*	2.3±0.6^{f}	N.D.	N.D.	43.7±4.0^{de}	44.3±5.9^{fgh}	N.D.	31.0±4.6^{e}
SI-16URAgr	*P. oxalicum*	12.3±1.5^{ef}	100.7±6.5^{c}	27.3±3.1^{a}	39.7±4.0^{def}	68.3±6.0^{defg}	696.3±54.8^{b}	1402.0±46.0^{b}

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 ($FePO_4$) supplemented medium

In iron phosphate (Fe-P) supplimented 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-5URAgr did not produce lactic acid. The oxalic acid was produced (1.5-811.0 µg/ml) by the strain SI-4URAgr, SI-9URAgr, SI-11URAgr and SI-12URAgr. Strains SI-3URAgr, SI-5URAgr, SI-7URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr SI-15URAgr and SI-16URAgr produced both citric and acetic acids, whereas SI-2URAgr, SI-13URAgr produced citric acid, SI-9URAgr and SI-14URAgr 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-10URAgr, SI-12URAgr, SI-13URAgr, SI-10URAgr, SI-4URAgr, SI-13 and SI-12URAgr, 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

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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 $AlPO_4$ by 16 phosphate solubilizing fungal strains*

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

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, Penicilium* and *Talaromyces* (Islam et al., 2019). Both type and the quantity of organic acids produced by fugal 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 supplimented medium

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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 $FePO_4$ by 16 phosphate solubilizing fungal strains*

Strains	Type of fungi	Organic acid (µg/ml)						
		Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
SI-1URAgr	*Penicillium sp.*	214.7±10.5^{d}	N. D	271.7±2.1^{b}	51.3±1.5^{fgh}	2.7±0.6^{jk}	56.0±1.0^{h}	N.D.
SI-2URAgr	*A. floccosus*	2.7±0.6^{f}	6.3±1.5^{gh}	26±1.0^{l}	12±1.0^{k}	7.3±0.6^{i}	566.3±3.5^{d}	N.D.
SI-3URAgr	*A. niveus*	15±1.0^{f}	40.7±1.5^{f}	70.7±1.5^{g}	44.3±3.8^{hi}	20.3±2.1^{g}	325.7±1.5^{e}	32.7±1.5^{f}
SI-4URAgr	*T. pinophilus*	N.D.	N.D.	17±1.0^{m}	47.7±4.0^{gh}	88.0±2.0^{a}	8.3±0.6^{k}	47.0±2.0^{e}
SI-5URAgr	*A. niveus*	1.7±0.6^{f}	432±6.0^{c}	115.7±0.6^{e}	29.3±2.5^{j}	N.D.	791.3±3.5^{c}	69.0±3.6^{d}
SI-6URAgr	*P. oxalicum*	14.0±1.0^{f}	N. D	16.7±1.2^{m}	27±2.6^{j}	72.7±2.5^{c}	225±9.5^{f}	258.7±4.2^{b}
SI-7URAgr	*Penicillium sp.*	3.0±0.6^{f}	349.3±7.5^{d}	127±2.0^{d}	17.3±0.6^{k}	26.7±3.5^{f}	17.3±1.5^{j}	37.3±3.5^{f}
SI-8URAgr	*Penicillium sp.*	2.0±0.0^{f}	N.D.	30±1.0^{k}	60.3±2.5^{e}	48.3±3.2^{d}	12.0±1.0^{jk}	N.D.
SI-9URAgr	*Penicillium sp.*	N.D.	N. D	14.7±1.5^{m}	43.7±1.5^{hi}	30.7±3.2^{f}	5.3±0.6^{k}	22.0±1.7^{g}
SI-10URAgr	*A. niger*	811.0±66.8^{a}	268.7±0.6^{e}	43±1.0^{i}	383.3±4.5^{a}	40±2.6^{e}	48.7±2.5^{hi}	208.3±6.0^{c}
SI-11URAgr	*A. niger*	N.D.	466±12.3^{b}	56.3±2.1^{h}	348±10.8^{b}	4.7±0.6^{ij}	93.0±2.0^{g}	18.0±2.0^{gh}
SI-12URAgr	*A. niger*	N.D.	955.7±9.5^{a}	78±1.0^{f}	218.7±2.1^{c}	14.7±1.5^{h}	46.0±2.0^{i}	342.3±8.5^{a}
SI-13URAgr	*Penicillium sp.*	104.7±4.5^{e}	7.7±0.6^{gh}	408.3±3.5^{a}	38.7±2.1^{i}	78.7±1.5^{b}	1082.7±4.7^{a}	N.D.
SI-14URAgr	*A. floccosus*	459±14^{b}	N. D	34±1.0^{j}	57.3±1.5^{ef}	41±2.0^{e}	871.7±14.0^{b}	43.3±4.9^{e}
SI-15URAgr	*T. pinophilus*	2.7±0.6^{f}	10.7±2.5^{g}	11.3±0.6^{n}	103.7±8.4^{d}	30.3±4.5^{f}	10.0±1.0^{jk}	17.7±2.1^{gh}
SI-16URAgr	*P. oxalicum*	270.7±6.5^{c}	4.3±0.6^{gh}	153±1.7^{c}	54.3±4.0^{efg}	28.7±1.5^{f}	45.7±0.6^{i}	15.0±1.0^{h}

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-12URAgr have the strongest organic acid production ability regardless of P substrates. Isolates *P. oxalicum* (SI-6URAgr and SI-16URAgr) produced higher amount of organic acids in the medium supplemented with TCP and Al-P. Whereas, *A. niger* SI-10URAgr and *A. flucossus* SI-14URAgr were capable to produce organic acids in the medium supplemented with Fe-P. In our previous study *A. niger* (strain SI-10URAgr, SI-11URAgr and SI-12URAgr) considered as the outstanding P solubilizing fungi due to their high capabilities to solubilized 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 accelarate 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

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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 penicilin 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-1URAgr	*Penicillium sp.*	1801.3	1344.3	596.3
SI-2URAgr	*A. floccosus*	546.0	22.3	620.7
SI-3URAgr	*A. niveus*	270.3	103.7	549.3
SI-4URAgr	*T. pinophilus*	102.0	75.3	208.0
SI-5URAgr	*A. niveus*	537.3	133.7	1439.0*
SI-6URAgr	*P. oxalicum*	2492.3*	2486.9*	614.0
SI-7URAgr	*Penicillium sp.*	788.0	613.3	578.0
SI-8URAgr	*Penicillium sp.*	110.7	74.8	152.7
SI-9URAgr	*Penicillium sp.*	141.7	553.7	118.7
SI-10URAgr	*A. niger*	520.7	499.0	1803.0*
SI-11URAgr	*A. niger*	1660.3	504.3	986.0
SI-12URAgr	*A. niger*	3630.7*	1555.0*	1655.3*
SI-13URAgr	*Penicillium sp.*	1264.0	2020	1720.7
SI-14URAgr	*A. floccosus*	293.3	285.7	1506.3*
SI-15URAgr	*T. pinophilus*	121.3	107.0	186.3
SI-16URAgr	*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 $FePO_4$ and $AlPO_4$ supplemented medium, which contributed to P solubilization. Among the isolates, *A. niger* (SI-12URAgr) considered as outstanding P solubilizer based on

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organic acids production potential 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. Future experiment is necessary to evaluate the performance of the outstanding strains on growth and yield of plant in the soils contain insoluble phosphates.

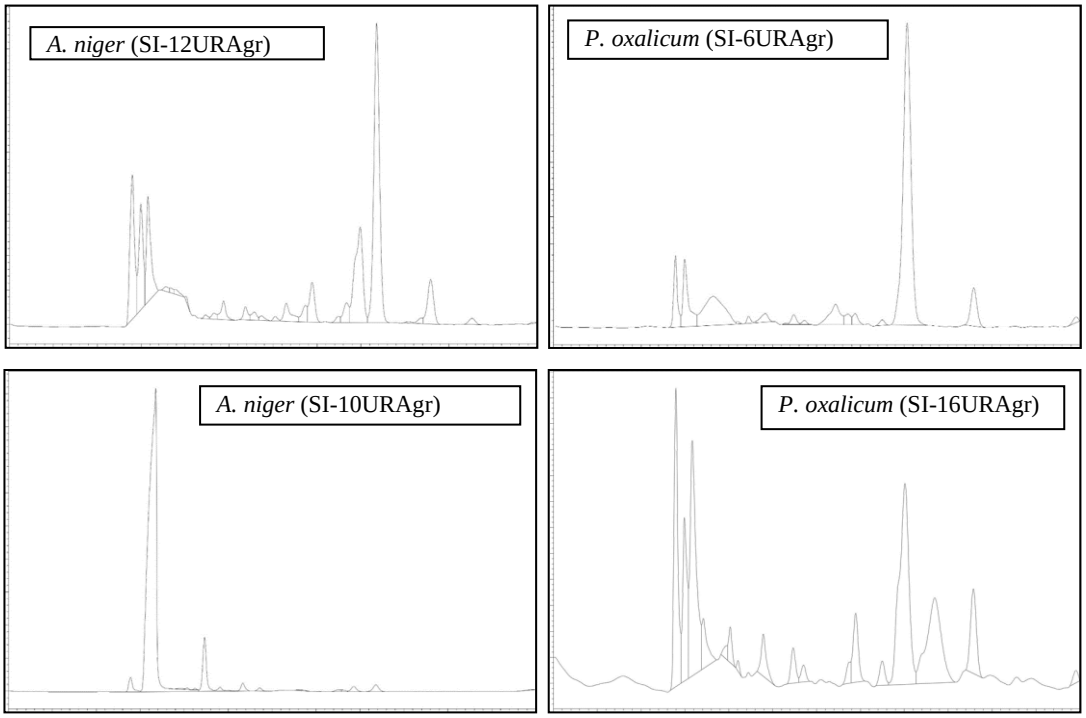

Figure 2. *Chromatograms of organic acids analyzed by HPLC. The acids were produced by outstanding P solubilizing fungal strains [A. niger (SI-12URAgr), P. oxalicum (SI-6URAgr, SI-16URAgr) and A. niger (SI-10URAgr)]*

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