INTRODUCTION

Onion (Allium ascalonicum L.) is a vegetable commodity that has high economic value. Onion has great economic importance due to its vegetable, spice, and medicinal values. Onion cultivation still has various problems, one of the technical problems in onion cultivation is plant disease. Plant diseases play an important role in decreasing production and quality. Purple blotch disease caused by Alternaria porri (Ell) Cif is the most destructive foliar disease in onion and other Alliaceae families (Kareem, Murthy, Hasanbab, & Waseem, 2012). Disease symptoms first appear as water-soaked lesions with small white centers, elliptical, in high humidity, the lesions enlarged and turn brownish. Furthermore, these lesions later become purplish with a darker margin surrounded by light yellow concentric rings. A. porri destructs the leaf tissue and reducing foliar production by 62-92%, necrotic lesions in the leaf tissues which interferes bulb initiation, furthermore delaying bulb formation and maturation. Moreover, it caused plant wilting and death (Black, Conn, Gabor, Kao, & Lutton, 2012). The average onion production in Indonesia only 9.29 t/ha, while the potential production can reach up to 20 t/ha (Central Bureau of Statistics, 2017). The productivity of onion is affected by several factors including plant disease. Production losses due to A. porri up to 80-100% (Abo-Elyousr, Abdel-Hafez, & Abdel-Rahim, 2014).

The control of purple blotch relies heavily on synthetic fungicides such as mancozeb, propineb and difenoconazole (Priya, Sataraddi, & Darshan, 2015). Fungicides are applied as a foliar spray to control A. porri but the present-day fungicides have failed to control the disease. This may be due to the arising genetic variability or introduction of new ANTIFUNGAL ASSAY OF ENDOPHYTIC FUNGI AS BIOCONTROL OF ONION PURPLE BLOTCH DISEASE CAUSED BY Alternaria porri (Ell) Cif In Vitro

Wita Firdausi, Liliek Sulistyowati* and Luqman Qurata Aini

Plant Pest and Disease Department, Faculty of Agriculture, Universitas Brawijaya, Malang, East Java, Indonesia

ARTICLE INFO

Keywords:
- Allium ascalonicum
- Antagonism
- Antifungal activity
- Biocontrol
- Endophytic fungi

ABSTRACT

Purple blotch disease caused by Alternaria porri is the main destructive foliar disease of genus Allium, causing significant losses in yield of the crops. Recently, purple blotch disease is controlled by synthetic fungicides. However, fungicides have negative effects on the environment. Endophytic fungi can be used as an alternative control because a close symbiosis with the internal tissue of the host can minimize competition in new and complex ecosystems. This study aimed to explore and identify endophytic fungi that have the highest inhibition ability against A. porri and investigate the antagonistic mechanism. The method used in this study is an exploration of endophytic fungi, isolation of A. porri, in vitro antagonism tests, observation of the antagonistic mechanism, extraction of crude protein, SDS-PAGE, and identification. The antagonistic fungi that had the highest inhibition ability were identified as Penicillium citrinum with an inhibitory of 60.04%. Crude protein extracted from P. citrinum which showed inhibitory activity against A. porri is saturation level of ammonium sulfate 80% with a molecular weight of 40 kDa. This study implies that P. citrinum can inhibit the growth of A. porri through its anti fungi compounds. Further in vivo assays or field trials will need to be conducted in future studies.

ISSN: 0126-0537 Accredited First Grade by Ministry of Research, Technology and Higher Education of The Republic of Indonesia, Decree No: 30/E/KPT/2018

races of the pathogen. The use of pesticides incurs high production costs, the emergence of resistant pathogenic strains, and the onion bulbs are at risk of containing pesticide residues (Bayoumi, Taha, Shalaby, Alshaal, & El-Ramady, 2019). Therefore, it is necessary to find alternative techniques to control purple blotch disease that are effective but environmental-friendly. Currently, many beneficial microorganisms are studied by many researchers to manage different plant diseases. Among the beneficial microorganisms which are used for biocontrol of plant diseases are endophytic fungi. Endophytic fungi colonize and grow asymptomatically within healthy plant tissues. The mutualism relationship between endophytic fungi and their host plants is that the host cell obtains protection against pathogens from compounds produced by endophytes while endophytic fungi obtain nutrients from the host. Entanglement endophyte with their hosts has the more beneficial endophyte compared to other biological agents for minimizing competition in the new and complex ecosystem. The mechanism of this biological control is based on ecological interactions, such as competition for space and nutrients, antibiosis, parasitism, and induction of plant defenses. The advantages of endophytic fungi apart from being antibiosis mechanisms (Lugtenberg, 2018). Previous studies have only focused on the inhibition ability of antagonistic fungi against A. porri. Furthermore, there were no reports of antagonistic mechanisms and potential antifungal substances produced by endophytic fungi against A. porri. This study aimed to identify the endophytic fungi that have the ability to inhibit A. porri, in addition to observing the antagonistic mechanism and the potential of antifungal substances produced by the endophytic fungi against A. porri.

MATERIALS AND METHODS

The study was conducted at the Plant Disease Laboratory 2, Faculty of Agriculture, and Animal Disease and Diagnostic (ADD) Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia from September 2018-February 2020.

Isolation of Endophytic Fungi

Endophytic fungi were isolated from the roots, stems, and leaves of healthy onion plants. Samples were sterilized in 2% sodium hypochlorite (NaOCl) solution for 1 minute, 70% alcohol solution for 30 seconds, rinsed with sterile distilled water then cut into 1 cm, and grown on a PDA medium.

Isolation of A. porri

Alternaria porri was isolated from onion infected with purple blotch disease. A. porri from the initial culture was grown on a PDA medium. After growing, it was observed using a microscope.

Screening of Antagonistic Fungi

The antagonist test was performed using a dual culture method. Observations were made every
day for 7 days after inoculation. The inhibition was calculated using the formula:

\[
P(\%) = \left( \frac{dc - dt}{dc} \right) \times 100\%
\]

Where: P is the percentage of inhibition, dc is diameter of *A. porri* without treatment (control) and dt is the diameter of *A. porri* with endophytic fungi treatment.

**Crude Protein Extraction**

Crude protein was extracted using ammonium sulfate (NH\(_4\))\(_2\)SO\(_4\) precipitation method. Ammonium sulfate was added to the suspension, the amount of which was adjusted to the target concentration, starting from 20%, 40%, 60%, and 80% (www.encorbio.com).

**Antifungal Activity of Crude Protein**

The inhibition of *A. porri* by crude protein was tested using the agar well diffusion method [10]. *A. porri* culture was taken using a cork borer and then placed right in the middle of the Petri dishes. Five sterile paper discs dripped with 10 µl of crude protein at different concentrations of ammonium sulfate (20%, 40%, 60%, 80%, and phosphate buffer as a control).

**SDS PAGE (Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis)**

The crude protein was separated based on its molecular weight using SDS-PAGE method.

**Identification of Antagonistic Fungi**

The endophytic fungi with an inhibition ability of more than 60% were identified based on macroscopic, microscopic, and molecular characteristics. Molecular Identification:

a. **DNA isolation.** The mycelium of antagonistic fungi was lyophilized and pulverized with liquid nitrogen using a mortar and a pestle until obtaining a fine powder. The mycelium that has been refined is given 500 µl of STES buffer and then was vortexed for 3 minutes. Samples were heated at 70°C for 2 hours then centrifuged at 13,000 rpm for 10 minutes. Then, 250 µl of supernatant was taken, and added 240 µl of chloroform (C) and 10 µl of isoamyl alcohol (I). Samples were centrifuged at 13,000 rpm for 5 minutes, 300 µl of supernatant was taken, and added 250 µl of CI. Samples were centrifuged at 13,000 rpm for 5 minutes, 250 µl of supernatant was taken, and added ammonium acetate 0.1 times the total volume and 2.5 times the absolute ethanol. Samples were incubated at -20°C for 2 hours, then centrifuged at 13,000 rpm for 15 minutes. Pellets were added 40 µl of TE buffer and stored at -20°C.

b. **Amplification using PCR.** The primers used for the amplification of the Internal Transcribed Spacer (ITS) region in the fungi samples were ITS 1 (forward: 5'-TCTGTAGGTGAACCTGCGG-3') and ITS 4 (reverse: 5'-TCCTCCGCTTATTGATGC-3').

c. **Sequencing and DNA Sequence analysis.** The DNA sequences of ITS regions 1 and 4 were determined using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA, USA). The sequences were compared with the National Center for Biotechnology Information (NCBI) databases.

**Statistical Analysis**

Data were analyzed using one-way variance (ANOVA). Probability values P<0.05 were considered statistically significant. The difference between treatments was followed by Duncan test.

**RESULTS AND DISCUSSION**

**Isolation of Endophytic Fungi**

The endophytic fungi that were explored from the onion cultivated land obtained 25 isolates. Fungi isolates were distinguished based on their macroscopic and microscopic appearance when cultured on PDA media. Each purified fungi isolate was tested for its inhibitory ability against *A. porri* using a dual culture method in vitro.

**Isolation and Colony Morphology Characterization**

**A. porri**

Morphological characters of *A. porri* colonies 3 days after inoculation were grayish-white on PDA medium after 7 days inoculation becomes blackish-gray and its margin was brownish-white. Mycelium texture is like cotton, flat, and circular. The hyphae of *A. porri* was irregularly branched and insulated. The conidia measured 150-300 µm, shaped like a club and a blunt tip, 8–12 transverse septa, 0–7 longitudinal or oblique septa. Conidiophores pale to mid-brown, up to 80-100 µm long, 7–10 µm thick, and straight.
grayish-white surface when it reaches the 4 day after inoculations it changes to blackish-gray with brownish margins. The *A. porri* colony had a smooth, texture like cotton when cultivated in PDA media, yellowish or dark orange on the reverse side, flat margins and was circular. Full plate growth of *A. porri* takes about 8-10 days. Madhavi, Kavitha, & Vijayalakshmi (2012) state that *A. porri* has hyphae that are branched and insulated. The mycelium, conidium, and conidiophores are brown. The conidium is shaped like a baffled club and a blunt tip measuring 145-350 µm. *A. porri* has unique characteristics, conidia which are brown and shaped like a club with a size of about 100-300 µm, a diameter of 15-20 µm, are straight to curved, have 7-9 transverse septa and 1-3 septa longitudinal. Conidia *A. porri* has septa and the tip is enlarged while at the other tip it is narrow.

**Pathogenicity Test**

The pathogenicity of *A. porri* was tested by spore inoculation method on 30-day old seedling of onions. Plants show the symptoms when entering 6 days after inoculation, the leaves appear small and white lesions that spread on the leaf surface, especially on old leaves. After 8 days of inoculation, the tips of the leaves began to dry out and caused some of the leaves to break. On the 10 days after inoculation, the lesion size grew and became white patches and formed concentric rings with a purplish center. Symptoms of a continuous attack cause discoloration not only in mature leaves but also in young leaves to turn a yellowish color. Muimba-Kankolongo (2018) stated *A. porri* attacks on onion plants were seen by the appearance of elliptical lesions and then turning brown. The lesion develops into patches that are larger and turn purple with a darker border surrounded by a yellow zone. The tips of the leaves will begin to dry out and break, especially on old leaves. In humid conditions such as rain and dew, it will worsen the symptoms of an attack and cause plant death (Muimba-Kankolongo, 2018).

**Antagonistic Test In Vitro**

The exploration obtained 25 endophytic fungi from onion roots, stems, and leaves. Each isolate was tested for inhibition against *A. porri* using dual culture methods. The inhibition appears on the 4 days after inoculation (Fig. 1), the average diameter of *A. porri* in the antagonist treatment ranging from 1.4 to 3 cm, while in control the diameter reached 3.7 cm. In observations up to 7 days after inoculation, the antagonistic fungi that had the highest ability to suppress *A. porri* growth was Y isolate which was able to inhibit the diameter to 1.7 cm, while the control reached 5.6 cm.

![Fig. 1. Inhibition by *P. citrinum* against *A. porri*: (A) 2 days after inoculation (dai), (B) 4 dai, (C) 7 dai, (D) control](image-url)
The antagonistic with the highest inhibition ability was found in the Y isolate, the percentage of inhibition was 60.04% and suppressed diameter of *A. porri* to 1.5 cm while in control diameter reached 3.5 cm (Table 1). The antagonistic fungi with the lowest inhibition ability were R isolate with an inhibition of 21.04%. The percentage of inhibition ability by other antagonistic fungi varied between 21.64%-53.45%. According to Noveriza & Quimio (2004) criteria for an antagonistic agent as potential biocontrol if it has an inhibition percentage was higher than 60%, but if it is less than 60%, it only has minimal inhibition of pathogens.

The antagonistic mechanism of *A. porri* by endophytic fungi was observed under the microscope. Based on observations, it showed that the growth of hyphae was abnormal such as lysis, enlargement, or swelling, the hyphae become curly and grows abnormally. Nunes & Philipp-Wiemann (2018) stated abnormal pathogenic hyphae in the antagonistic test showed the ability of biocontrol agents to produce chitinase enzymes; this enzyme causes abnormalities of target fungi hyphae such as perforation, lysis, and fragmentation. The antagonistic fungi spores colonize the hyphae of *A. porri* (Fig. 2a and Fig. 2b), it causes the growth of hypha is disrupted. In Fig. 2e, the antagonistic fungi hyphae were able to wrap around *A. porri* hyphae. The fungi hyphae of *A. porri* were abnormally enlarged or swollen when compared to hyphae without antagonist treatment (Fig. 2c). The inhibition mechanism that occurs in the antagonist test shows antibiotic activity, this is indicated by the formation of an inhibition zone between the colonies of endophytic fungi and *A. porri*. Antibiosis is the ability of microorganisms to produce secondary metabolites or toxins that can cause lysis in other microorganisms. The mechanism of antibiosis that occurs in the antagonist test is characterized by the formation of an inhibition zone as a zone of growth inhibition for pathogens (Köhl, Kolnaar, & Ravensberg, 2019). Endophytic fungi are known to produce bioactive compounds which are classified into several biochemical groups, including phenols, alkaloids, quinones, and flavonoids. These variations of the chemical structures have been examined to be the root cause of antimicrobial susceptibilities by different pathogens up to varying extents. Differences in the inhibitory ability of antagonistic fungi are influenced by differences in the concentration and type of secondary metabolite compounds secreted, the rate of biosynthesis equilibrium, the types and strains of antagonistic fungi, and the types of pathogens tested (Gokhale, Gupta, Gupta, Faraz, & Sandhu, 2017).

### Table 1. Inhibition by endophytic fungi

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate code</th>
<th>Inhibition (%)</th>
<th>Diameter <em>A. porri</em> (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>A</td>
<td>49.41</td>
<td>1.76 abcd</td>
</tr>
<tr>
<td>2.</td>
<td>B</td>
<td>52.37</td>
<td>1.66 abc</td>
</tr>
<tr>
<td>3.</td>
<td>C</td>
<td>49.77</td>
<td>1.74 abcd</td>
</tr>
<tr>
<td>4.</td>
<td>D</td>
<td>51.03</td>
<td>1.71 abc</td>
</tr>
<tr>
<td>5.</td>
<td>E</td>
<td>34.51</td>
<td>2.28 de</td>
</tr>
<tr>
<td>6.</td>
<td>F</td>
<td>41.31</td>
<td>2.05 bcd</td>
</tr>
<tr>
<td>7.</td>
<td>G</td>
<td>42.54</td>
<td>2.00 bcd</td>
</tr>
<tr>
<td>8.</td>
<td>H</td>
<td>47.57</td>
<td>1.83 abcd</td>
</tr>
<tr>
<td>9.</td>
<td>I</td>
<td>53.09</td>
<td>1.64 abc</td>
</tr>
<tr>
<td>10.</td>
<td>J</td>
<td>51.43</td>
<td>1.69 abc</td>
</tr>
<tr>
<td>11.</td>
<td>K</td>
<td>38.59</td>
<td>2.15 bcd</td>
</tr>
<tr>
<td>12.</td>
<td>L</td>
<td>39.06</td>
<td>2.13 bcd</td>
</tr>
<tr>
<td>13.</td>
<td>M</td>
<td>44.35</td>
<td>1.94 abcd</td>
</tr>
<tr>
<td>14.</td>
<td>N</td>
<td>36.88</td>
<td>2.21 cd</td>
</tr>
<tr>
<td>15.</td>
<td>O</td>
<td>42.34</td>
<td>2.02 bcd</td>
</tr>
<tr>
<td>16.</td>
<td>P</td>
<td>42.54</td>
<td>2.01 bcd</td>
</tr>
<tr>
<td>17.</td>
<td>Q</td>
<td>40.43</td>
<td>2.09 bcd</td>
</tr>
<tr>
<td>18.</td>
<td>R</td>
<td>21.04</td>
<td>2.76 e</td>
</tr>
<tr>
<td>19.</td>
<td>S</td>
<td>45.49</td>
<td>1.90 abc</td>
</tr>
<tr>
<td>20.</td>
<td>T</td>
<td>48.54</td>
<td>1.80 abcd</td>
</tr>
<tr>
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<td>U</td>
<td>52.21</td>
<td>1.67 abc</td>
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<tr>
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<td>2.75 e</td>
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<td>1.63 ab</td>
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<tr>
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<td>X</td>
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<td>1.88 abcd</td>
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<td>25.</td>
<td>Y*</td>
<td>60.04</td>
<td>1.40 a</td>
</tr>
<tr>
<td>26.</td>
<td>Control</td>
<td>0</td>
<td>3.50 f</td>
</tr>
</tbody>
</table>

Remark: (*) endophytic fungi with the highest inhibition and selected for further testing. The numbers followed by the same notation in the same column group show insignificant differences at the 5% level according to the Duncan Multiple Range Test.
Identification of Antagonistic Fungi

The antagonistic fungi with the highest inhibitory ability were identified macroscopically, microscopically, and molecularly. The antagonistic fungi isolate selected was Y isolate because it had an inhibition percentage value above 60%. Colonies of Y isolate grown on PDA medium have a bluish-green with white margins, the smooth, surface texture was velutinus (soft, velvety surface) and behind of container was yellow. Microscopic observations were carried out using a 400X magnification microscope, show the fungi produces septate, hyaline (clear, not pigmented), and insulated vegetative hyphae. Conidia was greenish, globose to sub-globose (round to off-round), and have a finely roughened surface. The conidiophores can branch and hyaline. The tip of conidiophores has a metula of about 12 x 25 µm. Tolulope, Adeyemi, Erute, & Abiodun (2015) mentioned that P. citrinum has a velvety surface texture. The mature colonies have a turquoise center with white margins. The reverse is a pale yellow to a light yellow-brown. P. citrinum had septate hyphae, hyaline (clear). The smooth conidiophores are rather long (100-300 µm). Metula has a length of 12-15 µm which is found in a circle of 3-5 different structures, the vegetative hyphae were hyaline and insulated and the conidia are round to oval in shape. At the tip of the conidiophore, there is a metula measuring 1.2 x 2.5 µm and greenish.

Endophytic fungi with the highest inhibitory ability against A. porri were identified molecularly to determine the identity of the isolates down to the species level. Fungi isolates were identified based on ribosomal DNA (rDNA) sequences in the ITS region. The PCR product was sequenced to determine the nucleotide sequence. The results of the sequencing are then aligned using the Basic Local Alignment Search Tool (BLAST) program to determine the homology with species that are similar to the sequence. The visualization of gel electrophoresis showed a single band of DNA with a clear thickness (Fig. 3). The results of electrophoresis which resulted in a thick and bright band qualitatively indicated that the concentration of the DNA isolation results obtained was high, while the thin band indicated that the resulting DNA concentration was low. DNA with high quality is shown by electrophoresis resulting in the high intensity of DNA and low smear intensity on the gel electrophoresis. The purity of DNA had an

Fig. 2. An antagonistic mechanism: (a) spores of Y isolate colonize A. porri hyphae; (b, c) hyphae are swollen; (d) curly hyphae; (e) Y isolate hyphae wrapped around the hyphae of A. porri; (f) hyphae are lysis; (g) abnormal hyphal (malformation); (h) hyphae of A. porri without treatment (control)
absorbance value of A260 / A280 in the range of 1.8–2.0. DNA purity that shows a value above 2 is likely to be contaminated with RNA, while below a value of 1.8 is due to contamination of protein and phenol solutions. The ratio of DNA purity measured was based on the absorbance ratio of DNA A260 with the absorbance value of protein (contaminant) A280 (O’Neill, McPartlin, Arthure, Riedel, & McMillan, 2011). Antagonistic fungi had a DNA concentration of 174.2 ng/µl. Based on the results of the visualization of gel electrophoresis, it showed that the fungi isolates had a DNA band size of 540 bp (Fig. 3). Based on the BLAST results, Y isolates had a 99% homology as *Penicillium citrinum*. DNA amplification in the ITS region obtained the length of *P. citrinum* fragment around 540 bp (Doan et al., 2019). The BLAST results with a homology value of ≥99% indicate the sequence is the same species, while the homology value of ≥99% indicates species that have similarities in the genus (Raja, Miller, Pearce, & Oberlies, 2017).

*P. citrinum* produces alkaloids and citrinins which are known as polyketides in antifungal activity because of their properties as mycotoxins (Lai, Brötz-Oesterhelt, Müller, Wray, & Proksch, 2013). The mechanism of action of antifungal substances in inhibiting the growth of pathogenic microorganisms in several ways, including damaging microbial cell walls, disrupting cell membrane permeability, inhibiting cell synthesis, disrupting microbial cell metabolism, inhibiting protein synthesis and microbial cell nucleic acids (Gubbins & Anaissie, 2009). *P. citrinum* produces two polyketides, emodin and citrinin. Antifungal effect of emodin causes changes in the cell membrane and structure, furthermore, this leads to morphological aberrations including swelling, distortion, increase in membrane permeability, and a decrease in the total cell protein content (Luo et al., 2019). Citrinin was reported to have broad-spectrum antifungal activity against *Fusarium* sp., *F. nivale*, and *Aspergillus niveus* (Hu et al., 2017). Emodin was reported to have antifungal activity against *Candida albicans*, *Penicillium vulpinum*, *Aspergillus flavus*, and other phytopathogenic.

**Antagonistic Activity of Antifungal (In Vitro)-Agar Disc Diffusion Assay**

The antifungal protein of *P. citrinum* was extracted using the ammonium sulfate precipitation method. The principle of the ammonium sulfate precipitation method is to reduce the solubility of the protein in water by a mechanism called salting in and salting out. Salting in is a protein ionization process by ammonium sulfate which causes the solubility of the protein in the solvent to increase, while salting out is a mechanism for protein deposition due to reduced solvent molecules needed to dissolve protein, due to increased salt concentration. The test was carried out using the direct opposition method. Inhibition test by the crude protein of *P. citrinum* against *A. porri* showed the inhibition occurred in crude protein with a saturation level of 80% ammonium sulfate (Fig. 4A). Based on the visualization of SDS PAGE gel a molecular weight of 40 kDa (Fig. 5).
Fig. 4. Antifungal inhibition test against *A. porri* (for 7 days after inoculation): (A). 1. control (phosphate buffer), 2. ammonium sulfate concentration 20%; 3. 40%; 4. 60%; 5. 80%; (B). *A. porri* culture without treatment (control)

Fig. 5. Protein profile of *P. citrinum*: (M) protein marker (20%, 40%, 60%, and 80% are the concentration of ammonium sulfate)
The inhibitory ability of antagonistic fungi is influenced by differences in the concentration and types of secondary metabolites secreted, the rate of biosynthesis equilibrium, the types and strains of antagonistic fungi, and the types of pathogens tested. The research conducted by Wen, Guo, & Chen (2014) proved that *P. citrinum* secretes an antifungal protein known as PcPAF and showed antifungal activity through inhibition of pathogenic fungi including *Alternaria*. Penicrtimertone compound, bioactive citrinin dimer is an antimicrobial agent from secondary metabolites secreted by *Penicillium* and has been shown antimicrobial activity against several pathogenic microbes (Li et al., 2020). Based on studies of Luo et al., (2019), *P. citrinum* has two groups most known antifungal compound to inhibit the growth of pathogens that citrinin and emodin. Citrinin mycotoxins have been shown to inhibit the growth of pathogens from the genus *Alternaria*. Emodin antifungal causes damage to cell membranes and changes in the structure of hyphal cells, further lysis, swelling, distortion, loss of hyphal pigment and disruption of membrane permeability.

CONCLUSION

The endophytic fungi obtained from the exploration were 25 isolates. Endophytic fungi had the highest inhibition was identified as *Penicillium citrinum* with an inhibition of 60.04%. The antagonistic mechanism between *P. citrinum* and *A. porri* was antibiosis, indicated by the presence of an inhibition zone formed during the antagonistic test and abnormal growth of *A. porri* hyphae. Crude protein of *P. citrinum* showed inhibition activity at a saturation level of 80% ammonium sulfate with a molecular weight of 40 kDa.

ACKNOWLEDGEMENT

The authors thank the Head of Plant Pest and Disease Department, Faculty of Agriculture, Universitas Brawijaya for helping in various aspects of this study. Financial support from the DGHE, Ministry of Research and Technology No. 147/SP2H/LT/DRPM/2018, is gratefully acknowledged.

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