RESEARCH ARTICLE

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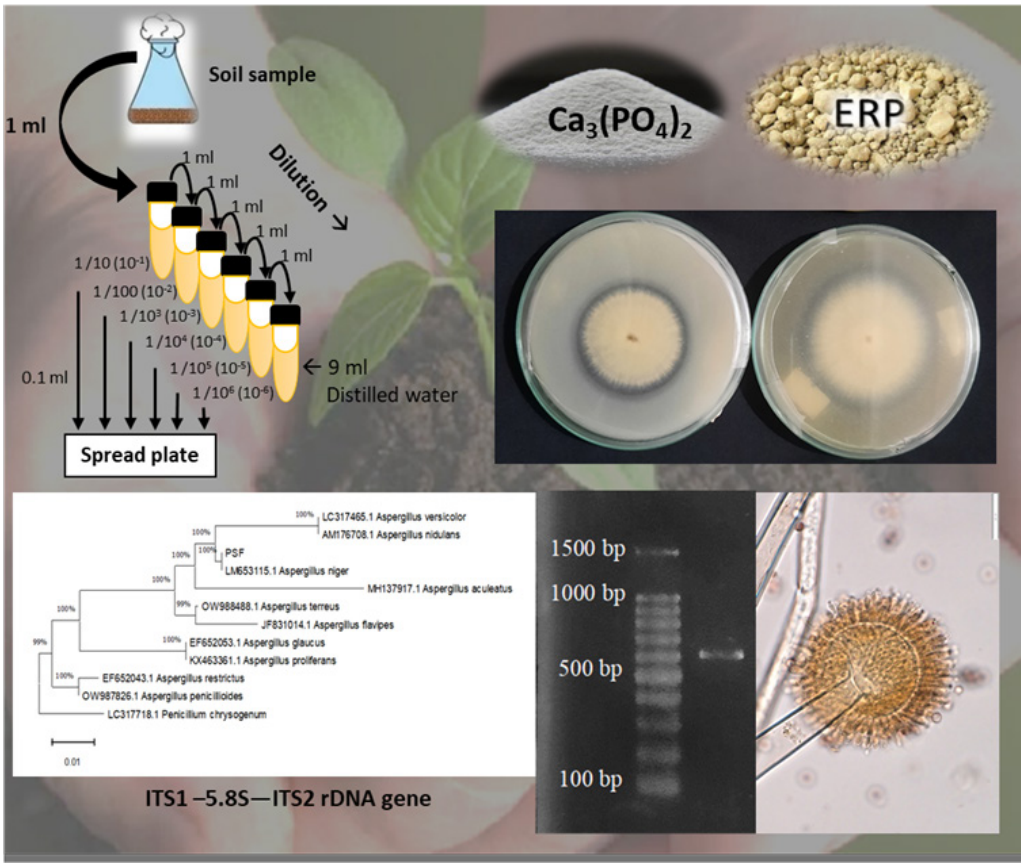
L. R. L. S. Kumari, A. Senaratne and W. R. P. Wijesinghe *



Highlights

- Phosphate solubilizing fungi were isolated from soil samples.
- Solubilization Index on Pikovskaya's (PVK) Agar with Eppawala Rock Phosphate (ERP) was determined.
- Phosphate solubilizing *Aspergillus* sp. was identified and characterized.

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Isolation of a potential rock phosphate solubilizing *Aspergillus* sp. towards development of biofertilizer

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Abstract: Phosphorus (P) is one of the key bio elements that limits agricultural production. Although Sri Lanka is endowed with rock phosphate deposits, practical means to utilize this resource are limited. Phosphate-solubilizing fungi (PSF) play an important role in enhancing the bioavailability of soil phosphorus to plants. In this study, fungi from eight soil samples in Kaikawala, Sri Lanka, were isolated using spread plate method and analyzed for its phosphate solubility capacity. One fungal isolate with significant halo zones on Pikovskaya's agar (PVK) plates, containing 0.5% tricalcium phosphate, was identified. After purification, the isolate was transferred to three media setups: PVK with 0.5% apatite (ERP) as the phosphate source, PVK with 0.5% tricalcium phosphate as a positive control, and PVK without P source as a negative control and analyzed for the solubilization index (SI). DNA sequences of the internal transcribed spacers with 5.8S region of ribosomal DNA (ITS1-5.8S-ITS2) was analyzed with universal primer pair ITS4-F/ITS5-R to determine the identity of the species. Fungal isolate PSF01 showed a phosphate solubilizing activity on both PVK media, ERP (1.07 ± 0.03 cm SI) and tricalcium phosphate (1.07 ± 0.01 cm), with similar effects ($p > 0.05$). However, the SI in the negative control was 1.00 ± 0.00 cm without halo zone ($p < 0.05$). The fungal strain is fast growing with initially white but quickly changing to black colonies after producing conidial spores on potato dextrose agar with distinctive conidial heads and pale-yellow lower surface characterized as *Aspergillus*. Sanger DNA sequencing identified the fungus as *Aspergillus niger* (99%) and a phylogenetic tree was constructed using MEGA11 software using reference data obtained from NCBI GenBank data to identify the isolated PSF01. Consequently, our preliminary studies demonstrate the importance of examining more soil samples to identify PSF for sustainable agricultural applications in the future.

***Keywords*:** *Aspergillus niger*; biofertilizer; phosphorous solubilization

INTRODUCTION

Phosphorous (P) is the most important key element next to nitrogen, which involves in all major metabolic processes in plants; photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, and respiration (Khan et al., 2010). P is a major growth-limiting nutrient. Approximately 95-99% of P is present in an insoluble form complexed with cations like iron, aluminum, and calcium that cannot be utilized by the plants (Son et al., 2006). Substantial deposits of cheaper and low grade Rock Phosphate (RP) are locally available in many countries of the world (Van straaten, 2002). Sri Lanka consists of two significant phosphate deposits in Eppawala and Ridigama (Dushyantha et al., 2017). Conventionally, RP is chemically processed by reacting with sulfuric or phosphoric acid although which involved with high cost and environmental pollution (Van Straaten, 2002).

The natural phosphorus cycle depends heavily on soil microbes, and recently, microbe-based methods have been suggested to increase the agronomic value of RP (Rodríguez et al., 2007). Consequently, recovering depleted soil with the application of P-solubilizing microorganisms is a far more affordable and practical solution. This could be an alternative strategy for lowering the environmental pollution caused by the conventional chemical processing and hence lowering the greater cost of producing phosphate fertilizer in industry.

Rhizobium, *Bacillus*, and *Pseudomonas* are three significant genera of P-solubilizing bacteria found in the rhizosphere (Chen et al., 2006; Hu et al., 2006). The two most common P-solubilizing filamentous fungi in the rhizosphere are *Penicillium* and *Aspergillus* spp (Kumar, 2007; Vassilev & Gamalero, 2006). Filamentous fungi play a crucial role in the solubilization of RP. They are frequently employed as organic acid manufacturers. The present study was focused on the isolation and molecular identification of RP solubilizing *Aspergillus niger* isolated from Kaikawela area in Matale, Sri Lanka.

MATERIALS AND METHODS

Collection and isolation of fungi from soil using soil dilutions

Eight soil samples were collected from the Kaikawela area, Matale District, Central Province, Sri Lanka. Kaikawela area represents the highest number of pegmatites and hydrothermal deposits located in the Matale district in Sri Lanka. Soil samples were taken and stored in plastic bottles and carefully transferred to the laboratory of Department of Botany, University of Peradeniya, Sri Lanka, and stored at 4 °C for further analysis. Ten grams of each soil sample was dissolved into 90 mL of sterilized distilled water in

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250 mL sterilized flasks and shaken at 120 rpm for 1 hr on a rotary shaker and allowed to settle for 10 min. Aliquots of 1 mL of the supernatant was transferred from the sample to 9 mL of sterile distilled water, dispensed into test tubes and serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. The soil dilutions were used to isolate fungi from soil samples. From each serially diluted soil suspension, 0.1 mL aliquots were transferred onto Potato Dextrose Agar (PDA) and Pikovskaya's agar (PVK) plates supplemented with 40 µg/mL chloramphenicol and spread using a sterile L-shaped glass spreader. One liter (1 L) of PVK agar contained the following ingredients (g/L): 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H2O$, 0.02 g NaCl, 0.02 g KCl, trace amount of $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$, 5 g of insoluble phosphate, 10 g glucose, 0.5 g yeast extract, 18 g agar, and 1000 mL distilled water (Pikovskaya, 1948). The Petri plates were incubated for 7 days at room temperature (28 °C). Phosphate solubilizing fungi in the soil samples were identified, based on the size of halo zones percent on PVK agar plates containing 0.5% tricalcium phosphate [TCP, $Ca_3(PO_4)_2$] as an insoluble phosphorus source. Fungal colonies were isolated by transferring a single hyphal tip on to PDA. The pure strains were maintained on PDA at 4 °C for further screening.

Determination of Solubilization Index on PVK Agar with Apatite

After incubation, fungal colonies showing clear zones around the colonies were further purified by transferring into (1) Pikovskaya's agar medium with apatite as the phosphate source, (2) PVK agar with $Ca_3(PO_4)_2$ as the phosphate source of the positive control, and (3) PVK agar without any P source as the negative control. The medium was autoclaved at 121 °C for 15 min and at a lower temperature it was suspended with 40 µg/mL chloramphenicol to suppress bacterial growth. Sterilized PVK agar was poured into sterilized petri plates. Fungal mycelium of each isolate grown on PDA at 28 ºC for 7 days were taken from the edges of each actively growing colony using a sterile toothpick (2 mm^3). Plugs of sterile PDA were used as controls. Three replicates were tested for each fungal isolate. The diameter of clear zones around the colony of each isolate was measured after the 7th day of incubation. The phosphate solubilization index (SI) was calculated according to the formula below (Premono et al., 1996).

$$\text{Solubilization Index (SI)} = \frac{\text{colony diameter} + \text{clear zone diameter}}{\text{Colony diameter}}$$

Statistical Analysis

Data from different treatments were calculated and statistically analyzed with a one-way analysis of variance (ANOVA) Tukey's test using R version 4.0.3 and significant differences were detected at $p < 0.05$ between the mean values of SI.

Morphological Studies

Fungal strains grown on PDA were identified using macroscopic colony characteristics and mounting in glycerol for microscopic examination. Morphological observations and photomicrographs were made with an Olympus' BX53 Light Microscope (Olympus Tokyo, Japan).

DNA Extraction, PCR Amplification, and Sequencing

The DNA was extracted from the mycelium grown on PDA at 28 °C for 7 days using a modified microwave based DNA extraction method (Dörnte & Kües, 2013). Fungal mycelia were directly used for DNA extraction in sterile distilled water. A pinhead sample of mycelium from fungal cultures (about 2 mg) was scraped from the surface of plates thereby taking care of agar contamination. Individual samples were transferred into 1.5 mL sterile micro-centrifuge tubes (Biologix, USA) and 500 µl sterile distilled water were added. Opened tubes were microwaved for 10 sec. at 600 W in a household microwave oven (2450MHz 850W, LG Electronics, Germany), shortly vortexed, stored for 30 sec at room temperature (RT) and microwaved again for 10 sec, 2 sec. and 10 sec. short intervals at 600 W. Afterwards, tubes were centrifuged at 8000 rpm for 5 min at RT. One µL of resulting supernatant was directly used as the template for the PCR. The ITS1 –5.8S—ITS2 rDNA gene with the two flanking internal transcribed spacers (ITS) regions were amplified and sequenced using primers ITS4/ITS5 (White et al., 1990).

The PCR amplifications were conducted in 10 µL final volumes which consisted of 1 unit Taq DNA Polymerase (Promega. USA), 0.1 mM dNTP Mixture each (dATP, dCTP, dGTP, dTTP) (Promega. USA), 1.5 mM $MgCl_2$ (Promega. USA), 0.5 µL of each primer (0.5 µM), 1 µL genomic DNA extract and 4.8 µL nuclease free water. The PCR reaction was carried out using a Thermal Cycler (Takara Bio Inc, Otsu, Shiga, Japan) under the following conditions: initial denaturation of 10 min at 94 °C, followed by 35 cycles of 1 minute at 94 °C, 30 s at 55 °C, 1 min at 72 °C and final extension period of 10 min at 72 °C. The PCR products were analyzed by electrophoresis on 1.5% agarose (Figure 2). Amplified PCR products were purified and sequenced by GENETECH, Sri Lanka. The DNA sequences of isolated strains were searched using basic local alignment search tool (BLAST) for species identification.

Phylogenetic Analyses

Consensus sequences were BLAST searched in the nucleotide database of GenBank to examine their closely related taxa. Twelve reference sequences and outgroups were selected from recent relevant literature and GenBank. The ITS1–5.8S—ITS2 rDNA regions were aligned and phylogenetic trees were inferred using the Maximum Likelihood method and the Jukes-Cantor model (Huber et al., 2004; Jukes & Cantor, 1969) using MEGA11 (Tamura et al., 2021).

RESULTS

One phosphate solubilizing fungus, PSF01 was isolated from soil samples collected from the Kaikawela area, situated in Matale, Sri Lanka. The PSF01 strain grown on PDA medium showed an initial growth of white color, which with time acquired a black coloration due to the production of spores (Figure 3). Underside of the colony

was light yellow as in the Figure 1. The conidial heads were dark brown to black and the conidia were globose. The conidial heads were formed by uniseriate phialides (Cruz, 2014). The hyphae are well-developed, profusely branched, septate, and hyaline near the apex (Pera & Callieri, 1997).

The solubilization index (SI) of the isolated phosphate solubilizing fungus was 1.07 after seven days of incubation at 28 ºC (Table 1). Results on Table 1 revealed that, the screened PSF isolate (*Aspergillus niger*), showed efficient phosphate solubilizing capability on PVK plates with $Ca_3(PO_4)_2$ and apatite significantly ($p < 0.05$) when compared to negative control without P-source (Figure 1).

The PCR amplification of the ITS region of the isolate PSF01 yielded PCR products of 600 bp (Figure 2). The BLAST search showed that the sequence data of the isolated strain PSF01 shared 99% similarity with *Aspergillus niger* (MT123512.1). The phylogenetic tree showed phylogenic relationship of the isolated strain with the reference strains (Figure 4). Twelve reference sequences were included in the analysis, which consisted of 206 characters including alignment gaps. The tree is rooted to *Penicillium chrysogenum* (LC317718.1) as the out group. The maximum parsimony analysis for the sequences had 17 parsimony informative, 179 constant, 27 variable characters. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test with 1000 replicates are shown next to the branches (Felsentein, 1985). The phylogeny analysis of isolated PSF01 strain showed 100% similarities with *Aspergillus niger* (LM653115.1).

Table 1: Solubilization index (SI) of each treatment. The lowercase superscript indicates the significant difference between the treatments ($p < 0.05$).

Treatment	Average SI value
PVK media with ERP	1.07 ± 0.03 [a]
PVK media with $Ca_3(PO_4)_2$	1.07 ± 0.01 [a]
PVK media without P	1.00 ± 0.00 [b]

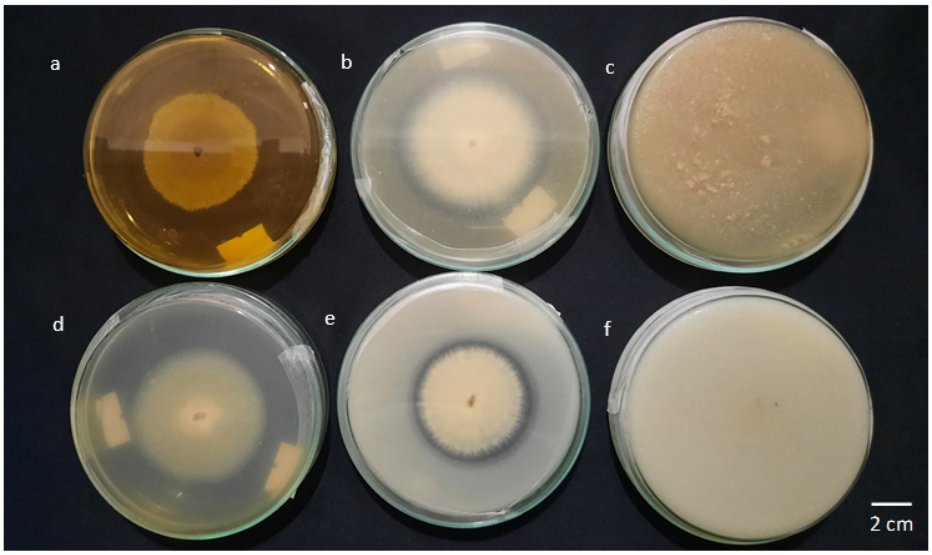

Figure 1: Seven-day old *Aspergillus niger* PSF01 colony on PDA (a), PVK media with apatite as the insoluble P source (b), PVK media with apatite without the fungal inoculum (c), PVK media without any P source (d), PVK media with $Ca_3(PO_4)_2$ as the insoluble P source (e) and PVK media with $Ca_3(PO_4)_2$ without the fungal inoculum(f), grown at 28 °C, respectively.

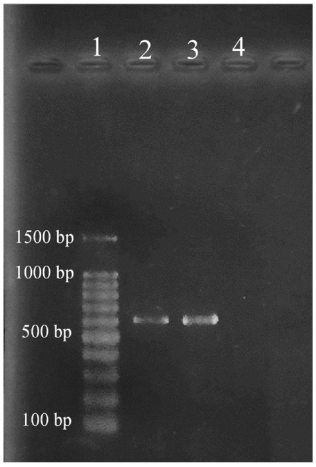

Figure 2: Agarose gel electrophoresis (1.5 % agarose) of the PCR amplified products using ITS4 (F)/ITS5 (R) PCR primer pair. Lane1:100 bp DNA size markers, Lane 2: PSF01 isolate replicate 1, Lane 3: PSF01 replicate 2, Lane 4: Non-template control.

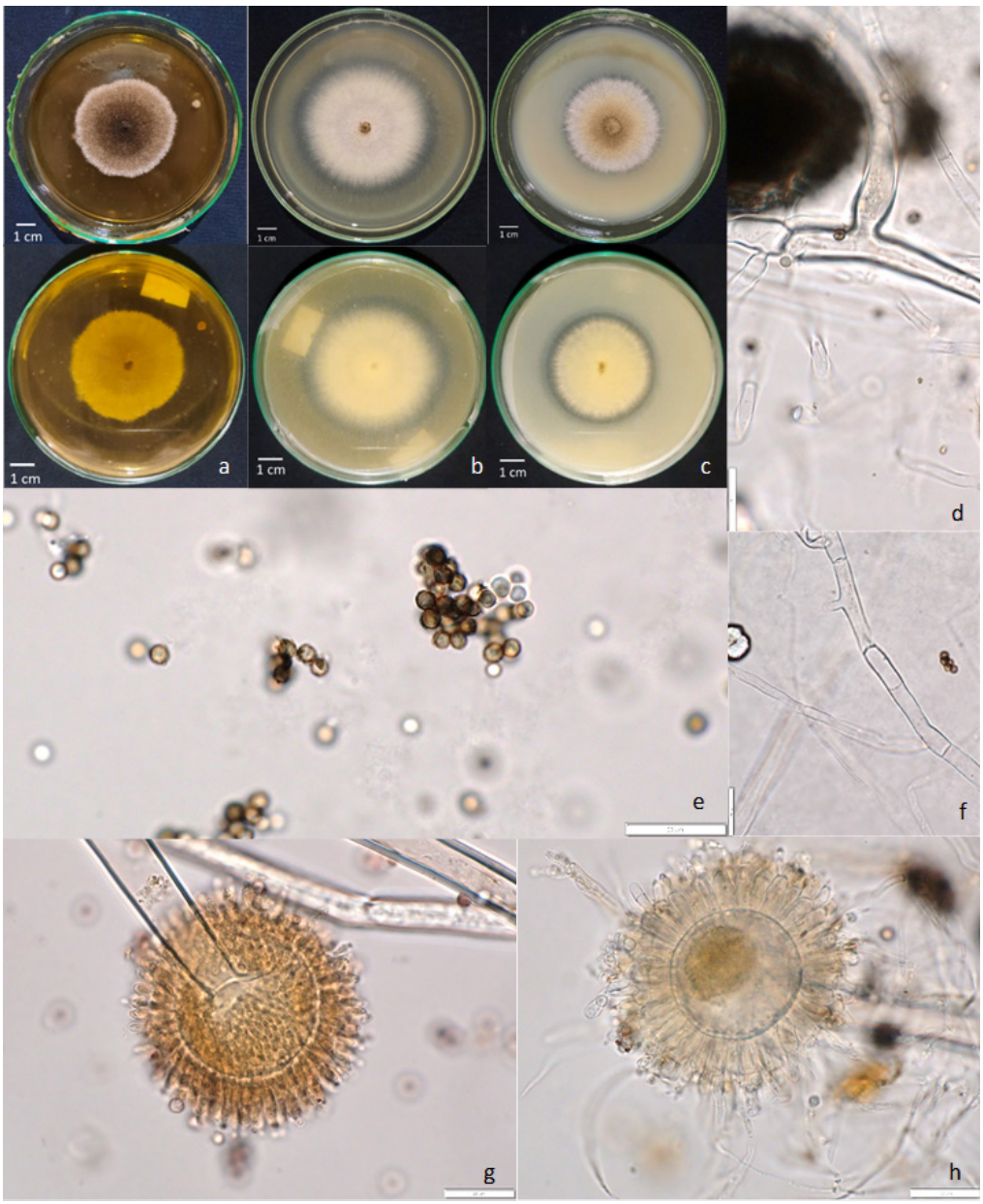

Figure 3: Morphology of *Aspergillus niger* PSF01. Colonies on PDA (a), PVK media with apatite as the insoluble P source (b), and PVK media with $Ca_3(PO_4)_2$ as the insoluble P source (c), at 28 °C, respectively. Conidiophore attachment (d), Conidia (e), Hyphae (f) and Close-up of conidiophores, vesicle, phialides, and conidia (g - h) (d - h: exhibiting colors of polarized light under OLYMPUS BX53 Light Microscope); Scale bars: (a – c) = 1 cm and (d - h) = 20 µm

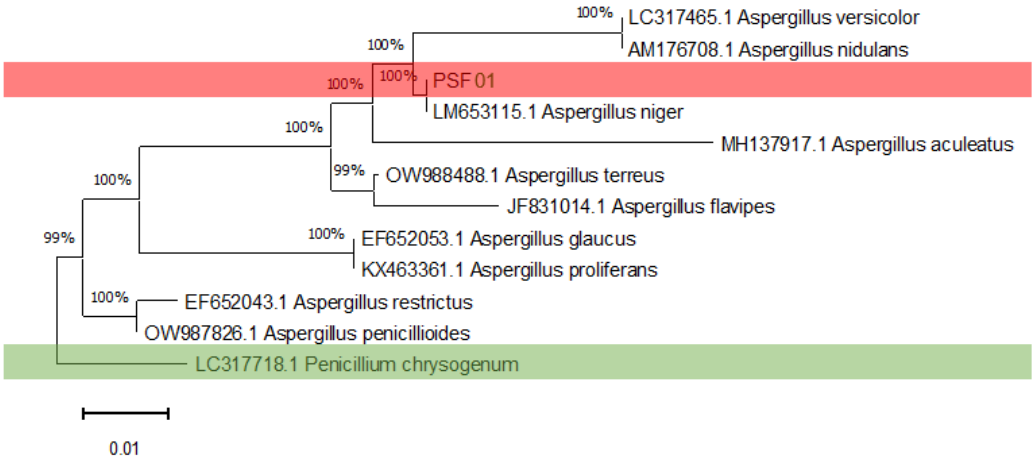

Figure 4: Phylogram generated from maximum likelihood analysis based on ITS sequence data. Out group is highlighted in green and the new isolate is in red.

DISCUSSION

Phosphate solubilization by soil-derived *Aspergillus* spp., *Penicillium* spp., and their teleomorphs *Talaromyces* spp. had been previously demonstrated (Mendes et al., 2014). Numerous authors have observed that *Aspergillus* and *Penicillium* have the ability to promote plant development in pot and field trials in addition to phosphate solubilization under laboratory conditions (Mittal et al., 2008; Whitelaw, 1999). Thus, the PSF may have the capacity to mobilize and increase plant nutrient uptake. Herein, we are showing how well PSF01 can dissolve phosphorus using the calculated SI value in PVK media. The PSF01 showed desired P-solubilizing capability in both tricalcium phosphate and apatite. The SI value of $Ca_3(PO_4)_2$ was similar to that of apatite. The screening of PSF could provide useful insights into plant phosphate solubilizers and may further the development of environmentally-friendly biofertilizers in P limitation areas.

The overall P consumption efficiency is low in agrarian soils because insoluble complexes develop following the application of phosphate fertilizer (Vassilev and Vassileva, 2003). As a result, crop production frequently requires the use of soluble forms of inorganic P, which leaches into the ground water and causes eutrophication of aquatic systems (Torrent, 2002). Research efforts are focused on creating techniques that utilize less expensive, but less bio-available plant nutrient sources, such as RP. The use of phosphate solubilizing microorganisms can enhance the effectiveness of these methods in agriculture. This is due to environmental concerns and recent developments in sustainability efforts (Pradhan & Sukla, 2005). By using the processes of acidification, chelation, exchange reactions, and the creation of organic acids, a range of soil fungi and bacteria can convert insoluble forms of inorganic phosphate into soluble forms. It has been recognized that natural phosphate rocks are an excellent substitute for phosphorus fertilizers. In recent years, there has been a lot of interest in the potential application of RP as fertilizers. Unfortunately, RP is not plant available for soils with a pH higher than 5.5 to 6.0, and even under ideal circumstances, plant yields are inferior even with soluble phosphate (Khasawneh & Doll, 1979). Traditionally, RP is chemically processed into soluble phosphate fertilizer by reacting with sulfuric acid or phosphoric acid. This method contributes to increased fertilizer prices and ecological damages (Reddy et al., 2002; Xiao et al., 2008).

From the present study, we demonstrate the potential of rhizosphere associated *Aspergillus* sp. to solubilize rock phosphate, which has the potential applications as a biofertilizer. However, follow up studies warrant the true applicability of the isolated strain. The advantage of using natural soil isolates over the genetically manipulated or the one which has been isolated from a different environmental set up is the easier adaptation and succession when inoculated into rhizosphere.

CONCLUSION

In this study, the isolated PSF01, was identified as *Aspergillus niger* based on morphological, molecular and phylogenetic characteristics. PSF01 had statistically significant P-solubilizing abilities with tricalcium phosphate, and apatite in PKV medium. Therefore, PSF01 is an ideal candidate to be utilized in filed experiments to test the potential as a biofertilizer.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Chen, Y. P., Rekha, P. D., Arun, A. B., Shen, F. T., Lai, W. A., & Young, C. C. (2006). Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Applied soil ecology*, **34**(1), 33-41. https://doi.org/10.1016/j.apsoil.2005.12.002

Cruz, R. (2014). Guía para el diagnóstico de laboratorio de enfermedad fúngica invasora por hongos filamentosos. *Cátedra de Micología*, 173–179. http://dx. doi. org/10.4067/S0716-10182014000200008.

Dörnte, B., & Kües, U. (2013). Fast microwave-based DNA extraction from vegetative mycelium and fruiting body tissues of Agaricomycetes for PCR amplification. *Current Trends in Biotechnology and Pharmacy,* **7**(4), 825-836.

Dushyantha, N. P., Hemalal, P. V. A., Jayawardena, C. L., Ratnayake, A. S., Premasiri, H. M. R., & Ratnayake, N. P. (2017). Nutrient characteristics of lake sediments around Eppawala phosphate deposit, Sri Lanka. *Journal of the Geological Society of Sri Lanka,* **18**(2), 33-42.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* **39**(4), 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Hu, X., Chen, J., & Guo, J. (2006). Two phosphate- and potassium-solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. *World Journal of Microbiology and Biotechnology,* **22**, 983–990. https://doi.org/10.1007/s11274-006-9144-2

Huber, T., Faulkner, G., & Hugenholtz, P. (2004). Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics*, **20**(14), 2317-2319. https://doi.org/10.1093/bioinformatics/bth226

Jukes, T. H., & Cantor, C. R. (1969). Evolution of protein molecules. In H. N. Munro (Ed.), *Mammalian Protein Metabolism* (pp. 21-132). Academic Press: New York.

Khan, M. S., Zaidi, A., Ahemad, M., Oves, M., & Wani, P. A. (2010). Plant growth promotion by phosphate solubilizing fungi - Current perspective. *Archives of Agronomy and Soil Science,* **56**(1), 73–98. https://doi.org/10.1080/03650340902806469

Khasawneh, F. E., & Doll, E. C. (1979). The use of phosphate rock for direct application to soils. *Advances in Agronomy,* **30**, 159-206. https://doi.org/10.1016/S0065-2113(08)60706-3

Kumar, B. (2007). Phosphate solubilization by *Penicillium spp.* isolated from soil samples of Indian Himalayan region. *World Journal of Microbiology and*

Biotechnology. https://doi.org/10.1007/s11274-007-9444-1

Mendes, G. D. O., Luiz, A., & Freitas, M. De. (2014). Mechanisms of phosphate solubilization by fungal isolates when exposed to different P sources. *Annals of Microbiology,* **64**(1), 239–249. https://doi.org/10.1007/s13213-013-0656-3

Mittal, V., Singh, O., Nayyar, H., Kaur, J., & Tewari, R. (2008). Stimulatory effect of phosphate-solubilizing fungal strains (*Aspergillus awamori* and *Penicillium citrinum*) on the yield of chickpea (*Cicer arietinum* L. cv. GPF2). *Soil Biology and Biochemistry,* **40**, 718–727. https://doi.org/10.1016/j.soilbio.2007.10.008

Pera, L. M., & Callieri, D. A. (1997). Influence of calcium on fungal growth, hyphal morphology and citric acid production in *Aspergillus niger*. *Folia Microbiologica, 42*, 551–556. https://doi.org/10.1007/BF02815463

Pikovskaya, R. I. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya*, ***17***, 362-370.

Pradhan, N., & Sukla, L. B. (2005). Solubilization of inorganic phosphates by fungi isolated from agricultural soil. *World Journal of Microbiology and Biotechnology,* **21**(5), 850–854.

Premono, M. E., Moawad, A. M., & Vleck, P. L. G. (1996). Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science,* **11**, 13-23.

Reddy, M. S., Kumar, S., Babita, K., & Reddy, M. S. (2002). Biosolubilization of poorly soluble rock phosphates by *Aspergillus tubingensis* and *Aspergillus niger*. *Bioresource Technology,* **84**(2), 187-189. https://doi.org/10.1016/S0960-8524(02)00040-8

Rodríguez, V. B., Vassilev, N., Alameda, E., & Fern, M. (2007). Simultaneous phytase production and rock phosphate solubilization by *Aspergillus niger* grown on dry olive wastes. *Industrial Crops and Products*. https://doi.org/10.1016/j.indcrop.2007.04.003

Son, H. J., Park, G. T., Cha, M. S., & Heo, M. S. (2006). Solubilization of insoluble inorganic phosphates by a novel salt- and pH-tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. *Bioresource Technology,* **97**(2), 204–210. https://doi.org/10.1016/j.biortech.2005.02.021

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution,* **38**(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Torrent, J. (2002). Long-term effectiveness of vivianite in reducing iron chlorosis in olive trees. *Month or Date of Publication*. https://doi.org/10.1023/A

Van Straaten, P. (2002). *Rocks for crops: Agrominerals of sub-Saharan Africa*. ICRAF, Nairobi, Kenya, 338 p.

Vassilev, N., & Gamalero, E. (2006). Simultaneous P-solubilizing and biocontrol activity of microorganisms: Potentials and future trends. *Applied Microbiology and Biotechnology*, **71**, 137-144. https://doi.org/10.1007/s00253-006-0380-z

Vassilev, N., & Vassileva, M. (2003). Biotechnological solubilization of rock phosphate on media containing agro-industrial wastes. *Applied Microbiology and Biotechnology,* **61**(5-6), 435–440. https://doi.org/10.1007/s00253-003-1318-3

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols* (pp. 315–322). Academic Press. https://doi.org/10.1016/b978-0-12-372180-8.50042-1

Whitelaw, M. A. (1999). Growth promotion of plants inoculated with phosphate-solubilizing fungi. *Advances in Agronomy,* **69**, 99-151. https://doi.org/10.1016/S0065-2113(08)60948-7

Xiao, C. Q., Chi, R. A., Huang, X. H., Zhang, W. X., Qiu, G. Z., & Wang, D. Z. (2008). Optimization for rock phosphate solubilization by phosphate-solubilizing fungi isolated from phosphate mines. *Ecological Engineering,* **33**(2), 187-193. https://doi.org/10.1016/j.ecoleng.2008.04.001