Research article

Isolation and Identification of Potential Bio-Inoculants Based on Phosphate Solubilizing Molds From Different Plant Rhizospheres

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Abstract.

In crop production, phosphorus (P) is the second most important limiting nutrient. However, due to precipitation reactions with Al3+, Fe3+ in acidic soil, or Ca2+ in alkaline soil, its availability in soil is severely limited. Microbes have recently been proposed as a means of increasing the bioavailability of soil phosphate for plants. The goal of this research was to isolate and identify phosphate solubilizing molds (PSM) from various plant rhizospheres, including gadung (*Dioscorea hispida Dennst*), maize (*Zea mays* L.), bamboo (*Dendrocalamus asper*), pineapple (*Ananas comosus L.*), and banana (*Ananas indica L.*). PSM was isolated in vitro and then diluted using the dilution plate technique with Pikovskaya's solid medium. Five colonies were confirmed as PSM, namely *Talaromyces aculeatus, Metarhizium anisopliae, Fusarium proliferatum, Mucor hiemalis,* and *Aspergillus niger*, out of fourteen colonies formed from those rhizospheres. In the PVK solid medium, these isolates were capable of solubilizing insoluble P with a solubility range of 2.05 to 3.03. *Talaromyces aculeatus* (125.6 mg L-1), *Metarhizium anisopliae* (80.76 mg L-1) and *Fusarium proliferatum* (41.59 mg L-1) were the best P solubilizers, followed by *Mucor hiemalis* (9.51 mg L-1), and *Aspergillus niger* (7.85 mg L-1), respectively. The bioinoculants *Talaromyces aculeatus* and *Metarhizium anisopliae* had the most potential.

Keywords: *Dendrocalamus asper,* Molds, Phosphate, Rhizosphere, Solubilizer

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1. Introduction

Plant growth and development is basically affected by genetic, environmental and management factors. In this regard, the availability of plant nutrients including phosphorus was indispensable for a number processes in plants. Phosphorus (P) was observed as a second essential bioelements after nitrogen that plays an important roles in plants. It plays a significant role in metabolic processes including energy transfer, respiration, photosynthesis, signal transduction and fixation of nitrogen in legumes [1]-[2]. The absence of this nutrient may affect the root development and disturbe the flowering processes and seed formation [3]. Plants absorb this nutrient in the ionic form $H_2PO_4^-$ or HPO_4^{-2} from soil. However, its availability was strictly limited due to high retention in soil. In general, the lack of phosphorus in agricultural soil was adressed with P fertilizers, but unfortunatly large amount of P applied is transformed to a fixed state by reaction of precipitation with Fe3 + and Al3 + in acid soils and Ca2 + in calcareous soils [4]-[5], becoming a potential source of contamination by P [6]. Therefore, there is an urgent need to find an alternative solution that can dissolve the insoluble phosphate in the soil instead of adding more phosphorus [7].

It is well known that soil microorganisms can dissolve insoluble phosphorus in soil [8]-[9]. Microorganisms produce secondary metabolites, some of which have the ability to dissolve phosphate [10]. Mechanisms, such as acid production, ion chelation, and exchange reactions in the growth environment to lower pH, have been reported to play a significant role in the release of insoluble phosphate by microorganisms [11]-[12]. Several authors report that molds work better in acid soils [13]-[14], improving soil availability in poor soils, and produced more acid than bacteria exhibit higher P-solubilization activity [15]-[16]. Mold hyphae form a larger substrate in the soil, thus providing better plant nutrients [17]. Some genera of *Aspergillus, Aureobasidium, Curvularia, Fusarium, Penicillium* and *Trichoderma* has been reported were capable of improving soil P avaliability in soil [18]. Soil microorganism like, molds, have been studied successfully about various phosphate dissolution efficiencies [6], [19], However, these microorganisms are limited in the soil [7]. So, it is prominent to recognise the characters of microorganism and its adaptation in particular condition which allows to be used as a biofertilizers [20]. In addition, using of various of molds as biofertilizers should be selected from a given environmental conditions. According to the problem mention before, the purpose of this study is to identify the phosphate-solubilizing molds isolated from different plants rhizosphere that could be developed as bio-inoculants agents to improve soil fertility.

2. Methodology

The samples of Gadung (*Dioscorea hispida* Dennst) and Bamboo (*Dendrocalamus asper*) rhizosphere were randomly collected from agricultural area near of Kendari town. The sites are located between 04^o 01'10, 2" S and 122^o 31' 58, 7" E with an altitude of 48 masl and between 04^o 00' 33, 3" S and 122^o 31' 27, 9" E with an altitude of 44 masl, respectively. The samples of Pineapple (*Ananas comosus* L.) and Banana (*Musa, sp*) rhizosphere were taken from Field Experiment Station, Faculty of Agricultural, Halu Oleo University. Meanwhile, soil samples of maize (*Zea mays* L.) rhizosphere were obtained from farmer's field in Konda, Konawe Selatan Regency, approximately 25 Km from Kendari, located between 04^o 06'46,3" S and 122^o 27' 06,7" E with an altitude of 57 masl. Five composite rhizosphere samples of soil, up to 25 cm in deep, were collected from different environment soil conditions. Approximately 1 kg samples of soil was served for isolation and identification of phosphate dissolution molds (PSM). The samples were air-dried, grounded and sieved through a 2-mm sieve for physical and chemical analysis. Parameters assessed were; pH, soil texture, organic carbon, total nitrogen, phospore and potassium. Used a pH meter equipped with a glass electrode to measure the pH of the soil. Measurement of organic carbon was performed by wet digestion (oxidation) method [21]. Total-N, P and K available were analysed using standard method.

Phosphate-solubilizing molds isolation is performed under *in vitro* condition, followed by the dilution plate technique. Then, 10 g of soil sample was taken and dilute serially 10^{-6} times in distilled water. Approximately 0.1 ml of the inoculum sample was transferred to a Petri dish containing Potato Dextrose Agar (PDA) by spread plate method and then incubated at room temperature for 7 days. The isolate was subcultured twice until pure colonies were obtained for morphological identification. Identification of macroscopic and microscopic characters was conducted by following the standard procedure as described by [22]. Colonies morphology observed involve texture, surface and reverse appearance, upper and lower side colonies color, growth zone and diameter of colonies. Cell morphology observed was asexual spore, septate and aseptate hyphae, type and color of conidia, conidiophore in term of type, size and color, surface of conidiophore and phialides.

Test the phosphate solubility of the template isolate was used a needle tip and 4-quadrant staining to collect pure mold colonies on a sterile solid medium from Pikovskaya containing; 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4$, 5 g $Ca_3(PO_4)_2$, 0.002 g $MnSO_4$, 0.002 g $FeSO_4$, 10 g glucose 0.5 g yeast exstract, 20 g agar, 0.2 g NaCl

and 1000 mL distilled water [23] and then incubated at room temperature for 7 days. Observe until a clear area is formed around the mold colony, indicating that phosphate solubilization has occurred. After incubation, the clear zone formed around the colony were estimated using Solubilitation Index (SI) formula as described by [24] :

$$Solubilization\ Index\ (SI)\ :\ \frac{Halozone\ diameter\ +\ Colony\ diameter}{Colony\ diameter}$$

The potential colonies were further purified using Potato Dextrose Agar (PDA) and maintained in nutrient agar slants at 4^{o}C for the further studies [25].

The ability of the molds isolates to dissolve insoluble P was estimated through growing the selected isolates in Pikovskaya liquid medium and incubated for 7 days. Then, pH medium was estimated with a pH meter equipped with a glass electrode and then the culture medium was centrifuged at 3500 rpm for 15 minutes. The supernatant was used for P analysis using UV spectrophotometer with an absorbance of 880 nm [26].

The total of organic acid produced by phosphate solubilizers was analyzed in term of total titrable acidity of molds cultures [27]. Some selected isolates were inoculated in the Pikovskaya's liquid medium and incubated for 7 days. The molds culture was centrifuged and removed its cell biomass, pipetted 1 mL of culture filtrate and added 1% phenolphthalein and then titrated with 0.1 N sodium hydroxide. The titration was stopped after a change in color and the total acid was calculated.

Phenetic characters of mold isolates were analyzed using Multi Variate Statistical Package (MVSP) 3.1 version to find out the similiarity index between isolates versus references molds and the isolates were identified using *Illustrated Genera of Imperfect Fungi* [22]. All measurement (P soluble, pH and organic acid) were conducted in triplicate and a analyzed using Analysis of Variance (Anova) and continue analyzed usin'g LSD's tests for the differences means.

3. Result and Discussion

In the present study, a total of 14 molds isolates were observed in 5 composite rhizosphere soil samples from different plants namely gadung, bamboo, maize, pineapple and banana collected from agricultural area near of Kendari town and farmer field in Konda, Konawe Selatan Regency. Collected rhizosphere soil samples were isolated on PDA medium and incubated at room temperature for 7 days. The ability of the isolate to dissolve phosphate in PVK [23] solid medium was verified, which was combined with tricalcium phosphate (Ca3 (PO4) 5).

Out of 14 isolates, five isolated were confirmed as phosphate solubilizing fungi as shown by the apperence of halozone around the colonies in PVK solid medium incubated at room temperature for 7 days. The preliminary analysis macroscopic and microscopic characters revealed that these isolates were belonging of the genera *Talaromyces, Metarhizium, Fusarium, Mucor and Aspergillus*. Selected colony morphology of molds isolates was presented in Figure 1 and 2.

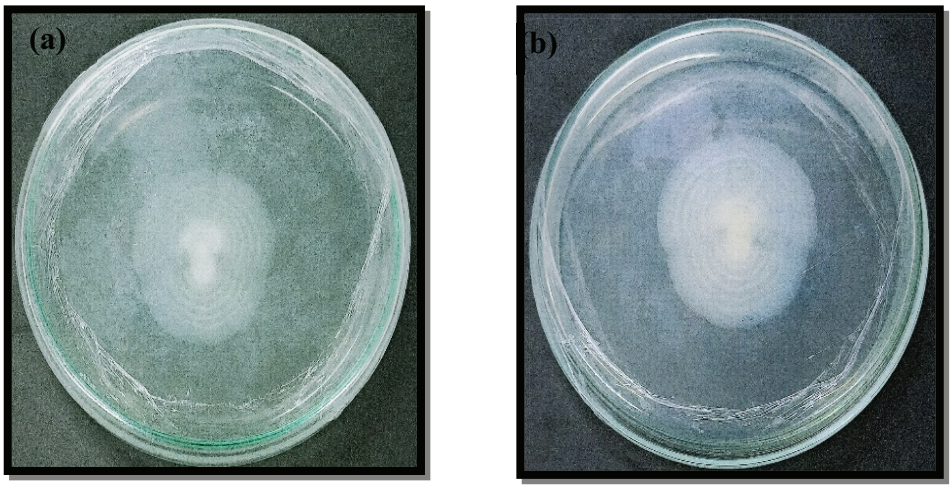

Figure 1: Colony morphology of *Mucor hiemalis* grown on PDA. Upper surface (a) and lower surface (b).

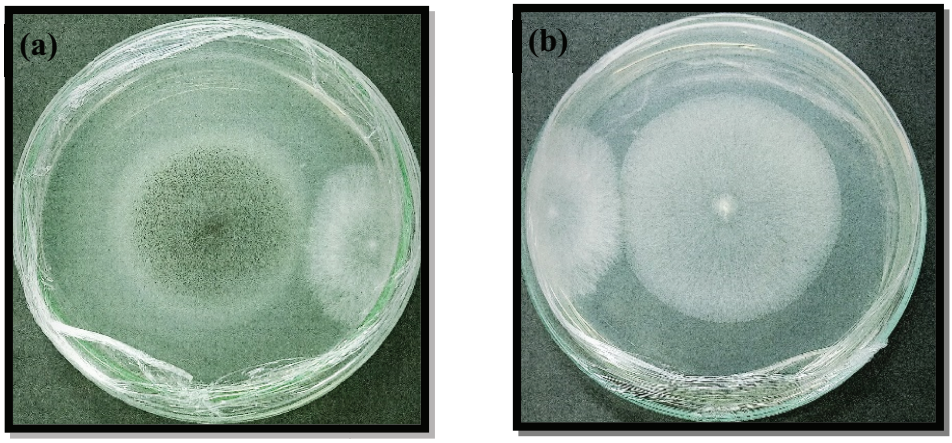

Figure 2: Colony morphology of *Aspergillus niger* grown on PDA. Upper surface (a) and lower surface (b).

Furthermore, the analysis of these characters was performed using Multi Variate Statistical Package (MVSP) 3.1 vesion to find out the similarity index between isolates versus references molds. Selected microscopic feature of molds isolates was presented in Figure 3 and 4. The result shown that these strains were identified as *Talaromyces acualeatus, Metarhizium anisopliae, Fusarium ploriferatum, Mucor hiemalis and Aspergillus niger* from gadung, bamboo, maize, pineapple and banana rhizosphere respectively. The different strain observed in the present study suggested that

the different variety and characteristic of the molds depend on many factors including substrate and the environmental conditions [28]-[31]. The different of physico-chemical properties of plant rhizospheres noted in the current study may be attributed to the variation of microbes associated with the plant roots. Another author reported that plant rhizospheres released some organic compounds such as organic acids, water soluble sugars, and amino acids, amino compounds, phenolics and sugar phosphate esters [32]. Different microbes living surrounding the rhizospheres indicated the different ecological conditions affecting the excudation and thereby rhizoshpere colonization by phosphate solubilizers [33].

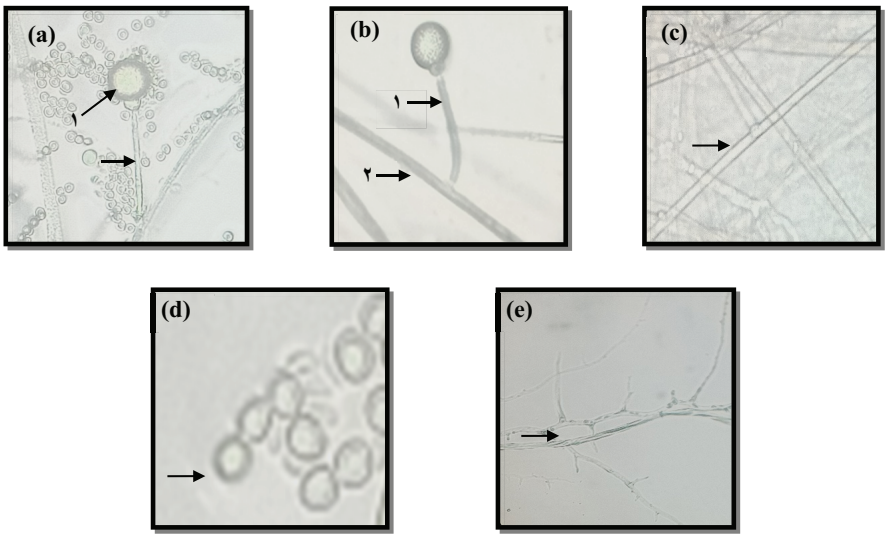

Figure 3: Microscopic features of *Mucor hiemalis* under compound microscope 400x. collumela (1a), sporangiophore (1b), hyphae (2b), non septate hyphae (1c), sporangia (1d) and stolon (1e).

The result shown that all isolates were capable of dissolving tricalcium phosphate by the appearance of clear zones on the isolates grown on Pikovskaya's solid medium after seven-days of incubation, see table 1. The solubilization index (SI) was varied between isolates. Solubilization index of these isolates ranged from 2.052 - 3.030. The maximum clear zone was observed by maize rhizosphere (M1) and the minimum was recorded by bamboo rhizosphere (B1).

Table 1: Solubilization index (SI) of different isolates in PVK solid medium.

No.	Plant Rhizosphere	Isolate Code	SI
1.	Gadung (*Dioscorea hispida* Dennst)	G1	2,405
2.	Bamboo (*Dendrocalamus asper)*	B1	2,052
3.	Maize (*Zea mays* L.)	M1	3,030
4.	Pineapple (*Ananas comosus* L.)	P4	2,163
5.	Banana (*Musa, sp*)	B1	2,083

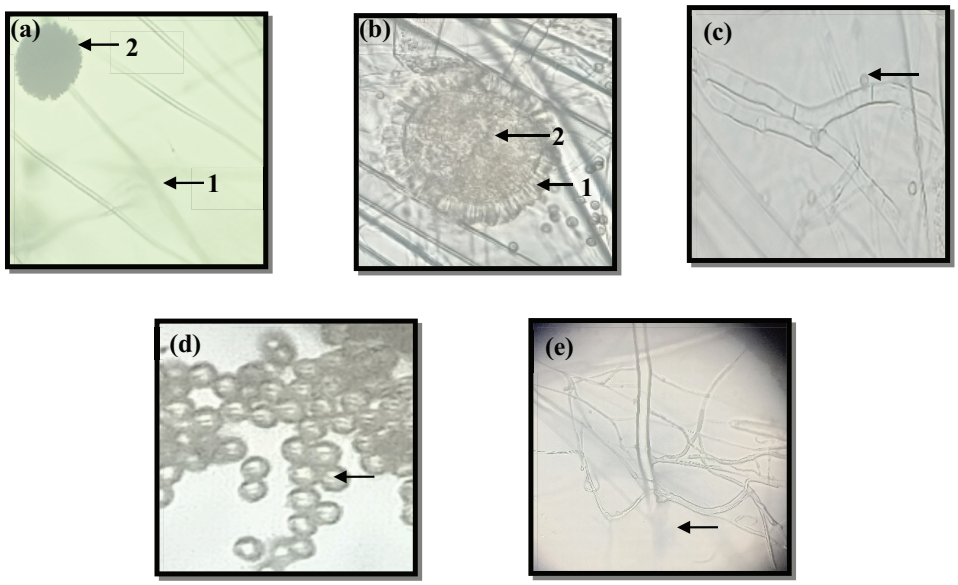

Figure 4: Microscopic characteristics of Aspergillus niger under a 400X compound microscope. conidiophore (1a), conidium (2a), fialid (1b), vesicle (2b), septate hyphae (1c), conidia (1d) and rhizoid (1e).

The different solubilization index may be attributed by the different soil sample physico-chemical properties, particularly pH and carbon content in soil. Some selected physico-chemical characteristics of three rhizosphere soil samples used in current research were: sandy loam in texture as described by USDA textural class triangle [34] and total-N was 0.09 %. The different characters was soil pH 5.7, total-C 2.13 %, P_2O_5 56 ppm and K_2O 84 ppm for gadung, and soil pH 6.29, total-C 0.96 %, P_2O_5 14 ppm and K_2O 65 ppm for bamboo, and soil pH 5.80, total-C 2.51 %, P_2O_5 64 ppm and K_2O 75 ppm for maize. It was observed that total carbon of maize rhizosphere was higher than others rhizosphere. The current result is according to the study reported by [32] that the capacity of microorganisms to dissolve phosphate mainly depends on the carbon-rich source of plant roots.

In the current study, it was investigated the decreasing of pH medium from the initial pH (6.7). The maximum dropping in pH was noted in G1 and M1 isolates, however, the decreasing of pH in both was not significantly different. The reduction of pH is correlated to organic acids release by P solubilizers molds in culture medium. This finding was according to be reported by the other workers [35]-[37]. It was observed that the solubilization of tricalcium phosphate was significantly different ($p<0.05$) after 7 days of incubations. The highest P solubilization was recorded by *Talaromyces aculeatus* isolates (125.60 ppm) from gadung rhizosphere, followed by *Metarhizium anisopliae* (80.75 ppm) from maize, and *Fusarium proliferatum* (41.59 ppm) from bamboo rhizosphere. The least was noted by *Mucor hiemalis and Aspergillus niger*. The increase in dissolution is consistent with the organic matter produced in the volume of NaOH titrated in the

medium after 7 days of culture, but the mean values were not significantly different, see table 2. The maximum organic production was noted by the molds isolated from gadung rhizosphere.

Table 2: Available-P, pH medium, and total organic acid in term of NaOH titrated after 7-days incubation.

Isolate Code	Molds Strain	pH medium	P-available (ppm)	Total organic acid (0.1N NaOH titrated)
		day-7		
G1	*Talaromyces aculeatus*	5.30a	125.60d	0.80a
B1	*Fusarium proliferatum*	5.49a	41.59b	0.53a
M1	*Metarhizium anisopliae*	5.42a	80.75c	0.73a
P4	*Mucor hiemalis*	5.64a	9.51a	0.75a
B1	*Aspergillus niger*	5.87a	7.85a	0.60a

Note : All values were the mean of 3 replicates. The mean values after the same letter in the column were not significantly different according to the LSD test ($p<0.05$).

In addition, a positive correlation was established between the release of P and the production of organic acids ($r = 0.67$). Some authors report that the P release mechanism was closely related to the release of organic acids from phosphate fungi solubilized. These fungi form chelates with cations (Al, Fe or Ca) and bind to P through hydroxyl chelation. and carboxyl, thus converting into available forms [9]; [38]. Another workers investigated that organic acids plays an important role in P solubilizing include oxalate, tartaric, citric, succinic, gluconic acids [38]-[39].

4. Conclusion

The current study concluded that different species of molds species was observed around the rhizosphere of gadung, bamboo, maize, pineapple and banana. All of molds selected were capable to dissolve insoluble-P under in vitro conditions, however, the amount varied between the isolates. The higher P-solubilizer molds was noted by *Talaromyces aculeatus* and *Metarhizium anisopliae* and these strains were the most potential to be proposed as a bio-inoculant in order to improve the soil fertility.

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