Pythium ultimum and Phytopythium vexans, 
the Potential Pathogen Isolated from 
Potato Rhizosphere in Central Java, Indonesia

Pythium ultimum dan Phytopythium vexans, Patogen Potensial yang Diisolasi dari Risosfer Kentang di Jawa Tengah Indonesia

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ABSTRACT

Phytophthora and Pythium are a group of Oomycetes that are widely associated with diseases in potato plants. Therefore, this study was conducted to identify the Oomycetes associated with the rhizosphere of infected potato plants showing leaf blight. Four isolates were collected from four regions in Central Java (UGM_St_TM, UGM_St_BK, UGM_St_BNJ, and UGM_St_KJ, the isolate from Temanggung, Bakal, Banjarnegara, Kejajar respectively), and UGM_St_NG isolate as culture collection from Laboratory of Plant Pathology. Molecular identification of all isolates was carried out using the internal transcribed spacer (ITS1/ITS4), nuclear large-ribosomal subunit (LSU), and cytochrome C oxidase subunit 1 (COX1) gene markers. Based on the results, the isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were identified as Phythium ultimum while UGM_St_KJ and UGM_St_NG isolate were identified as Phytopythium vexans.

Keywords: gene marker, identification, morphology characters, oomycetes
INTRODUCTION

Potato (Solanum tuberosum) is an important horticultural commodity with high economic value (Asgar 2013). Infection of fungal pathogens has been reported worldwide as one of the limiting factors for potato production. Phytophthora infestans from the class Oomycetes is known as the primary pathogen in potatoes that causes leaf blight which may lead to yield losses of up to 100% (Nathasia et al. 2014). Phytophthora is a new genus of Oomycetes that has been reported to cause disease in many crops (Baten et al. 2015). The genus Phytopythium belongs to the family Peronosporaceae and to the order Peronosporales (Beakes et al. 2014). It has similar morphological and physiological characteristics to the genera Pythium and Phytophthora, such as having a mechanism of zoospore release similar to Pythium, producing papillary sporangium with a globose to ovoid shape, and the presence of internal proliferation as in Phytophthora (Bala et al. 2010). de Cock et al. (2015) also reported that the genus Phytopythium was grouped between Pythium and Phytophthora based on phylogenetic analysis using ITS, LSU, SSU, and COI markers.

Phytopythium vexans was first reported as the cause of root rot in kiwifruit in Turkey (Polat et al. 2017), while Santosso et al. (2015) also reported an association of P. vexans and a disease in durian plants in Indonesia. Later on, Santika et al. (2021) identified P. vexans from the rhizosphere of potato plants in Ngablak, Magelang, Central Java. Therefore, this study aims to identify the Oomycetes from the potato rhizosphere associated with rot disease and determine its ability to infect potato plants.

MATERIAL AND METHODS

Exploration and Sample Collection

A disease survey was conducted in August 2020 in several potato-growing areas in Central Java, including Kledung (Temanggung), Bakal (Banjarnegara), Pejawaran (Banjarnegara), and Kejaraj (Wonosobo). Soil samples were taken at a 5–10 cm depth around the rhizosphere of potato plants showing leaf or stem blight symptoms. In addition, the isolate collection of the Laboratory of Plant Pathology, Universitas Gadjah Mada from Ngablak, Magelang (isolates UGM_St_NG) was included in this study. Nucleotide sequences of this isolate have been submitted to GenBank, i.e., MW898226 (ITS) and MW911663 (LSU).

Fungal Isolation and Purification

Fungal isolation was carried out following the modified soil baiting method (Santoso et al. 2015). Apples were the first surface disinfected using 70% alcohol and perforated using a cork borer (0.5 cm in diameter) at four opposite points. The hole was then filled with soil samples and covered with tape. Thereafter, incubation was carried out in containers at room temperature. Observations were made every day until the initial symptoms of soft brown spots appeared (more or less 2–3 days after inoculation). Symptomatic tissues were cut out at the border between healthy and symptomatic parts, around 2 × 3 mm in size, and then isolated on potato dextrose agar (PDA) medium and incubated for 2–3 days. Furthermore, the fungi that grew from the slices were transferred to a water agar (WA) medium and cultured as a pure culture. The morphology of each isolate, including sporangium, hyphae, chlamydospore, and colony shape, was observed according to Abad et al. (2019) 7 days after the incubation period.

DNA Extraction, PCR Amplification, and Sequencing

Five days old fungal cultures were cut using a scalpel and weighed up to 0.5 g extraction and amplification of DNA were carried out using the CTAB procedure (Doyle and Doyle 1990) and modified multigenic analysis method, respectively, based on polymerase chain reaction (PCR) using ITS1/ITS4 (Ochoa et al. 2012), LSU (Schurko et al. 2003), and COX1 (Martin and Tooley 2003) as shown in Table 1. Each amplification reaction was carried out in a total volume of 13 μL, which was composed of 1 μL DNA template for each isolate, 6.5 μL of PCR mix (My Taq HS Red Mix: Geneaid), 4.5 μL of dDH2O, and 0.5 μL each of primers pair. The PCR products were loaded into 1%
Table 1 Primers used for PCR amplification and DNA sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>ITS 1</td>
<td>TCCGTAGGTGAACCTGCGG</td>
<td>Ochoa et al. (2012)</td>
</tr>
<tr>
<td>ITS</td>
<td>ITS 4</td>
<td>TCCTCCGCTTTATGATATGC</td>
<td>Schurko et al. (2003)</td>
</tr>
<tr>
<td>LSU</td>
<td>UN-up28S40</td>
<td>5-GCATATCAATAAGCGGAGGAAAAG-3</td>
<td></td>
</tr>
<tr>
<td>COX1</td>
<td>OomCoxILevup</td>
<td>5-TCAWCMGATGCGTACACACTAC-3</td>
<td>Martin and Tooley (2003)</td>
</tr>
<tr>
<td>COX1</td>
<td>Fm85mod</td>
<td>5-RRHWACKTGACTDATRATACAAA-3</td>
<td></td>
</tr>
</tbody>
</table>

agarose gel (w/v), electrophorized with a voltage of 55 V for 50 minutes, and visualized with UV light after ethidium bromide staining. DNA sequencing analysis was carried out at the Integrated Laboratory for Researching and Testing of Gadjah Mada University, Yogyakarta.

Phylogenetic Analysis
Sequence data from the Oomycetes isolates were aligned and compiled using the ClustalW method. The results were used as input data to construct phylogenetic trees using the maximum likelihood method with 1000 bootstrap values. Furthermore, the phylogenetic tree was generated using Mega-X software (Kumar et al. 2016). The DNA sequence data were used to analyze the isolates’ similarities compared to data from GeneBank using the NCBI BLAST-N program (Basic Local Alignment Search Tool) on [http://www.ncbi.nih.gov/BLAST](http://www.ncbi.nih.gov/BLAST) (Table 2).

RESULTS

Morphological Characteristics of Isolate
There were 4 isolates successfully collected from the potato rhizosphere, namely UGM_St_TM (Kledung-Temanggung), UGM_St_BK (Bakal-Banjarnegara), UGM_St_BNJ (Pejawaran-Banjarnegara), and UGM_St_KJ (Kejajar-Wonosobo). All of the isolates, including UGM_St_NG (laboratory collection), showed rapid growth on the PDA medium or with an average of 3–4 days after incubation. General characteristics observed from all isolates were white colonies, aseptic hyphae, and thick-walled chlamydospores (Figure 1). Moreover, the isolates of UGM_St_TM, UGM_St_BK, and UGM_St_BNJ had cottony-shaped colonies, while UGM_St_KJ and UGM_St_NG had chrysanthemum and rosaceous stellate-shaped colonies, respectively. Sporangium grew on tenth day after incubation at 25 °C. While UGM_St_TM, UGM_St_BK, and UGM_St_BNJ isolates produced papillary and non-papillary sporangium with globose, ovoid, and obovoid shapes, UGM_St_NG isolate produced no sporangium. The morphological characteristics of isolates are presented in Figure 1 and Table 3.

Analysis of Phylogenetic Tree
Maximum likelihood analysis based on ITS, LSU, and COX1 with a bootstrap value of 1000 was presented in a phylogenetic tree (Figure 2). Redundant sequences of GenBank accessions were identified, and those with 100% identity to other included taxa were removed from the analyses. These duplicates are cataloged in Table 2. The cladogram showed that the isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were in the same group as other P. ultimum isolates. In contrast, the isolates UGM_St_KJ and UGM_St_NG (MW898226) were found in the group of P. vexans. Based on the LSU analysis, isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ have the closest relationship with P. ultimum from Japan (AB513047); while UGM_St_KJ and UGM_St_NG (MW911663) isolates have the closest relationship with P. vexans from Canada (HQ665090) and Iran (MT729990), respectively. Furthermore, phylogenetic analysis based on COX1 showed that isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were closely related to P. ultimum from the Netherlands (HG708919) and America (KF761145), while the isolates of UGM_St_
KJ and UGM_St_NG were closely related to *P. vexans* from Italy (MN510424). The phylogenetic tree analysis based on the three primers showed that UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were identified as *P. ultimum* with a similarity level of 99%–100%. Meanwhile, UGM_St_KJ and UGM_St_NG were identified as *P. vexans* based on ITS and LSU with a similarity level of 99%–100%, and based on COX1 with a similarity of 98%–99% (data not shown) (Table 4).

**DISCUSSION**

Five *Oomycetes* isolates from Central Java potato fields had similar morphological characteristics. The hyphae were generally hyaline and aseptate, while the chlamydospore had thick walls and was located intercalary and terminally. In addition, the isolates mostly had a globose to ovoid shape of the sporangium, while colony morphology varied from cottony to chrysanthemum and rossaceous-stellate. Therefore, morphological characteristics observed in this study were unable to distinguish the presence of different genus between the isolates. These morphology characters were in line with the characteristics of *Pythium* and *Phytopythium* described by Bala *et al.* (2010) and Uzuhashi *et al.* (2010). Other references showed that *P. vexans* produced stellate colonies when incubated at 18 °C on a PDA medium (Santika *et al.* 2021). *P. vexans* and several types of *Pythium* had a cottony aerial colony with a spreading pattern when grown on V8, PDA, and corn meal agar (CMA) medium at 25 °C (Nam and Choi 2019).

Further molecular identification of these *Oomycetes* isolates was carried out to observe their genetic relationship. The use of ITS, LSU, and COX1 as molecular markers for
Figure 1  Morphology of fungal isolates. a, e, i, m, q are colony patterns; b, f, j, n, and r are aseptate hyphae; c, g, k, o are sporangium; d, h, l, p are terminal and intercalary chlamydospores with thick walls. Bar scale 50 µm.

*Pythium* and *Phytophthium* has been widely reported. The markers have been used to identify and characterize, among others *P. vexans* in Vietnam (Thao et al. 2020), *Pythium* spp. from soil samples in Illinois, America (Radmer et al. 2017), and a new species of *Phytophthium* and *Pythium* in Korean waters (Nam and Choi 2019).

*Pythium ultimum* and *P. vexans* species have been identified from the rhizosphere of potato plants in Central Java by using three different molecular markers. Both species had the same microscopic morphological characteristics, such as sporangium, hyphae, and chlamydospore, but had different colony forms. The temperature was one of the factors...
Figure 2  Maximum likelihood phylogenetic trees of: a, ITS; b, LSU ribosomal RNA region; and c, cytochrome c oxidase subunit 1 COX. Maximum likelihood 1000 bootstrap.
Table 3  Morphological characteristics of *Oomycetes* isolates from Central Java

<table>
<thead>
<tr>
<th>Characters colony</th>
<th>UGM_St_TM</th>
<th>UGM_St_BK</th>
<th>UGM_St_BNJ</th>
<th>UGM_St_KJ</th>
<th>UGM_St_NG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pattern</td>
<td>cottony</td>
<td>cottony</td>
<td>cottony</td>
<td>Rossaceous-stellate</td>
<td>Chrysanthemum</td>
</tr>
<tr>
<td>Color</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Sporangium</td>
<td>25.69 ± 8.22 ×</td>
<td>25.23 ± 2.94 ×</td>
<td>24.36 ± 4.49 ×</td>
<td>25.52 ± 3.21 ×</td>
<td>No present</td>
</tr>
<tr>
<td>Size 1 × w (µm)</td>
<td>21.10 ± 6.72</td>
<td>23.00 ± 2.23</td>
<td>19.29 ± 2.23</td>
<td>23.00 ± 3.57</td>
<td></td>
</tr>
<tr>
<td>Description</td>
<td>Globose to ovoid with papillae</td>
<td>Globose to ovoid semi-papillae</td>
<td>Globose and non-papillary obovoid</td>
<td>Globose non-papillary and ovoid with papillae</td>
<td></td>
</tr>
<tr>
<td>Hyphae (µm)</td>
<td>4.19 ± 0.60</td>
<td>3.26 ± 0.79</td>
<td>3.60 ± 0.78</td>
<td>4.47 ± 1.37</td>
<td>4.07 ± 0.59</td>
</tr>
<tr>
<td>Chlamydospore</td>
<td>21.38</td>
<td>21.71</td>
<td>18.24</td>
<td>15.71</td>
<td>17.21</td>
</tr>
<tr>
<td>Size 1 × w (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Description</td>
<td>intercalar chlamydospores with thick walls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Isolate collection Laboratory of Plant Pathology Gadjah Mada University*

Table 4  Identification of isolates from potato plants based on analysis of the phylogenetic relationship

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Origin</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGM_St_TM</td>
<td>Kledung</td>
<td><em>Pythium ultimum</em></td>
</tr>
<tr>
<td>UGM_St_BK</td>
<td>Bakal</td>
<td><em>Pythium ultimum</em></td>
</tr>
<tr>
<td>UGM_St_BNJ</td>
<td>Pejawaran</td>
<td><em>Pythium ultimum</em></td>
</tr>
<tr>
<td>UGM_St_KJ</td>
<td>Kejajar</td>
<td><em>Phytopythium vexans</em></td>
</tr>
<tr>
<td><em>UGM_St_NG</em></td>
<td>Ngablak</td>
<td><em>Phytopythium vexans</em></td>
</tr>
</tbody>
</table>

*Isolate collection Laboratory of Plant Pathology Gadjah Mada University*

that influenced the growth of pathogens. The level of pathogenicity and resistance was influenced by the potato variety used. Potato varieties M07 and Grandia showed a lower level of resistance than the Granola variety.

**REFERENCES**


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