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Isolation and Characterization of Phosphate Solubilizing Activity of Endophytic Fungi from Zingiberaceous Species

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Abstract: Phosphorus (P), which is one of the less available soil macronutrients, is important for plant growth and metabolism. A promising strategy to increase the P uptake by plants is by introducing Phosphate-Solubilizing Microorganisms (PSM) into the P-deficient regions, to convert P into soluble forms through organic acids secreted by the PSMs. The aim of this study was to characterize the phosphate solubilizing activity of endophytic fungi in wild Zingiberaceous species which is still underexplored followed by the evaluation of their organic acids production. Out of 35 endophytic fungal isolates, 4 Phosphate-Solubilizing Fungi (PSF), namely, *Pestalotiopsis thailandica* He06, *Trichoderma atroviride* El01, *Trichoderma brevicrassum* Am08 and *Trichoderma scalesiae* Al01, displayed the highest solubilization index (SI >1.0) based on plate assay. The highest amount of solubilized P in Pikovskaya broth was detected from the culture filtrate of *P. thailandica* (4.61 mg/L), followed by *T. scalesiae* (1.85 mg/L), *T. brevicrassum* (1.38 mg/L) and *T. atroviride* (1.33 mg/L) based on molybdate blue colorimetric method. The culture filtrate of *P. thailandica* was profiled using Gas-Chromatography-Mass Spectroscopy (GC-MS), which revealed most of the organic acids, such as butyric, glutamic, lauric, margaric, oleic, palmitic, phthalic, propionic and stearic acid at their lowest pH (<4.0) at the end of incubation. Of the acids, phthalic acid (pKa = 2.76) was in the largest amount based on the GC-MS analysis revealing its possible contribution in P solubilization. The study then promoted *P. thailandica* He06 as potential P solubilizing agent for the future *in planta* test to assess its efficacy in the field trial.

Keywords: Endophytic Fungi, Inorganic P, Organic Acid, Phthalic Acid, *Pestalotiopsis Thailandica*

Introduction

Phosphorus (P) and Nitrogen (N) are the two main limiting and biocritical nutrients which greatly affect the horticultural and agricultural crops' productivity worldwide (Xiao *et al.*, 2011). Phosphorus (P) is one of the most indispensable macronutrients next to Nitrogen (N) required for plant growth and development within the optimum concentration (Relwani *et al.*, 2008).

Approximately >95% of soil phosphorus is in insolubly bound to complex cations such as aluminum, calcium and iron which hinder its availability and uptake by plant roots, therefore, it cannot be assimilated by plants for their growth (Zhou *et al.*, 1992).

The use of a natural source of phosphate materials, for example, Rock Phosphate (RP), as fertilizer for P-depleted soils has been considered as the gold standard in recent years due to its significant results. Also, RP is plentiful in many countries of the world (Reddy *et al.*, 2002).

However, its solubilization is limited to normal and non-acidic pH range of soils, commonly within the range of 5.5 to 6.0 pH (Khasawneh and Doll, 1978). During the manufacturing process of RP, phosphoric and sulphuric acids are processed to produce partially acidulated RP. As a result of the chemical reactions, the production of RP are considered as cost-ineffective and may produce by-products that may pose a threat to environmental health (Elias *et al.*, 2016).

An alternative strategy is to harness insoluble P in soils by P-solubilizing microorganisms, mainly recovered from the soils of rhizospheric or endophytic origins.

Thus, P-solubilizing microorganisms, used as inoculants in microphos technology, play a critical role in the natural P cycle in soils to improve the productivity of the soil and the yield of horticultural and agricultural crops (Sharma *et al.*, 2013).

The prominent mechanism by P-solubilizing microorganisms for P solubilization is through the release of organic acids which are the main feature from fungi (Vassilev *et al.*, 2007). Endophytic fungi are a group of fungal communities that live in the tissue of various healthy plants without causing any harm to their host (Gouda *et al.*, 2016). The symbiotic relationship between endophytic fungi and the host plant species may be discovered through the study of their mutually beneficial properties established by the continual metabolic exchange for stabilization under evolutionary pressure (Strobel and Daisy, 2003).

Medicinal plants are considered as one of the potential sources of isolation and elaboration of the diverse endophytic fungal species and strains which may have the potential for P-solubilization (Jia *et al.*, 2016). Five endophytic fungi belonging to *Penicillium chrysogenum* and *P. crustosum* were isolated from an Egyptian medicinal plant, *Teucrium polium* L. and displayed potential phosphate solubilization based on the plate assay (Hassan, 2017). Other studies also reported the phosphate solubilizing activity by the members of *Aspergillus* and *Penicillium* isolated from a Himalayan medicinal plant, *Taxus wallichiana* Zucc and their P solubilization were measured using the colorimetric method. (Adhikari and Pandey, 2019). Zingiberaceae, a reputable plant family extensively used for its medicinal aspect, is still less studied regarding the occurrence of endophytic PSF rather than the phosphate solubilizing bacteria (Chakraborty *et al.*, 2019; Zhang *et al.*, 2018).

The present study focuses on the potential Phosphate Solubilizing Fungi (PSF) from the collection of endophytic fungi isolated from wild Zingiberaceous species in North Sumatra and evaluates their inorganic P solubilizing mechanisms through the secretion of organic acids and detection of its major constituents.

Materials and Methods

Screening of PSF

A collection of 35 fungal isolates from the rhizomes of species belonging to five Zingiberaceous genera (*Alpinia, Amomum, Elettaria, Etlingera and Hedychium*) previously collected from the forest of North Sumatra were re-grown in fresh Potato Dextrose Agar (PDA) medium at 25-28°C (Lutfia *et al.*, 2019; Lutfia *et al.*, 2020; Munir *et al.*, 2020). An agar plug of active-growing colonies was inoculated onto a solid Pikovskaya medium containing 10 g glucose, 0.2 g NaCl, 0.2 g KCl, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.5 g $FeSO_4.7H_2O$, 0.5 g $MnSO_4.7H_2O$, 0.5g yeast extract, 5 g Ca_3PO_4, 18 g agar powder in 1 L of d istilled water (Pikovskaya, 1948). The PSF was indicated by the presence of clear halo around colonies after 7 d incubation at 25-28°C while their P-solubilizing activities were expressed in Solubilization Index (SI) (Mursyida *et al.*, 2015). Isolates with high potential, expressed as the highest SI, were maintained on PDA slants at 4°C for further experimentation.

Molecular Identification of Potential PSF

DNA extraction was performed using Wizard® Genomic DNA Purification Kit Protocol (United States). The protocol prescribed by the manufacturers was followed. The ITS region of the rDNA gene was amplified with primers ITS1: 5'-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' (Manter and Vivanco, 2007). Polymerase Chain Reactions (PCR) were conducted using 40-µL reaction mixture containing 2.0 µL of each primer (10 pmol/µL), 4.0 µL of genomic DNA (50 ng/µL), 20 µL of 2× PCR MasterMix buffer (0.4 mm dNTPs, 4 mm $MgCl_2$, 0.05 µg/µL Taq polymerase) and 12 µL of nuclease-free water. The PCR cycling conditions were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec and a final extension at 72°C for 7 min. After amplification, the PCR products were visualized in 1.0% agarose gel with a 100-bp DNA ladder (MBI Fermentas). Samples with a single obvious band were delivered to Macrogen, Inc., Singapore for DNA sequencing in both directions. The raw sequences were trimmed qualitatively and utilized to generate a consensus sequence using Unipro UGENE.

BLASTn search was employed to obtain the genera level of identification for each fungal isolate. A character-based method in the form of maximum-likelihood phylogenetic tree was constructed for species-level identification of PSF with other fungal ITS-rDNA database retrieved from previous BLASTn search results. The phylogenetic tree was generated using MEGA X based on the best-predicted DNA model of the Kimura-2 parameter and 1000× bootstrapping, with gaps treated as data for divergence (Kumar *et al.*, 2018). The ITS-rDNA sequence of each PSF was submitted to GenBank (https://www.ncbi.nlm.nih.gov/nuccore/?term=MW020161:MW020164[accn]).

Quantification of Soluble P in Pikovskaya Broth

Based on the solubilization index on solid medium, selected fungal isolates were further characterized for their P-solubilizing capacity in Pikovskaya Broth (PB) medium. An agar plug of each potential fungal isolates was inoculated into a 250-mL flask containing 50 mL of PB at 25-28°C for 7 d at 120 rpm. Control was made without fungal inoculation in the PB medium. All experiments were made in duplicates. Each day, an aliquot of fermentation medium was checked

for its pH using a pH meter. After incubation, fungal biomass was removed by centrifugation at 10,000 × g for 10 min at 4°C. One mL of supernatant was reacted with a color reagent prepared with 40% (w/v) ammonium molybdate, 0.1 M ascorbic acid, potassium antimonyl tartarate and 5 N sulphuric acid and measured for its absorbance at A880 (Adhikari and Pandey, 2019). The positive result of soluble P in samples was indicated by the formation of blue color while the P content was quantified by plotting the sample absorbance into the linear regression formula from standard KH_2PO_4 solution.

Detection of Organic Acids

Detection of organic acids from each culture filtrate of PSF was performed without pre-derivatization with GC-MS detector GC-MS-QP2010 Ultrasystem (Shimadzu Europa GmbH, Dusseldorf, Germany), fitted with RTX-5MS (30 × 0.25 × 0.10 m) column. The proportion of organic acids was represented in % area with the identification of compounds based on the spectrum provided in WILEY7 library.

Results

Out of the 35 isolated fungi, 13 fungal isolates (37%) displayed P-solubilizing activities detected through the plate assay by the formation of clear zones around their colonies (Table 1). Also, Solubilizing Index (SI) was measured in duplicates which designated four fungal isolates namely Al01, Am08, El01 and He06 with the SI ranging from 1.2 to 1.5 as potential P-solubilizers for further experimentation. According to the BLAST search results of ITS-rDNA sequence of four fungal isolates and phylogenetic constructions, three isolates were identified as members of *Trichoderma* (Hypocreaceae) while one isolate belonged to *Pestalotiopsis* (Sporocadaceae). GenBank accession numbers were also generated for each isolate e.g. *Trichoderma scalesiae* Al01 (MW020164.1), *Trichoderma brevicrassum* Am08 (MW020162.1), *Trichoderma atroviride* El01 (MW020163.1) *and Pestalotiopsis thailandica* He06 (MW020161.1) (Fig. 1). The P-solubilizing capacities of four potential PSF were assayed in Pikovskaya's broth as the growth medium. After 7-d incubation, the fermentation medium was observed to be varying in terms of clarity due to the different P-solubilizing activities of fungal isolates. Based on visual observation, *T. scalesiae* Al01 and *P. thailandica* He06 proved more efficient P solubilizers, while *P. thailandica* He06 showed better growth as indicated by its biomass after incubation (Fig. 2).

The amount of phosphates solubilized by each PSF was detected and measured by the colorimetric molybdate-blue method with the positive results of blue color formation. In this study, the blue color of the highest intensity was detected after 7-d incubation. Greater intensity of blue colour indicated more P solubilized by fungal isolates in the liquid medium (Fig. 3).

The absorbance of each sample (A_{880}) was quantified and expressed in the amount of soluble P in mg/L. The results showed that all fungal isolates solubilized more P than the uninoculated control (Fig. 4).

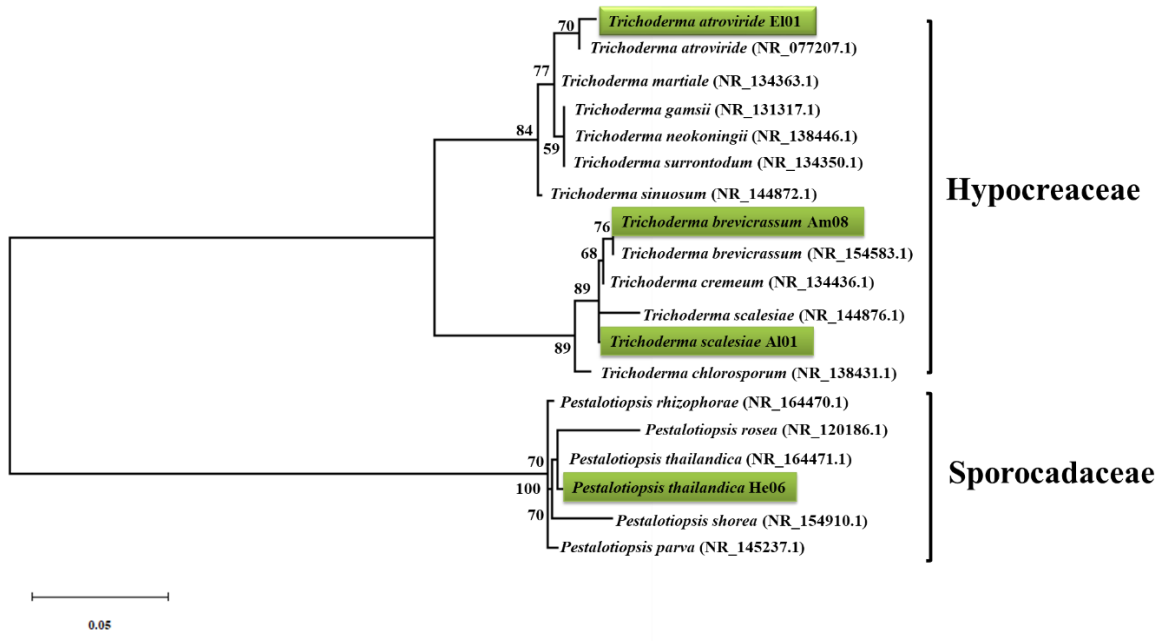

Fig. 1: Phylogenetic tree of the ITS sequence of Al01, Am08, El01 and He06. The ML phylogram was inferred from Kimura-2 parameter model with bootstrap percentages of >70% derived from 1000 replicates as indicated at the nodes. Bar = 0.05 substitutions per nucleotide position

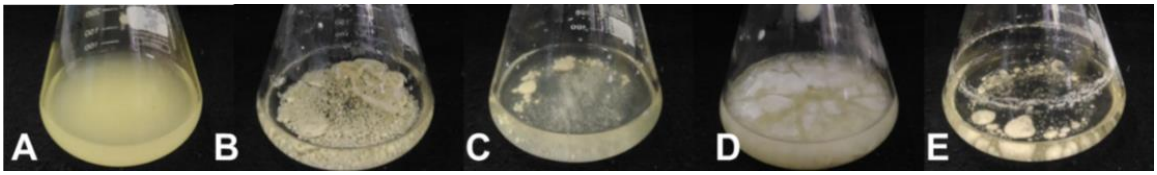

Fig. 2: Medium condition of pikovskaya broth after 7 d-fermentation by PSF at 25–28 ºC. (A) Control, (B) *Pestalotiopsis thailandica* He06, (C) *Trichoderma brevicrassum* Am08, (D) *Trichoderma atroviride* El01, (E) *Trichoderma scalesiae* Al0

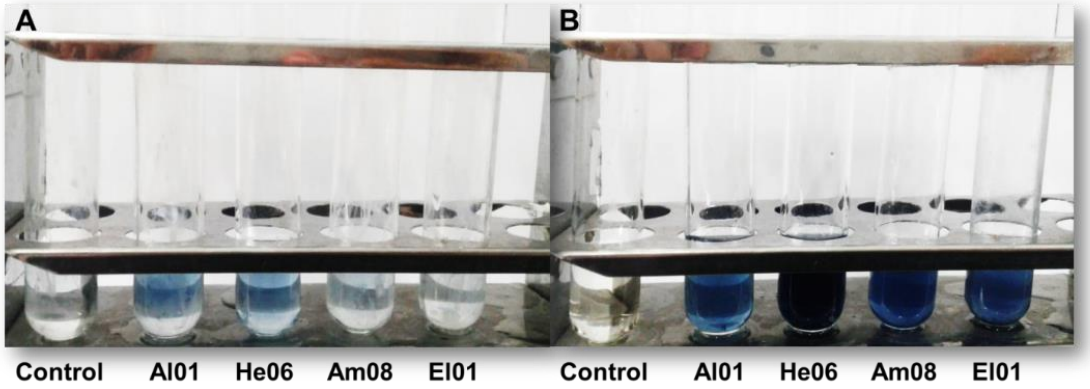

Fig. 3: Detection of soluble P by using the Na_2MoO_4 colorimetric method as indicated from the blue color formation in solution. (A) 1-d incubation, (B) 7-d incubation.

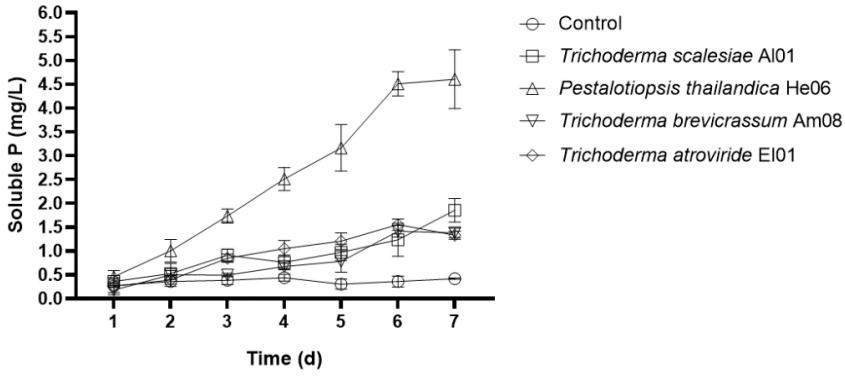

Fig. 4: Soluble P content of each culture filtrate of PSF on pikovskaya broth at 25–28°C

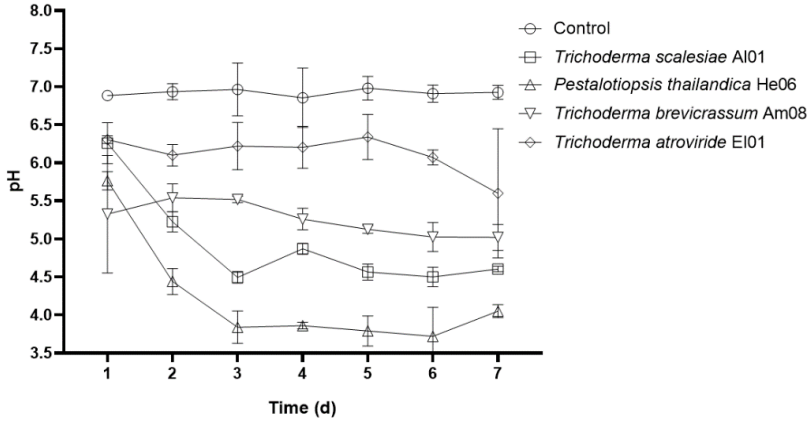

Fig. 5: pH condition of pikovskaya broth during fermentation by PSF at 25–28°C

Table 1: Solubilizing Index (SI) of endophytic fungi from zingiberaceous species

No.	Source	Code	Solubilizing ındex	No.	Source	Code	Solubilizing ındex
1	*Alpinia* sp.	Al01	1.5	19	*Etlingera* sp.	Et01	-
2		Al02	-	20		Et02	0.3
3		Al03	0.5	21		Et03	-
4		Al04	-	22		Et04	0.5
5		Al05	-	23		Et05	0.4
6	*Amomum* sp.	Am01	-	24		Et06	0.4
7		Am02	-	25	*Hedychium* sp.	He01	0.5
8		Am03	-	26		He02	0.4
9		Am04	-	27		He03	-
10		Am05	-	28		He04	0.5
11		Am06	-	29		He05	-
12		Am07	-	30		He06	1.3
13		Am08	1.4	31		He07	-
14	*Elettaria* sp.	El01	1.2	32		He08	-
15		El02	-	33		He09	-
16		El03	0.5	34		He10	0.4
17		El04	0.6	35		He11	-
18		El05	-				

Table 2: List of organic acids, its proportion and pKa(s) detected secreted by PSF

			Proportion of % area			
No.	Compound	pKa[a]	*T. scalesiae* Al01	*T. brevicrassum* Am08	*T. atroviride* El01	*P. thailandica* He06
1	Butyric acid	4.82	0.00	0.00	0.00	0.33
2	Carbazic acid	-3.50	2.20	0.00	0.00	0.00
3	Glutamic acid	9.60	0.00	0.00	0.00	3.36
4	Lauric acid	5.30	0.00	0.00	0.00	1.43
5	Linoleic acid	4.77	0.00	0.70	0.00	0.00
6	Malonic acid	2.85	0.00	0.59	0.00	0.00
7	Margaric acid	4.95	3.42	0.00	0.00	2.68
8	Myristic acid	4.90	0.00	53.87	0.00	0.00
9	Oleic acid	5.02	5.50	3.58	7.49	17.05
10	Palmitic acid	4.95	2.86	40.78	27.97	29.80
11	Phosphonic acid	5.30	0.00	0.00	1.49	0.00
12	Phthalic acid	2.76	54.64	0.00	63.05	39.79
13	Propionic acid	4.88	0.00	0.00	0.00	3.84
14	Stearic acid	4.75	31.38	0.48	0.00	1.73

[a)]Data retrieved from Kim *et al*. (2019)

The highest amount of solubilized P was recorded from the culture filtrate of *P. thailandica* He06 (4.61 mg/L) followed by *T. scalesiae* Al01 (1.85 mg/L), *T. brevicrassum* Am08 (1.38 mg/L) and *T. atroviride* (1.33 mg/L) after 7-d incubation. The majority of PSF decreased the pH of the medium as detected during fermentation. The results showed that there was a significant decrease in pH, to <4.0 by *P. thailandica* He06, while other PSF displayed lesser decreases in pH, 4.5, 5.2 and 5.5, respectively by *T. scalesiae* Al01, *T. brevicrassum* Am08 and *T. atroviride* El01 (Fig. 5). Meanwhile, the uninoculated control did not decrease the pH to any significant extent during the period of observation.

Fourteen organic acids were detected in the filtrate from the cultures of the PSF based on the GC-MS spectra and compound identification by search in the WILEY7 library database (Table 2). The highest number of organic acids (N = 9) was produced by *P. thailandica* He06, namely, butyric, glutamic, lauric, margaric, oleic, palmitic, phthalic, propionic and stearic acids. *T. brevicrassum* Am08 produced six (N = 6), *T. scalesiae* Al01 (N = 5) and *T. atroviride* El01 (N = 4). Phthalic acid (pKa = 2.76) formed the largest percentage (39.79%–63.05%) of all acids produced by three PSF. The exception was *T. brevicrassum* Am08. It produced a high proportion of myristic acid (53.87%). In general, the organic acids produced by PSF are considered as medium to weak acids, while three of them, i.e., carbazic, malonic and phthalic acids are considered as strong acids that may aid the P-solubilizing activity of the PSF.

Discussion

The four fungal strains, *P. thailandica* He06, *T. atroviride* El01, *T. brevicrassum* Am08 and *T. scalesiae* Al01, were regarded as phosphate solubilizing fungi. Phosphate solubilization activity by rhizospheric and

endophytic fungi including *Trichoderma* spp. had been previously demonstrated due to their diverse habitat and association with numerous plant species (Resende *et al.*, 2014; Bononi *et al.*, 2020). However, we have not come across any recent information on any phosphate-solubilizing fungus of the genus *Pestalotiopsis* in which our isolate *Pestalotiopsis thailandica* stood out as the strongest among the P-solubilizers found in this study.

The four PSF may improve the P uptake of the other plants by solubilizing P and mobilizing the insoluble P toward the roots of their Zingiberaceous host. The screening of PSF could provide useful insights that other wild and unreported Zingiberaceous species may harbor prospective biofertilizers for application in P-deficient soils. In general, PSF produced more organic acids than bacteria that exhibit higher P-solubilizing activity (Venkateswarlu *et al.*, 1984).

The main mechanisms that PSF employ in inorganic P solubilization is by exudating the organic acids which lower the pH and increase cationic chelation. Thus, it provides more P adsorption sites to release P more readily in the soil to compete with other metal complexes (Al, Ca, Fe) (McGill and Cole, 1981). Depending on the species, a variety of organic acids may be secreted by PSF such as citric acid, gluconic acid, 2-ketogluconic acid, lactic acid, oxalic acid, succinic acid and tartaric acid (Satyanarayana *et al.*, 2017; Wang *et al.*, 2018).

Our results showed that the four PSF displayed distinct P-solubilizing capacity as indicated by the soluble P concentration. The highest soluble P was recorded on the final day of incubation (7 d) along with the acidification of fermentation broth. The low pH of the fermentation broth directly influenced the growth of PSF which also gave us insight for the selection of acid-tolerant strains of PSF. However, some studies suggested no correlation between solubilized P and pH of the medium (Chaiharn and Lumyong, 2009; Xie *et al.*, 2009).

In this study, we then investigated the production of those organic acids by PSF which may contribute to the reduction of pH owing to the release of protons, which is the basic principle of phosphate solubilization (Sperber, 1958; Marra *et al.*, 2015). Based on the GC-MS analysis, organic acids were of medium to weak acids based on their acid dissociation constant or pKa (Kim *et al.*, 2019). Several fatty acids were also detected through the GC-MS analysis, which indicated the possibility of cellular disruption releasing the components of the fungal cell membrane into the culture filtrate. Phthalic acid was detected as the dominant constituent in the culture filtrate of PSF.

Phthalic acid or 1,2 benzenedicarboxylic acid is a member of benzoic acid with limited information on its capacity in solubilizing P, especially that derived from a fungal source. The only other report that discussed the role of phthalic acid in P solubilization was from a study on diazotrophic cyanobacteria of *Westiellopsis prolifica* and *Anabaena variabilis*, which produced and secreted phthalic acid into their culture filtrates as mineral P-solubilizing agent (Yandigeri *et al.*, 2011).

Other solubilization mechanisms may be involved besides the production of organic acids, for example, the Exopolysaccharide (EPS) produced by PSF which manifested in the high biomass at the end of incubation (Yi *et al.*, 2008). However, we did not record the yield of fungal biomass which may correspond to the accumulation of EPS responsible for the P solubilization in the liquid medium. Based on our study, we then promoted *Pestalotiopsis thailandica* He06 as a prospective biofertilizer agent for agricultural application in the future. Future perspective is to characterize the selected PSF's biomass and EPS production under various initial pH levels to understand another possible P-solubilization capacity of the isolate.

Conclusion

Exploration of Phosphate Solubilizing Fungi (PSF) from our collection of endophytic fungi from Zingiberaceous species revealed four isolates as potential candidates for P-solubilizers. *Pestalotiopsis thailandica* solubilized the highest P and secreted the most numerous organic acids. Phthalic acid was the major constituent which may be responsible for efficient P solubilizing activity. The long-term objectives are to validate the field efficacy of *P. thailandica* He06 before their formulation as bio-inoculants.

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Author's Contributions

Erman Munir: Conceived the original idea, designed the study and manuscript approval.

Yurnaliza: Data analysis and experimental development.

Anisa Lutfia: Designed research methodology, conducted field sampling and data interpretation.

Adrian Hartanto: Materials and equipment engagement, literature search and manuscript writing.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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