Cultivable Endophytic Fungi Producing Phosphatase of Rhizophora mucronata

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Abstract. Low availability of phosphorus (P) is a major constraint for optimal crop production as in the soil it is mostly present in insoluble form. Therefore, the availability of phosphate in the soil can be improved by the utilization of phosphate-solubilizing microorganisms. The existence of Rhizophora mucronata as the dominant species in the Wanatirta mangrove forest, Kulon Progo is estimated to be inseparable from the role of endophytic fungi that produce important compounds for the growth and development of host plants including production of phosphatase enzyme. This study aimed to determine the diversity of endophytic fungi from R. mucronata root and to evaluate their potential in producing phosphatase enzyme. Isolation of endophytic fungi was conducted by growing the pieces of the root on the Pikovskaya medium containing insoluble phosphate, namely calcium phosphate (Ca₃(PO₄)₂). Endophytic fungal isolates were purified on Potato Dextrose Agar medium. The screening test of phosphatase activity was performed to determine the ability of each isolate to produce phosphatase enzymes. The characterization of endophytic fungi isolates was carried out by observing the microscopic and macroscopic appearance of each isolate and then comparing it with the fungi identification book. The results obtained six endophytic fungi isolates of R. mucronata root, namely U2A, T2A, T3B, P2, P3A and P3B. The P2 isolate displayed a phosphatase activity characterized by the formation of a clear zone around the fungi isolate with the phosphate solubility index (PSI) is 2.04. Based on its morphological characteristics, P2 isolate belongs to the genus Penicillium.

Keywords: Endophytic fungi; Mangrove; Phosphatase; Root of Rhizophora mucronata

1. Introduction
Phosphate is the second essential element after nitrogen [1] which plays an important role in plant growth and development. Soil phosphate (P) is classified into inorganic and organic P, and 60–80% of the total P is inorganic P, since P is an element of the sedimentary cycle [2]. However, its present in an insoluble form resulted in it cannot be directly used by plants because most of the phosphate ions is absorbed by colloidal soil reducing their mobility in the soil [3]. Low availability of phosphorus (P) is a major constraint for optimal crop production as in the soil it is mostly present in insoluble form [4]. The use of natural phosphate-bearing materials such as rock phosphate (RP) as fertilizer for P-deficient soils has received due attention in recent years since substantial deposits of cheaper and low grade RP are locally available in many countries of the world. Conventionally, RP is chemically...
processed by reacting with sulphuric acid or phosphoric acid to produce partially acidulated RP [5]. This acidification of rock phosphates (RPs) with strong acid for production of phosphate fertilisers is high cost and makes the environmental health worse [6]. Therefore, the availability of phosphate in the soil can be improved including by the utilization of phosphate-solubilizing microorganisms to support the growth and development of plants [7]. In recent years, microbial solubilisation of RPs has gained increasing attention, since this approach is simple, economic, and environmental friendly. Bio-solubilisation of RPs is based on the ability of autotrophic and heterotrophic microorganisms to recover P from insoluble phosphate compounds [6]. Biologically dissolved phosphates occur in the process of mineralization of organic matter, i.e., organic phosphate compounds are broken down into inorganic phosphates available to plants with the help of the enzyme phosphatase. The phosphatase enzyme is secreted by plant roots and endophytic microorganisms (Joner et al., 2000) in response to the low availability of phosphate in the soil. Among the P-solubilising microorganisms, filamentous fungi, especially black Aspergilli including A. aculateus, A. awamori, A. niger, A. tubingensis, as well as Penicillium have shown potential for solubilisation of insoluble P compounds. The phosphate solubilising ability of fungi has been accompanied with acidification, complexolysis, and acid phosphatase production [6]. Phosphate solubilising fungi (PSF) are able to reach greater distance and show good attachment to insolubilised P particles as a result of its hyphal structure compared to bacteria and actinomycetes, which do not form hyphae [8]. Phosphate-solubilizing fungi have been reported obtained from different ecological niches such as agricultural fields, arctic region, forest, mangrove, mine areas, vermicompost, etc (Sahoo & Gupta, 2014). Microbial communities, including fungi, possess the ability for phosphate solubilization and mineralization. In soil, P-solubilizing fungi about 0.1-0.5% of the total fungal population. Among them endophytic fungi are the major contributors.

Attention to endophytic fungi has been increased in recent years due to its potential to increase the uptake of plant nutrients, fight pathogen infections, and as a source of secondary metabolites. The potential of endophytic fungi in producing phosphatase has been revealed in various studies, especially in mangrove ecosystems. Khastini et al., (2015) has successfully isolated as many as 20 isolates of root endophytic fungi from the mangrove ecosystems of Pulau Dua Nature Reserve in Banten and as many as 4 isolates showed the activity of phosphatase. Elfiati et al., (2016) succeeded in isolating the phosphate solvent fungi from 4 different areas including the mangrove forest area. Fungi isolates from the four regions positively showed the activity of the phosphatase enzyme with a variety of phosphate solubility indexes. Sawitri (2012) states that Rhizophora mucronata is one of seven mangrove species that contribute greatly to the Wanatirta mangrove ecosystem, Kulon Progo. The existence of these dominant species, certainly not separated by the role of endophytic fungi producing important compounds for the growth and development of host plants. So it is necessary to explore endophytic microorganisms through isolation and characterization of R. mucronata root endophytic fungi to determine the diversity and potential in producing phosphatase enzymes. The present work focuses on isolation and identification of potent endophytic fungi solubilizing-P under in vitro condition through production of phosphatase enzyme.

2. Methodology

2.1 Isolation of endophytic fungi producing phosphatase enzyme from R. mucronata root.

The plant root samples of R. mucronata were collected from Wanatirta Mangrove Forest in Kulonprogo, Yogyakarta. Samples were collected in the form of pieces from the roots of the base, middle and tip of R. mucronata. Samples were put in plastic bags and stored in the ice box. R. mucronata root endophytic fungi were isolated by the direct planting technique proposed by Nakagiri et al., (2005). Plant roots were washed in tap running water for removing excess soil. The root pieces were then sterilized following washing in 70% ethanol (2 min), rinsing with sterilized water (1 min), immersing in 1% solution of NaOCl 1% (2 min), and finally rinsing 3 times in sterilized water. The roots were dried using sterilized tissue paper and then cut into small pieces with a length of 1 cm x 1 cm. The roots then were further processed for isolation of endophytic fungi producing phosphatase on Pikovskaya medium, incubating at room temperature for 3-7 days. The pure fungal culture were
characterized on the basis of phenotypic (colony morphology and microscopy). The fungal endophyte were maintained on PDA slants at 4 °C (Adhikari & Pandey, 2019).

2.2. Comparison of phosphatase activity.
The potential of endophytic fungi from *R. mucronata* root in producing phosphatase can be determined by comparing the clear zone size formed by each fungi isolate. The clear zone was determined by measuring of the diameter of the colony and the diameter of the clear zone 2-3 times using the calipers in different positions, then the measurement results are averaged. The phosphate solubility index is calculated using the formula according to Islamiati & Zulaika (2015):

\[
\text{Phosphate Solubility Index} = \frac{dk + dzb}{dk},
\]

where \(dk\) is colony diameter and \(dzb\) is clear zone diameter.

2.3. Characterization of endophytic fungi producing phosphatase enzymes.
Purified fungi isolates were then observed morphologically and microscopically. Macroscopic fungi morphological observations was conducted by observing the colony's color and surface, texture, growth area, radial and concentric lines, and exudate drops. The microscopic fungi morphological observations is done by observing the presence of spores, the shape of spores and the presence of septa in hyphae under a microscope. The results of macroscopic and microscopic fungi morphological observations were then compared with literature or monographs to determine the fungal identity (Hafsari & Pertiwi, 2017).

3. Result and Discussion
There were six isolates of endophytic fungi obtained from *R. mucronata* root. The isolates were: U2A, T2A,T3B, P2, P3A, and P3B.

![Fungal isolates of R. mucronata roots on PDA media: (a) U2A; (b) T2A; (c) T3B; (d) P2; (e) P3B; and (f) P3B](image)

Each microorganism has different capacity in P solubilization and in this study it was only P2 that having capability in solubilizing P by phosphatase production. It was indicated by the formation of clear zone around of the isolate. P2 isolate has a medium capacity in solubilization of P (Marra et. Al., 2011).
Figure 2. Clear zone formation in the screening of fungal endophytes (x)

Table 1. The measurement of clear zone

| Av. Diameter of colony (cm) | Av. Diameter of clear zone (cm) | Phosphat Solubility Index (SPI) |
|-----------------------------|---------------------------------|--------------------------------|
| 2.62                        | 2.73                            | 2.04                           |

Table 2. Level criteria of Phosphate Solubility Index [10]

| No | Criteria | Phosphat Solubility Index (SPI) |
|----|----------|---------------------------------|
| 1  | Low      | < 2.00                          |
| 2  | Medium   | 2.00 ≥ SPI ≥ 4.00               |
| 3  | High     | > 4.00                          |

Figure 3. Microscopic observation of P2 isolate (a) conidia; (b) phialide; (c) metula; (d) conidiophore; (e) hyphae

P2 was identified as Penicillium. Its colony is yellow-in shades of green. The surface of the colony is velvety smooth or sometimes like cotton and emits yellow exudates (Akmalasari et al., 2013). It has conidiophores which form vesicles at the tip with varying amounts depending on the species. It has hyphae-septa, phialide and conidia with a round or ovoid cell (Barnett & Hunter, 1998).

The major endophytic P-solubilizing fungi belong to the genera Penicillium, Aspergillus and another class of endophytic symbionts arbuscular mycorrhizal (AM) fungi. P-solubilizing endophytic fungi are more competitive and aggressive colonizers than non-endophytic microbes (Mehta, Sharma, Putatunda, & Walia, 2019). Endophytic microorganisms are greater in producing phosphatase. It has a significant role in rhizosfer area, such as as solubilizing P agent causing the increase of P availability in andisol soil that affected by Merapi’s eruption (Suandi et al., 2015).

4. Conclusion

One out of six endophytic fungi from *R. mucronata*, P2 isolate, showed its ability in producing phosphatase. Based on morphological characterization, P2 was identified as Penicillium.
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