Phosphorous Solubilizing Fungi Isolated from Soils in Subtropical Okinawa, Japan

（亜熱帯に属する沖縄県の土壌より分離されたリン可溶化真菌）

MOHAMMAD KABIRUL ISLAM

March 2020

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The Dissertation submitted to The United Graduate School of Agricultural Sciences, Kagoshima University, Japan in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
IN AGRICULTURE

By

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THE DISSERTATION SUBMITTED TO THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES, KAGOSHIMA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN AGRICULTURE

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The dissertation hereto attached, entitled "**Phosphorous Solubilizing Fungi Isolated from Soils in Subtropical Okinawa, Japan**（亜熱帯に属する沖縄県の土壌より分離されたリン可溶化真菌）" prepared and submitted by **MOHAMMAD KABIRUL ISLAM** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agriculture is hereby accepted.

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Phosphorous Solubilizing Fungi Isolated from Soils in Subtropical Okinawa, Japan

（亜熱帯に属する沖縄県の土壌より分離されたリン可溶化真菌）

By

Mohammad Kabirul Islam

Abstract: Phosphorus is the second most important nutrient element required for plant growth and development. Phosphorus forms insoluble complexes with aluminum and iron in acidic soil and with calcium in alkaline soil. For sustenance of crop production, farmers rectify phosphorus deficiency by applying large amounts of phosphate fertilizers to the soil, but a small fraction of the applied phosphate is available to plants and the rest of the portion becomes immobilized which increases production costs and leads to environmental pollution. Furthermore, phosphorus pollution sometimes occurs not only with damage of salts by high tide waves but also by desertification accompanied with global warming. On the other hand, some bacteria have ability to dissolve insoluble phosphorus compounds in soil, however, there are a few reports on fungal species with phosphorus solubilizing abilities. The present study aimed to isolate efficient phosphate solubilizing fungi that could be utilized as potential bio inoculants for resolving the phosphorous deficiency in subtropical soil as well as industrial benefits.

Sixteen strains from the grey soil (pH 7.9), dark-red soil (pH 6.7) and red soil (pH 6.1) correspondent to the typical Okinawan soils so-called as Jaagaru, Shimajiri maaji and Kunigami maaji having solubilizing ability of insoluble $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ were isolated from Okinawa, Japan located at subtropical area cultured on phosphorus agar plates at 25^{o}C for 1 week. The colonies with the transparent zone on the medium were picked up and used for the investigations. Based on morphological and the molecular biological identifications using β-tubulin and/or Calmodulin sequences, the isolates were confirmed as *Aspergillus* spp., *Penicillium* spp. and *Talaromyces* spp. Four representative *Aspergillus* spp. isolates showed higher phosphate solubilization ability followed by another genus regardless of phosphate substrates.

Phosphate solubilization ability was evaluated using $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$.The highest quantity of organic acids was found when $Ca_3(PO_4)_2$ was used as

substrate followed by $FePO_4$ and $AlPO_4$. The phosphate solubilization ability was influenced by the potential of hydrogen (pH). Some isolates showed strong phosphate solubilization ability in lower pH.

The salt and high osmophilic stresses on the fungal strains were evaluated in order to correlate to the salt damages and or desert soil contaminated with phosphate. A series of salt concentration from zero to 20%, and that of sucrose one from 0 to 50% supplemented on routine culture media were evaluated. Interestingly, 3 isolates could grow at 12% of salt concentration, and 5 ones at 10%. Furthermore, all isolates survived with 35% sucrose in medium, and 5 isolates could grow on 50% sucrose condition.

The mechanism of phosphorus solubilization by the fungi mostly depend on the synthesis of organic acids (mainly oxalic, tartaric, malic, citric, acetic, tartaric and formic acids). The acid productions are depending on both phosphate minerals and the strains of fungi. In this study, 16 fungal strains have been identified from the soils in subtropical Okinawa for phosphorous solubilization. There were some candidate isolates for industrial use, such as an isolate of *A. niger* and 2 of *P. oxalicum*. On the other hand, considering both salt tolerance and phosphorous solubilizing abilities, a *Panicillium* sp. isolate was considered to be the candidate for industrial usage. The application for industrial usages of the isolates showing higher phosphorous solubilizing abilities in or not in the salt-rich conditions could be the further studies.

In conclusion, industrially important fungal strains were isolated from the subtropical soils in Okinawa, Japan.

亜熱帯に属する沖縄県の土壌より分離されたリン可溶化真菌

（Phosphorous Solubilizing Fungi Isolated from Soils in Subtropical Okinawa, Japan）

By

モハマド カビル イスラム

リンは、窒素に次いで植物の成長と発達に必要な栄養素で、酸性土壌ではアルミニウムと鉄、アルカリ性土壌ではカルシウムと不溶性の複合体を形成する．農家は作物生産を維持するために，大量のリン酸肥料を土壌に散布してリン欠乏状態を改善するが、植物はそのごく一部を利用するに過ぎず，利用されずにそのまま土壌に固定化されていくため，さらなる生産コストを増加させ、環境汚染をもたらす．また地球温暖化に伴い，リン過給状態の土壌は，高潮による塩害を被っている地域や砂漠化している地域でも発生している．過給状態のリンを分解し，土壌保全に役立つ微生物として土壌細菌の働きが報告されているが，真菌によるリン分解能についての報告は少ない．本研究は亜熱帯地域に属する沖縄県の土壌からリン分解能を有する真菌を分離し，その活性と，将来的に産業用有用微生物として利用する可能性について研究することを目的とした．沖縄県を代表とするジャアガル（灰色の土壌，pH 7.9)、島尻マージ（暗赤色の土壌．pH 6.7)、および国頭マージ（赤色の土壌，pH 6.1）の土壌懸濁液を作成し，リンを分解するとその周囲が透明になる特殊培地（because 培地）に塗布し， 25℃で 1 週間培養し，特徴的な集落を釣菌し，リン分解能を有する 16 株を得た．菌種は形態と分子生物学的同定（β-チューブリンおよび/またはカルモジュリン配列）に基づいて同定したところ，*Aspergillu*s 属、*penicillium* 属または *Talaromyces* 属のいずれかの菌種であった． リン酸可溶化能力の測定は基質として、$Ca_3(PO_4)_2$、$FePO_4$、および $AlPO_4$ を用いたところ，Ca_3（PO_4）$_2$ を基質として使用されたときに分解産物である有機酸量が最も高いことが判明した．さらにリン酸可溶化能力は、水素イオン濃度（pH）の影響

を受けることも判明し，一部の株は低 pH でも強いリン酸塩可溶化能力を示した．また塩害や砂漠化との関連を探るために塩ストレス及び高調液ストレスを測定した．培地に 0 から 20%の食塩および 0-50%のショ糖を加えたときの発育を調べたところ，3 株は塩濃度の 12%で成長し、5 株は 10%まで発育可能であった．さらに、全分離株は 35%ショ糖添加培地で生存し、さらに 5 株は 50%ショ糖添加培地での生育が可能であった.

真菌によるリンの可溶化のメカニズムは、主に有機酸（主にシュウ酸、酒石酸、リンゴ酸、クエン酸、酢酸、酒石酸およびギ酸）の合成によって行われている．有機酸の生産量は基質による違いだけでなく，菌株の生物活性能による違いも反映していると推測した．本研究で亜熱帯沖縄の土壌から分離したリン分解能を有した真菌 16 株のうち，*A. niger* 株 1 株および *P. oxalicum* の 2 株を産業用有用微生物候補としてあげられると考えた．また、耐塩性，耐高調性とリン可溶化能力を総合すると、*Penicillium* 属菌種に同定された株もその候補となる可能性がある。塩害汚染地域や砂漠地域でのリン酸分解有用微生物の研究は今後の課題である．結語として，本研究では沖縄県の亜熱帯土壌より産業有用微生物としてリン酸分解能を有する真菌を分離した.

Chapter I

General Introduction

Phosphorus (P) is second the most essential plant nutrient required for plant growth and yield (Chai *et al*. 2011; Li *et al*., 2015a; Ram *et al*., 2015). However, soils have substantial reserve of total phosphorus, but most of the natural soils are typically phosphorous deficient, especially in highly weathered soils in which phosphorus forms insoluble complexes with aluminum, iron, and hydroxides (in acidic soils) and with calcium (in alkaline soils) (Mendez 2014). To ensure adequate phosphorus supply for plants growth by applying large amounts of phosphate fertilizers to the soil. However, only a small fraction of applied phosphate is available to plants and rest of the amount becomes immobilized rapidly (Singh 2011). This not only increases production costs but also leads to environmental pollution. Which often 'run off' into water bodies causing eutrophication (Knobeloch *et al*., 2009).

Our dependence on chemical fertilizers has encouraged the rapid growth of some industries which are producing life-threatening chemicals. Their products are not only hazardous for human health but also destroy the ecological balance and enhances the risk of climate change (Chun *et al*., 2014). In fact, attention is now shifting from consuming food grown with chemical fertilizers to food grown with organic fertilizers. Microbial inoculant can help to mitigate the food demand of global population by increasing crop yield. It is an eco-friendly and cost-effective agricultural input (Khosru *et al*. 2012). The exploitation of beneficial microbes has become paramount importance in agriculture due to their potential role in food safety and food quality. They lead to enhance the availability of essential plant nutrients, growth and tolerance of plants to biotic and abiotic stresses (Deepak *et al.,* 2014). The application of beneficial microorganisms improves soil properties. They possess a functional relationship and constitute a holistic system with plants (Vessey *et al*., 2003).

Application of beneficial microorganisms in agricultural practices started about 60 years ago and most of the research were conducted on growth promoting bacteria. A few works have done on fungi, but fungi have a more pronounced stable genetic traits than bacteria such as *Aspergillus* and *Penicillium* (Gyaneshwar *et al*., 2002). It is now evident that these microbes can enhance plant growth providing all kinds of macro and

micronutrients via nitrogen fixation, phosphate and potassium solubilization, release of plant growth promoting substances (Sinha *et al.,* 2014). Microbial inoculant when applies in soil, they multiply and participate in nutrient cycling that leads to crop productivity and healthy environment (Adesemoye *et al.,* 2009). They have long lasting effects due to their slow nutrient releasing capabilities. As a result, long term use of these beneficial microbes leads to the buildup of nutrients in the soil thereby increasing the overall soil fertility (Mahimaraja *et al*., 2008).

Phosphorus-solubilizing activity of microorganisms to be considered the most important among their multiple properties that promote plant growth and nutrient absorption (Rodríguez and Fraga, 1999). However, the growth-promoting effects of these microorganisms are also influenced by several environmental factors, including temperature, moisture content, salinity and pH (Ponmurugan and Gopi, 2006). Some microorganisms have stress tolerant properties. They could survive under stressed condition using various biochemical and molecular mechanisms. Inoculation of these microbes give benefit for crop production under stressed environment (Ahmad *et al*., 2011, Spence and Bais, 2015).

There are several things that needs to be considered for choosing effective microorganisms as bio inoculant for successful crop production in phosphorous deficient agricultural soils. Beside this, their potentiality, adaptability in environment, able to compete with other soil microflora and ability to successful colonization in the rhizosphere should be considered (Khosru *et al*., 2012). In this regard, there is a substantial advantage in using natural soil isolates as potential inoculants in the same area from which they were isolated. The present studies aim to the following specific objectives,

1. Isolate and identify potential P solubilizing fungi from different soils in subtropical Okinawa, Japan as indigenous bioresource.
2. Investigate the secondary metabolites or organic acid production potentials of fungal strains that could facilitate and mechanize P solubilization process.
3. Examine the effect of abiotic stresses on the growth of fungal strains.
4. Evaluate P solubilizing ability of potential strains under saline condition and
5. Evaluation of the pH on the growth and survival of fungal strains.

Chapter II

Isolation and Identification of Phosphate Solubilizing Filamentous Fungi from Subtropical Soils in Okinawa, Japan

Abstract. Phosphorus (P) is an essential nutrient element required for plant growth and development. Low phosphorus availability in soil is one of the majors constrains for crop production. Phosphate solubilizing fungi enhance available phosphorous released from soils and contribute to fulfil the plants phosphorous requirement. This study aimed to isolate and identify potential phosphate solubilizing fungi from subtropical soils for environment friendly biofertilizer development. Sixteen fungal strains were isolated and identified as *Aspergillus* spp., *Penicillium* spp. and *Talaromyces* spp. from subtropical dark red soil, red soil and grey soil based on phosphate solubilization index, morphological studies and the sequences of β-tubulin and/or Calmodulin. Subsequently, fungal isolates having excellent phosphate solubilization efficiency were selected by their potential in broth containing insoluble $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$. Interestingly, *Aspergillus niger* isolates (strain SI-10URAgr, SI-11URAgr and SI-12URAgr) have marked phosphate solubilization ability regardless of the substrates followed by *Penicillium oxalicum* and *Talaromyces pinophilus.* In addition, there was inverse proportion between the pH and phosphate solubilizing capacities. The fungal isolates with higher phosphate solubilizing abilities may have great potentials for agricultural utilization as environmentally sound biofertilizer. In this study, phosphate solubilization by filamentous fungi is reported for the first-time in subtropical Okinawa.

Introduction

Phosphorus (P) is one of the major nutrients for crop production (Reena *et al*., 2013). This nutrient play important physiological and biochemical activities of plants, like, photosynthesis, energy and sugar production, nucleic acid synthesis, and promotes nitrogen fixation in legume plants (Saber *et al*., 2005). Phosphorus promotes the strength of grain crop straw, flower initiation and fruit settings, root development and seed formation (Sharma *et al*., 2013) and disease resistance capacity of plants (Richardson *et al*., 2007). In soils, only 0.1% of the total P exists in a soluble form available for plant uptake because of its fixation into an unavailable form (Zhou *et al*., 1992; Khan *et al*., 2010). In order to provide this nutrient, farmers use chemical fertilizers. The most widely used fertilizers are obtained from the acidification of rock phosphates with strong acids which not only represent a major cost of agricultural production but also impose adverse environmental impacts on overall soil health, terrestrial, freshwater and marine resources (Sing *et al*., 2011; Tilman et al., 2001).

The major soils in sub-tropical Okinawa are, red soil, dark-red soil and grey soil which are phosphorous deficient (Oshiro *et al*., 2016). The red soil and dark red soil have low pH value (5.4 and 6.6 respectively), one the other hand grey soil has high pH value (8.4). Large amount of soluble P fertilizers is widely used in order to increase agricultural production world widely (Bo *et al*., 2011). Moreover, the efficiency of applied P fertilizers in chemical form rarely exceeds 30% due to its fixation, either in the form of iron/aluminium phosphate in acidic soils or in the form of calcium phosphate in neutral to alkaline soils (Norish *et al*., 1983; Lindsay *et al*., 1989). According to the latest estimates, the global reserve of P could become depleted within 50-100 years (Heppel *et al*., 2016). Besides the efficient use of P reserves, it is also important to reduce the current wastage of P fertilizers and to recover applied P. The realization of all these potential problems associated with chemical P fertilizers has led to the search for environmentally compatible and economically feasible alternative strategies for improving crop production in low or P-deficient soils (Zaidi *et al*., 2009).

The microbial inoculants (biofertilisers) function as key player in sustainable agriculture by improving soil fertility and crop productivity (Deepak *et al*., 2014). Especially, fungi can penetrate into deep underground and show good attachment to

insolubilized P particles as results of its hyphal structure compared to bacteria and actinomycetes. Furthermore, fungi are good acid producer and consequently show greater phosphate solubilization activity than bacteria (Deepak *et al*., 2014; Jose *et al*., 2010). Among these, *Aspergillus* spp., *Penicilium* spp., *Talaromyces* spp. and *Eupenicilium* spp. are considered "key organisms" in the P cycle (Jose *et al*., 2010). However, most of the fungal species solubilize inorganic calcium phosphate and have a limited capacity to solubilize aluminium or iron phosphate. There are few in vitro studies concerning the solubilization of other phosphates by fungal species. To address this limitation, the present study aims to isolate and identify new isolates of indigenous phosphate solubilizing filamentous fungi which could be potential to solubilize both tricalcium phosphate, aluminium phosphate and iron phosphate.

Materials and Methods

Soil samples collection

The study was carried out in the Mycology Laboratory, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan during August 2017–November 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.

The sampling area located at 26.5000°N and 128.0000°E (Fig. II.1.). 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 soil, red soil and grey soil in this area.

Zero to fifty cm depth soil samples were collected from ten different locations of three type soils 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 stored at room temperature. Then microbiological study was done as early as possible.

Isolation of phosphate solubilizing fungi

For isolating phosphate solubilizing fungi Pikoveskaya's (PKV) agar medium was used. Pikoveskaya's (PKV) agar 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 $MgSO_4$.$7H_2O$, 0.02 g NaCl, 0.02 g KCl, 0.003 g $FeSO_4$.$7H_2O$, 0.003 g $MnSO_4 \cdot H_2O$ 0.5 g yeast extract, 15.0 g agar and 1000 mL distilled water (Rao, 1982). In this medium Ca_3 $(PO_4)_2$ was used as a source of insoluble phosphate. The medium was autoclaved at 121 °C for 15 min. About 20 ml of the sterilized medium poured into each petri dish and allowed to solidify before inoculation. Chloramphenicol was also used to avoid bacterial growth.

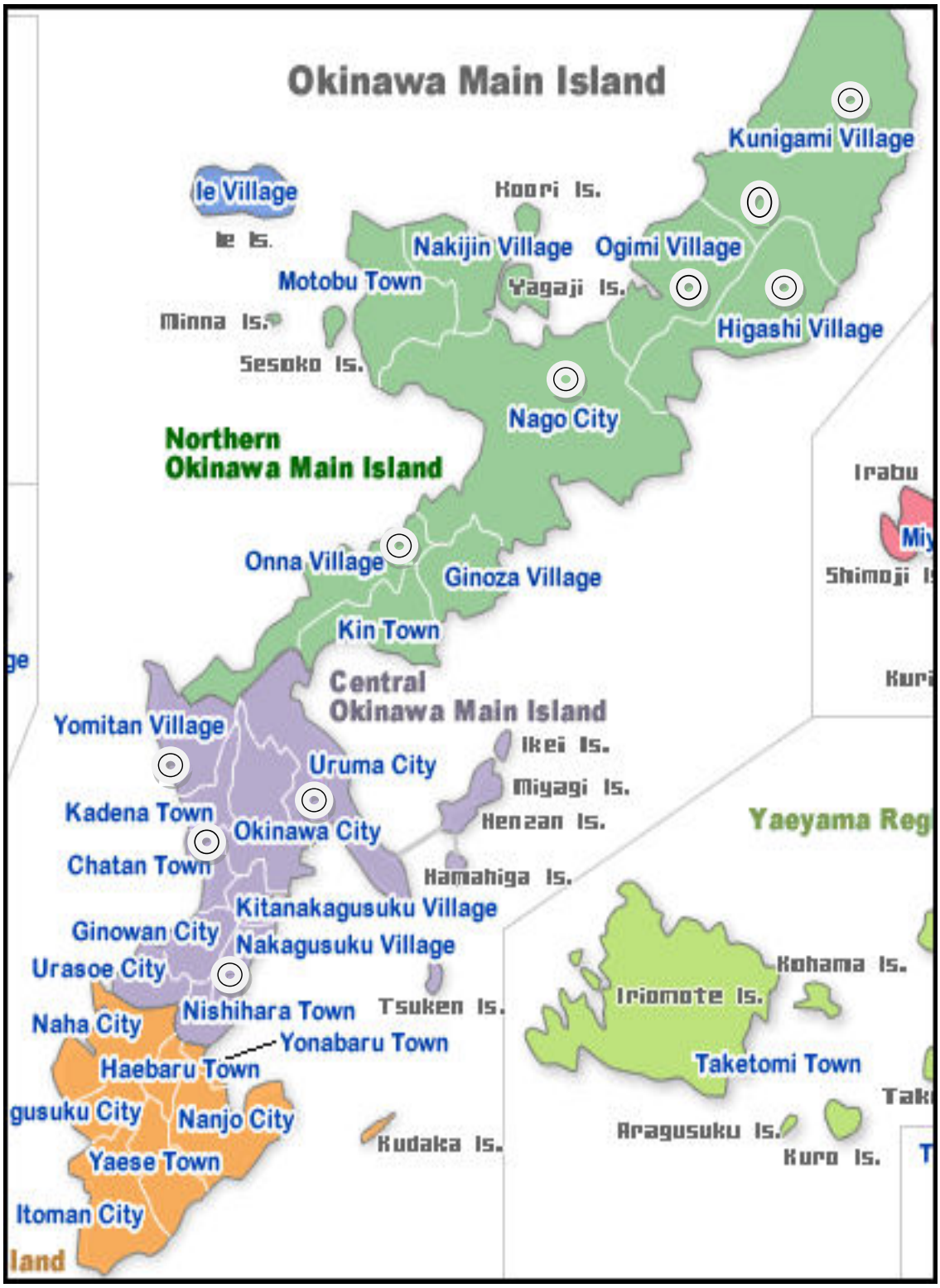

Figure II.1. Geographical map of the study area indicated sampling location

Isolation of phosphate solubilizing fungi using serial dilution plate technique. Five-gram soil sample was diluted in to 50 ml of sterile water. It was vigorously shaken until to get homogenous suspension and serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. From each dilution, 200 µL was plated on Pikovskaya's agar. The phosphate solubilizing fungi were identified by the presence of a clear halo around the colonies after 7 days incubation at 25 °C (Rao, 1982). The experiment was performed in triplicate. Phosphate solubilizing fungi of the soil samples were isolated and purified by transferring into new plates. The pure cultures were preserved on potato dextrose agar slants at 4 °C for further study. Phosphate solubilisation index was measured using the following formula (Birhanu et al., 2017).

SI = [Colony diameter + Halo zone diameter] / colony diameter

Identification of phosphate solubilizing fungi

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 (Fig.II.2) with an OLYMPUS BX50 microscopy equipped with image Analysis system (Olympus Corporation, Tokyo, Japan).

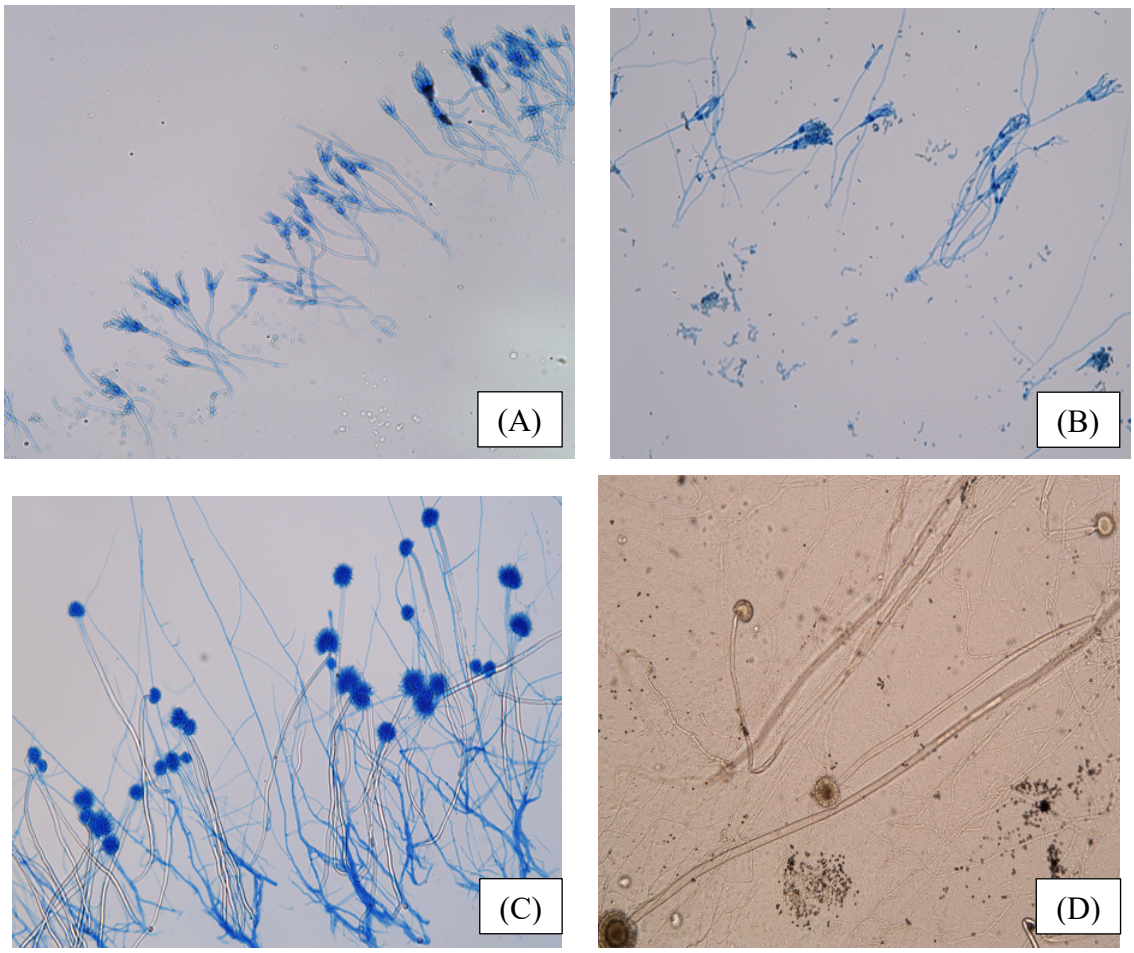

Fig. II. 2. Microscopic view of fungal fruiting body under photoelectron microscope with an imaging system

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 genes amplified with primers (Invitrogen by Thermo Fisher Scientific, USA) Bt2aF/ 5′-GGTAACCAAATCGGTGCTGCTTTC-3′ and Bt2b-R/5′-ACCCTCAGTGTAGTGACCCTTGGC-3′ and Calmodulin genes amplified with primers CMD5-F/5′-CCGAGTACAAGGARGCCTTC-3′ and CMD6-R/5′-CCGATRGAGGTCATRACGTGG-3′.CF1 F/5′-GCCGACTCTTTGACYGARGAR-3′ and CF4-F/5′-TTTYTGCATCATRAGYTGGAC-3′(Fig.II.3) were determined followed by the method (Samson et al., 2014)

Sequences were analyzed by the NCBI BLAST (http://www.**ncbi**.nlm.nih.gov/**blast**) 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.

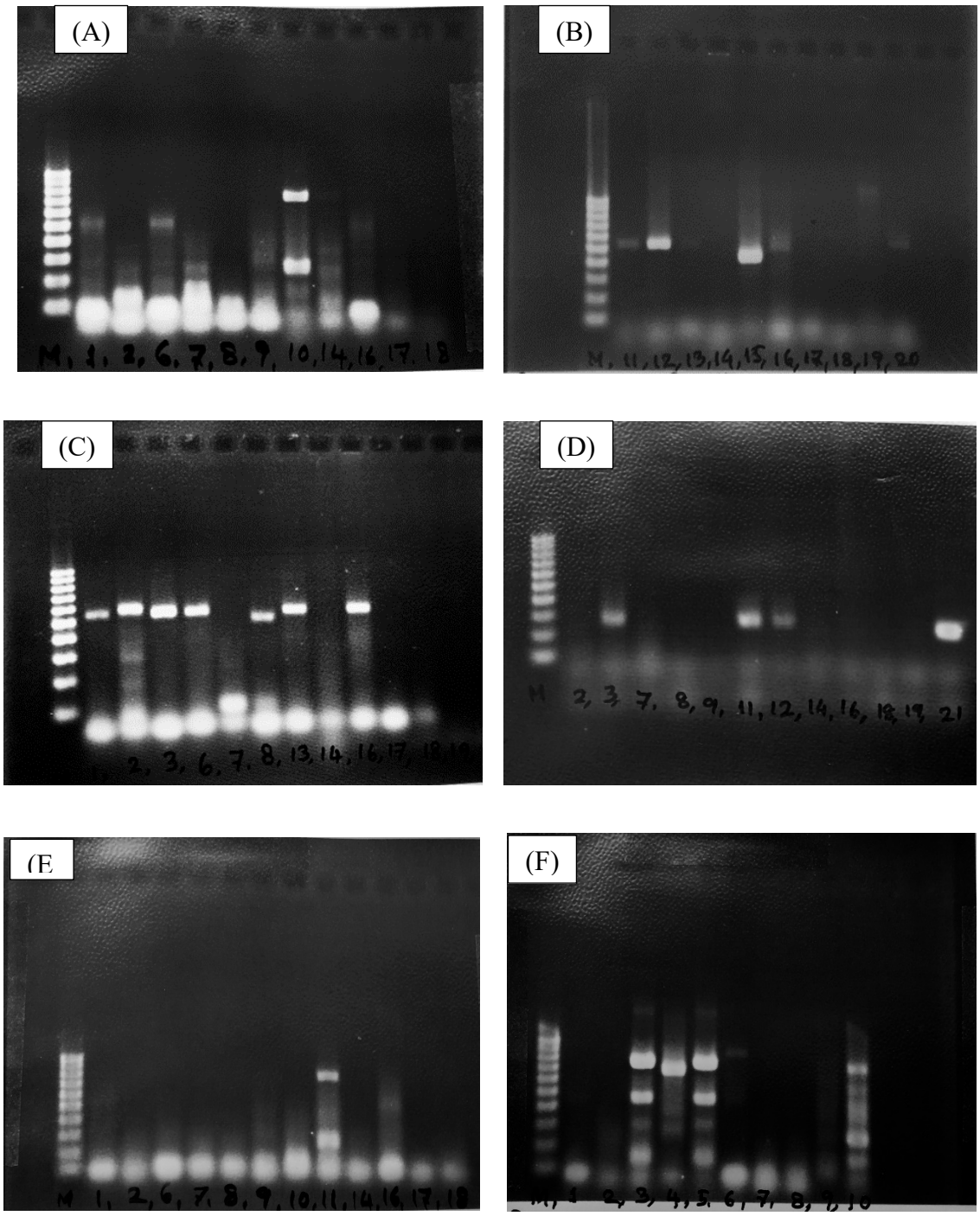

Fig. II. 3.DNA band appears under UV transilluminator after gel electrophoresis (A, B, C, D, E, F)

Preparation of spore suspension

Sporulated pure fungal cultures in PDA slants (incubated at 25^0C for 7 days) were selected for preparation of spore suspension by using standard procedure. A total volume of 5 ml sterile water with twin 80 (Wako Pure Chemical Corporation, Osaka, Japan) was added in culture slants and the fungal colony surface was lightly scraped by sterile bamboo stick. The cultures were passing through a syringe with staff cotton (Kyualet, Kawamoto Sangyo, Osaka, Japan). Spore count was done by a haemocytometer and the suspension was adjusted to approximately10^6 spores mL^{-1}.

Quantitative estimation of phosphate solubilization

It was 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 isolate containing 10^6 spores mL^{-1}. Sterile distilled water inoculated flaks was treated as control. Three replicates were maintained for each test isolate and mean value was recorded. Incubation was done at 25°C in an incubator shaker at 120 rpm up to 9 days. The samples were autoclaved and centrifuged at 5000 rpm for 25 min to remove any suspended solids and mycelial parts. Then the cultures were filtered through 0.45 µm pore size syringe filter unit (Advantech, japan). The filtrates were used for analysis of soluble phosphate and pH value. The pH value of the culture supernatants was determined by a pH meter (Horiba, Japan) equipped with a glass electrode. The amount of soluble phosphorus in culture supernatants was measured by molybdenum blue method and expressed as mg/L (Murphy and Riley, 1962). Samples cultured for 3, 6 and 9 days were compared. After calculation of mean phosphate degradation ability from 16 isolates of each day, we selected the adequate period for the comparison depending on the substrate.

Effect temperature on the growth and survival of isolates

The isolates were cultured on PDA slants in triplicate and incubated at temperature at 35, 37 and 42 °C for 7 days to evaluate the growth of mycelia. Growth of isolates at 25 °C (room temperature) treated as positive control.

Statistical analysis

All experiments were conducted in triplicate and data were analysed using Microsoft Excel program. The mean values were compared by Fisher test and significant differences were detected at $p < 0.05$ level. Correlation between solubilized phosphate and pH of the medium was determined by using Pearson correlation studies.

Results

Screening and identification of phosphate solubilizing fungi

A total of 16 fungal isolates showed phosphate solubilizing activities. The isolates were 6 *Aspergillus* spp., 6 *Penicillium* spp. and 2 *Taleromyces* spp. identified based on colony morphology, microscopic observation, Beta-tubulin and/or calmodulin sequences (Table II.1.).

Table II.1. List of fungal strains with gene bank accession number isolated from dark red, red and grey soils of subtropical environment

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

Qualitative phosphate solubilization

Sixteen fungal isolates showed significant phosphate solubilization in Pikovskaya agar medium using tricalcium phosphate as the substrate. The phosphate solubilization index (PSI) ranged from 1.42 to 2.24 (Table II.2.). Isolate SI-16URAgr (*Penicillium oxalicum*) produced highest PSI; 2.24 (Fig. II. 4), whereas; the smallest PSI of 1.42 was achieved from SI-3URAgr (*Aspergillus nevius*)

Table II.2. In vitro phosphate solubilization in solid medium by 16 fungal strains

Sl. No.	Fungal strain	Type of fungi	PSI
1	SI-1URAgr	*Penicillium sp.*	1.6 ± 0.03^{d}
2	SI-2URAgr	*Aspergillus floccosus*	1.67 ± 0.08^{d}
3	SI-3URAgr	*Aspergillus niveus*	1.42 ± 0.02^{e}
4	SI-4URAgr	*Talaromyces pinophilus*	1.8 ± 0.04^{c}
5	SI-5URAgr	*Aspergillus niveus*	1.67 ± 0.05^{d}
6	SI-6URAgr	*Penicillium oxalicum*	1.78 ± 0.03^{c}
7	SI-7URAgr	*Penicillium sp.*	1.5 ± 0.05^{d}
8	SI-8URAgr	*Penicillium sp.*	1.56 ± 0.08^{d}
9	SI-9URAgr	*Penicillium sp.*	1.42 ± 0.04^{e}
10	SI-10URAgr	*Aspergillus niger*	1.91 ± 0.03^{b}
11	SI-11URAgr	*Aspergillus niger*	1.66 ± 0.04^{d}
12	SI-12URAgr	*Aspergillus niger*	1.64 ± 0.04^{d}
13	SI-13URAgr	*Penicillium sp.*	2.02 ± 0.24^{b}
14	SI-14URAgr	*Aspergillus floccosus*	1.62 ± 0.09^{d}
15	SI-15URAgr	*Talaromyces pinophilus*	1.72 ± 0.04^{d}
16	SI-16URAgr	*Penicillium oxalicum*	2.25 ± 0.06^{a}

PSI: Phosphate solubilization index
Values given are the mean ± standard deviation of three independent replicates
Same letter in the column are not significantly different at $p < 0.05$ by Fisher's test

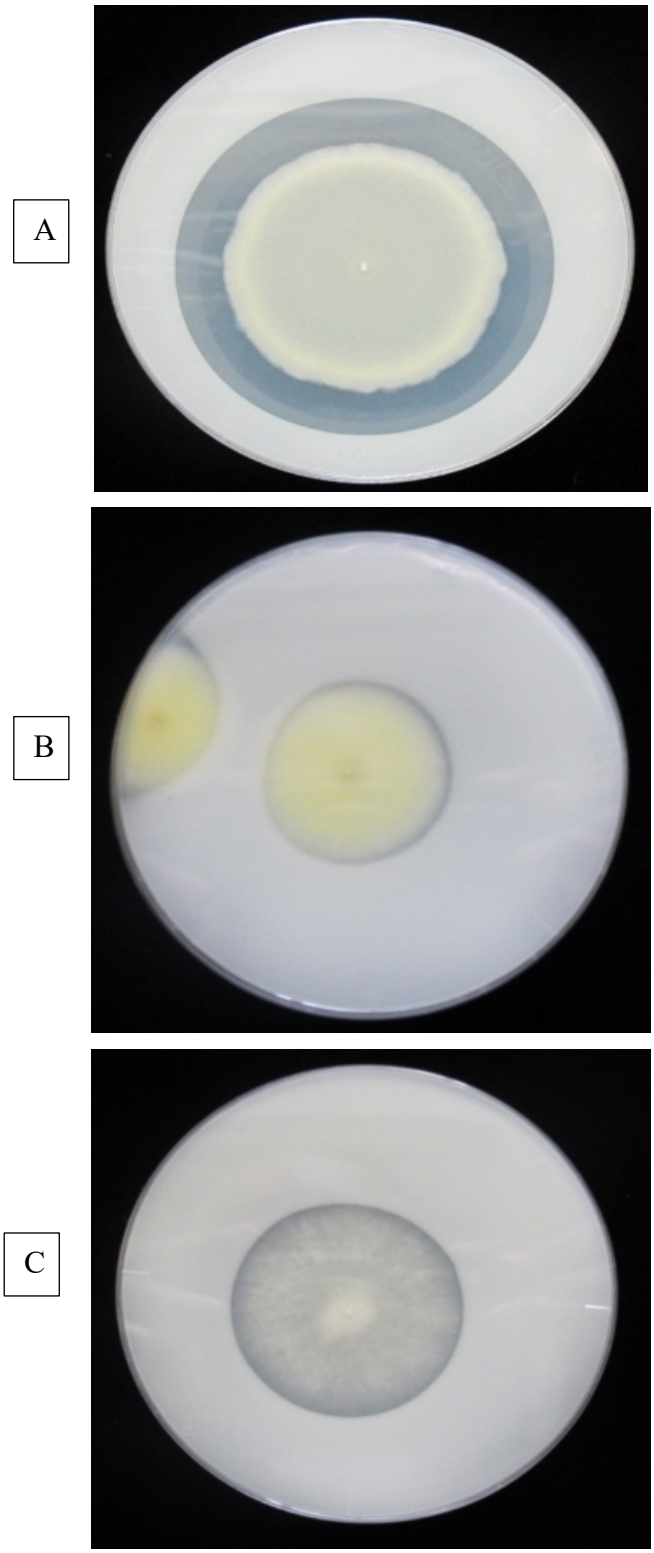

Figure II.4. Clear halo formation by representative fungal isolates in Pikovskaya agar plates (A: Penicillium oxalicum, B: T. pinophilus and C: Aspergillus niger)

Quantitative phosphate solubilization

Phosphate solubilizations by the isolated fungi were analyzed in Pikovskaya broth medium using three substrates of recalcitrant phosphate compounds: tricalcium phosphate [$Ca_3(PO_4)_2$], aluminium phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). The P-solubilizing ability of fungal isolates varied with incubation period and substrates. The best period of observation was selected considering their mean P solubilization, which 9 days for $Ca_3(PO4)_2$ and 6 days for both $AlPO_4$ and $FePO_4$ (Table II.3.).

Table II.3. Selection for the best period of phosphate solubilization by fungal strains

Solubilized phosphate (mg/L)								
Tricalcium phosphate (TCP)			**Aluminium phosphate (Al-P)**			**Iron phosphate (Fe-P)**		
3 days	6 days	9 days*	3 days	6 days*	9 days	3 days	6 days*	9 days
192.2±106.2	245.4±101.5	303.4±216.3	81.4±31.6	236.0±167.6	194.2±192.5	93.6±93.7	173.4±212.0	156.5±171.7

Values given are the mean ± standard deviation of P solubilized by 16 fungal isolates

An asterisk (*) indicated the highest solubilization day

The strongest phosphate (P) solubilization effect was found in the medium containing $Ca_3(PO_4)_2$ followed by $AlPO_4$ and $FePO_4$ (Table II.4.). The solubilized P ranged between 73.2-759.4 mg/L, 85.6-599.6 mg/L and 36.6-663.8 mg/L from $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ respectively. Among the isolates the highest amount of P was solubilized by *Aspergillus niger* followed by *Penicillium oxalicum* and *Talaromyces pinophillus.* Finally, *Aspergillus niger* strain SI-10URAgr, SI-11URAgr and SI-12URAgr were considered as outstanding isolates because solubilized P was higher than sum of the mean and standard deviation of P solubilized by 16 isolates. The amount of solubilized P from $Ca_3(PO_4)_2$ was 759.5, 647.8 and 670.5 mg/L; from $AlPO_4$ was 388.0, 558.3 and 599.7 mg/L and from $FePO_4$ was 663.8, 555.9 and 517.0 mg/L respectively (*Table 4*). SI-10URAgr showed outstanding performance in both $Ca_3(PO_4)_2$ and $FePO_4$ solubilization but in case of $AlPO_4$, it was very close to the outstanding.

Table II.4. Comparison of phosphate solubilization from different substrate by phosphate solubilizing fungal strains

Sl. No.	Fungal strain	Type of fungi	Solubilized phosphate (mg/L)		
			TCP	Al-P	Fe-P
1	SI-1URAgr	*Penicillium sp.*	295.5	166.7	176.0
2	SI-2URAgr	*Aspergillus floccosus*	83.5	190.3	42.4
3	SI-3URAgr	*Aspergillus niveus*	73.3	96.3	41.4
4	SI-4URAgr	*Talaromyces pinophilus*	175.9	194.9	36.1
5	SI-5URAgr	*Aspergillus niveus*	126.1	102.6	39.4
6	SI-6URAgr	*Penicillium oxalicum*	240.5	370.0	41.9
7	SI-7URAgr	*Penicillium sp.*	157.9	108.0	40.5
8	SI-8URAgr	*Penicillium sp.*	84.2	256.7	38.4
9	SI-9URAgr	*Penicillium sp.*	207.7	90.6	37.4
10	SI-10URAgr	*Aspergillus niger*	759.5*	388.0	663.8*
11	SI-11URAgr	*Aspergillus niger*	647.8*	558.3*	555.9*
12	SI-12URAgr	*Aspergillus niger*	670.5*	599.7*	517.0*
13	SI-13URAgr	*Penicillium sp.*	305.4	101.8	86.9
14	SI-14URAgr	*Aspergillus floccosus*	321.9	333.2	227.5
15	SI-15URAgr	*Talaromyces pinophilus*	308.0	134.8	48.0
16	SI-16URAgr	*Penicillium oxalicum*	397.2	85.7	182.9
		Mean±Sd	303.4 ± 216.3	236.0 ± 167.6	173.4 ± 212.0

TCP: tricalcium phosphate; Al-P: aluminium phosphate and Fe-P: iron phosphate
An asterisk (*) indicated outstanding values of solubilized phosphate. It was higher than sum of mean and standard deviation of P solubilized by 16 fungal isolates
Values given are the mean ± standard deviation of P solubilized by 16 fungal isolates

pH value of the culture medium

pH of the culture medium exhibited the opposite changes. It decreased with the increased amount of soluble P in the medium. Correlation studies showed a significant inverse relationship between soluble P and pH of the culture medium. The strongest negative correlation was observed in all fermented broth culture and correlation coefficient (r) was -0.88, -0.74 and -0.84 in TCP (Fig. II.5 A.), Al-P (Fig. II.5B.) and Fe-P (Fig. II.5C.) respectively.

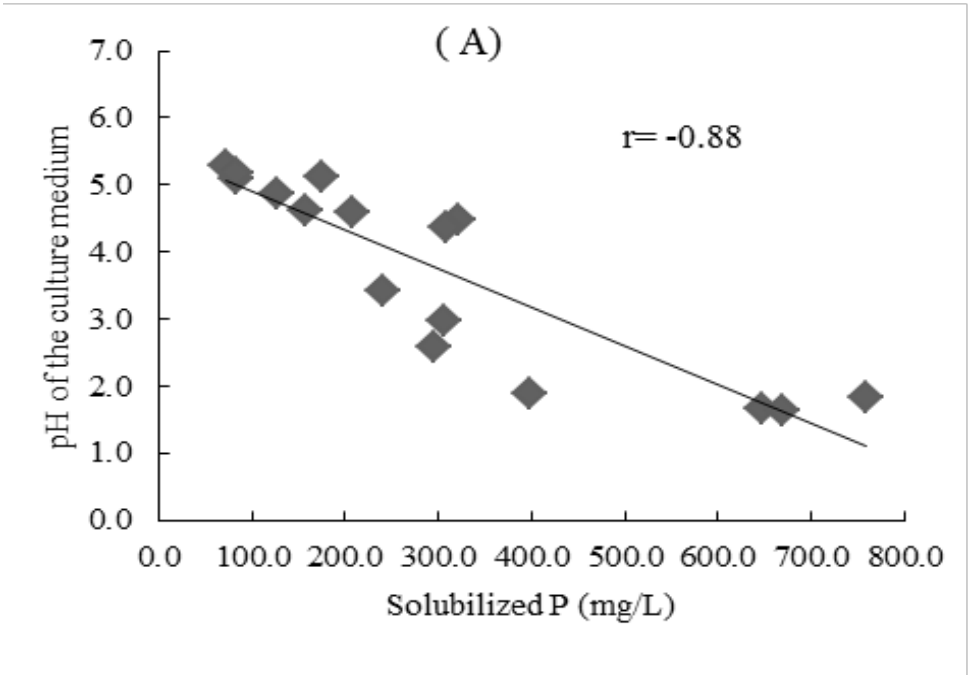

Figure II.5A. Pearson's correlation between soluble phosphate and pH of the culture medium supplemented with $Ca_3(PO_4)_2$ and inoculated by 16 fungal strains

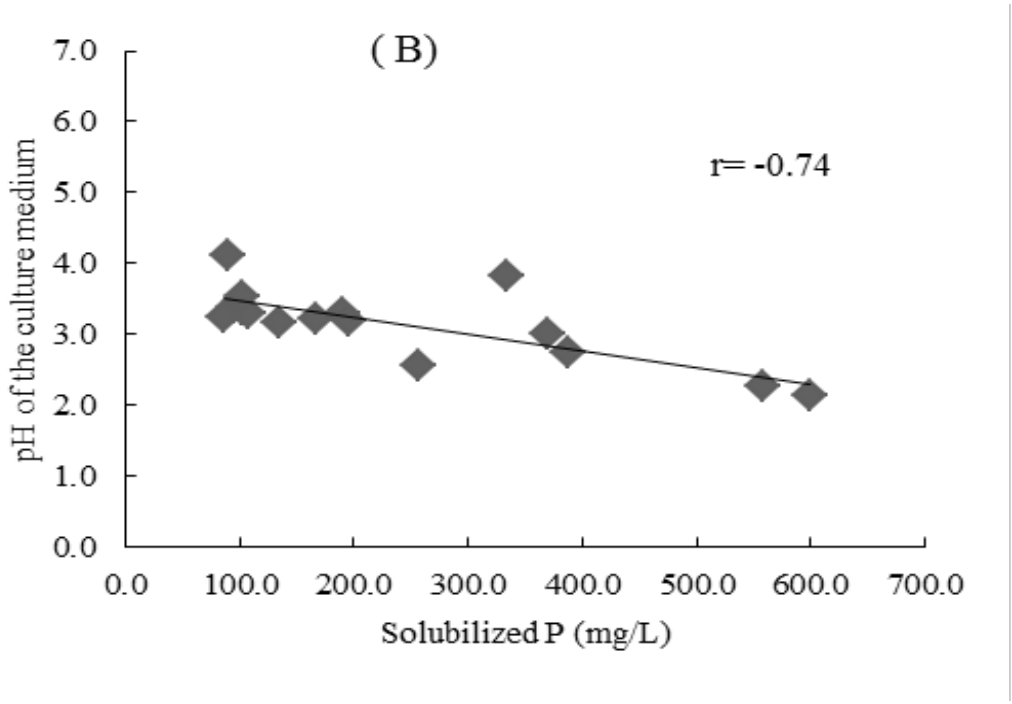

Figure II.5B. Pearson's correlation between soluble phosphate and pH of the culture medium supplemented with $AlPO_4$ and inoculated by 16 fungal strains

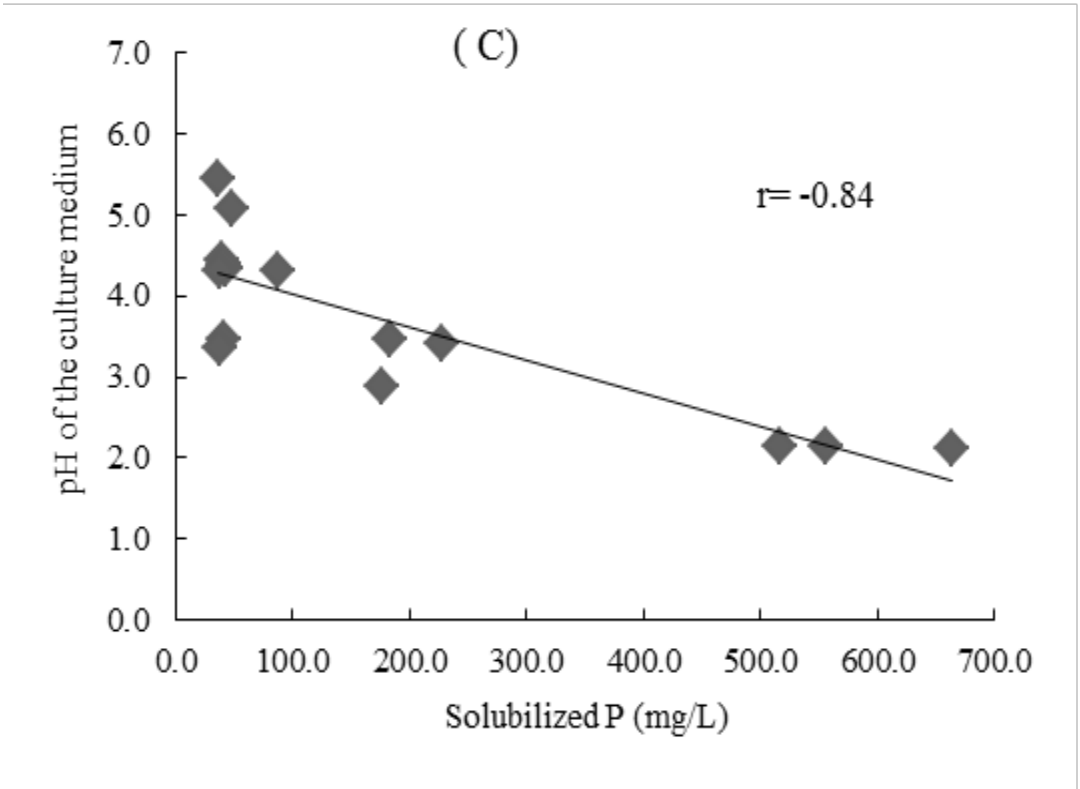

Figure II.5C. Pearson's correlation between soluble phosphate and pH of the culture medium supplemented with $FePO_4$ and inoculated by 16 fungal strains

Temperature effects on isolates

Survival of the isolates at different temperature was tested (Table II.5.). SI-7URAgr, SI-8URAgr and SI-9URAgr could grow and survived up to 35 °C while SI-1URAgr and SI-13URAgr survived up to 37 °C and other isolates were capable to grow at 42 °C.

Table II.5. The growth and survival of isolated phosphate solubilizing fungal strains at different temperature

Sl. No.	***Fungal strains***	***Name of the fungi***	***Growth temperature***			
			Positive control 25 °C	35 °C	37 °C	42 °C
1	SI-1URAgr	*Penicillium sp.*	+	+	+	-
2	SI-2URAgr	*Aspergillus floccosus*	+	+	+	+
3	SI-3URAgr	*Aspergillus niveus*	+	+	+	+
4	SI-4URAgr	*Talaromyces pinophilus*	+	+	+	+
5	SI-5URAgr	*Aspergillus niveus*	+	+	+	+
6	SI-6URAgr	*Penicillium oxalicum*	+	+	+	-
7	SI-7URAgr	*Penicillium sp.*	+	+	-	-
8	SI-8URAgr	*Penicillium sp.*	+	+	-	-
9	SI-9URAgr	*Penicillium sp.*	+	+	-	-
10	SI-10URAgr	*Aspergillus niger*	+	+	+	+
11	SI-11URAgr	*Aspergillus niger*	+	+	+	+
12	SI-12URAgr	*Aspergillus niger*	+	+	+	+
13	SI-13URAgr	*Penicillium sp.*	+	+	+	-
14	SI-14URAgr	*Aspergillus floccosus*	+	+	+	+
15	SI-15URAgr	*Talaromyces pinophilus*	+	+	+	+
16	SI-16URAgr	*Penicillium oxalicum*	+	+	+	-

(+) indicated growth of fungi, (-) indicated no growth.

Discussion

The sixteen P solubilizing fungal strains were isolated from the dark red, red and grey soils of subtropical Okinawa. The isolates were belonging to the genera of *Aspergillus, Penicilium* and *Talaromyces*.

According to Zang et al. (2018), Mendes et al. (2014) and Ruangsanka (2014), there were diversities on the isolation rate of phosphate solubilizing fungi depending on area. They reported that the most dominant genera of P solubilizing filamentous fungi are *Aspergillus, Penicilium* and *Talaromyces*, however, there were large variations in phosphate solubilizing abilities among fungal species (Barrooso et al., 2006; Surange et al., 1985). At the present studies, strains SI-10URAgr, SI-11URAgr and SI-12URAgr identified as *A. niger*, showed excellent P solubilizing abilities regardless of the phosphate substrates. It suggested that phosphate solubilizing abilities in *A. niger* is a universal property.

Among the filamentous fungi *Aspergillus* spp. are widely used for the production of fermented foods, organic acids and enzymes (Wongwicharn et al., 1999). Especially, *A. niger* has a long history of industrial usage, which means many strains already have a GRAS ("generally regarded as safe") status (Wongwicharn et al., 1999). It has been used for commercial production of many enzymes, e.g. pectinase, glucose oxidase, glucoamylase, hemicellulase, glucanases, acid proteinase, catalase (Aguilar and Huitron, 1993; Liu et al., 1999; Garhartz, 1990) and citric acid (Friedrich et al., 1989; Gokhale et al., 1991; Lee et al., 1989).

Interestingly, beside *A. niger* species, *P. oxalicum* (SI-14URAgr) also showed an excellent phosphate degradation ability on halo assay. According to Jain et al. (2014), Johri et al. (1999), Alam et al. (2002), Elias et al. (2016) and Jain et al. (2017) solid medium using agar plates performed better phosphate degradation ability than those in liquid media. Thus, it is impossible to ignore the *P. oxalicum* isolate SI-16URAgr. *Penicillium* spp. is important in the natural environment as well as food and drug production. Some members of the genus produce penicillin, a molecule that is used as an antibiotic, which kills or stops the growth of certain bacteria spp. Other species are used in cheese industries (https://en.wikipedia.org/wiki/*Penicillium*). *Penicillium*

oxalicum produces secalonic acid D, chitinase and oxalic acid (https://en.wikipidia. Org/wiki/Penicillium_oxalicum).

The solubilization of $Ca_3(PO_4)_2$ was the highest, followed by $AlPO_4$ and $FePO_4$ because $AlPO_4$ and $FePO_4$ have complex structure than $Ca_3(PO_4)_2$. Zang et al. (2018) and Son et al. (2006) reported that fungi exhibited low P solubilizing ability in media containing $AlPO_4$ and $FePO_4$. The mechanisms of phosphate solubilization by microorganisms are very complex and are not completely known yet (Bo et al., 2011). The very common mechanisms are acidification, chelation and exchange reactions (Bo et al., 2011). Organic acids play an important role in phosphate solubilization processes, which can help the release of P by providing protons and complexing anions, or ligand exchange reactions or complexion of metal ions release to solution. Zang et al. (2018) and Scervino et al. (2013) reported that organic acids production depends on the interaction of P source and fungi.

In this study, *A. niger* showed the highest efficiency in P solubilization by decreasing pH of the culture medium, which indicated higher amount of organic acid production. Silva et al. (2014), Li et al. (2016) and Barroso et al. (2006) reported that *A. niger* produce higher amount of organic acids and enhance phosphate solubilization. Zang et al. (2018) reported that solubilization of the different P sources mostly depended on the amount of organic acids production by fungi. Tricarboxylic acids such as citric acid, oxalic acid and other lower molecular weight organic acids are considered to be the main contributors to phosphate solubilization and a decrease in pH of the medium (Bo et al., 2011).

It was observed that phosphate solubilization was negatively correlated with pH of the medium. There are several reports where such correlation was documented (Pandey et al., 2008; Jain et al., 2012; Xio et al., 2015). The activities in lower pH indicated that the increase of organic acids in the medium (Pradhan and Sukla, 2005; Saxena et al., 2013). However, soluble P was increased without changing pH in some occasion because of other mechanism (Jain et al., 2012 and 2017), such as chelation and exchange reactions (Bo et al., 2011).

Conclusion

Isolates *A. niger* strain SI-10URAgr, SI-11URAgr and SI-12URAgr have unique capabilities to solubilized three insoluble phosphate compounds and may become an important bio resource for soil fertility management as well as sustainable crop production and pollution free environment.

This study indicated that isolated fungi are capable to solubilize three kinds of insoluble phosphate compounds in broth medium. Findings also indicated that pH of the fermented broth negatively corelated with the pH of fermented broth culture. It is assuming that some microbial exudates or secondary metabolites such as organic acids were produced during incubation, these acids act as the main contributor of P solubilization and pH dropping. Therefore, next study will be carried out to investigate the secondary metabolites or organic acid production potentials of isolated fungi that could facilitate P solubilization process. It will be helpful to know the mechanism of phosphate solubilization as well as to select the most effective phosphate solubilizers based on acid production potentials.

Chapter III

Evaluation of Organic Acid Production Potential of Phosphate Solubilizing Fungi Isolated from Soils in Okinawa, Japan

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 depended 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.

Introduction

Phosphorous is the second major nutrient after nitrogen that limits plant growth and yields (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 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 subtropical soils in Okinawa, Japan to select outstanding strains that could facilitate the P solubilization process.

Materials and Methods

Isolates used in this study

Sixteen phosphate solubilizing fungi were isolated from different soils in Okinawa, Japan and identified based on both mycological studies and sequences of Beta tubulin and Calmodulin (Chapter-1I, page 10 to 14). Isolates were SI-1URAgr, SI-2URAgr, SI-3URAgr, SI-4URAgr, SI-5URAgr, SI-6URAgr, SI-7URAgr, SI-8URAgr, SI-9URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-13URAgr, SI-14URAgr, SI-15URAgr and SI-16URAgr used in this study.

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 ^{0}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 4^0C. 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™ , 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 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.III.1.). 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 5000rpm 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).

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

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 (300mm x 7.8mm), auto sampler (LC-20AD) and fraction collector (FRC-10A). The injection volume, temperature and flow rate was 50 µl, 50^{o}C and 0.5ml/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 [$Ca_3(PO_4)_2$] supplimented medium

All the strains produced oxalic acids and lactic acids. The amount ranged from 2.3-342.0 and 26.3-320.7µg/ml respectively. Except SI-3URAgr and SI-5URAgr, other strains produced malic acid ranged from 22.7-139.7µg/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 1.7-27.3µg/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-9,URAgr, SI-15URAgr, and most of the strains produced acetic acid except SI-2URAgr, SI-3URAgr, SI-5URAgr and SI-8URAgr. It was ranged from 16.7-1102.7µg/ml and 9.7-2812.3µg/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 III.1.).

Table III.1. 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 and organic acid calculated as micrograms per milliliter.

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 5.7-119.0µg/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 III. 2.).

Table III.2. Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with insoluble $AlPO_4$ by 16 phosphate solubilizing fungal strains

Strains	**Organic acid (µg/ml)**							
	Type of fungi	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 and organic acid calculated as micrograms per milliliter.

Organic acid production by fungal strains in Fe-P ($FePO_4$) supplimented medium

In iron phosphate (Fe-P) supplimented medium, all strains showed the production of tartaric acid (11.3-408.3µg/ml), malic acid (12.0-383.0µg/ml), lactic acid (2.7-88µg/ml) and formic acid (8.3-1082.0µg/ml), whereas SI-5URAgr did not produce lactic acid. The oxalic acid was produced (1.5-811.0µg/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 (811.0µg/ml), citric (955.7µg/ml), tartaric (408.3µg/ml), malic (383.3.0µg/ml), lactic (88.0µg/ml), formic (1082.7µg/ml) and acetic (342.3µg/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 III.3.).

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 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 III.4.). HPLC chromatograms of outstanding fungal strains shown in (Fig. III.2.).

Table III.3. Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with insoluble $FePO_4$ by 16 phosphate solubilizing fungal strains

		Organic acid (µg/ml)						
Strains	Type of fungi	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}	11.7±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 and organic acid calculated as micrograms per milliliter.

Table III.4. Comparison of organic acid production form different P sources by 16 phosphate solubilizing fungal strains

		Organic acid (µg/ml) from		
Strains	**Type of fungi**	**TCP**	**Al-P**	**Fe-P**
SI-1URAgr	*Penicillium* sp.	1801.3	1344.3	596.3
SI-2URAgr	*Aspergillus floccosus*	546.0	22.3	620.7
SI-3URAgr	*Aspergillus niveus*	270.3	103.7	549.3
SI-4URAgr	*Talaromyces pinophilus*	102.0	75.3	208.0
SI-5URAgr	*Aspergillus niveus*	537.3	133.7	1439.0*
SI-6URAgr	*Penicillium 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	*Aspergillus niger*	520.7	499.0	1803.0*
SI-11URAgr	*Aspergillus niger*	1660.3	504.3	986.0
SI-12URAgr	*Aspergillus niger*	3630.7*	1555.0*	1655.3*
SI-13URAgr	*Penicillium* sp.	1264.0	2020	1720.7
SI-14URAgr	*Aspergillus floccosus*	293.3	285.7	1506.3*
SI-15URAgr	*Talaromyces pinophilus*	121.3	107.0	186.3
SI-16URAgr	*Penicillium oxalicum*	2346.7*	2026.0*	571.7
	Mean ± SD	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 acids. It was higher than sum of the mean and standard deviation of organic acids produced by 16 fungal strains. It also indicated the best substrate for organic acid production.

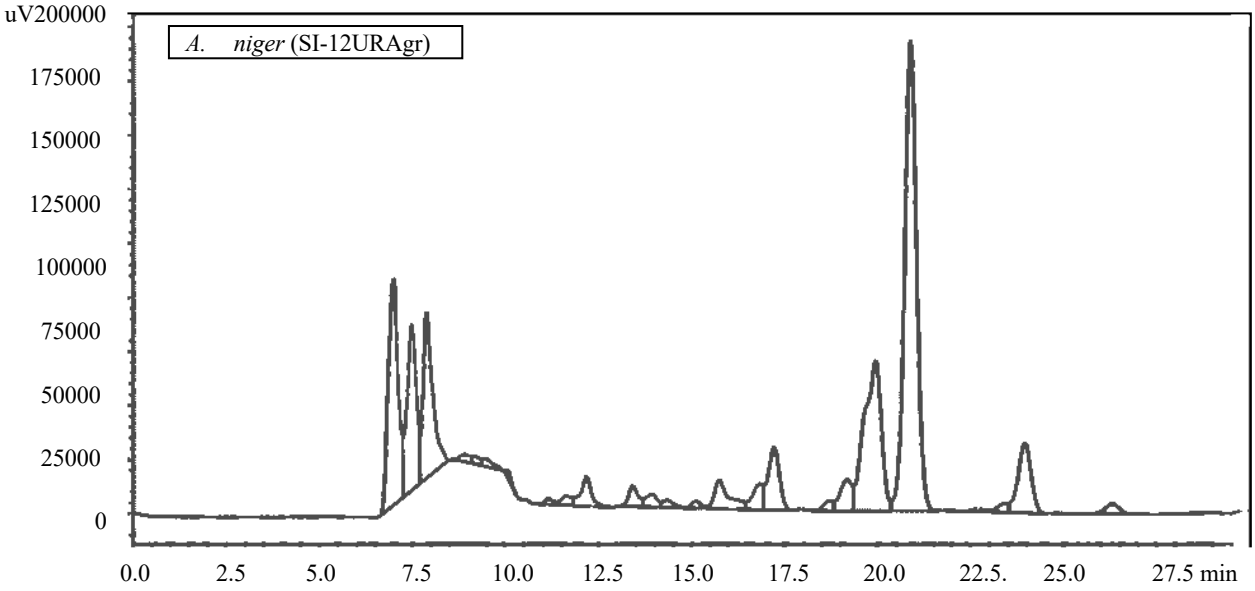

Fig.III.2. Chromatograms of organic acids analyzed by HPLC. The acids were produced by outstanding P solubilizing fungal strain A. niger (SI-12URAgr).

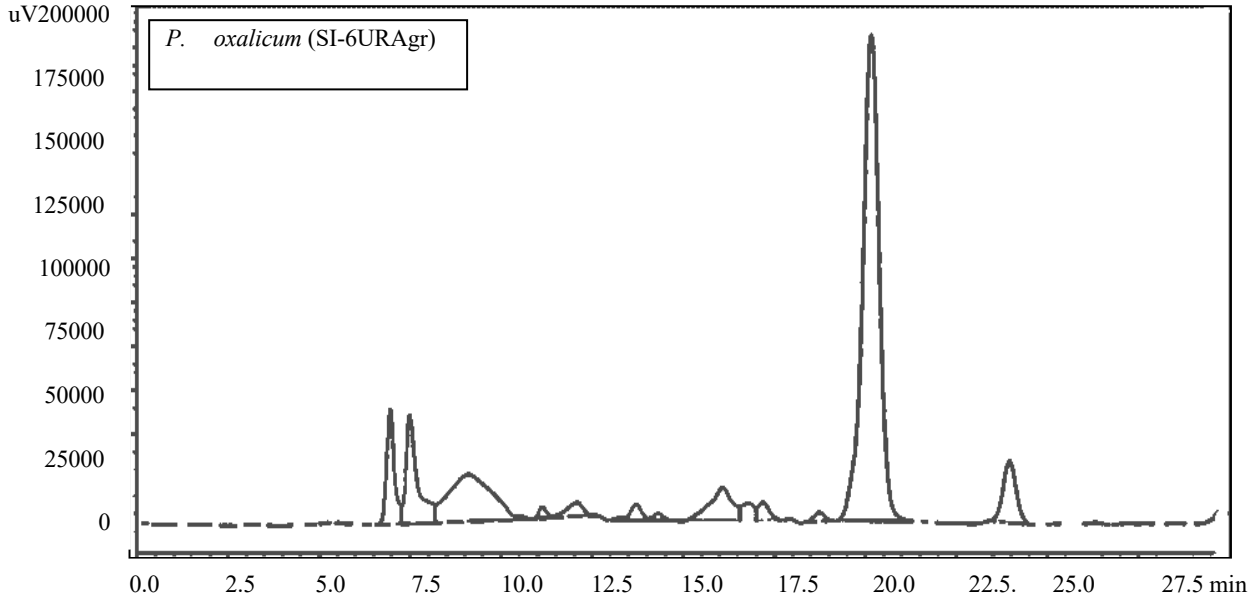

Fig.III.3. Chromatograms of organic acids analyzed by HPLC. The acids were produced by outstanding P solubilizing fungal strain P. oxalicum (SI-6URAgr).

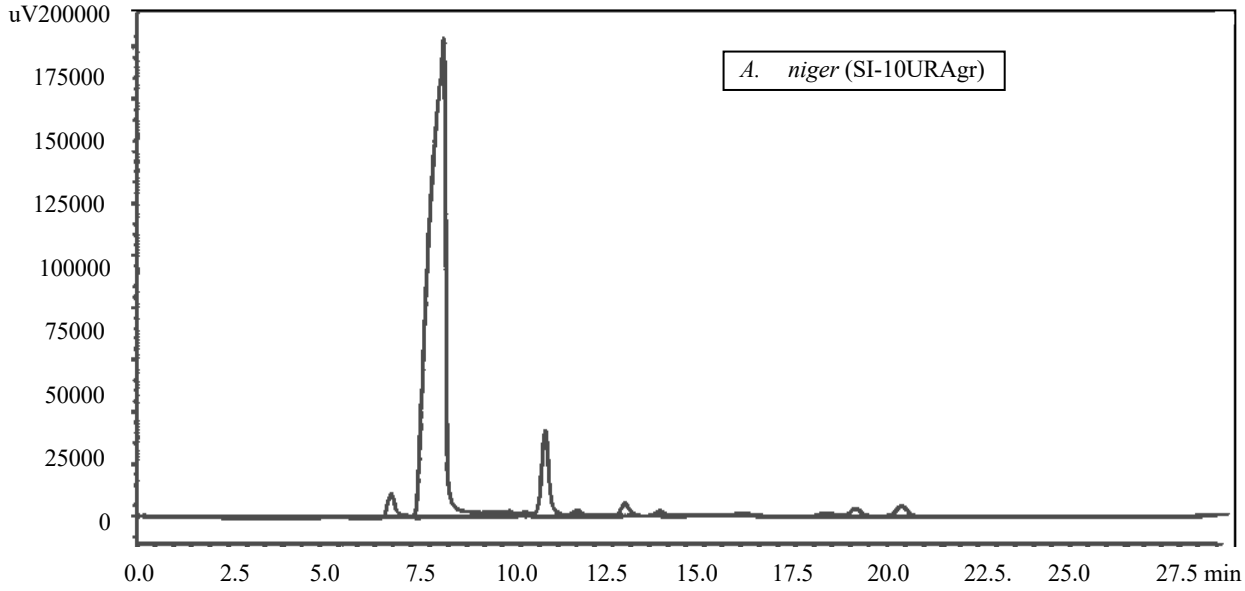

Fig.III.4. Chromatograms of organic acids analyzed by HPLC. The acids were produced by outstanding P solubilizing fungal strain A. niger (SI-10URAgr).

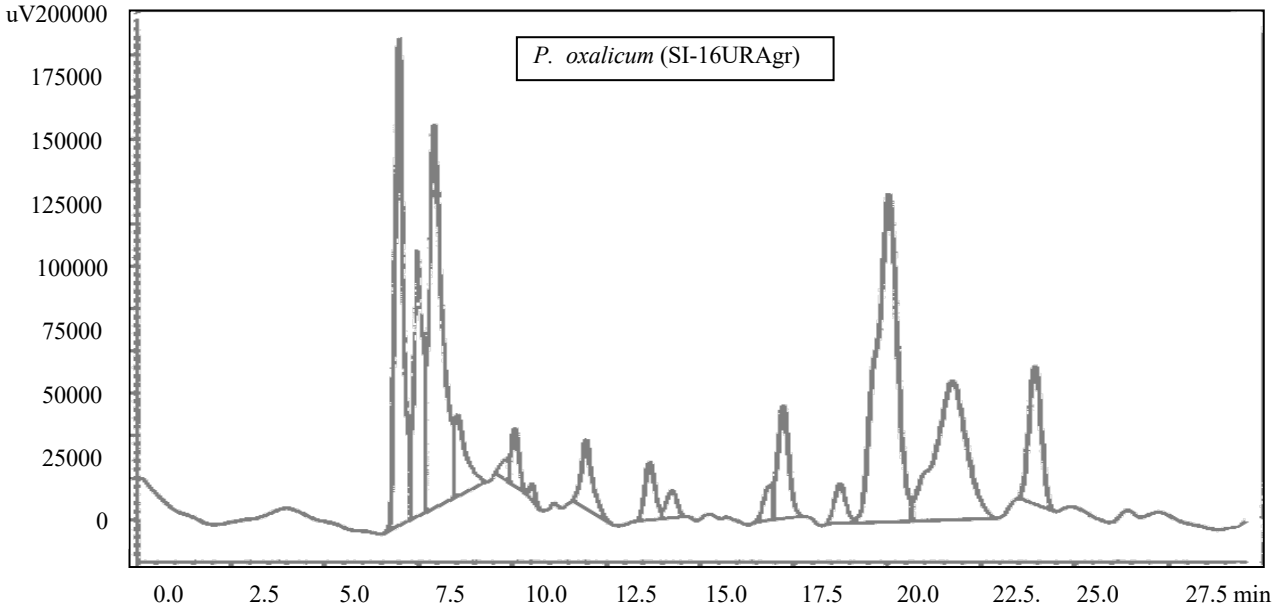

Fig.III.5. Chromatograms of organic acids analyzed by HPLC. The acids were produced by outstanding P solubilizing fungal strain P. oxalicum (SI-16URAgr).

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 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).

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 was 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 and 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.

Conclusion

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 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.

Present studies showed that organic acids play a key role to phosphate solubilization and it is considered as the main mechanism. Based on the mechanism and some strains have promising performance in phosphate solubilization These strains may consider as effective bioinoculant for phosphorus nutrition in plants. Though, some environmental stresses may hamper and reduce the phosphate solubilization efficiency of the selected strains. In this regard, it is essential to know about their stress tolerant abilities. Therefore, next study will be carried out to evaluate the abiotic stress tolerant properties of fungal strains. It will be helpful to select and identify effective strains that could be survive in stress condition.

Chapter IV

Evaluation of Abiotic Stress Tolerant Properties of Phosphate Solubilizing Fungi Isolated from Soils in Okinawa, Japan

Abstract: Sixteen indigenous phosphate solubilizing fungal strains were isolated from three types (dark-red, red and grey) soils in Okinawa, Japan and examined their phosphate solubilizing potential at *in-vitro* condition previously. The fungi were identified based on both mycological and molecular studies. Present study aimed to evaluate the abiotic stress (such as drought and salinity stress) tolerant properties of these fungal strains. Results revealed that several the tested fungal strains exhibited good activities on these stress tolerances. Among them, SI-6URAgr, SI-1URAgr, and SI-14URAgr could grow at 12% NaCl concentrate medium and to be considered as the highest salt tolerant strains followed by SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-15URAgr and SI-16URAgr (10% NaCl). Beside this, SI-6URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-14URAgr were considered as most osmophilic fungal strains followed by SI-1URAgr, SI-4URAgr and SI-16URAgr. They could survive in the 50% sucrose and 35% glycerine amended medium. These strains possessing a crucial function to improve plant growth at adverse situation of environment. They could be utilized as bioinoculant to establish a sustainable crop production system in subtropical, tropical and arid region.

Introduction

World population is increasing at an alarming rate and expected that it will be reached about six billion by the end of year 2050. Although, food productivity is decreasing by the influence of some abiotic stresses such as salinity, low water activity and temperature which adversely affect plant growth, development and productivity (Mahajan *et al.,* 2005). It also affects the crop quality (Yang *et al*., 2009). According to the United Nations Food and Agriculture Organization, approximately 20% irrigated lands are affected by salinity (Rozema and Flowers, 2008). In Europe, twenty-six countries have reported cases of salinization with higher frequency in Mediterranean coastal areas (Flowers, 2004). Salinity leads to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm Saudi Arabia resulting in a reduction of the cytosolic and vacuolar volumes of pant body. Plants survive under the above stress environment using multiple biochemical pathways that facilitate retention and/or acquisition of water. It also helps to synthesis of osmotically active metabolites and enzymes that supports plants to habituate that condition (Parida *et al.,* 2005).

Water scarcity or drought stress is another most significant abiotic stresses that affect plant growth and development (Xu *et al*., 2010). Both drought stress and salt stress are global issues to ensure survival of agricultural crops and sustainable food production (Gosal *et al*., 2010). It occurs when available water in the soil is reduced to such critical levels and atmospheric conditions adds to continuous loss of water. Salinity and drought primarily cause disruption of ionic and water homeostasis of plant cells with consequent deleterious effects on growth and eventually plant death (Maggio *et al*., 2007). Therefore, identifying and developing eco-friendly strategies that can ameliorate plant growth in response to abiotic stresses are an immediate need in agricultural systems that have to cope with the jeopardies of climate change increasingly.

Microorganisms may influence plant growth and development under both favorable and unfavorable environments directly or indirectly. They could facilitate plant tolerance to abiotic stresses has emerged as a promising strategy to improve plant adaptation and resource use efficiency in hostile environments (Yang *et al*., 2009). This strategy gives benefit to plant in stress environment by producing some substances which promote plant growth and increase nutrient availability in soil as well as plants

uptake (Ribeiro and Car-doso, 2012). Some strains of *Aspergillus, Penicillium* and *Talaromyces* have been reported as phosphate solubilizing fungi isolated from different soils in subtropical Okinawa (Islam *et al*., 2019). These fungal strains have excellent phosphate solubilizing abilities. However, environmental stress may influence their potentiality in respective field. If, they have stress tolerant properties beyond their characterized function, it will increase their efficiency and give additional benefit to farmers and other users. On these bases, we aimed to evaluate the abiotic stress tolerant properties of indigenous P solubilizing fungal strains isolated from soils in subtropical Okinawa, Japan for effective use in stress induced agricultural soils for sustainable crop production.

Materials and Methods

Fungal strain used in this study

Sixteen phosphate solubilizing fungi were isolated from different soils in subtropical Okinawa, Japan and identified based on mycological studies and molecular biological techniques (Chapter-2, page 7 to 9). Fungal strains were SI-1URAgr, SI-2URAgr, SI-3URAgr, SI-4URAgr, SI-5URAgr, SI-6URAgr, SI-7URAgr, SI-8URAgr, SI-9URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-13URAgr, SI-14URAgr, SI-15URAgr and SI-16URAgr used in this study.

Abiotic Stress Tolerance test

Some important abiotic stress tolerant tests (halotolerant/halophilic and osmotolerant/osmophilic) were conducted using fresh cultures of the isolated P solubilizing fungi. The salt stress tolerant properties of fungal cultures were checked by observing their growth on the medium amended with different concentration (0-20% w/v) of sodium chloride (NaCl) at 28°C for 7 days. Drought stress tolerant ability of fungi were evaluated by following the method of Leo Daniel et al. (2011), using 0-50% glycerin and 0-50% sucrose medium.

Statistical analysis

Microsoft Excel 2016 was used to accomplish statistical analysis. All the experiments were carried out in triplicate.

Results

Abiotic stress tolerance of fungi

In the present study, we have assayed important abiotic stress such as salt stress and drought stress tolerant traits of the isolated fungi by inspection of their growth in the medium containing different levels of salt, sucrose and glycerin. Sucrose and glycerin plate assay were performed to evaluate drought stress tolerant properties of the isolates.

Almost all the fungal strains were able to grow at 6% of salt amended medium. Among the tested fungal strains, the most salt tolerant properties revealed by SI-6URAgr, SI-1URAgr and SI-14URAgr (12%), followed by SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-15URAgr and SI-16URAgr, whereas SI-3URAgr, SI-4URAgr and SI-5URAgr could grow at 8% NaCl concentration (Table IV.1.: Fig. IV.1.).

Table IV.1. *Salt tolerance property of phosphate solubilizing fungi isolated from soils in Okinawa, Japan.*

Sl. No.	***Fungal strains***	***Name of the fungi***	***Growth of fungi in salt enriched PDA medium (NaCl %)***										
			0	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
1	SI-1URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	-	-	-	-
2	SI-2URAgr	*Aspergillus floccosus*	+	+	+	+	-	-	-	-	-	-	-
3	SI-3URAgr	*Aspergillus niveus*	+	+	+	+	+	-	-	-	-	-	-
4	SI-4URAgr	*Talaromyces pinophilus*	+	+	+	+	+	-	-	-	-	-	-
5	SI-5URAgr	*Aspergillus niveus*	+	+	+	+	+	-	-	-	-	-	-
6	SI-6URAgr	*Penicillium oxalicum*	+	+	+	+	+	+	+	-	-	-	-
7	SI-7URAgr	*Penicillium sp.*	+	+	+	+	-	-	-	-	-	-	-
8	SI-8URAgr	*Penicillium sp.*	+	+	+	+	-	-	-	-	-	-	-
9	SI-9URAgr	*Penicillium sp.*	+	+	+	+	-	-	-	-	-	-	-
10	SI-10URAgr	*Aspergillus niger*	+	+	+	+	+	+	-	-	-	-	-
11	SI-11URAgr	*Aspergillus niger*	+	+	+	+	+	+	-	-	-	-	-
12	SI-12URAgr	*Aspergillus niger*	+	+	+	+	+	+	-	-	-	-	-
13	SI-13URAgr	*Penicillium sp.*	+	+	+	+	+	-	-	-	-	-	-
14	SI-14URAgr	*Aspergillus floccosus*	+	+	+	+	+	+	+	-	-	-	-
15	SI-15URAgr	*Talaromyces pinophilus*	+	+	+	+	+	+	-	-	-	-	-
16	SI-16URAgr	*Penicillium oxalicum*	+	+	+	+	+	+	-	-	-	-	-

(+) indicated growth of fungi, (-) indicated no growth

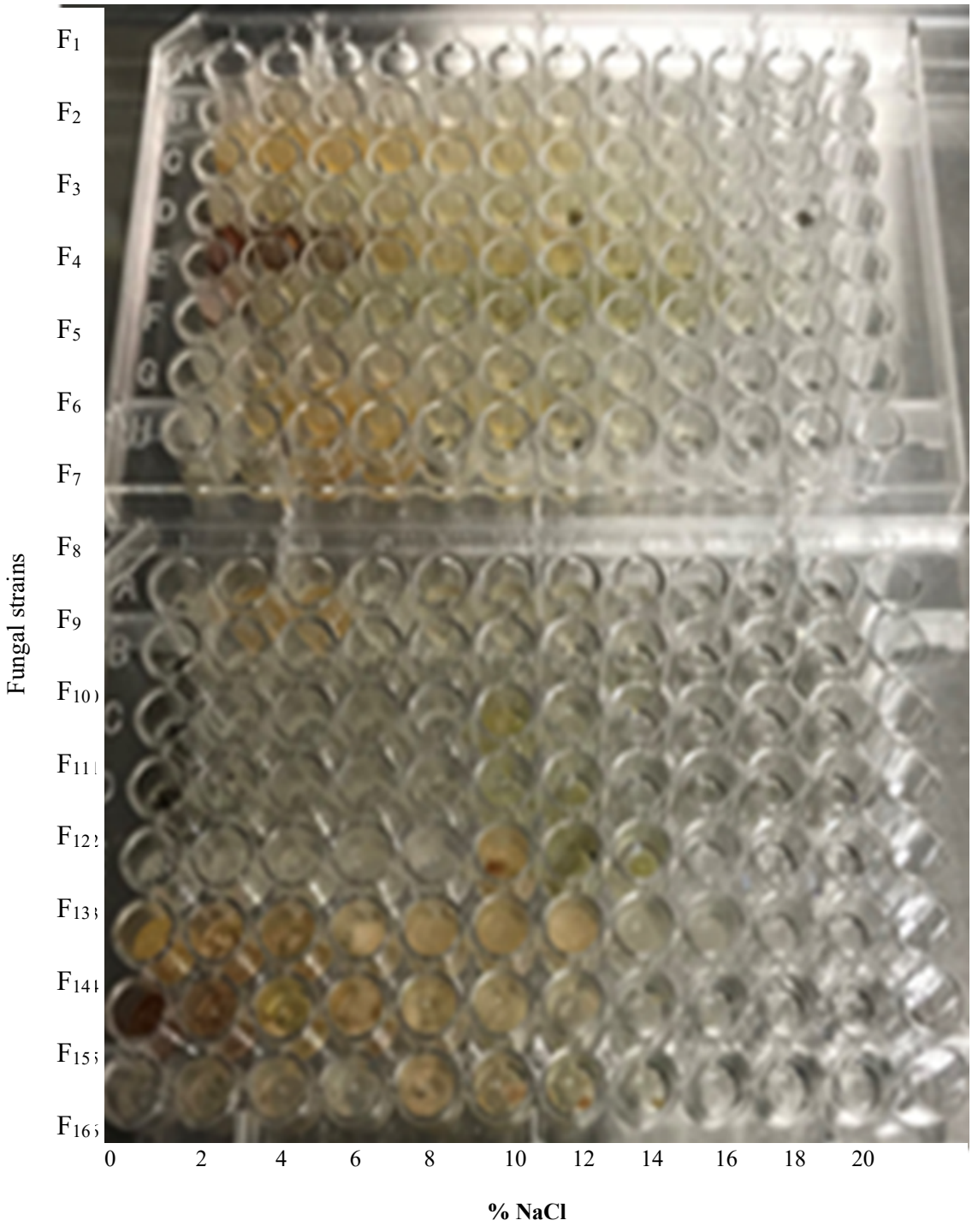

Fig. IV. 1. Growth of phosphate solubilizing fungi in NaCl amended

growth medium in microplate.

All the tested fungal strains could survive 40% sucrose in medium except SI-13URAgr. The Strains SI-6URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr and SI-14URAgr were found to be more resistant (50%) to sucrose (Table IV.2.; Fig.IV.2.), whereas, SI-1URAgr, SI-4URAgr and SI-15URAgr could grow at 45% sucrose concentration.

Table IV. 2. *The growth and survival of isolated phosphate solubilizing fungal strains at different sucrose enriched growth medium*

Sl. No.	***Fungal strains***	***Name of the fungi***	***Growth of fungi in Sugar enriched PDA medium (Sucrose concentration %)***										
			0	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
1	SI-1URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	+	+	+	-
2	SI-2URAgr	*Aspergillus floccosus*	+	+	+	+	+	+	+	+	+	-	-
3	SI-3URAgr	*Aspergillus niveus*	+	+	+	+	+	+	+	+	+	-	-
4	SI-4URAgr	*Talaromyces pinophilus*	+	+	+	+	+	+	+	+	+	+	-
5	SI-5URAgr	*Aspergillus niveus*	+	+	+	+	+	+	+	+	+	-	-
6	SI-6URAgr	*Penicillium oxalicum*	+	+	+	+	+	+	+	+	+	+	+
7	SI-7URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	+	+	-	-
8	SI-8URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	+	+	-	-
9	SI-9URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	+	+	-	-
10	SI-10URAgr	*Aspergillus niger*	+	+	+	+	+	+	+	+	+	+	+
11	SI-11URAgr	*Aspergillus niger*	+	+	+	+	+	+	+	+	+	+	+
12	SI-12URAgr	*Aspergillus niger*	+	+	+	+	+	+	+	+	+	+	+
13	SI-13URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	+	-	-	-
14	SI-14URAgr	*Aspergillus floccosus*	+	+	+	+	+	+	+	+	+	+	+
15	SI-15URAgr	*Talaromyces pinophilus*	+	+	+	+	+	+	+	+	+	+	-
16	SI-16URAgr	*Penicillium oxalicum*	+	+	+	+	+	+	+	+	+	+	-

(+) indicated growth of fungi, (-) indicated no growth

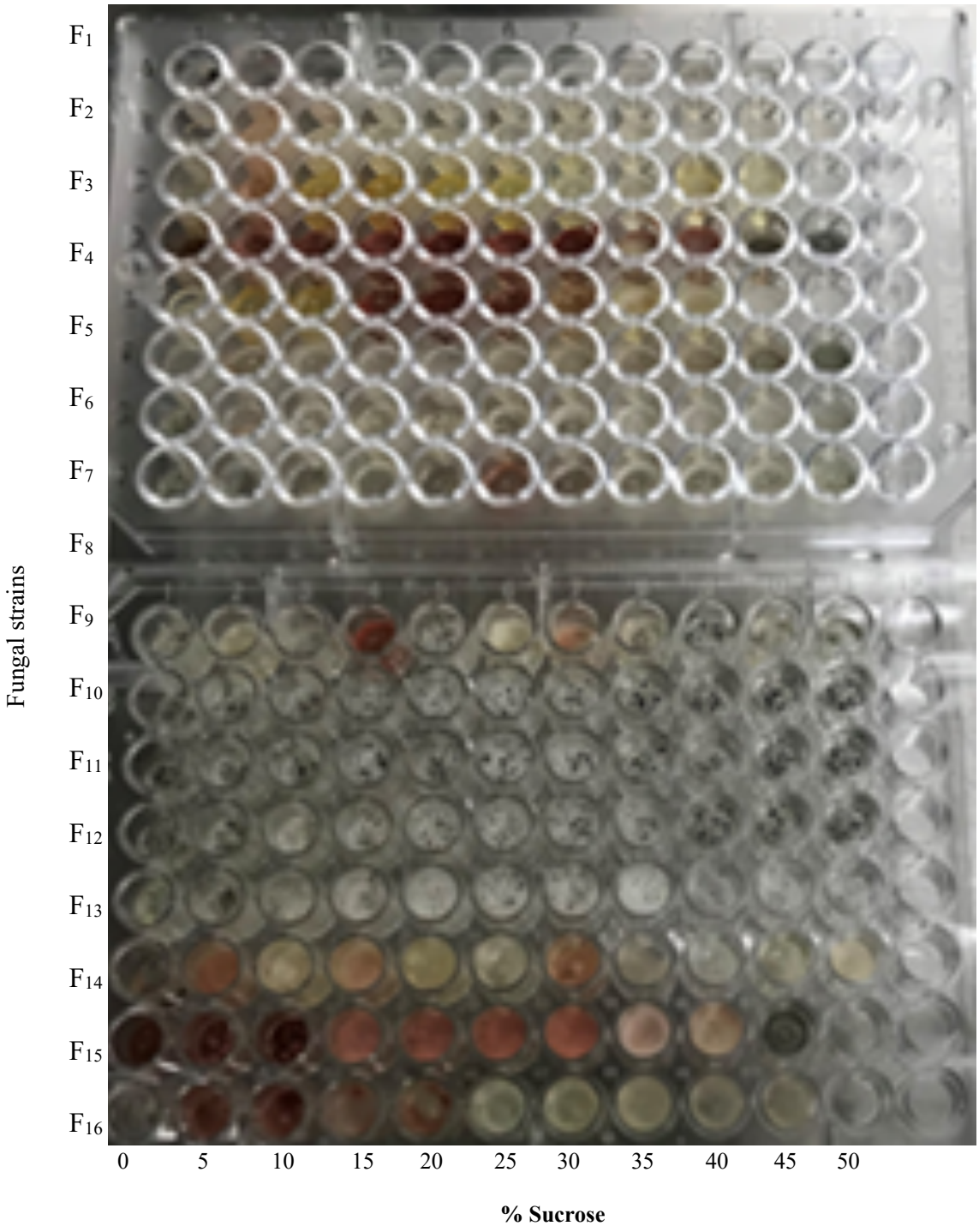

Fig. IV.2. Growth of phosphate solubilizing fungi in sucrose amended growth medium in microplate.

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Again, in case of glycerin amended medium, sporulation was observed in presence up to 35% of glycerin in medium. All the fungal strains could grow 25% glycerin concentration except SI-8URAgr. The strain SI-6URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr and SI-14URAgr could grow 35% glycerin in medium and considered as the highest drought stress tolerant strains followed by SI-2URAgr, SI-5URAgr, SI-8URAgr, SI-13URAgr and SI-15URAgr, (Table IV.3.; Fig.IV.3.).

Table IV.3. *The growth and survival of isolated phosphate solubilizing fungal strains at different glycerine concentration*

Sl. No.	***Fungal strains***	***Name of the fungi***	***Growth of fungi in glycerine enriched PDA medium (Glycerine concentration %)***										
			0	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
1	SI-1URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	-	-	-	-
2	SI-2URAgr	*Aspergillus floccosus*	+	+	+	+	+	+	+	-	-	-	-
3	SI-3URAgr	*Aspergillus niveus*	+	+	+	+	+	+	-	-	-	-	-
4	SI-4URAgr	*Talaromyces pinophilus*	+	+	+	+	+	+	+	-	-	-	-
5	SI-5URAgr	*Aspergillus niveus*	+	+	+	+	+	+	-	-	-	-	-
6	SI-6URAgr	*Penicillium oxalicum*	+	+	+	+	+	+	+	+	-	-	-
7	SI-7URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	-	-	-	-
8	SI-8URAgr	*Penicillium sp.*	+	+	+	+	+	-	-	-	-	-	-
9	SI-9URAgr	*Penicillium sp.*	+	+	+	+	+	+	+	-	-	-	-
10	SI-10URAgr	*Aspergillus niger*	+	+	+	+	+	+	+	+	-	-	-
11	SI-11URAgr	*Aspergillus niger*	+	+	+	+	+	+	+	+	-	-	-
12	SI-12URAgr	*Aspergillus niger*	+	+	+	+	+	+	+	+	-	-	-
13	SI-13URAgr	*Penicillium sp.*	+	+	+	+	+	+	-	-	-	-	-
14	SI-14URAgr	*Aspergillus floccosus*	+	+	+	+	+	+	+	+	-	-	-
15	SI-15URAgr	*Talaromyces pinophilus*	+	+	+	+	+	+	-	-	-	-	-
16	SI-16URAgr	*Penicillium oxalicum*	+	+	+	+	+	+	+	-	-	-	-

(+) indicated growth of fungi, (-) indicated no growth

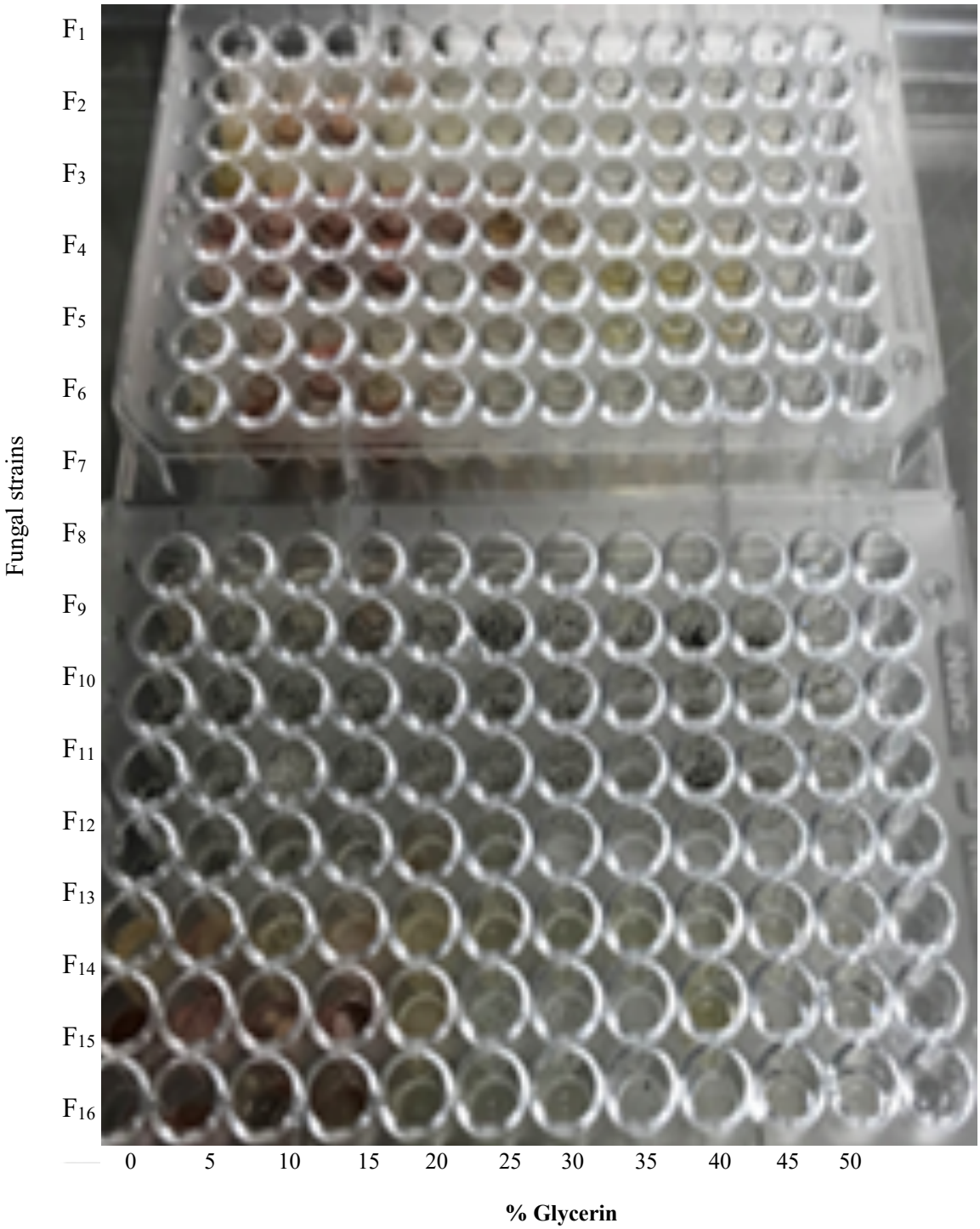

Fig. IV. 3. Growth of phosphate solubilizing fungi in glycerine amended growth medium in microplate.

Discussion

The need to increase food production by ecofriendly approaches including the use of beneficial fungi for plant growth promotion have great importance. A wide range of fungi contributes plant growth and development at stressed environment (Larran *et al*., 2007, Vacheron *et al*. 2013). By inspiring with the previous research, current investigation was designed to screen out abiotic stress tolerant fungi based on salt, sucrose and glycerin test by microplate assay from 16 phosphate solubilizing fungal stains isolated from different soils in subtropical Okinawa, Japan (Islam *et al*., 2019). Most of the isolated fungus exhibited stress tolerance properties, such as salt stress and drought stress.

Salt stress tolerant microbes are a prospective bioresource for saline prone areas. Isolates SI-6URAgr (*P. oxalicum*) SI-1URAgr (*Penicillium* sp.) and SI-14URAgr (*A. flucosus*) have higher salt tolerant ability (12% NaCl) compare with other strains used in this study. Nayak et al., (2012) reported that a diverse genus of halophilic or salt tolerant fungi present in mangrove area of Goa, India, such as, *Aspergillus* spp. and *Penicilium* spp. They could tolerate 25% and 20% raw salt respectively. Okinawa is island surrounded by the Pacific Ocean and East-China Sea. Its climate is subtropical. In this area salinity induced by evaporation of sea water and accumulated in soil. For this reason, most of the isolates have salt tolerant ability (8% NaCl).

Crop plant-associated microbes having good drought tolerance property are recently getting increased attention. By influencing plant morphology, development, and physiological and biochemical responses to stress, fungi can provoke mechanisms of drought escaping, drought lenience, and drought recovery in their hosts (Vurukonda *et al*., 2016, Bashan and Holguin 1998). In our investigation, we have observed that all the selected fungal strains are able to resist drought in variable range but *Aspergillus niger* (SI-10URAgr, SI-11URAgr and SI-12URAgr), *Aspergillus flucosus* (SI-14URAgr) and *Penecilium oxalicum* (SI-6URAgr) have ability to survive in 50% sucrose and 35% glycerin amended medium. These strains are osmophilic in nature. Abdel-Hafez et al., identified 22 species of *Aspergillus* from Egyptian soils by 60%

sucrose-Czapek's agar plates. They also reported that *A. niger* have the highest osmophilic potential among the identified fungi. Mostafa and Al-Musallam isolate and identified some *Penicillium spp. which could grow and survive under high osmotic pressure induced environment.*

Conclusion

From the above discussion, we concluded that all the fungal isolates gave positive result on stress tolerances. Among them, strain SI-1URAgr, SI-6URAgr and SI-14URAgr have higher salt tolerant ability followed by SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-15URAgr and SI-16URAgr. These strains could grow in presence of 12% and 10% NaCl respectively. On the other hand, SI-6URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-14URAgr could grow and survive in the presence of 50% sucrose and 35% glycerine amended medium followed by SI-1URAgr, SI-4URAgr and SI-16URAgr. These strains could be utilized as bio inoculants to establish a sustainable crop production under salt and drought stress environment respectively.

Salinity is a severe problem worldwide in agriculture. It is closely related to the global warming and climate change. Present study showed that some strains could grow well in saline environment. Salt concentration did not influence their growth. Although, we have no information about their phosphate solubilization potential in the presence of salt. Therefore, next study will be carried out to examine the effect of salt on phosphate solubilizing potential and growth of selected fungal strains. It will be helpful to identify the effective strains for improve available P status in salt affected agricultural soils.

Chapter V

Influence of Salt on the growth and Phosphate Solubilizing Ability of Potential Fungi Isolated from Soils in Okinawa, Japan

Abstract: Salt stress is a major agricultural problem all over the world, which influences the physiological and regular functioning of plants. It also greatly affects the uptake of essential plant nutrients and survivals of microbes. In this regard, some fungi have ability to survive in saline environment and enhance the supply of essential mineral nutrients like P that facilitate the plant growth and development in such condition. This study aimed to examine the influence of salinity on the growth and phosphate solubilizing ability of fungal strains that were isolated from soils in subtropical Okinawa, Japan. Results reveled that fungal strain SI-6URAgr (*Penicillium oxalicum*) showed the maximum P solubilization in present of 1-4% NaCl as salt followed by SI-11URAgr *(A. niger)* and SI-15URAgr (*T. pinophilus*) which was significantly superior over all other isolates. Mycelial dry weight measurement revealed that salt concentration did not affect the growth of fungal strain. These strains could grow well in saline environment and solubilized P perfectly. They could be utilized as salt tolerant phosphate solubilizing bioinoculant for crop production in salt affected agricultural soils.

Introduction

The global climate change creates major problem concerning our agriculture as well as crop production, which considered as major global threat to future of food security (Battisti and Naylor, 2009). When world current population reached to 8.9 billion from 7 billion by 2050, it will be create adverse condition (Singh *et al*., 2011). Our agroecosystem is continuously affected by some abiotic and biotic stress which directly influence the crop productivity, soil health and fertility. Various stress such as drought, temperature and salt stress negatively affect the growth, yield and quality of crop plants. About 50% and 30% yield loss occurred by abiotic and biotic stresses. Salinity is one of the major abiotic stresses limiting crop growth and yield (Lei *et al*., 2015). It creates threat for agricultural sustainability (Wassmann *et al*., 2009). Plant physiological biochemical and gene regulation activities are mostly affected by soil salinity. It has negative effects on soil microbial population, diversity and soil fertility (Klimek *et al*., 201 6).

Only the possible alternatives is plant associated beneficial microbes such as plant growth promoting fungi and bacteria which enhance plant growth and development under different stresses. The application of efficient microorganisms are helpful in enhancing and improving agricultural and environmental stability . The soils in such kind of environment have tendency to fix phosphorus (Johri *et al*., 1999). Microorganisms have the ability to solubilize the insoluble phosphates and maintain the nutrient status in soil (Richardson, 2001).

The plant growth promoting microorganisms maintain plant fitness and health under stress environment (Vimal *et al*., 2017). However, most of the plant growth promoting microbes (PGPM) are unable to tolerate drought, salinity, and heavy metal stress. So, it is a very challenging work for the farmers as well as the scientists to find out efficient microbes, which are capable to survive under salt stress environment. Therefore, the present study aimed to select potential fungi that could survive and solubilize phosphate in the presence of higher salt concentration to obtain efficient strains for application as a potential bioinoculant in saline problematic soils.

Materials and Methods

Fungal strain used in this study

Sixteen phosphate solubilizing fungi were isolated from different soils in subtropical Okinawa, Japan. Fungal isolates were identified based on both mycological studies and molecular biological techniques (Chapter 2, page 7 to 9). Higher salt tolerant fungal strains from 16 isolates by microplate assay were selected for this study. Strains were SI-1URAgr, SI-2URAgr, SI-3URAgr, SI-4URAgr, SI-5URAgr, SI-6URAgr, SI-7URAgr, SI-8URAgr, SI-9URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-13URAgr, SI-14URAgr, SI-15URAgr and SI-16URAgr.

Influence of NaCl on P solubilization and growth of fungal strains

Seven days old homogenized cultures of fungal strains were inoculated into 40 ml of Pikov's broth medium containing a series of salt concentration (0.1, 1, 2, 3 and 4 % NaCl). Incubation was done at 28 ± 2 °C with 150 rpm shaking speed for seven days with three replicates (Fig. V.1, V.2). After that the cultures were filtered through pre-weighed Whatman No.2A filter papers and mycelial biomass were dried at 65 °C in a hot air oven for 24 hours. The dry mycelial weight was recorded for each culture. The culture filtrates were filter again through 0.45 mm nylon filter (Advantech, Japan). Then the phosphorous released by the fungal strains in the extract was estimated by Inductively Coupled Plasma Emission Spectrometer (ICP-AES). It was expressed as μg/ml.

Figure V. 1. Pekovskaya's medium amended with different (0.1-1%) NaCl and inoculated with 7 phosphate fungal strains.

Figure V.2. Pekovskaya's medium amended with (2-4%) NaCl and inoculated with 7 phosphate solubilizing fungal strains.

Statistical analysis

All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program (version 2016). The mean values were compared by Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

Results

Role of NaCl on P-solubilization by fungal isolates

The selected phosphate solubilizing fungal strains were tested for their ability to solubilize P from insoluble tricalcium phosphate in the presence of different concentrations of NaCl (0.1-4%) in Pikovskaya's broth medium. The data regarding the P released in broth medium at 7 days after incubation are presented in tableV.1. A significant variation was observed considering the P solubilization ability of fungal strains in the different salt concentration. It was ranged from 60.10-620.06, 66.46-670.5, 67.51-599.28, 59.50-562.89 and 74.30-638.12 µg/ml in 0.1, 1.0, 2.0, 3.0 and 4% NaCl respectively. Strain SI-6URAgr (*Penicillium oxalicu*m) solubilized the highest amount of P considering all tested concentration of NaCl except 0.1% followed by SI-11URAgr and SI-15URAgr (Fig.V.4-V.7). The amount was 670.51ppm (1%), 599.28(2%), 562.89(3%) and 638.12 (4%) µg/ml. It was significantly superior over all other strains used in this study. Besides these, strain SI-16URAgr solubilized significantly higher amount of P in only the NaCl concentration was 0.1% (Fig.V.3) SI-10URAgr solubilized highest amount of P when NaCl concentration was 3% (Fig.V.6) which was statistically at per with strain SI-6URAgr.

Table V.1. Solubilization of P from TCP by phosphate solubilizing fungi as influenced by NaCl concentration in Pikovskaya's broth.

Fungal strains	NaCl concentration in broth medium				
	0.1 %	1%	2%	3%	4%
	Solubilized P (mg/L				
SI-1URAgr	583.3b	572.68d	428.96d	422.04c	339.87d
SI-6URAgr	519.76c	670.51a	599.28a	562.89a	638.12a
SI-10URAgr	504.00c	578.88d	425.37d	543.34a	438.54c
SI-11URAgr	465.94d	640.96b	459.29c	445.51b	567.03b
SI-14URAgr	60.10e	66.46f	67.51e	59.50e	74.30e
SI-15URAgr	518.63c	612.86c	491.98b	405.39c	570.54b
SI-16URAgr	620.06a	494.87e	423.53d	365.37d	419.82c

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

Effect of NaCl on fungal growth

The data on mycelial mat weight (dry) of the tested fungal strains as influenced by different concentrations of NaCl are presented in table V.2. A significant variation was observed considering the growth of fungi based on mycelial dry weight in the different salt concentration. It was ranged from 1.02-3.90, 1.88-3.34, 1.32-4.42, 1.72-7.65 and 2.70-6.43g/L in 0.1, 1.0, 2.0, 3.0 and 4% NaCl respectively. Strain SI-14URAgr produce the significantly highest amount of mycelial biomass when NaCl concentration was 0.1 and 1% (Fig.V.3, V.4). The fungal strain SI-10URAgr produce the highest amount of mycelial biomass when salt concentration was 2%. It was statistically similar with SI-11URAgr, SI-1URAgr and SI-6URAgr (Fig.V.5). SI-10URAgr also produced the highest amount of biomass in 3% NaCl concentration (Fig.V.6). SI-1URAgr produced the highest amount of mycelial biomass in the 4% NaCl concentration followed by SI-6URAgr, SI-14URAgr and SI-15URAgr (Fig.V.7).

Table V. 2. Mycelial dry weight of phosphate solubilizing fungi as influenced by NaCl concentration in Pikovskaya's broth medium.

	NaCl concentration in the broth medium				
	0.1%	**1%**	**2%**	**3%**	**4%**
Fungal strain	**Mycelial Dry Wt. g/L**				
SI-1URAgr	1.17c	2.15cd	3.64abc	5.46c	6.43a
SI-6URAgr	1.54c	2.47bc	4.11ab	4.78e	5.52b
SI-10URAgr	3.17ab	2.77b	4.42a	7.65a	2.82cd
SI-11URAgr	2.50b	2.82b	4.23a	7.25b	2.70d
SI-14URAgr	3.90a	3.34a	3.13bc	5.08d	5.08b
SI-15URAgr	1.02c	1.99c	1.32d	4.47f	5.53b
SI-16URAgr	3.14ab	1.88d	3.07c	1.72g	3.46c

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

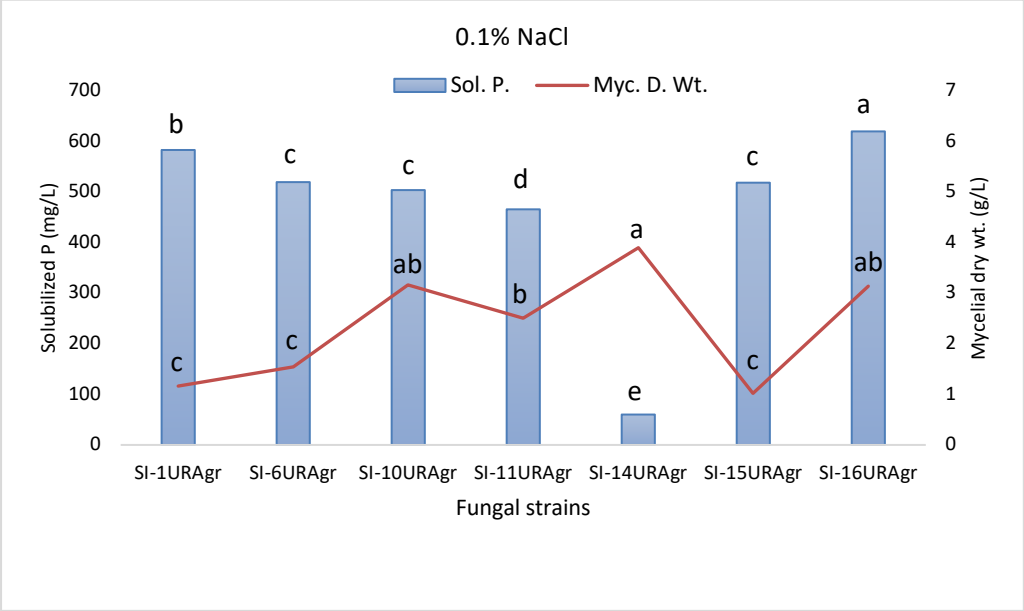

Fig.V.3. Solubilization of P and growth of phosphate solubilizing fungi influenced by NaCl (0.1%) concentration.

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

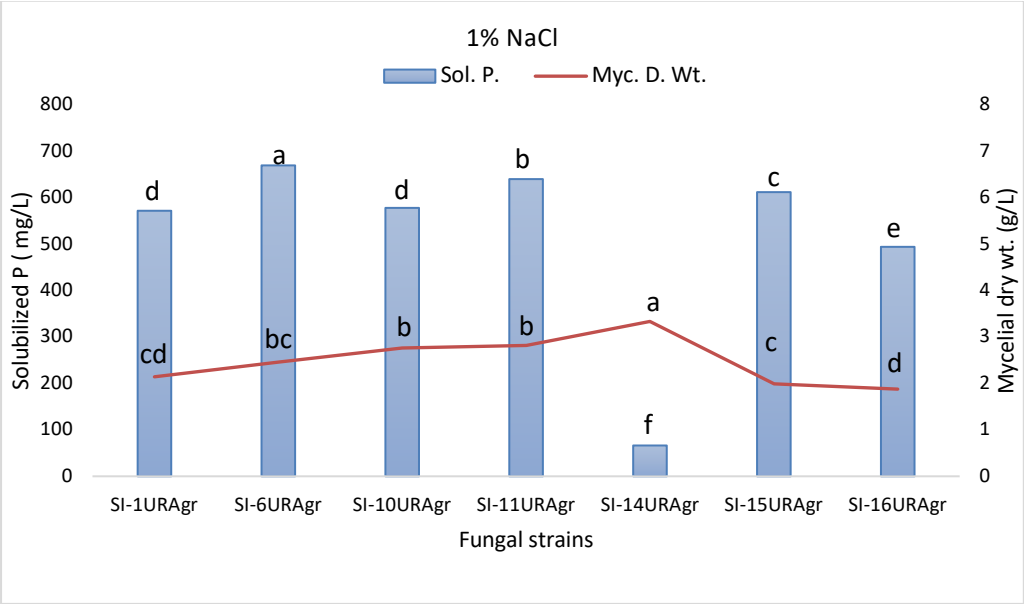

Fig.V.4. Solubilization of P and growth of phosphate solubilizing fungi influenced by NaCl (1%) concentration.

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

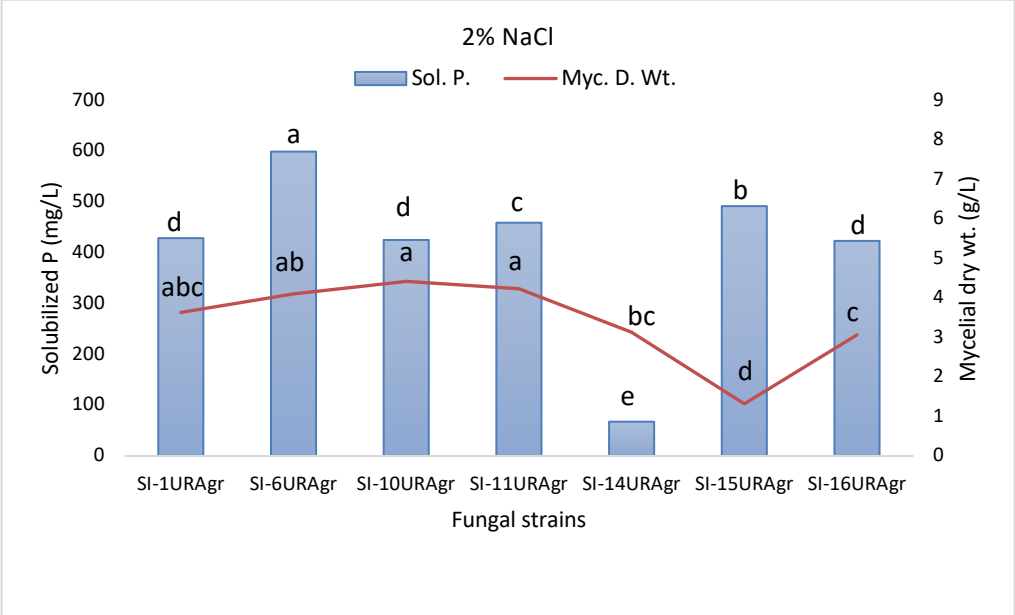

Fig.V.5. Solubilization of P and growth of phosphate solubilizing fungi influenced by NaCl (2%) concentration.

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

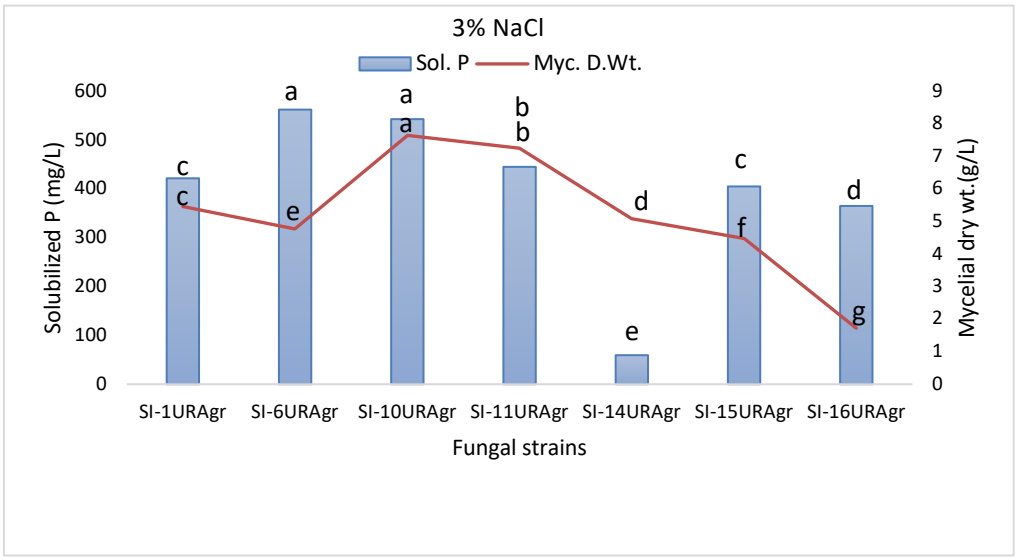

Fig.V.6. Solubilization of P and growth of phosphate solubilizing fungi influenced by NaCl (3%) concentration.

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

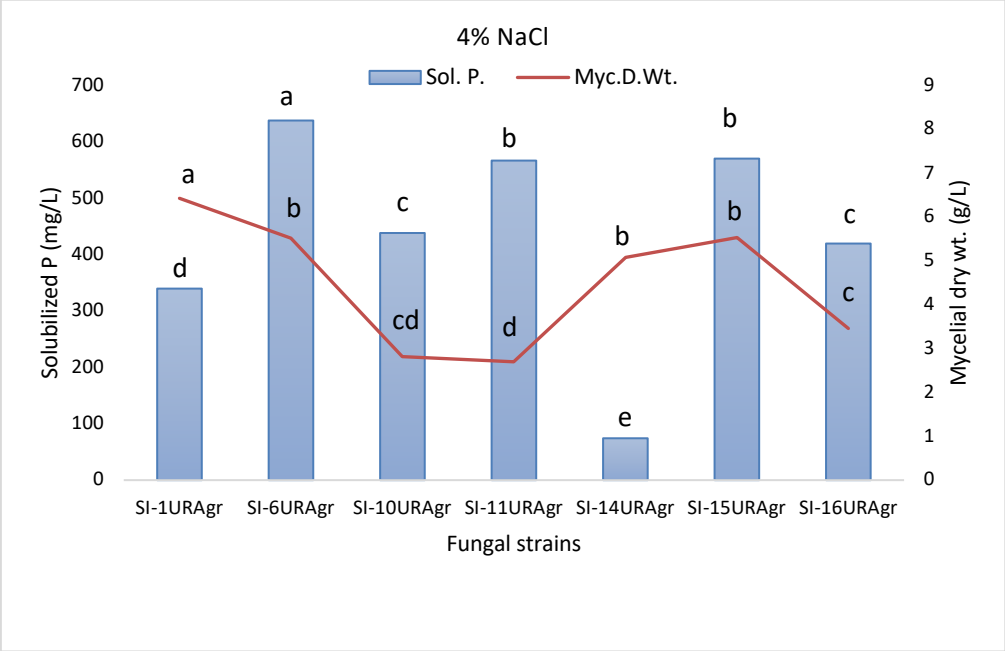

Fig.V.3. Solubilization of P and growth of phosphate solubilizing fungi influenced by NaCl (4%) concentration.

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

Discussion

Phosphate solubilizing fungi used in this study were isolated from soils in Okinawa under subtropical environment. An attempt was made to evaluate phosphate solubilizing ability of these fungal strains as well as their growth and survival under salt stress environment.

When the isolates exposed to different concentration of NaCl, phosphate solubilization did not affect greatly. It is indicating that the fungal strains may give benefit in maintaining available phosphorous level in salt affected agricultural problem soils. Our isolated *P. oxalicum* (SI-6URAgr), *A. niger* (SI-11URAgr) and *T. Pinophilus* (SI-11URAgr) have prominent phosphate solubilizing ability regardless of salt concentration (0.1-4%). Our findings in agreed with Yadav *et al*., (2011). They reported that *Penicillium citrium* isolated from the sugarcane rhizosphere could solubilize phosphorous under saline environment, although that study was perform using only 1% NaCl as salt. The *Eupenicillium parvam* and some *Aspergillus* spp. (Narsian and Palet 2000; Rinu and Pandey, 2010; Srinivasan *et al*., 2012) are able to solubilize phosphate at various level of salinity. Khan *et al*., (2011), reported that plant growth promotion activities of *Talaromyces* spp. strain LHLO6 was not affected by salinity stress.

The mechanism of salt tolerant by fungi is still now unknown. It is assuming that fungi could survive in salt stress environment using different mechanism, such as synthesis of compatible solutes or accumulation of potassium against NaCl to overcome the Na^+ toxicity. Although, a study on arbuscular mycorrhizal fungi *(Glomus intraradicas)* from saline soils showed that adaptation to salinity was related to up-regulation of fungal genes encoding chaperones or aqua porins (Estada *et al*., 2013). The possibility that chaperones and aquaporins may have a key role in the salinity stress tolerance of phosphate solubilizing fungi is a research area that needs investigation.

I observed that mycelial biomass dry weight sometimes higher in higher salt concentration. Generally, it was decreased with the increase of NaCl concentration. It suggested that these fungal strains are halophilic in nature. The reduced growth of fungi in the presence of NaCl due to disturbance of their normal functioning of synthesis

pathways. It has been shown that some mineral ions inhibit the activity of specific enzymes (Boots ford, 1984). Another point is that presence of Na^+ inhibit the activity of enzymes related to the normal growth of fungi. Salt tolerant or halophilic fungi have ability to overcome all the barriers related to survival in such stress environment.

Yokyama *et al*., (1992) observed that growth of some fungi was isolated from saline soils mostly affected by NaCl concentration in liquid medium. It was decreased when NaCl concentration was increased. They also reported that the mycelial dry weight of *Aspergillus* sp. appears almost half at 90g/L NaCl concentrate culture medium compared to the culture without NaCl. In this study, *Penicillium* sp. solubilized the highest amount of P from TCP followed by *Aspergillus* sp. It indicated that the better adaptability of these strains in slat stress condition. Furthermore, these strains have great importance in industrial application. They could be utilized as important bioresource for saline prone areas.

Conclusion

In conclusion, as it is able to solubilize insoluble tricalcium phosphate in vitro under saline conditions, fungal strains, SI-6URAgr (*Penicillium oxalicum*) showed significantly higher P solubilizing ability in presence of higher salt concentration (NaCl 1-4%) in the medium followed by SI-11URAgr, SI-10URAgr and SI-15URAgr. Finally, SI-6URAgr (*Penicillium oxalicum*) is considered as a suitable candidate for maintaining available phosphorous level in saline agricultural soils and needs future research in soil conditions with a view towards development as bio inoculant.

Present study showed that some strains have promising phosphate solubilizing ability under saline condition. Another most important abiotic factor is pH. Soil pH greatly influence the growth and proliferation of fungi. Therefore, next study will be carried out to investigate the influence of pH on the growth of phosphate solubilizing fungi. It will be helpful for choosing the effective strains that could be survive in acidic or alkaline environment.

Chapter VI

Influence of pH on the Growth of Phosphate Solubilizing Fungi Isolated from Soils in Okinawa, Japan

Abstract: The influence of pH on the growth and survival of phosphate solubilizing fungi were investigated. The three representative fungal strains were cultured for seven days with the initial pH in the medium ranged from 1.5 to 8.5. We estimated the fungal growth by measuring dry matter of mycelial biomass. The growth-based measurements revealed that all the tested fungal strains were capable to grow and survive in a wide range of pH (2.5-8.5). Among them, SI-10URAgr (*A. niger*) enhanced the highest acidity in all tested pH values followed by *P. oxalicum* (SI-16URAgr) and SI-14URAgr. The fungal growth mostly depends on the pH. SI-10URAgr showed the highest growth (0.28g) at pH 3.5. Besides this, SI-14URAgr and SI-16URAgr showed the maximum growth (0.43g and 0.20g) when initial pH value was 5.5 and 7.5 respectively. These findings suggested that *A. niger* have strongest adaptability to acidic environment followed by *P. oxalicum.* Although, these fungal strains could grow and survive in higher pH also. It may give an extra advantage to utilize these strains in any pH condition in soil.

Introduction

The soil microbial community is responsible for most nutrient transformations in soil. Availability of these mineral nutrients limit soil fertility and plant productivity. The activities and survival of microbes influence by various properties such as biomass composition (De Ruiter *et al*.,1993), nutrient demand (De Vries *et al*., 2006; Rousk *et al*., 2007; Van *et al*., 2007), metal tolerance (Rajapaksha *et al*.,2004), temperature and pH dependence (Pietikinen *et al*., 2005). Beside these, anthropogenic impacts, such as changes in nutrient input, climate change, and soil management, have the potential to directly or indirectly affect the fungal composition, with consequent impacts on soil function.

Soil pH is one of the most influential factors affecting the microbial community. It also influences other abiotic factors, such as availability of carbon, nutrients and solubility of metals (Andersson *et al*., 2000; Kemmitt et al., 2006; Aciego *et al*., 2008; Firestone et al., 1983; Flis *et al*., 1993). In addition of these, soil pH may control the biomass composition of fungi and bacteria in both forest and agricultural soils (Fierer *et al*., 2006; Baath *et al*., 2003; Blagodatskaya *et al*., 1998; Arao *et al.*, 1999; Bardgett *et al*., 2001). So, it is important to studying the influence of soil pH on growth and survival of fungi as an inherent problem that influences multiple parameters. Therefore, present study aimed to evaluate the influence of pH on the growth of selected fungal strains which were isolated from different soils in subtropical Okinwa, Japan as phosphate solubilizing fungi.

Materials and Methods

Fungal Strains used in this study

In our previous study, sixteen phosphate solubilizing fungal strains were isolated from different soils of Okinawa, Japan and identified (Chapter 2, page 10). The three representative fungal strains *Aspergillus niger* (SI-10URAgr), *Aspergillus flucosus* (SI-14URAgr) and *Penicillium oxalicum* (SI-16URAgr) were selected to determine the influence of pH on their growth and survival.

Preparation of inoculum for Biomass measurement of fungi under different pH conditions

Sporulated pure fungal cultures slants of 10URAgr, SI-14URAgr and SI-16URAgr were selected for preparation of spore suspension by using standard procedure. A total volume of 5 ml sterile water with twin 80 was added in culture slants and the fungal colony surface was lightly scraped by sterile bamboo stick. The cultures were passing through a syringe with staff cotton. Spore count was done by a haemocytometer and the suspension was adjusted to approximately10^6 spores mL^{-1}. The potato dextrose liquid medium was precisely regulated to pH 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 with adding hydrochloric acid for lower pH and sodium hydroxide for higher pH adjustment. Then sterilized at 121 °C for 20 min. The 1 ml spore suspension of each fungal strains were inoculated in Erlenmeyer flask containing 50 ml Potato Dextrose broth medium with various acidity (Fig. VI.1.). These flasks were incubated at 28 ± 2 °C for seven days under shaking speed 150 rpm. Then the cultures were filtered through Whatman No.2A filter paper and the mycelium was weighed after drying 24 h at 65 °C.

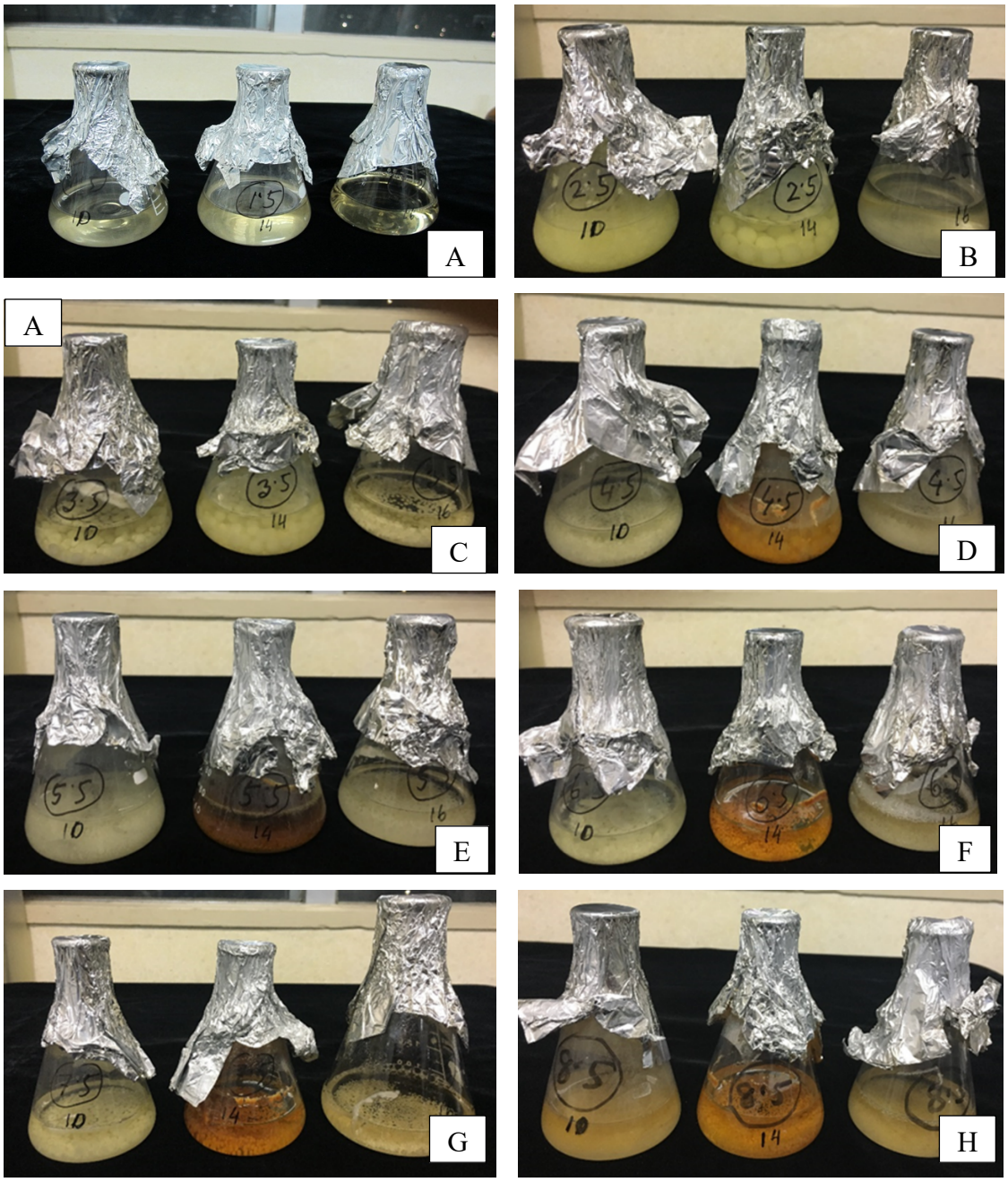

Figure VI.1: Effect of pH on the growth of phosphate solubilizing fungi isolated from subtropical soils in Okinawa, Japan (A: pH 1.5; B: pH 2.5; C: pH 3.5; D: pH 4.5; E: pH 5.5; F: pH 6.5; G: pH 7.5; H: pH 8.5).

pH values measurements

The pH values of fermented broth culture were measured after filtered through a 0.45 mm filter unit by pH meter (Horiba, Japan) furnished with glass electrode

Statistical analysis

All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program (version 2016). The mean values were compared by Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

Results

Influence of initial pH on the acidification ability of fungi

After seven days of incubation, the pH value of the all culture filtrates were significantly decreased compared to original (8.5, 7.5, 6.5, 5.5, 4.5, 3.5 and 2.5). Results also showed that SI-10URAgr dropped the maximum pH when initial pH was 8.5, 7.5, 6.5, 5.5and 4.5 followed by SI-6URAgr (Table VI.1). The final pH value was 1.70, 1.84, 1.86, 1.78 and 1.90 respectively. In the initial pH 7.7-5.5, all fungal strains have steady state condition in pH dropping (Fig.VI.2). Considering all the tested fungal strains, SI-10URAgr has the highest acid producing ability compare to other strain based on pH reduction.

Table VI.1. Variation of acidity of phosphate solubilizing fungal strains in the culture medium isolated from soils in subtropical Okinawa, Japan.

Fungal strains	Original pH of the medium							
	8.5	**7.5**	**6.5**	**5.5**	**4.5**	**3.5**	**2.5**	**1.5**
	Final pH (After 7 days incubation)							
SI-10URAgr	1.70c	1.84c	1.86c	1.78c	1.90c	1.86a	1.96a	1.02b
SI-14URAgr	6.21a	5.05a	5.01a	4.94a	2.71a	1.56b	1.37c	1.06a
SI-16URAgr	2.38b	2.36b	2.33b	2.40b	2.12b	1.91a	1.87b	1.08a

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

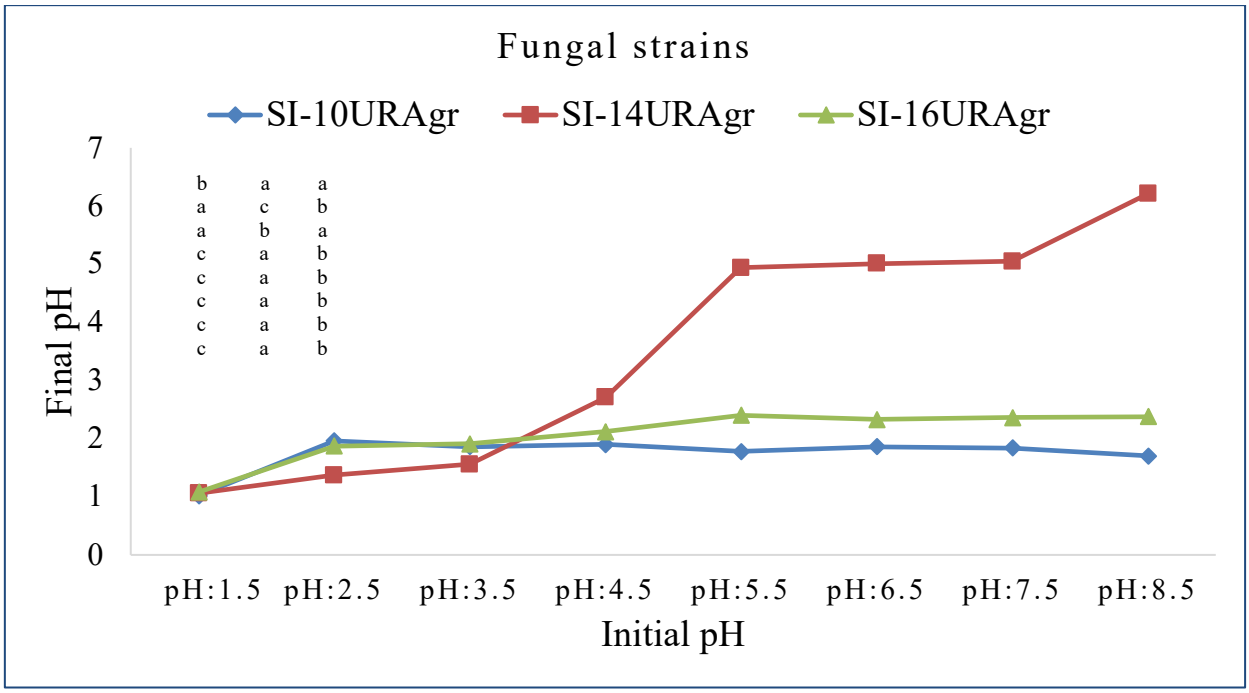

Fig. VI.2. Effect of pH on acidification ability of phosphate solubilizing fungi isolated from soils in subtropical Okinawa, Japan.

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

Influence of pH on the growth of fungi in relation to their mycelial dry weight

All the tested fungal strains were cultured in the medium with initial pH value 8.5, 7.5, 6.5, 5.5, 4.5, 3.5, 2.5 and 1.5. Mycelial biomass is a significant parameter to directly evaluate the growth of fungi. The tested fungal strains showed distinct biomass change under various acidic environment (Table VI.2.). SI-10URAgr produced significantly the highest amount of biomass in extreme acidic environment (pH 3.5, 2.5 and 1.5) followed by SI-16URAgr and SI-14URAgr (Fig.VI.3). SI-14URAgr produced the significantly highest amount of biomass in pH 8.5, 7.5, 6.5, 5.5 and 4.5. This strain has better adaptability in both alkaline and acidic environment. Results also demonstrated that few dispersive mycelia can be identified at pH 1.5. The biomass of SI-10URAgr (0.283g), SI-14URAgr (0.433g) and SI-16URAgr (0.206g) arrived peak value when initial pH 3.5, 5.5 and 7.5 respectively (Fig.V1.3).

Table VI.2. Influence of pH on the growth of representative phosphate solubilizing fungal strains isolated from subtropical Okinawa, Japan.

Fungal strains	pH of the medium							
	8.5	**7.5**	**6.5**	**5.5**	**4.5**	**3.5**	**2.5**	**1.5**
	Produced biomass(g/50ml)							
SI-10URAgr	0.16b	0.183bc	0.060c	0.033c	0.203b	0.283a	0.270a	0.150a
SI-14URAgr	0.246a	0.22a	0.230a	0.433a	0.266a	0.200b	0.160b	0.100b
SI-16URAgr	0.126c	0.206b	0.146b	0.186b	0.166b	0.204b	0.123b	0.103b

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

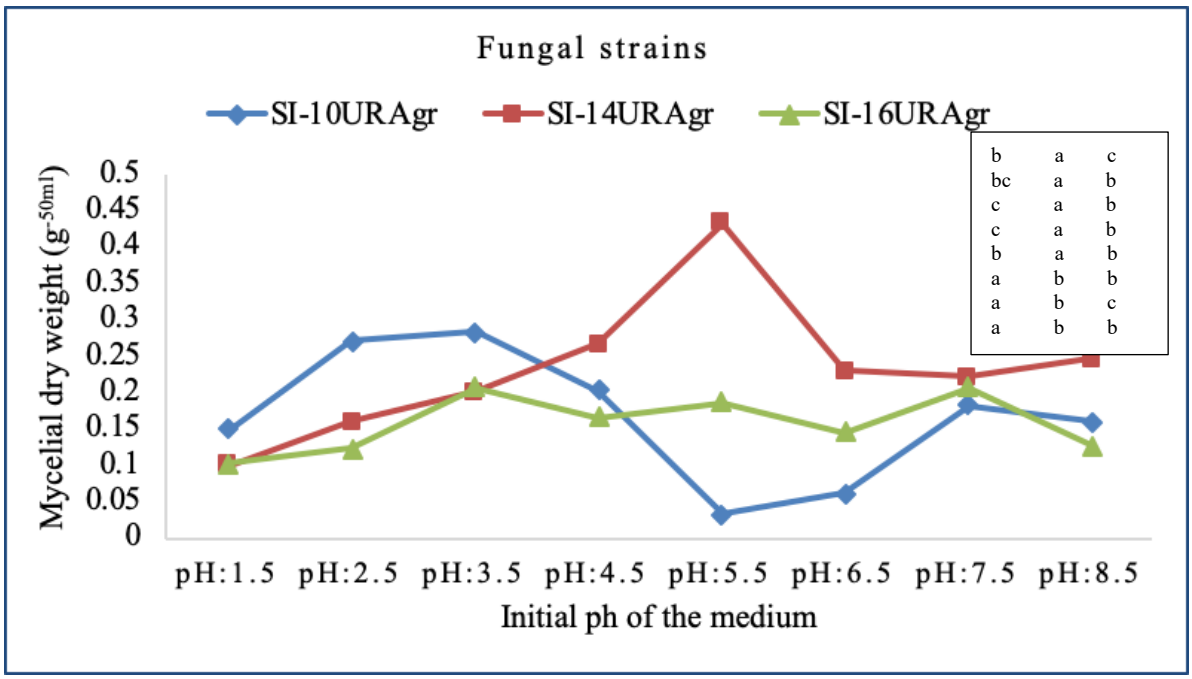

Fig.VI.3. Effect of pH on the growth of phosphate solubilizing fungi isolated from soils in subtropical Okinawa, Japan.

Values given are the mean of three replicates. Means with the same letter are not significantly different according to the Tukey's Honest Significant Difference (HSD) Test at $p<0.05$ level.

Discussion

The sixteen phosphate solubilizing fungi were isolated from different soils in subtropical Okinawa, Japan and identified as *Aspergillus* spp. *Penicillium* spp. and *Talaromyces* spp. (Islam *et al*., 2019). To investigate the mechanism of phosphate solubilization, organic acids were determined by HPLC previously. Considering phosphate solubilization in solid and broth medium amended with different insoluble phosphate compounds as well as their organic acid production potential, *Aspergillus niger* and *Penicillium oxalicum* were to be selected as prominent isolates followed by *Penicillium* sp. and *Aspergillus flucosus*. Three representative isolates were used in this study to evaluate their acid production potential and growth in different pH environment.

Seven days culture in this study showed that both SI-10URAgr and SI-16URAgr have impressive potentials in changing pH within such a short time compare with SI-14URAgr. The organic acids secreted by fungi contribute to pH dropped. It indicated the higher phosphate solubilizing ability of fungi. In the present study, *A. niger* and *P. oxalicum* were isolated from the soils in Okinawa, Japan and were identified as the fungi with prominent phosphorous solubilizing ability in the pools of *Penicillium* and *Aspergillus* (in our lab) respectively.

The *A. niger* shows higher solubilizing ability and organic acids production potentials compared to *P. oxalicum* and *A. flucosus* (Islam *et al*., 2019). Considering the biomass production, *A. niger* and *Penicillium oxalicum* produced lower amount of biomass compared to that of *Aspergillus flucosus* (Table 2; Fig.VI.3.). *A. niger* and *P. oxalicum* shows high efficiency in acid secretion per unit of biomass (Zhen *et al*., 2016). The pH reduction can be attributed to diffusion of various organic acids secreted by the PSF (Singh *et al*., 2011). In soils, the organic acids are more adventitious compared to inorganic acids because they have a low acidity constant (Zhen *et al*., 2016).

A serial of organic acids has various acidity constants, which determine their ability in changing acidity of the environments. The acidity has great significance on activities of microorganisms. The fungal species used in this study have eosinophilic

characteristics, and their growth was relatively good in acidic environment (pH 3.5-5.5) but they also could grow in higher pH (8.5). However, under the extreme acidic condition (pH < 2.5), only the biomass of *A. niger* (SI-10URAgr) (Fig.VI.3.) was visualized. *Aspergillus niger* (SI-10URAgr) has more acid production potential and higher adaptability to acidic environments, compared to *Penicillium oxalicum* (SI-16URAgr) and *Aspergillus floccosus* (SI-14URAgr).

Conclusion

All the tested fungi could grow a wide range of pH (1.5-8.5) but pH vale <2.5, their growth seriously hampered. Among the tested fungal strains, *Aspergillus niger* (SI-10URAgr) has more acid production potential and higher adaptability to acidic environment compared to *Penicillium oxalicum* (SI-16URAgr) and *Aspergillus floccosus* (SI-14URAgr). Besides this, the fungal strains also could grow well in higher pH level. Finally, we concluded that *Aspergillus niger* is a better candidate followed by *Penicillium oxalicum* as bio inoculant for phosphate solubilization in phosphate deficient problem soils.

Chapter VII

General Discussion

Phosphorus (P) is an essential nutrient element responsible for important functioning of plant growth, development and yield. Its availability in soils mostly depend on the soil properties. In higher pH condition, P becomes unavailable due to the formation of complex with calcium and in lower pH condition, it becomes unavailable due to the formation of complexed with iron and aluminium. As a result of these phenomenon and shortage of available phosphorous, plants growth seriously hampered and finally we failed to achieve yield goal. Farmers or crop growers addressed this problem using chemical fertilizer. The near about 70% of applied fertilizer immediately converted to unavailable form. It is not only financial loss but also destroy our environment as soil pollutants.

A group soil microorganism such as bacteria and fungi can solubilize phosphate from soil pools and ensure phosphorus supply for plants. These organisms are known as phosphate solubilizing microbes. Among these, some filamentous fungi are important component at phosphate solubilization. They are capable to release phosphorus from soil complex to soil solution perfectly than bacteria. In our agriculture, these beneficial microbes may use as alternative of chemical fertilizer for upgrade the available phosphorous status in soil. This strategy is environment friendly and cost effective. For these purposes, we isolate and identified 16 potential filamentous from different soils in subtropical Okinawa, Japan. They could solubilize three kinds of insoluble phosphate minerals such as TCP, Al-P and Fe-P. The fungi were identified as *Aspergillus* spp., *Penicillium* spp. and *Talaromyces* spp. based on both mycological and molecular biological studies. Previously Zang *et al*., (2018), Mendes *et al*., (2014) and Ruangsanka (2014), found that the most dominant genera of P solubilizing filamentous fungi are *Aspergillus, Penicilium* and *Talaromyces*. We demonstrated that there were large variations in phosphate solubilizing abilities among fungal species. The Aspergillus spp. more effective to phosphate solubilization in broth culture but in solid medium Penicillium spp. achieves the highest result. Considering the phosphate solubilizing performance in both solid and broth medium, *A. niger* showed excellent P

solubilization potential regardless of the substrate followed by *P. oxalicum* and *T. pinophillus*. *Aspergillus* spp. have long history as beneficial fungi that widely used in industries for making fermented foods, production of organic acids and some enzymes like, pectinase, glucose oxidase, catalases etc (Wongwicharn *et al*., 1999). On the other hand, *Penicillium* spp. have a potential role to phosphate solubilization. This fungus are widely used as lifesaving drug like antibiotic in pharmaceutical industries (https://en.wikipedia.org/wiki/*Penicillium*). *Talaromyces* spp. are widely used in dying industries as pigment producing agents.

To investigate the mechanism of phosphate solubilization, we demonstrated that isolated fungi produce different types of organic acids in the medium. Acid production depends on the fungal species and the composition of insoluble phosphate compound used in this study. These acids are the main contributor of phosphate solubilization. We observed that *A. niger* have strongest acids production ability compare to *P. oxalicum* and *T. pinophillus*. 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. Based on the quantity of organic acids produced, TCP medium produces the highest followed by followed by Fe-P and Al-P. This variation occurred due to the result of interaction between fungal strains and the phosphate substrates (Zang *et al*., 2018; Scervino *et al*., 2013) The genetic variation of fungal strains also influenced the organic acid production potential of fungi (Protiva *et al*., 2009).

We evaluate the abiotic stress tolerant properties of isolated fungi. Results revealed that most of the isolates have stress tolerant abilities, specially *Penicillium* sp. and *A. flucosus* showed the highest salt tolerant ability. Besides this, *A. niger* have excellent drought tolerant ability. A wide range of fungi contributes plant growth and development at stressed environment (Larran *et al*., 2007, Vacheron *et al*., 2013). By inspiring with the previous research, current investigation could play a vital role to use microbial inoculum in stress pound areas. Okinawa is island surrounded by the Pacific Ocean and East-China Sea. Its climate is subtropical. In this area salinity induced by evaporation of sea water and accumulated in soil. For this reason, most of the isolates have salt tolerant ability (8% NaCl). Our findings in agreed with Nayak *et al*., (2012).

They reported that halophilic *Aspergillus* spp. and *Penicilium* spp isolated from mangrove area of Goa, India could tolerate higher concentration of raw salt. Crop plant-associated microbes having good drought tolerance property are recently getting increased attention. By influencing plant morphology, development, and physiological and biochemical responses to stress, fungi can provoke mechanisms of drought escaping, drought lenience, and drought recovery in their hosts (Vurukonda *et al*., 2016, Bashan and Holguin, 1998). In our investigation, we have observed that all the selected fungal strains are able to resist drought in variable range. Among them *A. niger* and *A. flucosus* and *P. oxalicum* have ability to survive in 50% sucrose and 35% glycerin amended medium. These strains are osmophilic in nature. Abdel-Hafez et al., reported that *A. niger* have the highest osmophilic properties among the fungi. Other researchers reported that (Moustafa and Al-Musallam) *Penicillium* occupied the second position among the osmophilic fungi.

When the isolates exposed to different concentration of NaCl, phosphate solubilization did not affect greatly. It indicated that the fungal strains may give benefit in maintaining available phosphorous level in salt affected soils, specially Penicillium spp. These strains could tolerate higher concentration of salt and solubilized P from salt contaminated environment (4% NaCl). Yadav *et al*., (2011), was isolated a *Penicillium sp.* from the rhizosphere of sugarcane that could solubilize phosphorous under saline environment. The *Eupenicillium parvam* and some *Aspergillus* spp. (Narsian and Palet 2000; Rinu and Pandey 2010; Srinivasan *et al*., 2012) are able to solubilize phosphate at various level of salinity. Khan *et al*., (2011), reported that growth promotion of soybean by *Talaromyces* spp. strain LHLO6 was not affected by salinity stress. The mechanism of salt tolerant by fungi is still now unknown. It is assuming that fungi could survive in salty environment using different mechanism. They synthesized compatible solutes or acumulated of potassium against NaCl to overcome the Na^+ hazards. Another study showed that adaptation of fungi to salinity stressed environment was related to up-regulation of fungal genes encoding chaperones or aqua porins (Estada *et al*., 2013). The possibility that chaperones and aquaporins may have a key role in the salinity stress tolerance of phosphate solubilizing fungi is a research area that needs more investigation.

To investigate the effect of pH on the growth of fungi, we observed that tested strains have impressive potentials in changing pH within such a short time. In this study, *A. niger* dropped the highest pH followed by *P. oxalicum* and *A. fluccosus*. Considering the acid production potential. *A. niger* and *P. oxalicum* shows high efficiency in acid secretion per unit of biomass (Zhen *et al*., 2016). The pH reduction can be attributed to diffusion of various organic acids secreted by the PSF (Singh *et al*., 2011). In soils, the organic acids are adventitious compared to inorganic acids because they have a low acidity constant (Zhen *et al*., 2016). The fungal species used in this study have eosinophilic characteristics, and their growth was relatively good in acidic environment (pH 3.5-5.5) but they also could grow in higher pH (8.5). However, under the extreme acidic condition (pH < 2.5), only *A. niger* is a better candidate compared to *P. oxalicum* and *A. floccosus*.

These strains would be potential bioresource in subtropical areas for successful crop production in P deficient agricultural soils and also industrial utilization.

Chapter VIII

Summary and Conclusion

Phosphorus is the second most important nutrient element required for plant growth and development. Phosphorus forms insoluble complexes with aluminum and iron in acidic soil and with calcium in alkaline soil. For sustainable crop production, farmers are widely using a huge amount of phosphate fertilizers to the soil, but a small fraction of the applied phosphate is available to plants and rest of the portion becomes immobilized within a very short time. Excessive use of fertilizer not only increase the production costs but also leads to environmental pollution. The microbes have ability to dissolve insoluble phosphorus compounds from soil native phosphorous pols and also increase phosphorous use efficiency. Such activity of microbes basically depends on their genetic variation and environment. Therefore, studies were aimed to identify efficient phosphate solubilizing fungi that could be utilized as potential bio resource for resolving the phosphorous deficiency by ecofriendly way in subtropical soil.

Sixteen fungal strains were identified from the grey soil, dark-red soil and red soil having pH value 7.9, 6.7 and 6.1 respectively. These strains could solubilize insoluble $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$. Based on morphological and the sequences of β-tubulin and/or Calmodulin studies, the isolates were identified as *Aspergillus* spp., *Penicillium* spp. and *Talaromyces* spp. The *Aspergillus* isolates (strain SI-10URAgr>SI-12URAgr>SI-11URAgr>SI-14URAgr) showed higher phosphate solubilization ability followed by *Panicillium* isolates (SI-16URAgr>SI-6URAg>SI-1URAgr) and *Talaromyces* isolates (SI-15URAgr>SI-4URAgr) regardless of phosphate substrates.

The mechanism of phosphorus solubilization by the fungi mostly occurred by the synthesis of organic acids (mainly oxalic, tartaric, malic, citric, acetic, tartaric and formic acids). These acids production depend on both composition of phosphate minerals 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 acid production potentiality, the sequence of the fungal strains was SI-12URAgr>SI-6URAgr>SI-13URAgr>SI-16URAgr>SI-1URAgr>SI-11URAgr>SI-10URAgr>SI 14URAgr>SI-7URAgr>SI-5URAgr>SI-2URAgr>SI-3URAgr>SI-9URAgr>SI-

15URAgr>SI-4URAgr>SI8URAgr.

Abiotic stress tolerant properties of fungal strains were examined for their growth and survival under stressed environment. All fungal strains except SI-7URAgr, SI-8URAgr and SI-9URAgr were survive with 0-8% of salt (NaCl) in the medium. Among the tested fungi, SI-14URAgr, SI-6URAgr and SI-1URAgr showed the highest salt tolerant (12% NaCl) ability followed by SI-10URAgr, SI-11URAgr, SI-12URAgr, SI-15URAgr and SI-16URAgr (10% NaCl). The strain SI-6URAgr has highest phosphorous solubilizing ability under different levels of NaCl concentration (1-4%) in the medium followed by SI-1URAgr, SI-11URAgr, SI-10URAgr, SI-15URAgr and SI-16URAgr.

All the fungal strains survived with 35% sucrose amended medium. The strains SI-6URAgr, SI-10URAgr, SI-11URAgr, SI-12URAgr and SI-14URAgr were the most drought (50% sucrose) resistant followed by SI-1URAgr, SI-4URAgr and SI-16URAgr (45% sucrose).

From the results of the influence of pH on the growth and survival of tested fungi, *Aspergillus niger* strains have better adaptability in acidic environment compared to *Penicillium oxalicum* and *Aspergillus floccosus*. They produced maximum biomass at pH 3.5, 5.5 and 7.5, respectively.

In this study, 16 fungal strains have been identified from the soils in subtropical Okinawa as potential phosphate solubilizing fungi. Based on the phosphorous solubilization potential and total organic acid production abilities, *A. niger* (SI-12URAgr) was considered as the best strain followed *P. oxalicum* (SI-6URAgr, SI-16URAgr) regard less of phosphate substrates $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$. Considering salt tolerance and phosphorous solubilizing abilities under saline condition *Panicillium oxalicum* (SI-6URAgr) was considered the best strain. These fungal strains were considered as potential for phosphorus solubilization in different types of soils which could contribute significantly in agriculture and environment. These strains also have industrial importance.

Chapter IX
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