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Solubilization of inorganic phosphate by phosphate solubilizing fungi isolated from oil palm empty fruit bunches of Central Kalimantan

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A study that was aimed to isolate and identify phosphate solubilizing fungi from oil palm empty fruit bunches and to assess their ability to solubilize inorganic phosphate sources was conduted using dilution plate methods on Pikovskaya's medium. The isolation of phosphate solubilizing fungi was done by the pouring method on Pikovskaya medium with tri-calcium phosphate [$Ca_3(PO_3)_2$] as an inorganic phosphate source. Sixteen fungi isolates potential for solubilizing phosphate were found in the oil palm empty fruit bunches. From the sixteen isolates, twelve isolates were included in the genus of *Acremonium, Aspergillus, Hymenella, Mucor,* and *Moniliella*. The iisolates of TB1 (*Acremonium kiliens*), TB7 (*Aspergillus oryza*), TM1 (*Hymenella Fr.),* and TM8 (*Neosartorya fischeri*) were able to increase soluble P by 451%, 400%, 216% and 114%, repectively, at day 5. The four isolates were the efficient phosphate solubilizing fungi that can potentially increase phosphate solubilization in soils.

Keywords: phosphate, fungi, oil palm fruit bunches, solubilization

INTRODUCTION

The main dominant soil type in Central Kalimantan, Indonesia, is Ultisol that covers about 25% of the total land (Sudaryono, 2009). Based on the coverage, the soil has the potential to support the expansion of agricultural development in Indonesia. However, the production of plants grown on the soil is generally very low due to low fertility level of the soil. Ultisols have relatively high acidity with pH of less than 4.5 (Ohta et al. 1993), and low availability of phosphorus due to fixation (Hilman et al. 2007). Efforts to improve the availability of phosphorus in Ultisols can be done with phosphorus fertilization periodically, but the application of inorganic fertilizers to the soil does not always improve soil productivity because phosphate substances from fertilizers react quickly with iron, aluminum and manganese in acid soils so that phosphorus is not available for plant uptake (Podile and Kishore, 2006). Efforts to improve the availability of phosphorus in Ultisols of Central Kalimantan have been done by application of organic materials in the form of oil palm empty fruit bunches (Ariani, 2009, Ichriani et al. 2016). This is because Central Kalimantan has 1.09 million ha of oil palm plantation with total crude palm oil (CPO) production of 3.3 million tons in 2014 (Central Bureau of Statistics, 2015). From the total solid waste generated from the production process of CPO, oil palm empty fruit bunches contribute about 23% of biomass (Indriyati, 2008). Therefore, with 3.35 million tons of CPO production, the potential for oil palm empty fruit bunch biomass in Central Kalimantan reached 3.6 million tons in that year. Beside from being a source of biomass, oil palm empty fruit bunch has potential microorganism biodiversity. Microorganisms isolated from organic wastes can be used as bio fertilizers to improve nitrogen and protein percentages in crop seeds (El-Din Mekki, 2016). In addition, various groups of microorganisms include fungi, bacteria, and actinomycetes, known as efficient phosphorus solubilizers (Sundara et al. 2002) and convert them into $H_2PO_4^-$ and HPO_4^{2-} that are available for plants (Coutinho et al. 2012). Microbial phosphate solubilization occurs by different mechanisms, such as acidification, chelation, ion exchange reactions and the formation of polymer compounds (Delvasto et al. 2006). It is by the solubilization process (Rodrıguez et al. 1999), and from the accumulation of phosphorus in the biomass of microorganisms (Oehl, 2001). Fungi are important soil microbial components that make up more soil biomass than bacteria, depending on the depth of soil and nutrient conditions. Fungi have been reported to have greater ability to dissolve unavailable phosphate than bacteria (Nahas, 1996). In Sudan, inoculation of soils with indigenous fungi isolates greatly enhanced sorghum plant growth under greenhouse condition (Alsamowal et al. 2013). The most important genera of phosphate solubilizing fungi are *Aspergillus* (Vassilev et al., 2007) and some *Penicillium* species (Saber et al. 2009). The phosphate solubilizing fungi do not lose phosphate solubilizing activity in repeated culture in the laboratory (Kucey, 1983). *Penicillium oxalicum* isolated from the rhizosphere of rock phosphate mine and tested for its ability to solubilize rock phosphate could increase wheat and maize growth; the solubilization of the increased concentration of rock phosphate (Singh and Reddy, 2011). The phosphate solubilizing ability of the phosphate solubilizing fungi (*Aspergillus sp.*) in vitro is larger and more stable than the phosphate solubilizing bacteria (*Pseudomonas sp.*) (Sudadi et al. 2013). Phosphate solubilizing fungi can be isolated from rhizospheric soils, non-rhizospheric soils, phyllospheres, organic wastes, soil phosphate deposits and contaminated soils (Zaidi et al. 2009). The purpose of this study was to isolate and identify the most efficient phosphate solubilizing fungi from the oil palm empty fruit bunches and to assess their ability to solubilize inorganic phosphate sources so that they can be used as an alternative way to improve phosphorus availability in Ultisols of Central Kalimantan.

MATERIALS AND METHODS

Materials used for fungi isolation

The study was conducted in the soil biology laboratory of the Faculty of Agriculture, Brawijaya University from March to August 2016. Oil palm empty fruit bunches were obtained from PT Surya Inti Sawit Kahuripan (Makin Group) oil palm plantation, Parenggean Subdistrict, East Kotawaringin Regency, Central Kalimantan. The collection of oil palm empty fruit bunches was done in the area of oil palm solid waste processing in the form of (a) fresh oil palm empty fruit bunches, (b) half composted oil palm empty fruit bunches (oil palm empty fruit bunches that have been crushed and mixed with oil palm liquid waste for 2 weeks, immature compost), and (c) compost of oil palm liquid waste (crushed oil palm empty fruit bunches mixed with oil palm liquid waste for 6 weeks, mature compost). Chemical compositions of the three types of oil palm empty fruit bunches are presented in Tabel 1. Samples of each type of oil palm empty fruit bunch were brought to the laboratory, dried, and used for further study.

Isolation of phosphate solubilizing fungi

The isolation of phosphate solubilizing fungi from each type of oil palm empty fruit bunches was done by the pouring method on Pikovskaya (1948) medium with tri-calcium phosphate [$Ca_3(PO_3)_2$] as an insoluble organic phosphate source. In 1 liter of distilled water, the Pikovskaya agar medium contained 10 g glucose; 5 g [$Ca_3(PO_3)_2$]; 0.5 g $(NH_4)_2SO_4$; 0.2 g KCl; 0.1 $MgSO_4.7H_2O$; small amount of $MnSO_4$ and $FeSO_4$; 0.5 g of yeast extract, 15 g of bacto agar, and bactericidal (0.03 g Streptomycin) (Subba Rao, 1977, modified). Ten grams of each type of oil palm empty fruit branch was extracted with 90 mL of 0.85% NaCl solution and diluted to 105. The dilution results were poured aseptically onto the Pikovskaya medium. Fungi that have grown about 6-7 days on the Pikovskaya medium and had a clear zone (phosphate solubilization zone) around the colonies were then purified. The purification was carried out to obtain isolates of phosphate solubilizing fungi from oil palm empty fruit brunches.

Table 1. Chemical compositions of three types of oil palm empty fruit bunches

Oil palm empty fruit bunch	Water content (%)	pH H_2O (1:5)	Organic-C (%)	Total N (%)	Total P (%)	Total K (%)
Fresh oil palm empty fruit bunches	13.43	5.61	0.74	0.014	0.07	0.057
Immature compost of oil palm empty fruit bunches	29.58	6.72	0.8	0.017	0.29	0.065
Mature compost of oil palm empty fruit bunches	22.46	6.63	0.63	0.016	0.27	0.097

Each different fungi colony was purified by a scratch method on a the Pikovskaya agar medium. After 6 days the colony of fungi was purified again to obtain a single isolate. The purified fungi isolates were pinpointly inoculated onto solid Pikovskaya medium in petri dish and incubated at room temperature for 7 days. The phosphate solubilization index (SI) was calculated according to the ratio of total diameter (colony and halo zone) and colony diameter (Edi-Premono et al. 1996).

Identification of phosphate solubilizing fungi

Identification of phosphate solubilizing fungi isolate was done by using slide culture method (Cappucino and Sherman, 2005) to determine the phosphate solubilizing fungi obtained in the mold or yeast group. Identification was done by sampling the fungal mycelium from pure culture using ose needle and inoculating on Potato Dextrose Agar (PDA) medium above glass object and covered with cover glass. The growth of the fungi was observed using a trinoculer microscope BX41, DP26 camera with 400x magnification to obtain microscopic features such as mycelium, conidiophores shape, and growing of fungi conidia.

Ability of phosphate solubilization

The phosphate solublization ability was observed using liquid Pikovskaya medium. The phosphate solubilizing fungi isolates were grown for 7 days in a test tube containing PDA medium. In the test tube containing isolates of the phosphate solubilizing fungi were added 10 mL of sterile distilled wáter and centrifuged. The solution in the test tube was taken as 1 mL and inoculated on 100 mL of liquid Pikovskaya medium containing 5000 ppm tricalcium phosphate [$Ca_2(PO_4)_2$]. The Pikovskaya medium that had been inoculated with the phopstae solubilizing fungi was then shakedfor 4 hours daily at 125 rpm. On the 1st day - 5th day, 20 mL Pikovskaya medium solution was taken and filtered by Whatman No. 42 filter paper. The filtrate obtained was used for the determination of the P-soluble change and the pH of the liquid Pikovskaya medium. The change of the P-soluble of the medium was determined by the P reducing reagent (Mo-blue) and measured by a Spectrophotometer at 660 nm. The change in pH of the media was determined by a pH meter.

RESULTS AND DISCUSSION

Isolation and identification of phosphate solubilizing fungi

Isolation of phosphate solubilizing fungi from three types of oil palm empty fruit bunches using Pikovskaya soil media resulted in sixteen isolates having clear zone around their colonies. Two isolates (PS2 and PB4) were obtained from fresh oil palm empty fruit bunches (A), seven isolates (TB1, TB2, TB4, TB5, TB6, TB7, and TB10) were from immature compost of oil palm empty fruit bunches (B), and seven isolates (TM1, TM2, TM4, TM5, TM 6, TM7, and TM8) were from mature compost of oil palm empty fruit bunches (C) (Table 1).

Results of macro and micro morphological identification on each isolate indicated that PS2, PB4, TB5 and TB6 isolates were identified as yeast while the other twelve isolates (TB1, TB2, TB4, TB7, TB10, TM1, TM2, TM4, TM5, TM6, TM7, and TM8) were identified as mold. The mold fungi were then further tested and referred to as the phosphate solubilizing fungi. The results of identification presented in Table 1 showed that the isolates of TB1, TB2, TB4, TB10 and TM2 were included in the genus of *Acremonium*. Isolates of TB7, TM6, TM7, and TM8 were included in the genus of *Aspergillus*. Isolates of TM1, TM4, and TM5 were sequentially belonging to the genus of *Hymenella, Moniliella*, and *Mucor*.

Table 1. Phosphate solubilization fungi isolated from oil palm empty bunches

Isolate origin	Name of isolate	Phosphate Solubilization Index	Species
Fresh oil palm empty fruit bunches	PS2	1.33	Yeast
(A)	PB4	1.30	Yeast
Immature compost of oil palm	TB1	1.67	*Acremonium kiliense*
empty fruit bunches (B)	TB2	1.10	*Acremonium kiliense*
	TB4	1.10	*Acremonium kiliense*
	TB5	1.23	Yeast
	TB6	1.09	Yeast
	TB7	1.11	*Aspergillus oryzae*
	TB10	1.13	*Acremonium kiliense*
Mature compost of oil palm empty	TM1	1.13	*Hymenella Fr.*
fruit bunches (C)	TM2	1.02	*Acremonium strictum*
	TM4	1.00	*Moniliella acetoabutens*
	TM5	1.00	*Mucor circinelloides*
	TM6	1.00	*Eurotium herbariorum*
	TM7	1.00	*Aspergillus fumigatus*
	TM8	1.17	*Neosartorya fischeri*

The genus of *Aspergillus, Mucor,* and *Acremonium* are commonly found in oil palm organic wastes (Shahriarinour et al. 2013; Soleimaninanadegani and Manshad, 2014) as cellulolytic microbes. The genus of *Aspergillus* is also widely used as a phosphate solubilizing fungus (Fitriatin et al., 2014). The twelve isolates of phosphate solubilizing fungi could be classified into nine species of fungi (TB1, TB7, TM1, TM2, TM4, TM5, TM6, TM7, and TM8) (Figure 1). The nine isolates were further identified and tested for phosphate solubilization.

Phosphate solubilization index

Phosphate solubilizing index values of the isolates ranged from 1.00 to 1.67 after 5 days of inoculation (Table 1).Large phosphate dissolution indexes were found in TB1 isolate (*Acremonium kiliens*, 1.67), TM8 (*Neosartorya fischeri*, 1.17), TM1 (*Hymenella Fr.*, 1.13), and TB7 (*Aspergillus oryzae*, 1.1) (Table 1). The clear zones in other isolates were less visible because they coincided with the diameter of the fungus colony. Microorganisms capable of producing a clear zone due to phosphate solubilization in the surrounding medium were selected as potential phosphate solubilizers.Clear zones were formed around the colonies after 5 to 7 days of incubation on solidified Pikovskaya medium supplemented with tricalcium phosphate, indicating phosphate solubilizing ability of the fungal isolates. The clear zone was due to solubilization of tricalcium phosphate which was the result of combined effect of pH decrease and organic acids production (Fankem et al. 2006).

Phosphate solubilization and pH change of liquid Pikovskaya medium

The results of phosphate solubilization test by nine isolates of phosphate solubilizing fungi showed that from day 1 to day 5 there was an increasing trend of soluble Pin the Pikovskaya liquid medium. After 5 days, the highest soluble P (2363.28 ppm) was observed in the medium inoculated with TB1 isolate, and the lowest (380.86 ppm) was for the medium inoculated with TM5 (Table 2). Data presented in Table 2 show that the ability of nine isolates of phosphate solubilizing fungi in dissolving P compounds in Pikovskaya liquid medium were in the following order TB1> TM1>TM8> TB7> TM6> TM7> TM7> TM4> TM2>TM5.When the amount of soluble P in the media inoculated with fungsi isolates compared with the amount of soluble P in the media without incolation of fungi isolates, the TB1, TM1, TM8 and TB7 isolates were considered as the most efficient fungsi isolates as they increased soulble P by 451%, 400%, 216% and 114%, repectively, at day 5 (Table 2). The ability of fungi isolates to dissolve inorganic phosphates is thought to be due to the presence of organic acid compounds secreted by the fungi isolates (Osorio and Habte, 2013). The organic acids produced by the phosphate solubilizing fungi help chelating the metal compound or lower the pH so that they can dissolve the soil P (Pradhan and Shukla, 2005).

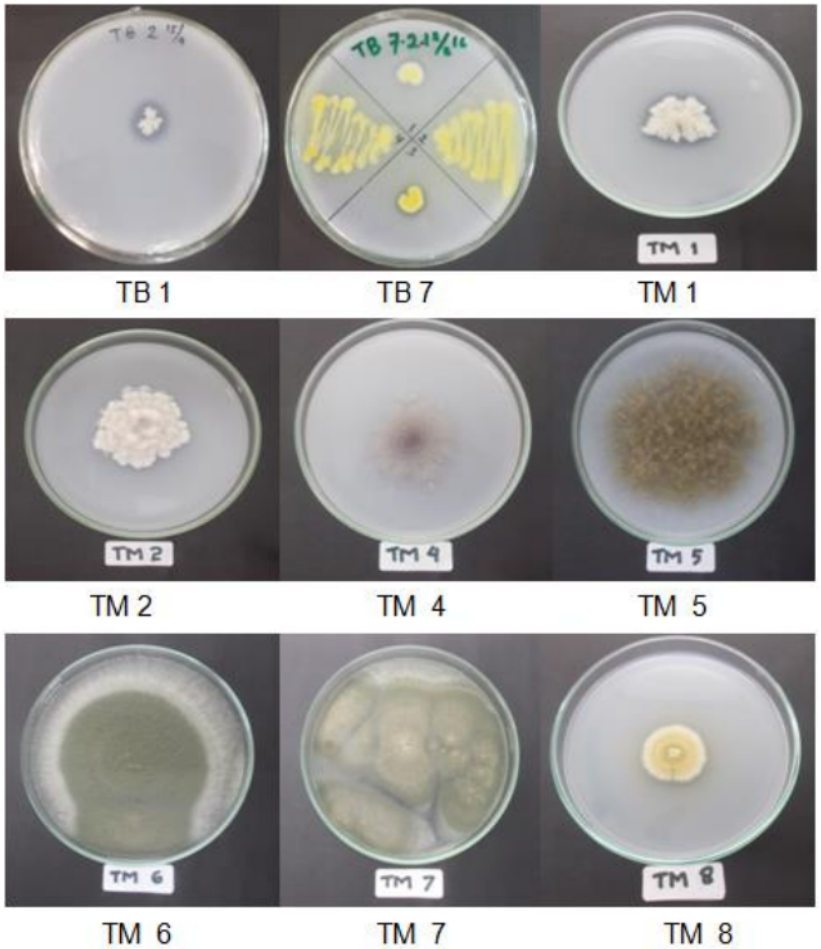

Figure 1. Phosphate solubilizing fungi isolates having clear zone of phosphate solubilization on Pikovskaya agar medium

TB 1 = *Acremonium kiliens,* TB 7 = *Aspergillus oryzae,* TM 1 = *Hymenella Fr.,* TM 2 = *Acremonium strictum,* TM 4 = *Moniliella acetoabutens,*TM 5 = *Mucor circinelloides,* TM 6 = *Eurotium herbariorum,* TM 7 = *Aspergillus fumigates,* TM 8 = *Neosartorya fischeri*

The presence of the organic acid compounds can be seen with the trend of decreasing the pH of liquid Pikovskaya media inoculated with the fungi isolates (Table 2). At day 5, the pH of the liquid Pikovskaya media inoculated with TB1, TM1, TM8 and TB7 isolates slightly decreased 1-3% compared to the pH of media without fungi isolates (Table 2).The pH of the liquid Pikoskaya media inoculated with TM2, TM4, TM5, TM6 and TM7 isolates tended to increase until day 5. The increase of soluble P in the media inculated with these four isolates, however, were much smaller than that inculated with TB1, TM1, TM8 and TB7 isolates.The pH decrease in phosphate solubilizing fungi liquid cultures resulted in this study was supported the study of El-Katatny (2004). Pradhan and Sukla (2005) reported that *Aspergillus* spp. showed much higher drop in pH and high P solubilization when compared to other genera.Therefore, any microorganism that acidifies its external medium will show some level of phosphate solubilizing activity.

Table 2. Soluble P and pH of the liquid Pikovskaya medium inoculated with nine isolates of phosphate solubilizing fungi

Isolates	Soluble P (ppm)					pH				
*)	D-1	D-2	D-3	D-4	D-5	D-1	D-2	D-3	D-4	D-5
M0	468.75	449.22	449.22	410.16	429.69	4.95	4.95	4.94	4.95	4.90
TB1	390.63	488.28	986.33	1767.58	2363.28	4.81	4.75	4.39	3.89	4.81
TB7	371.09	712.89	625.00	712.89	917.97	5.21	5.25	5.45	4.72	4.84
TM1	419.92	800.78	898.44	1396.48	2148.44	4.93	5.25	4.73	4.33	4.74
TM2	351.56	371.09	400.39	458.98	517.58	4.83	4.95	5.23	5.34	5.33
TM4	351.56	380.86	419.92	634.77	585.94	5.16	4.96	5.11	5.35	4.93
TM5	332.03	332.03	341.80	380.86	390.63	5.02	5.37	5.36	5.79	5.85
TM6	312.50	332.03	439.45	546.88	732.42	4.90	4.97	5.31	5.34	4.96
TM7	263.67	380.86	380.86	625.00	625.00	5.02	4.98	5.27	5.33	4.97
TM8	449.22	869.14	927.73	1318.36	1357.42	5.18	5.11	4.93	4.45	4.86

***) see Figure 1: D = day, M0 = media without inoculation of fungi isolates**

CONCLUSION

Sixteen fungi isolates potential for solubilizing phosphate were isolated from three types of oil palm empty fruit bunches. Four isolates were identified yeast and twelve isolates were identified as molds that were included in the genus of *Acremonium, Aspergillus, Hymenella, Mucor,* and *Moniliella*. Based on phosphate solubilization index and ability to solubilize phosphate in the Pikovskaya medium, isolates ofTB1(*Acremonium kiliens*), TB7(*Aspergillus oryza*), TM1(*Hymenella Fr.),* And TM8 (*Neosartorya fischeri*) were the most efficient phosphate solubilizing fungi that can potentially increase phosphate solubilization in Ultisols. The TB1, TM1, TM8 and TB7 were able to increase soluble P by 451%, 400%, 216% and 114%, repectively, at day 5. Further studies are being conducted with these fungal isolates to observe their potential to improve the productivity of plants grown on Ultisols.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest".

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AUTHOR CONTRIBUTIONS

GII and EH designed and performed the experiments and also wrote the manuscript. SF and YN reviewed the manuscript. All authors read and approved the final version.

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