Screening and Evaluation of Various Carbon Sources on the Ability of *Trichoderma harzianum* InaCC to Solubilize Insoluble Phosphate (Penapisan dan Evaluasi Beragam Sumber Karbon terhadap Kemampuan *Trichoderma harzianum* InaCC Melarutkan Fosfat Tidak Terlarut)

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ABSTRACT

Our previous works revealed that *Trichoderma harzianum* InaCC (Indonesian Culture Collection) produced volatile organic compounds that inhibited the growth of *Fusarium oxysporum*. The objective of this study was to evaluate the ability of *T. harzianum* InaCC to solubilize insoluble phosphate and optimize the carbon sources condition. Screening was conducted to 10 isolates of *T. harzianum* on Pikovskaya's broth in 100-mL conical flasks. The cultures were incubated at 30 ± 1°C for 7 days on a rotary shaker at 80 r/min. All *T. harzianum* isolates were observed for the solubility of insoluble tricalcium phosphate with the highest ability was performed by *T. harzianum* InaCC F88. Using this isolate, the optimization of insoluble phosphate solubilization was conducted on modifed Pivoskaya medium in 500-mL conical flasks in similar screening incubation condition with applying of different combination of phopshate sources (tricalcium phosphate and rock phosphate) and carbon sources (dextrose, lactose, and sucrose). When tricalcium phosphate was substituted by rock phosphate, less soluble phosphate was produced. Glucose was the best carbon source used for solubilization of both tricalcium phosphate and rock phosphate, then followed by lactose and sucrose. Acidification of medium was not the major mechanism of phosphate solubilization by *T. harzianum*. Based on the ability of high phosphate solubilization, *T. harzianum* InaCC F88 is the most potential strain as a plant growth promoting fungus among the 10 isolates of *T. harzianum* Ina CC.

Key words: biocontrol, Pikovskaya medium, phosphate solubilization, *Trichoderma harzianum*

ABSTRAK

Telah diketahui bahwa *Trichoderma harzianum* InaCC (Indonesian Culture Collection) menghasilkan senyawa organik yang mudah menguap yang menghambat pertumbuhan *Fusarium oxysporum*. Tujuan dari penelitian ini adalah untuk mengevaluasi kemampuan sepuluh isolat *T. harzianum* InaCC untuk melarutkan fosfat yang tidak larut dan mengoptimalkan kondisi sumber karbonnya. Skrining dilakukan untuk semua sepuluh isolat pada media cair Pikovskaya dalam 100 ml labu erlenmeyer. Kultur diinkubasi pada 30 ± 1 ° C selama 7 hari pada *rotary shaker* pada 80 r / menit. Semua isolat *T. harzianum* diamati untuk melarutkan trikalsium fosfat dengan kemampuan pelarutan tertinggi oleh *T. harzianum* InaCC F88. Dengan menggunakan isolat ini, optimalisasi solubilisasi fosfat yang tidak larut dilakukan pada media cair Pivoskaya termodifikasi dalam labu erlenmeyer berukuran 500 mL dalam kondisi yang sama dengan inkubasi skrining dengan menerapkan kombinasi yang berbeda dari sumber fosfat (trikalsium fosfat dan fosfat batuan) dan sumber karbon (dekstrosa, laktosa, dan sukrosa). Ketika trikalsium fosfat digantikan dengan fosfat batuan, fosfat terlarut yang dihasilkan lebih sedikit. Glukosa adalah sumber karbon terbaik yang digunakan untuk solubilisasi baik trikalsium fosfat maupun fosfat batuan diikuti oleh laktosa dan sukrosa. Pengasaman medium bukanlah mekanisme utama pelarutan fosfat oleh *T. harzianum*. Karena kemampuan pelarutan fosfatnya yang tinggi, *T. harzianum* InaCC F88 berpotensi sebagai jamur pendorong pertumbuhan tanaman.

Kata Kunci: biokontrol, media Pikovskaya, pelarutan fosfat, *Trichoderma harzianum*

INTRODUCTION

Microorganisms play key central role on the plant growth promotion as well as crop disease suppression (Van Geel *et al.* 2016). *Trichoderma harzianum* is known to suppress growth of *Fusarium oxysporum* notorius fungi which causes several plant diseases of tomato, potato, chili pepper, and banana (Ma *et al*. 2013). Indonesian Culture Collection (InaCC) has been continuously enriching the collections through microbial survey and isolation of specific targetted microorganisms.

We have isolated various *Trichoderma* spp. from soil and other natural resources. *Trichoderma harzianum* is one of InaCC collections which

are intensively studied. *Trichoderma* group was reported to increase plant growth and suppress plant diseases. The efficacy of microbes to enhance plant growth or suppress plant pathogen is strain dependent (Berg 2009), it is then crucial to evaluate the physiological characters of deposited cultures. In this study we selected 10 isolates of *T. harzianum* orginated from soil and other natural resources. A previous *in vitro* bioassay study showed these strains produced volatile organic compounds that inhibited the growth of *Fusarium oxysporum* (Napitupulu *et al.* 2019). Up to now the physiological mechanism by which *T. harzianum* reduce plant disease and stimulate plant growth are not well understood. It was indicated that several physiological mechanisms of plant-microbes interaction might contribute to enhance plant growth and to reduce disease incident (Saharan & Nehra 2011), which include production of plant growth hormone (Ahemad & Kibret 2014), increasing nutrient availability (Vacheron *et al*. 2013), production of Fe-chelating agent (Hayat *et al.* 2010), and increasing drought tolerance as well as water avalibility for plant growth (Chaparro *et al.* 2012).

We suspect that *T. harzianum* (collection of InaCC) could be important plant growth promoter through increasing posphate availibility. This is particularly true since the most of introduced phosphate fertilizer are fixed by soil matrices, hence phosphate availability for plant growth is limited (Singh *et al.* 2011). Though phosphate solubilizing microbes is ubiquitous in soil but since the growth of *T. harzianum* is very rapid therefore they could be important soil microbes. The objective of this study was to verify the phosphate solubilizing ability of *T. harzianum* (collection of InaCC) and to determine effect of carbon sources on the phosphate solubilizing profile.

MATERIALS AND METHODS

The investigation was carried out with 10 isolates of *Trichoderma harzianum* strains that were obtained from Indonesian Culture Collection (InaCC). The strains have been labelled as InaCC F86, InaCC F87, InaCC F88, InaCC F89, InaCC F90, InaCC F91, InaCC F92, InaCC F115, InaCC F116, and InaCC F144. These *T. harzianum* strains were isolated from leaf litters and soils of mount Bromo and mount Salak, Indonesia. The fungi were maintained on plates containing Potato Dextrose Agar (PDA) at 27^{o}C prior to use in the experimental procedures. At third day, the growth of each strains was monitored as percent occupation in petri dish. The image of culture isolates was captured and analyzed using ImageJ software.

All 10 isolates of *Trichoderma harzianum* InaCC strains were tested for their ability to solubilize inorganic phosphate from tricalcium phosphate (TCP) in Pikovskaya's broth (Himedia Laboratory, India). For this assay, 5-mm mycelial disc of each isolate was cut from the margin of an actively growing a-week-old culture and inoculated into 40 mL of fresh Pikovskaya's broth in 100-mL conical flasks. The cultures were incubated at 30 ± 1°C for 7 days on a rotary shaker at 80 r/min. Uninoculated control was also maintained. Three replicates were maintained for each treatment. Then, spores and mycelia of *Trichoderma* strains were separated from broth culture by centrifugation at 8,000 RPM for 10 min. The supernatant of each culture was analyzed for pH and phosphate concentration.

After screening, a strain that has highest ability for solubilization insoluble phosphate was used. Using this isolate, the optimization of insoluble phosphate solubilization was done on 200 mL modified Pikovskaya broth medium

Table 1. Modified Pivoskaya medium with different combination of phopshate sources and carbon sources

Ingredients	g/L
Yeast Extract	0.500
Carbon source (Dextrose/Lactose/Sucrose)	10.000
Phosphate Source (Tri Calcium phosphate/Rock Phosphate)	5.000
Ammonium sulphate	0.500
Potassium chloride	0.200
Magnesium sulphate	0.100
Manganese sulphate	0.0001
Ferrous sulphate	0.0001

500-mL conical flasks (Table 1) in similar screening incubation condition applying different combination of phosphate sources and carbon sources. Then, spores and mycelia of Trichoderma strains were separated from broth culture by centrifugation at 8,000 RPM for 10 min. The supernatant of each culture was analyzed for pH and phosphate concentration.

Phosphate concentration in the uninoculated control and supernatants was estimated by spectrophotometric method (Fiske & Subbarow, 1925) with some modifications. An aliquot of 1 mL culture supernatant was mixed with 3 mL of distilled water and 1 mL of color reagent containing ammonium molybdate ($(NH_4)d_6Mo_7O_{24}\cdot 4H_2O$) 1.5% (w/v), sulfuric acid (H_2SO_4) solution 5.5% (v/v) and ferrous sulfate ($FeSO_4$) solution 2.7% (w/v). The mixture was vigorously shake for 5 seconds then measured by a UV-Vis spectrophotometer (JK-VS-721N, JKI, China) at 600 nm. The same procedure was applied to control as well. The level of phosphate concentration was determined by using a standard graph of potassium dihydrogen phosphate (KH_2PO_4) and expressed as $H_2PO_4^-$ concentration in µg/mL after correction with $H_2PO_4^-$ concentration in uninoculated control.

Uninoculated control and supernatants collected after centrifugation of 7-day-old broth cultures were transferred to test tubes and the pH was measured using pH meter (WA-2015 Lutron).

RESULTS

Trichoderma harzianum strains that were used in the study consists of 10 strains and all of them obtained from Indonesian Culture Collection (InaCC). Macroscopic views of the cultures that were incubated for 5 days at temperature 27°C is shown in Figure 1. There are presence variations among strains, not only source of sample but also macroscopic morphology including the growth profile of each strains (Table 2).

All *Trichoderma harzianum* strains were screened for the potency to solubilize insoluble phosphate in Pikovskaya Broth (Table 3). All strains demonstrated diverse levels of phosphate

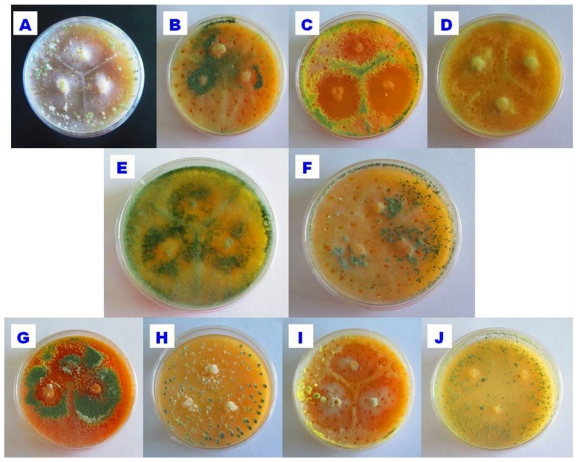

Figure 1. *Trichoderma harzianum* strains were used in the study (A) InaCC F86 (B) InaCC F87 (C) InaCC F88 (D) InaCC F89 (E) InaCC F90 (F) InaCC F91 (G) InaCC F92 (H) InaCC F115 (I) InaCC F116 and (J) InaCC F144

Tabel 2. The summarized profile of each *T. harzianum* strains

T. harzianum strain	Source of Sample	Macroscopic Morphology in PDA	% Occupation in Petri Dish after 3 days
InaCC F86	leaf litter	high wooly, white	70.38
InaCC F87	leaf litter	high wooly, greenish white	75.95
InaCC F88	soil	wooly, greenish white	73.95
InaCC F89	leaf litter	high wooly, off white	84.83
InaCC F90	leaf litter	high wooly, greenish white	82.21
InaCC F91	leaf litter	wooly, greenish white	54.94
InaCC F92	leaf litter	high wooly, greenish white	71.64
InaCC F115	soil	high wooly, greenish white	82.60
InaCC F116	leaf litter	high wooly, white	74.69
InaCC F144	leaf litter	wooly, greenish white	39.70

solubilization activity from very high to negative. According to the result, *T. harzianum* InaCC F88 has the highest ability as phosphate solubilizing isolate. Therefore, the strain F88 was subjected to evaluate for carbon source effects on phosphate solubilization.

Table 3. Phosphate solubilizing activity screening of ten isolates of *Trichoderma harzianum* in Pikovskaya's broth

T. harzianum strain	The ability to solubilize insoluble phosphate*
InaCC F86	+
InaCC F87	++
InaCC F88	+++
InaCC F89	+
InaCC F90	+
InaCC F91	++
InaCC F92	+
InaCC F115	+
InaCC F116	-
InaCC F144	++

*The degree of ability is marked as level concentration of K_2HPO_4 in supernatant: - means not detected; + means 1 – 20 µg/mL; ++ means 20 – 80 µg/mL; +++ means above 80 µg/mL

Table 4. Effect of variation of carbon source on phosphate solubilizing of *T. harzianum* InaCC F88 in two different phosphate sources determined as $H_2PO_4^-$ concentration in the culture filtrate

Carbon Source	Available $H_2PO_4^-$ in the culture filtrate (µg/mL)	
	Tri Calcium Phosphate	Rock Phosphate
Dextrose	340.84 ± 43.91	68.26 ± 13.12
Sucrose	ND*	29.20 ± 5.93
Lactose	162.03 ± 23.14	ND

*Not Determined

Table 4 shows the effect of carbon source on phosphate solubilizing of *T. harzianum* F88. In two different phosphate sources, Tri Calcium Phosphate (TCP) and Rock Phospate (RP). The results revealed that there is a difference in the phosphate solubilization when the dextrose, sucrose, and lactose were used as carbon source. The amount of solubilize phosphate is measured as concentration of dihydrogen phosphate ($H_2PO_4^-$). In general, concentration of solubilized phosphate is higher by applying TCP as phosphate source instead of RP. Dextrose is the best carbon source in this evaluation then followed by lactose and sucrose.

Except for sucrose, the degree of acidity of the medium after incubation for 7 days is slightly increased by using dextrose and lactose as carbon source in both phosphate sources (Table 5). The initial pH for medium containing TCP and RP is approximately 6 and 7-8, respectively, but the shifting of pH is only about 1 from the initial pH (control).

The relationship among selected strains is shown in Figure 2. The strains were selected due to the screening result in Table 2. The representative strains of each degree of ability was selected in order to compare them. ITS rDNA sequences of all strains are obtained from Indonesian Culture Collection (InaCC) database. InaCC F88 which have the high ability to solubilize insoluble phosphate has more close relation to InaCC F116 than InaCC F86 and InaCC F91 strain.

DISCUSSION

There are variations between isolates of *Trichoderma harzianum* InaCC strains such as macroscopic appearance as well as growth

Table 5. Influence of variation of carbon source on acidity of culture filtrate of *T. harzianum* InaCC F88 in two different phosphate sources

Carbon Source	Tri Calcium Phosphate		Rock Phosphate	
	Initial pH (Control)	pH of culture filtrate	Initial pH (Control)	pH of culture filtrate
Dextrose	6.03	5.27 ± 0.25	8.08	6.99 ± 0.34
Sucrose	6.03	7.44 ± 0.02	8.73	8.91 ± 0.28
Lactose	5.98	5.15 ± 0.06	7.84	6.33 ± 0.62

profile of isolates in solid media (Table 2, Figure 1). A very distinct difference is shown by the difference growth of each strains. Most of the strains have occupied the petri dish above 70% in third day, while InaCC F91 and InaCC F144 are slower with only occupied 54.94% and 39.70%, respectively. Similar result is also obtained in some *Trichoderma* strains in difference environmental parameter conditions (Petrisor *et al.* 2016). The presence of these variations has led to the study of differences in physiological characteristics, for instance phosphate solubilizing activity.

The ability of *T. harzianum* strains to solubilize insoluble phosphate shows variation (Table 3), even though all the isolates are grouped in one species (Figure 2). Similar with our result, *in vitro* evaluation between *Trichoderma aspereloides* strains shows significant difference in the ability of solubilizing insoluble phosphate (Borgeset *et al.* 2015). This variation among the strains could be resulted by the genetic factor that leads to degree of producing certain metabolites. A Study for the biocontrol purpose, Ghisalberti *et al.* (1990) reported that *T. harzianum* strains shows diversity of their ability to antagonize the take-all fungus (*Gaeumannomyces graminis*) due to the difference in production of metabolite pyrones.

Carbon source, along with other macronutrient sources, plays a very important role in the metabolism and development of cells. According to the result in Table 3, the influence of different carbon source has an impact in the ability of the fungi to solubilize the insoluble phosphate, in which dextrose is the most efficacious source then followed by lactose and sucrose. Similarly, some phosphate solubilizing microbes are also utilize dextrose as main carbon source to achieve maximum phosphate solubilization. Yadav *et al.* (2011) reported that *Aspergillus niger* showed maximum significant solubilization of TCP containing carbon source glucose followed by glycerol, maltose and sucrose. Our result showed that sucrose as carbon source, in compare to dextrose and lactose, gives poor ability of phosphate solubilization for *T. harzianum*. Study in *Burkholderia cepacia*, the phosphate solubilization ability was found to be two times less in a medium containing sucrose relative than in glucose (Song *et al*. 2008). However, for other microbes, this disaccharide composed of dextrose and fructose is excellent carbon source for phosphate solubilizing microbes compared to dextrose. Set side by side to maltose and dextrose, sucrose appeared to be the best carbon source for the solubilization of hydroxyapatite and $FePO_4$ by *Penicillium rugulosum* (Reyes *et al*. 1999).

Rock phosphate (RP) or also known as phosphorite is a sedimentary rock that contain high phosphate bearing mineral. It approximately contains 30% of phosphate and 3 – 8 % available phosphate. The lesser free phosphate in culture media containing RP in comparison to TRC is due to this phosphate proportion reason. Due to the availability, high phosphate content, and economic matters, RP is an excellent source of natural fertilizer and a very reliable source of phosphate for agriculture. For *in vitro* evaluation and screening of the phosphate solubilizing activity of microbes, TCP is more reliable than

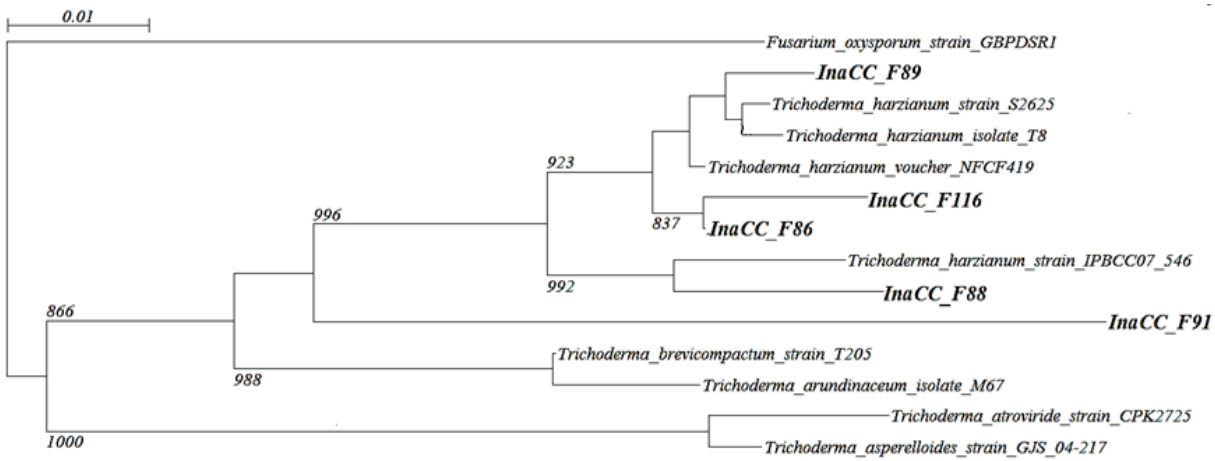

Figure 2. Phylogenetic tree of selected strains of *T. harzianum* from InaCC based on ITS rDNA sequence using neighbor-joining method and *Fusarium oxysporum* as outgroup.

RP because of its purity. However, it is necessary to further assess the capability of microbes to solubilize phosphate in media contain RP because most agricultural practice use it as phosphate source.

As source of energy, carbon source such us cellulose, sucrose, mannitol, and starch are abundantly presence in soil and released as photosynthetic products by plants. In general, simple sugars are uptaken into the cells and utilized in catabolic pathway for sustainability of the cells. During this process, various amounts of intermediate products and organic acids (OAs) such as citric acid, oxalic acid, and malic acid are produced and secreted outside the cells. The secreting of OAs is one of main mechanism of phosphate solubilizing efficiency of microorganism including fungi. When dextrose is used as main carbon source, microbes produced higher amounts of OAs that leads to more insoluble phosphate solubilization (Nautiyal 1999). However, according to our data (Table 4), acidification of medium was not the major mechanism of phosphate solubilization by *T. harzianum* since the pH of cultures are not significantly decrease and never fell below 5.0. Contradictory, some fungi produce high amount of OAs that help to solubilize environmental insoluble phosphate such as *Aspergillus niger* (Martin & Steel, 1955) and *Penicillium* sp. (Asea *et al.*1988). Some studies report the proposed mechanism how *T. harzianum* is able to solubilize insoluble phosphate. Li *et al.* (2015) suggested that phytase released by *Trichoderma* played an important role in solubilizing organic phosphate (phytate). Similar to our result, a previous study by Altomare *et al.* (1999) reported that OAs are not detected in the culture filtrate of *T. harzianum* T -22. On the other side, Adams *et al.* (2007) noted that metal chelation via OAs and proteins are the main mechanisms *T. harzianum* increase zinc desorption. The mechanism by which how *T. harzianum* process occurs are unclear and still remain topics of ongoing investigation.

CONCLUSION

There is significant variation among the ability of *Trichoderma harzianum* InaCC strains to solubilize insoluble phosphate. Glucose was the best carbon source for solubilization of tricalcium phosphate and rock phosphate followed by lactose and sucrose. When tricalcium phosphate was substituted with rock phosphate less soluble phosphate was produced. Acidification of medium was not the major mechanism of phosphate solubilization by *T. harzianum*. Due to the high phosphate solubilization ability, *T. harzianum* InaCC F88 is the most potential as a plant growth promoting fungus among the 10 isolates of *T. harzianum* Ina CC.

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