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Antimicrobial activity and molecular characterization of endophytic fungi strain isolated from dahlia (*Dahlia variabilis*)

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ABSTRACT

Dahlia variabilis has many beneficial effects to human health, such as antibacterial and antifungi agent. This research aimed to discover the potency of endophytic microbesisolated from Dahlia as antimicrobial agents. Screenings were carried out using agar diffusion method against pathogens with concentration of 50µl/disc. Based on our previous study, four isolates of endophytic fungi were isolated and characterized morphologically as follows, LBKURCC40 identified as Monilia sp., LBKURCC41 identified as Aureobasidium sp., LBKURCC42 identified as Moniliella sp., and LBKURCC42 identified as Sporothrix sp. The inhibition zone of amoxsan was 12.55 ±0.38 mm and ketoconazole was 17.67±0.57 mm. LBKURCC41 showed the most significant inhibition zones against Eschericia coli and Staphylococcus aureus which those were 19.33±1.15 mm and 17.00±1.00 mm respectively. LBKURCC41 was then characterized genetically. The molecular weight of chromosomal DNA of LBKURCC41 obtained was 11,706 bp. Its rDNA was amplified with PCR using ITS5 and ITS4 as DNA primers and the result of phylogenetic analysis at ITS-1, ITS-2 and 5.8S rDNA showed that LBKURCC41 was Fusarium oxysporum with 99% similarity, which was different from previous morphological characterization.

Keywords: antimicrobial activity, Dahlia, DNA, *Fusarium oxysporum*, inhibition zone

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INTRODUCTION

During the last 20 years, it has been observed that much of the wealth of microbial biodiversity with novel biochemistry and secondary metabolite production resides in plant tissues [1,2]. Interests in such endophytic microorganisms, increased immensely with the discovery of an endophytic fungi, from Taxus brevifolia, producing the billion dollar anti-cancer drug, taxol [3]. Numerous bioactive molecules have been isolated from endophytic fungi since this ground breaking discovery [1,4,5]. Endophytes are metabolically more active than their free counterparts due to their specific functions in nature and activation of various metabolic pathways to survive in the host tissues [1,6,7].

As the previous research on endophytes mainly focused on search for the host-plant metabolites in the endophytic partner [3,8,9], the theory of horizontal transfer from the host plant to its microbial symbiont received much impetus [1]. However, the sequencing of the taxadiene synthase gene from the taxol-producing endophyte revealed that endophytes possess biosynthetic pathways independent of the plant host [10]. This indicates that microorganisms have much more biosynthetic proficiency than previously thought. Thus, microorganisms may be screened for a wide range of biological activities and explored for useful chemical entities consistently produced by them.

Dahlia tubers are known to contain compounds which have high bioactivity. In some regions in Sumatra with the mountainous topography, Dahlia can naturally grow well. For instance, in the province of Sumatera Barat, such as in Solok, Padang Panjang, Payakumbuh, and Batu Sangkar; Brastagi (Sumatera Utara), Curup (Bengkulu), and Lubuk

Linggau (Sumatera Selatan) Dahlia thrives in an environment of settlements. While in Kalimantan, Dahlia are relatively difficult to find due to environmental factors affecting the growth of Dahlia [11]. The leaves, stems, and tubers of Dahlia contain a number of potential bioactive compounds that can be functioned as anti-microbial compounds [12].

Production of antimicrobial compounds from plants in large quantities requires a very large number of Dahlia plants, and it takes large area and long growing-type plants as well. On the other hand, plants generally contain endophytic microbes that can produce bioactive compounds as what can be found in its host plant [1]. Some microbes such as fungi, yeast, and bacteria can be associated with the plant because they help the host plant metabolism and produce potential secondary metabolites [13]. Some studies also show that the endophytic microbes provide benefits for the host plant, such as protection against insects, pathogens, and herbivores [14]. Therefore, it is necessary toisolate antimicrobial compounds from endophytic microorganisms that live inside plant tissues.

In previous study, four isolates of endophytic fungi from Dahlia tubers had been found; LBKURCC40 from orange-floweredDahlia, LBKURCC41 from yellow-flowered Dahlia, LBKURCC42 fromred-floweredDahlia, and LBKURCC43 from purple-floweredDahlia[15]. It will be very necessary to make an initial screening of the endophytic fungi isolated from *Dahlia variabilis*tubers. Screening of four endophytic fungi was carried out to see their antimicrobial activity against pathogenic *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. The media used is a Huang media with a 15-day incubation period [16].The endophytic fungi which have great antimicrobial activity will be analysed by phylogenetic identification. Therefore, it needs to make a more appropriate way of identification based on ribosomal DNA sequences (rDNA) in the area of ITS (internal transcribed spacer) [17].

The antibiotic resistance of bacterial pathogens has become a serious health concern and encourages the search for novel and efficient antimicrobial metabolites. These isolates of endophytic fungi are indigenous microbes from Dahlia and very potential to be developed as antimicrobial agents. This study was undertaken to discover the potency of endophytic microbes from Dahlia as antimicrobial agents. In this study, a proper identification will be carried out based on ribosomal DNA sequences of the ITS region equipped with a phylogenetic analysis of the fungi. Wiese *et al.*[18]reported that to identify the species of fungi, sequencingin the ITS-1 and ITS-2 rDNA region can be done.

EXPERIMENTAL SECTION

Fermentation media preparation

The Huang media [16] was used as a fermentation medium production of antimicrobial compounds, with its composition can be seen in Table 1. All the materials were dissolved in 250 ml of distilled water and heated, and sterilized by autoclaving at 121^{o}C for 20 minutes (vapor pressure was 15 lbs). The media was allowed a day to measure whether or not contamination happened. If no visible contamination, the media was ready for use.

Table 1.The Huang media composition

Composition	Weight(grams)
Sucrose	30
$NaNO_3$	3
KH_2PO_4	1
Yeast extract	1
KCl	0,5
$MgSO_4.7H_2O$	0,5
$FeSO_4.7H_2O$	0,001
Aquadest	1000

Fermentation of endophytic fungi

A total of 1 ml endophytic fungi (5%) was inoculated into 250 mL of media Huang, and incubated for 20 days at room temperature with a stirring speed at 150 rpm. Antimicrobial test was carried out each interval of 5 days. Fermented fungal cultures were taken and centrifuged at a speed of 5000 rpm for 20 minutes to separate the supernatant and cell mass.Fermented culture was filtered using 0,2 µm millipore syringe filter, thus crude endophytic fungiextract of Dahlia plant was obtained. This crude extract of endophytic fungi was used to test the antimicrobial.

Subculture of pathogenic bacteria and fungi

Escherichia coli and *Staphylococcus aureus* were transferred from agar slant to new Nutrient Agar (NA) media aseptically and incubated for 24 hours. Growing colonies were then inoculated into Nutrient Broth medium and

incubated for 24 h at 37°C. Bacteria were ready for antibacterial tests when the optical density reached 0.08 to 0.1 (equivalent to 10^7 CFU/ml). Whilst*Candida albicans* was inoculated into PotatoDextroseAgar(PDA) medium and incubated for 4 x 24 hours. Furthermore,the growing colonies were inoculated back into the Sabouroud Dextrose Broth (SDB) media and incubated for 48 hours.If optical densityof those bacteria and fungi was greater than 0.1, it should be diluted using 0.85% NaCl solution.

Antibacterial and antifungal test

Pathogen inoculum with OD_{600nm} = 0.1 or equivalent to 10^7 CFU/ml (Martin, 2011) was inoculated into a test tube containing a liquid NA media at 50°C and homogenized, then poured into petri dish and allowed to solidify.Each 50 µl sterile crude endophytic fungi extract was dropped on 6 mm-diameterpaper discs and allowed to dry. Amoxsan 30µg was used as a positive control and a sterile fermentation medium as negative controls. For anti-bacterial test, the paper disc was placed on NA medium containing *Escherichia coli* or *Staphylococcus aureus.* Whilst, for antifungal test, the paper disc was placed on PDA medium containing*Candida albicans.*The entiremediums wereincubated at 37° C with reversed-plate condition. Diameter of clear zone around the paper disc was measured after incubation for 24 hours with three replications.

Identification of endophytic fungi

Endophytic fungi which had antimicrobial activity, would be identified by phylogenetic trees after screening test was undertaken. A kit used for identification contained of SDA media (Sabouraud 4% dextro order) from Merck, Lyticase (SIGMA-Aldrich Chemical Co.St. Louis, USA catalog No. L-2524), Ethidium Bromide (10 mg/ml,Bio-rad catalog No. 161-0433), Kit Wizard Genomic Purification (Promega, Madison, Wi, USA catalog No. A-1120), 1 Kb DNA Ladder (Promega, Madison, WI, USA catalog No. G-5711) as the standard DNA. Go Taq^{TM} PCR Core TM system I (Promega, USA No. Kat. M7660) for DNA amplification reactions. The Internal Transcribe Spacer (ITS) primers included ITS-1, ITS-2, ITS-3, ITS-4, and ITS-5 used were produced by PT. Sentra Biosciences Dynamics, Jakarta, according to the sequence published by White *et al.*, [19], tris-base buffer, EDTA, CH_3COOH, HCl, NaOH, bromphenol blue, and agarose gel.

The fungal cultures were subcultured on growth medium. DNA isolation was conducted using Wizard genomic kit including litikase enzymes and ingredients from the Wizard genomic DNA purification kit (Promega). The DNA extract was then electrophoresed using 0.8% agarose gel and the DNA separation photographed using a 14.1 megapixels Sony Cyber-shot DSC-W610 digital camera. DNA isolated was determined based on DNA bands which were visible and those bands were used as a template in the amplification by Polymerase Chain Reaction (PCR). Primers of ITS-4and ITS-5 were used to determine the region sequences of the ITS-1 and ITS-2, orby using another ITS primer for a primer pair matching. The matching primers pairs could be seen based on the DNA bands which were seen in the UV transimulator after electrophoresed with 1.2% agarose gel. This PCR fragment was then determined its DNA sequence based on the method of Sanger sequencing process. All of this sequencing data were required for species identification and analysis of phylogenetic.

RESULTS AND DISCUSSION

Screening for antimicrobial activity of endophytic fungi from Dahlia

The crude extract of endophytic fungi LBKURCC 41 showed antimicrobial activity both against *E. coli* (Table 2) and *S. aureus* (Table 3)in the Huang media on the 15 and 20th day of incubation, withon 15thday, inhibition zones against *E. coli* and *S. aureus* were (19.33 ± 1.15) mm and (17.00 ± 1.00) mm. Meanwhile, inhibition zones against *E. coli* and *S. aureus* on the 20thday were (15.56 ± 0.50) mm and (15.11 ± 0.19) mm consecutively.

Figure1. Screening for antimicrobial activity against *E. coli* (a), *Staphylococcus aureus* (b), and *Candida albicans* (c) at the 15th day
***Notes**: (1) = positive control, (2) = negative control, (3) = LBKURCC 40, (4) = LBKURCC 41, (5) = LBKURCC 42, and (6) = LBKURCC 43*

Whilst, all crude extracts of endophytic fungi fermented with media production showed negative results for antifungal activity against pathogenic *Candida albicans* (Table 3).

Table2.Antimicrobial activity against Eschericia coli on the Huang media

Extracts	Inhibition zones (mm)			
	5th day	10th day	15th day	20th day
Amoxsan	(12,55 ± 0,38)	(11,67 ± 0,33)	(12,00 ± 0,33)	(11,66 ± 1,45)
Control (-)	-	-	-	-
LBKURCC 40	-	-	-	-
LBKURCC 41	-	-	(19,33 ± 1,15)	(15,56±0,50)
LBKURCC 42	-	-	-	-
LBKURCC 43	-	-	-	-

Tabel 3.Antimicrobial activity against *Staphylococcus aureus* on the Huang media

Extracts	Inhibition zones (mm)			
	5thday	10th day	15th day	20th day
Amoxan	(11,55 ± 0,50)	(11,66 ± 0,57)	(11,67 ± 0,33)	(11,77 ± 0,50)
Control (-)	-	-	-	-
LBKURCC 40	-	-	-	-
LBKURCC 41	-	-	(17,00 ± 1,00)	(15,11±0,19)
LBKURCC 42	-	-	-	-
LBKURCC 43	-	-	-	-

Tabel 4.Antimicrobial activity against *Candida albicans* on the Huang media

Extracts	Inhibition zones (mm)			
	5th day	10th day	15th day	20th day
Nizoral	(15,77 ± 0,69)	(16,44 ± 0,38)	(16,77 ± 0,38)	(17,67 ± 0,57)
Kontrol (-)	-	-	-	-
LBKURCC 40	-	-	-	-
LBKURCC 41	-	-	-	-
LBKURCC 42	-	-	-	-
LBKURCC 43	-	-	-	-

Isolation of DNA

Mycelia best for DNA isolation were those at 3 days aged. Moreover, the existence of a single DNA band which was visible with the help of UV light on gel electrophoresis showed that the presence DNA band was at range of 10,000 to 12,000 bp, indicating that DNA of LBKURCC41 have been successfully isolated (Fig. 2).

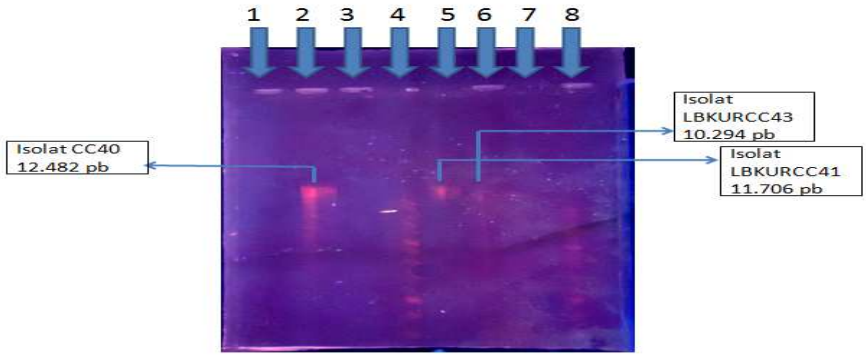

Figure 2. Electrophoreses result of endophytic fungi isolates

Notes: Line 1 : Empty
Line 2 : DNA isolate ofLBKURCC40
Line 3 : Empty
Line 4 : DNA Ladder
Line 5 : DNA isolate of LBKURCC41
Line 6 : DNA isolate of LBKURCC43

From migration data of DNA standards and the isolates, charts of the relationship between the log base pairs with distance migrationcan be made. The regression equation obtained was y = -0,279x + 5.017,and from thisequation the number of base pairs of LBKURCC41 DNA was 11,706 bp.

DNA amplification

The results of PCR amplification were electrophoresed on 1.2% agarose gel and stained with ethidium bromide in order to fluorescence under UV light. Similar to determination of the molecular weight of the isolates, PCR amplification results were obtained by measuring the standard migration distance. The regression equation obtained for LBKURCC41 was y = -0,381x + 4,428 and it was implemented in determining the molecular weight of the PCR product.From this equation, the molecular weight of LBKURCC41 was 616 bp with the migration distance of 43 mm.Electrophoreses result of some DNA amplification products can be seen in Fig.3.

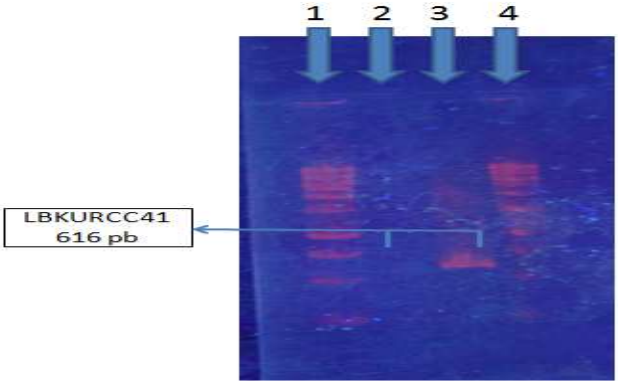

Figure3. Electrophoreses ofDNA amplification product of LBKURCC41in annealingat 47ºC

Notes: Line 1 : DNA *Ladder*
Line 2 : DNA of LBKURCC41
Line 3 : DNA of LBKURCC41
Line 4 : DNA *Ladder*

DNA sequencing of PCR Amplification Products

Molecular identification based on ribosomal DNA sequences of the ITS region was a step to identify and unlock the genetic information of fungi. After PCR amplification products obtained, then its DNA sequence was determined in the ITS1 and ITS2 rDNA region using ITS4 and ITS5 combined primers. Verification of ITS-1 rDNA region used primer pairs ITS5 (forward) and ITS2 (reverse), while in the ITS-2 rDNA regions, ITS3 used as forward and ITS4 as reverse. Alignment of DNA sequence can be seen in Fig.4.

> DNA Seq of LBKURCC-41 (5’ – 3’)

TGGAAGTAAAAAATCGTAACCAAGGTCTCCGTTGGTGAACCAGCGGAGGGAT
CATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTC
GGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGAACCCCT
AAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACT
TTCAACCAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTTCGAGCGTCATTTCAAC
CCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTG
ATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGG
TAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGAT
CAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Figure4.DNA sequencing of LBKURCC41 usingBioedit verification

Phylogenetic analysis in sequence region of ITS-1 and ITS-2 rDNA

Sequence regions of ITS-1 5,8S rDNA, and ITS-2 LBKURCC41 were compared to the same area owned by seven strains of *Fusarium* sp. and close relative whichwere accessed fromNCBI GenBank data (Table 5), on August 29,

2014. The Neighbour Joining bootstrapping in tree generation was displayed by TreeView software as a tree dendogram and non-rooted filogram (Fig.5).

Tabel 5.Data from Gen Bank of NCBI: species and similarity of LBKURCC41

Nama spesies	Access Code Gen Bank	Strain	References	% Similarity
Fusarium oxysporum f. sp. vasinfectum	AF322075.1	Ag149-1	Ying *et al.* [20]	99 %
Fusarium oxysporum	EU326216	BD	Bukovska *et al.* [21]	99 %
Fusarium andiyazi	KC954400.1	CBS 134430	Kebabci *et al.* [22]	98 %
Fusarium solani	KF918565	DE21	Wafa &Latiffah[23]	95 %
Fusarium culmorum	GU566271.1	G5	Bukovska*et al.* [21]	93 %
Fusarium armeniacum	KF944456.1	NEFU12	Zhao[24]	93 %
Fusarium poae	AF414967.1	BBA 70810	Yli-Mattila *et al.*[25]	93 %
Nectria haematococca	AF150468.1	SUF1223	Suga *et al.* [26]	91 %

Screening for antimicrobial activity

Huang media consists of sucrose as a carbon source. Sucrose is a disaccharide formed from a molecule of α-D-glucose and β-D-fructose and linked by β-1,2 glycosidic bonds. When β-1,2 glycosidic bond was interrupted by a hydrolysis reaction, it will form a mixture of glucose and fructose. Media which can produce secondary metabolites contain complex carbon sources as one of the factors that influence the microorganisms to produce secondary metabolites. The complexity of medium is a critical condition in which microbes will generally produce secondary metabolites to sustain their life [13].Based on our previous study[15], four isolates of endophytic fungi were isolated and characterized morphologically as follows, LBKURCC40 identified as *Monilia* sp., LBKURCC41 identified as *Aureobasidium* sp., LBKURCC42 identified as *Moniliella* sp., and LBKURCC42 identified as *Sporothrix* sp.

There are several possibilities why the crude extracts of endophytic LBKURCC40 and LBKURCC43 could not produce antimicrobial compounds. First, both isolates had genes that encode for the formation of secondary metabolites indeed, but those genes were not expressed in the media production that has been used. The genes will be expressed only when induced in advance [27]. In the three production medium used, there could be no inducers that could express the gene of metabolites formation of LBKURCC40 and LBKURCC43. This possibility also explained why all crude extracts of endophytic fungi gave no antifungal activity against *Candida albicans*.

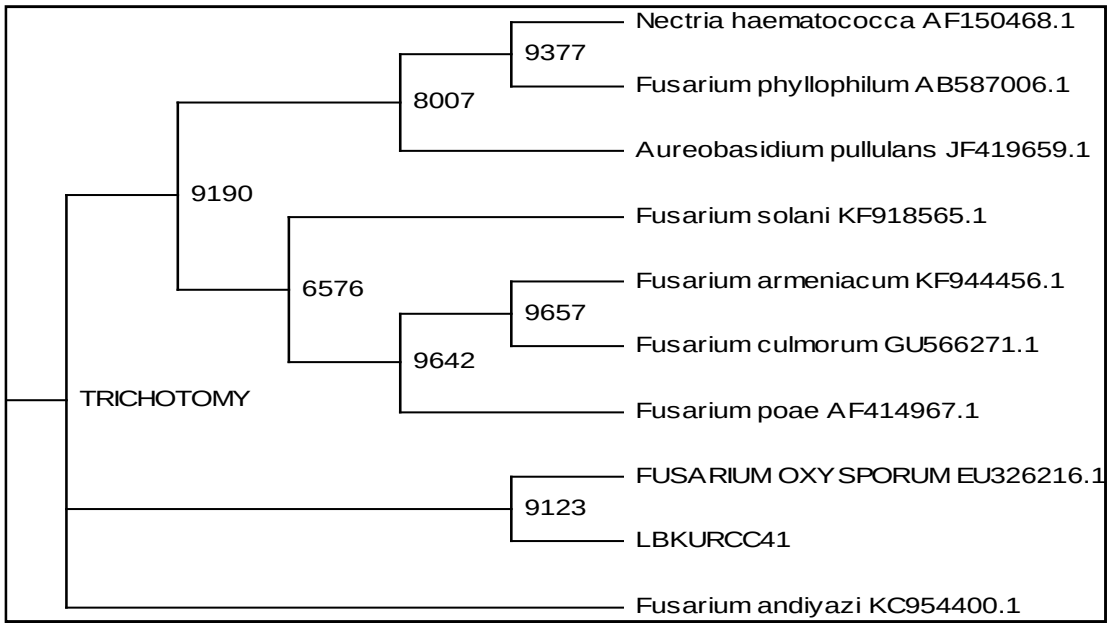

Figure5. Phylogenetic relationship between LBKURCC41 and 9 closest relatives, based on sequence of DNA ITS-1, 5,8S rDNA and ITS-2

Identification of LBKURCC41

A genetic information contained in fungal DNA which was sequenced in areas 5.8S ITS-1 and ITS-2 could be used to distinguish one species tothe others. PCR products obtained from rDNA amplification of LBKURCC41 were followed by sequencing process using ITS2, ITS3, ITS4, and ITS5. DNA sequences were visually checked back by adjusting the peaks with neucleotide bases existing on the spectrogram. DNA resulted from sequences of pairwise alignment can be seen in Fig.3. LBKURCC41 isolate was compared with 9 lines and it could be classified in three subclusters and 1 strain which did not form any subcluster. The three subclusterswhich can be seen in Fig. 4 are:

1. Subcluster A included 3 strains:*Nectria haematococco, Fusarium phyllophilum* dan *Aureobasidium pullulans*.
2. Subcluster Bincluded4strains:*Fusarium armeniacum, Fusarium culmorum, Fusarium poae,* dan *Fusarium solani*.
3. Subcluster Cincluded2strains:*Fusarium oxysporum*and LBKURCC41.

Subcluster Cwas a close relative to both*F. oxysporum* and LBKURCC41 with similarity percentage of 99%(Table 5). Neighbour Joining bootstrapping tree test at 8 strains with LBKURCC41 by 10,000 repetitions was performed to test the branching and the validity of the phylogenetic tree. LBKURCC41 isolate hadbranching number of 6,355 with *F. oxysporum*. This background can be estimated that the LBKURCC41 isolate contained in dahlia tubers was *F. oxysporum*. This research had a different result from Lorenita *et al*.[15] which had identified the same species as *Aureobasidium* sp. This difference occurred due to the each method used in both research, which the previous research used macroscopic observation while this research used phylogenetic analysis.

Fusarium is a soil-borne pathogen and a weak parasite as well. This fungi is transmitted through soil, tubers, and rhizomes which are derived from plants and it infects plants through wounds on the rhizome. The injury can occur due to seeds transport, weeding, or insects. *F. oxysporum*goes through pathogenic phase and saprogenesis. In the phase of pathogenesis, this fungi lives as a parasite on host plants. If no host plants, the pathogen lives in the soil as a saprophyte on crop residues and then enters saprogenesis phase which can be a source of inoculum to cause disease in other plants. Some *Fusarium* species are pathogenic to wheat and maize [28].

CONCLUSION

Crude extract of endophytic fungi, LBKURCC 41 (*F. oxysporum*), showed the most significant antimicrobial activity on the Huang media. The antimicrobial activity on the 15^{th} day was greater than thaton 20^{th} day. Furthermore, the results of phylogenetic analysis concluded that DNA isolate of endophytic fungi LBKURCC41 had a molecular weight of 11,706 bp. rDNA regions containing ITS-1, 5,8S rDNA, and ITS-2 could be amplified using PCR with ITS-4 and ITS-5 primers andthe amplification results of primer pairs obtained for LBKURCC41 was 616 bp.The DNA sequence was identified as *Fusarium oxysporum* with a 99% similarity. Allignment with 8 closest species strains indicated that there was phylogenetic relationship among species of *F. oxysporum*, shown by high bootstrapping level with 10,000 repetitions on each branch.

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