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Identification of endophytic fungi in nutgrass (*Cyperus rotundus* L.) as solubilizing phosphate and indole-3-acetic acid producers

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ABSTRACT. Low phosphate content in the soil causes insufficient plant needs. Meanwhile, endophytic fungi in nutgrass have great potential as a phosphate solvent and produce indole-3-acetic acid (IAA). Therefore, this study aims to determine the solubilizing phosphate and IAA production by the endophytic fungi of nutgrass and identify the isolates based on rDNA-ITS sequences. Endophytic fungi isolates were cultured in 10 ml of Pikovskaya broth media with $Ca_5(PO_4)_3$ as the inorganic phosphate source. The PCR results were analyzed using 1.5% agarose gel electrophoresis followed by sequence analysis. The isolation and purification results showed five isolates coded URT1, URT2, URT3, URT4, and URT5, while the solubilizing phosphate levels ranged from 54.03-87.83 ppm, with the highest levels found in the URT4 isolate. Furthermore, the IAA levels ranged from 5.58-45.50 ppm, with the highest levels produced by the URT1 isolate. The molecular analysis with rDNA-ITS sequences showed that URT4 had 97.42% similarity to *Aspergillus terreus* species, while UTR1 had 100% similarity to *Fusarium oxysporum* species. Based on the results, the endophytic fungi of nutgrass from *A. terreus* and *F. oxysporum* species have great potential as biofertilizers due to the high solubilizing phosphate and IAA levels.

Keywords: *Aspergillus terreus*; biofertilizers; *Fusarium oxysporum*; phylogenetic tree analysis; rDNA-ITS sequences

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INTRODUCTION

Most tropical countries usually have a very large swamp area divided into low and tidal swamps. Meanwhile, the expansion of agricultural areas to lands with acid sulfate soils is not an option, but a demand for the future. However, the common problem with acid sulfate soils is the low availability of phosphate nutrients. Phosphate constitutes one of the macromolecular nutrients needed in the soil to meet plant needs. Furthermore, it is very important, especially for root growth stimulation, starch metabolism, assimilation process, elevation of homogeneous microspores, viable pollen and ovules, acceleration of flowering, and ripening of seeds or fruits (Ponnu *et al*., 2011; Kasno & Sutriadi, 2012; Ghanem *et al*., 2014). The phosphate requirement is inversely proportional in the soil, meanwhile, the availability of dissolved phosphate is usually very low therefore, it is not sufficient to meet plant needs. The application of phosphate fertilizer is a common alternative often used to overcome this problem but it has negative effects. Besides, the use of long-term chemical phosphate fertilizers above normal dosage has negative impacts on the environment, such as a decrease in soil quality, soil fertility, and crop yield (Jiao *et al*., 2012; Kaur & Reddy, 2015; Ning *et al.*, 2017).

George *et al.* (2019) and Simarmata *et al.* (2020), stated that endophytic microorganisms have great potential to promote growth and do not cause any pathogenic reaction to the host plant. Furthermore, phosphate-solubilizing microorganisms synthesize phosphates which are chemically and biologically undissolved. Zega *et al*. (2018) reported that 15 endophytic bacteria of nutgrass (*Cyperus rotundus* L.) dissolved phosphate and produced indole-3-acetic acid (IAA), but the levels were only moderately high. Nutgrass is a commonly known agricultural weed in the world due to its fast reproduction, high survival ability, wide distribution, and difficult growth control (Dor & Hershenhorn, 2013; Coleman *et al*., 2015; Peerzada, 2017). However, it contains several important compounds, such as polyphenols,

flavonoids, tannins, and sterols which are useful for antimicrobials, fungicides, bactericides, antigenotoxic, and antioxidants due to the symbiotic endophytic fungi present in the plant (Kilani-Jaziri *et al.*, 2011; Seo *et al*., 2011; Peerzada *et al.*, 2015; Masfria & Permata, 2018; Horn & Vediyappan, 2021).

Endophytic fungi are microorganisms with higher phosphate solubility compared to other microorganisms (Klaic *et al*., 2017; Mehta *et al*., 2019). George *et al.* (2019) reported that rhizome endophytic fungi produce useful active compounds for plant growth and development, such as dissolving phosphate and releasing IAA phytohormones. However, the potential of rhizome endophytic fungi in nutgrass as phosphate solvents and IAA producers has never been analyzed and published. Aside from this analysis, molecular markers are also used to analyze endophytic fungal species based on the rDNA-Internal Transcribed Spacer (ITS) sequences which located in 5-28S rDNA and the main evolutionary area. Furthermore, it is also used in species-level comparison (Bokulich & Mills, 2013; Elías-Román *et al*., 2018). Therefore, this study aims to analyze the potential of endophytic fungi in *C. rotundus* L. as phosphate solvents and IAA producers. In addition, the molecular analysis of the species found was also performed using rDNA-ITS sequences.

MATERIALS AND METHODS

Isolation of endophytic fungi. *C. rotundus* L. rhizome was washed for 10 min and then cut in a 2-3 cm (Hasyyati *et al.*, 2017). The samples were immersed in 75% alcohol for 1 min, 5.3% NaOCl for 3 min, and 75% alcohol solution for 30 s. Furthermore, the samples were dried using sterile tissue, split using a sterile knife, and placed in a Petri dish containing PDA media (Widowati *et al*., 2016).

Analysis of phosphate solubilizing. The obtained endophytic fungi isolates were cultured in 10 ml of Pikovskaya broth media with $Ca_5(PO_4)_3$ as the phosphate inorganic source. The Pikovskaya broth medium contains $Ca_5(PO_4)_3OH$ 5 g, glucose 13 g, $(NH_4)2SO_4$ 0.5 g, NaCl 0.2 g, $FeSO_4 \cdot 7H_2O$ 0.0002 g $MgSO_4 \cdot 7H_2O$ 0.1 g, yeast extract 0.5 g, KCl 0.2 g, $MnSO_4$ 0.0002 g dissolved in 1 L sterile water. Furthermore, the cultured isolates were incubated in a shaker at 25°C and a speed of 160 rpm for 7 days. The isolates were then filtered using sterile Whatman filter paper and the filtrate was centrifuged at 10000 rpm for 15 min, while 0.4 ml of the supernatant was taken and placed into a test tube. Molybdate phosphate color reagent was added 0.16 ml of 2.5% sodium molybdate, 0.04 ml of 10 N sulfuric acids, 0.1 ml of 0.5 M hydrazine sulfate, and 2.3 ml of sterile distilled water, and left to stand for 10-35 min. A positive result is indicated when the solution changes to blue color (Syamsia *et al*., 2015). The absorbance was analyzed using a UV-VIS spectrophotometer with a wavelength of 840 nm, while the data were processed using the formula y = 0.0271x + 0.0623 (Hutton & Gregory, 1837). Moreover, the phosphate concentration was calculated based on the standard phosphate curve in ppm units and the applied standard curve was KH_2PO_4 with a concentration of 0, 2.5, 5, 10, 20, and 50 ppm (Lynn *et al*., 2013).

Analysis of IAA production. Potato dextrose broth (PDB) medium was sterilized using an autoclave with a pressure of 1 atm and 121°C for 15 min. The media were then added with a precursor L-tryptophan solution (10 g glucose, 1 g L-tryptophan, 0.1 g yeast extract dissolved in 100 ml sterile water) in a ratio of 20:1 for each tube. Furthermore, the endophytic fungi were inoculated in PDB at 25°C, and 160 rpm for 5 days, while the isolates were filtered using Whatman filter paper. The filtered endophytic fungi colonies and PDB media were centrifuged at 6000 rpm for 15 min, while 2 ml of the supernatant was taken, placed into a test tube and then added with 4 ml of Salkowski reagent (36.8 ml H_2SO_4 96%, 1.8 ml $FeCl_3.6H_2O$ 0.5 M, and 61.4 ml distilled water) and incubated in a dark room for 60 min. A positive result is indicated when the solution changes to pink color. The IAA content was analyzed using a UV-VIS spectrophotometer with a wavelength of 540 nm. The blank solution used was PDB media added with L-tryptophan, meanwhile, the concentration of IAA produced was determined using the

formula y = 0.0221x + 0.039 (Hutton & Gregory, 1837). Furthermore, the IAA concentration was based on pure IAA in the standard curve of the sample. The applied concentrations include 0, 2.5, 5, 10, 20, and 50 ppm (Dewi *et al*., 2015).

Morphological characterization. The morphological characteristics were analyzed by macroscopic and microscopic observation. Microscopic observations were performed using the basic slide culture method by (Rosana *et al*., 2014). Potato dextrose agar (PDA) medium was cut 0.5-by-0.5 cm block and placed on the glass slide, meanwhile, the inoculation was carried out using a sterile wire needle from the culture plate to four sides of the PDA block. Sterile water was added to the tissue in the Petri plate, the cover was replaced, and the basic slide culture was incubated at 30°C. Observation on the morphological characteristics was based on an identification guide book titled description of medical fungi (Kidd *et al*., 2016), pictorial atlas of soil and seed fungi, morphologies of cultured fungi and key to species (Watanabe, 2010).

DNA extraction and quantification. A total of 100 mg of endophytic fungi mycelium aged 7 days was taken, crushed in a sterile mortar, transferred into a 2 ml tube, added with 1000 µl of 2x CTAB buffer, vortexed, and incubated in a water bath at 65°C for 60 min. Furthermore, it was added with 900 µl of chloroform:isoamyl alcohol (24:1), incubated for 1 h at room temperature, and then centrifuged for 10 min at 13000 rpm. The supernatant was pipetted in a 1.5 ml tube, added with 1x volume of chloroform:isoamyl alcohol (24:1) and centrifuged for 10 min at 13000 rpm. The precipitation stage was performed using isopropanol with a dose of 2/3 from the volume of the supernatant. The solution was left to stand overnight at a temperature of -4°C, and then centrifuged for 10 min at 13000 rpm. Furthermore, the pellets were washed using 500 µl of absolute ethanol and then centrifuged for 5 min at 13000 rpm. The samples were dried in an oven at 25°C and then added with 20 µl of elution buffer and stored at -4°C (Aamir *et al*., 2015). DNA quantification was conducted using nano drops AE-Nano200 Nucleic Acid Analyzer version 2.0, while, the purity value was determined by calculating the absorbance ratio Å260/Å280. Meanwhile, DNA qualitative analysis was determined using 0.8% agarose gel electrophoresis (Tripathy *et al.*, 2017).

Amplification of Polymerase Chain Reaction (PCR). The PCR amplification was carried out using the molecular target in primer R-ITS1 (`TCCGTAGGTGAACCTGCGG`) and F-ITS4 (`TCCTCCGCTTATTGATGC`) (Zhang *et al.*, 2010). The composition of a total PCR volume is 25 µl according to (Geisen *et al*., 2017). Furthermore, PCR was carried out using the tool, My CyclerTM Cycler BIO-RAD Thermo Cycler, while ITS1 and ITS4 rDNA sequences amplification applied an annealing temperature of 55°C. The PCR results were analyzed using 1.5% (v/v) agarose gel electrophoresis for 30 min with a voltage of 100 v, 400 mA. The gel from electrophoresis was immersed in ethidium bromide (EtBr) for 15 minutes and was visualized using the Gel DocTM XR Imaging System BIO-RAD.

Sequencing analyses. The sequencing result data were read using Sequence Scanner 1.0 and compared to Gen Bank via the BLAST menu on NCBI. Furthermore, the alignment was carried out using the Clustal-X program, while the data were imported directly to MEGA 5.0 for phylogenetic tree analysis. The phylogenetic tree was constructed using the Neighbor-Joining algorithm with 1000 Bootsrap based on p-distance.

Data analysis. The quantitative data on dissolved phosphate levels and IAA were analyzed using SPSS 16.0. In addition, the data were tested for normality using the Shapiro-Wilk and homogeneity using Levene's test. When the data were normal and homogeneous, then it was followed by the ANOVA and DMRT test when there are differences in the effect of the isolates.

RESULTS AND DISCUSSION

Phosphate solubilizing levels produced by endophytic fungi. The endophytic fungi obtained from the isolation and purification of nutgrass (*C. rotundus* L.) produced five isolates coded URT1, URT2, URT3, URT4, and URT5. The solubilizing phosphate in the endophytic

fungi of nutgrass showed positive results indicated by the blue color with varying density in each isolate (Fig. 1).

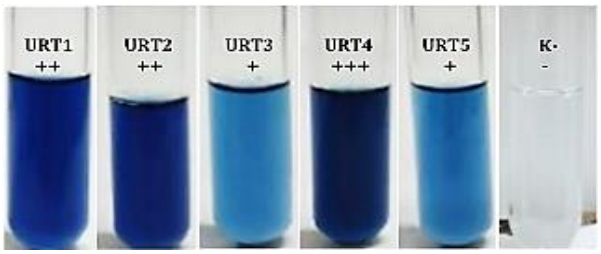

Fig. 1. The reaction of blue molybdenum with endophytic fungi isolates of nutgrass: (K-) negative control; (-) does not change in color; (+) light blue; (++) dark blue; (+++) deep blue.

Based on the results, URT4 produced the strongest color, URT1 and URT2 were dark blue, while, URT3 and URT5 produced a light blue color. Meanwhile, Pradhan & Pokhrel (2013) reported that the blue color density from the blue molybdenum reaction indicates the level of dissolved phosphate concentration produced by the sample. The blue color was produced due to a chemical reaction. Phosphoric acid (dissolved phosphate) binds to ammonium molybdate and form heteropoly acid which is then reduced by hydrazine sulfate to form phosphomolybdenum indicated by a blue color solution.

The quantitative analysis showed that the endophytic fungi of nutgrass produced dissolve phosphate ranging from 54.03-87.83 ppm (Fig. 2). Furthermore, the statistical analysis indicates that each isolate and negative controls were significantly different ($p<0.05$). The highest dissolved phosphate level was produced by the URT4 isolate, while the lowest was produced by the URT3. These results indicate a higher score compared to Syamsia *et al.* (2015), which reported that the endophytic fungi isolate of Enrekang aromatic rice produced dissolve phosphate ranging from 8.92-10.86 ppm. Furthermore, the quantitative and qualitative analysis of the nutgrass endophytic fungi also showed that the URT4 isolate had the highest ability to dissolve phosphate.

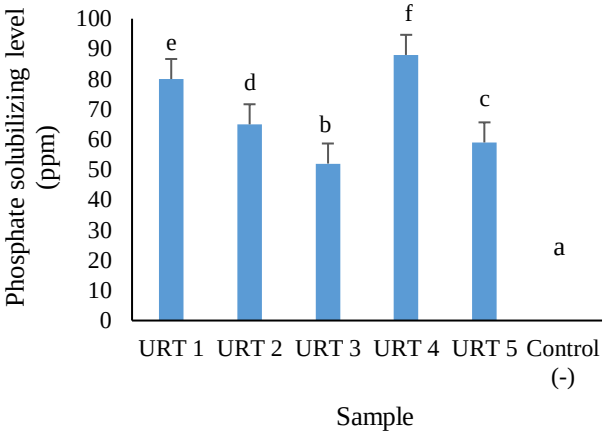

Fig. 2. Dissolved phosphate levels of endophytic fungi of nutgrass. Different superscripts indicate the comparison of means that are significantly different ($p < 0.05$).

Indole-3-acetic acid levels produced by endophytic fungi. Five isolates showed positive results in the test of IAA production (Fig. 3). The positive results were indicated by a change in the color of the supernatant solution from dark yellow to pink, meanwhile, this solution was reacted with Salkowski's reagent. Lestari *et al.* (2015) reported that the formed IAA concentration is characterized by a change in the color of the supernatant solution reacted with Salkowski's reagent from yellow to pink. In the negative control, no color change was observed. This is because the L-tryptophan in PDB media was not converted into IAA hence, the indole reaction was not achieved by Salkowski's reagent. Dewi *et al.* (2015) stated that the higher the color density, the higher the IAA production. Based on the color of the solution formed, the highest qualitative IAA levels were found in the URT1 isolate. Furthermore, URT3, URT4, and URT5 showed a change in color into pink, indicating that the level of IAA production was moderate. Meanwhile, in the URT2 isolate, the reaction color was yellow which indicates a low level of IAA production. The concentration of L-tryptophan is presumably one of the factors that determine the optimal level of IAA produced, meanwhile, the optimal level is determined by the color density produced by each isolate.

Fig. 3. The reaction of Salkowski's reagent with endophytic fungi isolates of nutgrass: (K-) negative control; (-) does not change in color; (+) yellow; (++) pink; (+++) deep red.

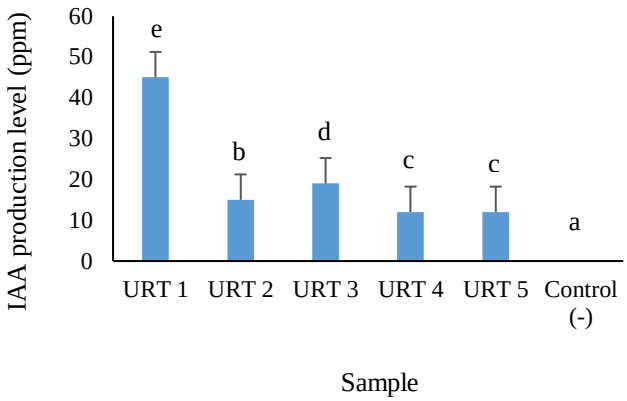

Fig. 4. The concentration of IAA production from endophytic fungi of nutgrass. Different superscripts indicate the comparison of means that are significantly different ($p < 0.05$).

The quantitative analysis showed that endophytic fungi of nutgrass produced IAA. Based on statistical analysis, the IAA level produced showed that each isolate (except URT3 and URT5) and negative controls were significantly different ($p < 0.05$) (Fig. 4), while URT3 and URT5 showed no significant difference ($p > 0.05$). Furthermore, the URT4 isolate produced the highest levels of IAA, which varied between 5.58-45.50 ppm. The highest IAA level was produced by the URT1 isolate, while the lowest was produced by the URT2. However, this result was also higher compared to Mehmood *et al*. (2018) which stated that the IAA production of *F. oxysporum* ranged from 1.5-2.5 mg/L.

Morphological characterization. Based on the results, URT4 and URT1 had high levels in both dissolved phosphate and IAA production tests. Therefore, morphological observations focused on both isolates. The fungal colony of URT1 and URT 4 have distinctive characteristics as presented in Fig. 5. The colony was white with aerial hyphae (URT1), zonate between brown and green with floury (URT4), macroconidia 29.15 µm (URT1), conidia 9-10 µm in diameter (URT4), conidial head 235 x 163 µm in diameter.

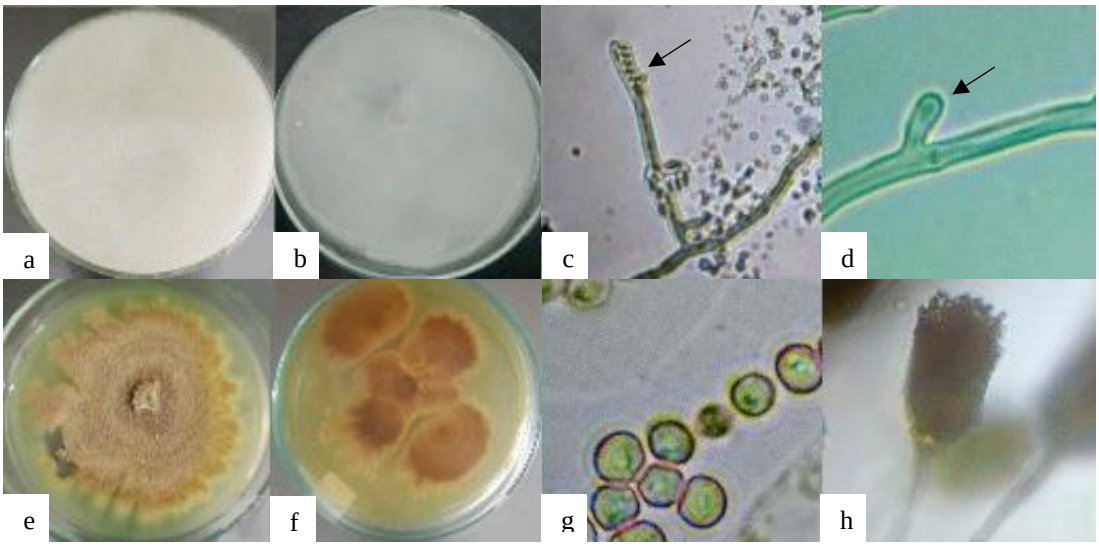

Fig. 5. The morphological (colony appearance, conidial head, and macroconidia) from endophytic fungi of nutgrass: a. URT1 (upper side); b. URT1 (reverse side); c. URT1 (macroconidia); d. URT1 (chlamydospore); e. URT4 (upper side); f. URT4 (reverse side); g. URT4 (conidia); h. URT4 (conidial head).

Visualization of PCR. The visualization of URT1 and URT4 PCR amplification is presented in Fig. 6. The results showed that both isolates were parallel to the marker with a size of 550 and 625 bp respectively.

Meanwhile, Poczai & Hyvönen (2010) showed that the ITS area varies between 500-750 bp.

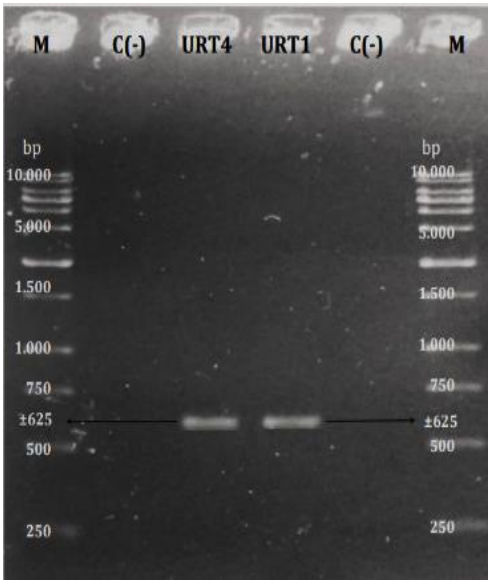

Fig. 6. Visualization of DNA bands from PCR amplification on 1.5% agarose gel: M= Marker; C (-)= negative control.

Sequencing and phylogenetic analysis. Data sequencing analysis for URT4 and URT1 isolates with other sequences in the NCBI was conducted using the BLAST program and the results are presented in Table 1. The URT4 isolate had 97.42% similarity with *A. terreus* AGE while URT1 had 100% similarity with *F. oxysporum* AS13-D89.

Table 1. BLAST results of URT1 and URT4 isolates of the endophytic fungi of nutgrass rhizome (*C. rotundus* L.).

Isolate code	BLAST Species	Similarity (%)	Seq. id.
URT4	*Aspergillus terreus* AGE	97.42	MT152989.1
URT1	*Fusarium oxysporum* AS13-D89	100	MN153518.1

The reconstruction of relationships and diversity of organisms based on the level of similarity was analyzed using phylogenetic and the results are shown in Fig. 7. This reconstruction based on rDNA-ITS sequences indicated that there were two clades with a bootstrap of 76% and 100%.

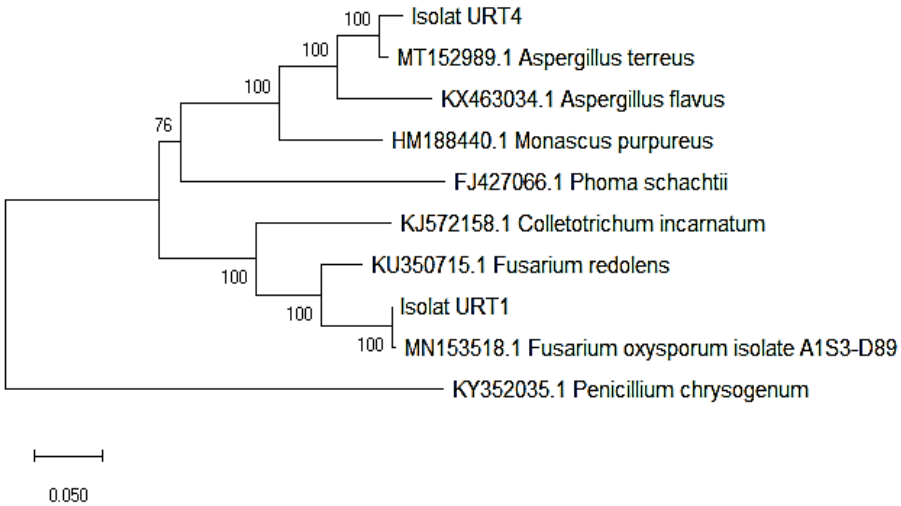

Fig. 7. The reconstruction of the phylogenetic tree for endophytic fungi of nutgrass isolates using the neighbor-joining method and the bootstrap with 1000 replications.

The URT4 isolate had 97.42% similarity percentage with *A. terreus* and a genetic distance of 0.026, indicating a high similarity value. Meanwhile, the URT1 isolate had 100% similarity with *F. oxysporum* and a genetic distance of 0.00. Furthermore, URT4 showed similarities to *A. terreus*, while the URT1 showed similarities to *F. oxysporum*. This implies that *A. terreus* and *F. oxysporum* have great potential to dissolve phosphate and produce IAA. *Aspergillus* genus is one of the fungi that has been shown to effectively dissolve phosphate (Srinivasan *et al*., 2012). This is due to its ability to survive in conditions of low humidity, extreme temperatures, and low pH since the growth of phosphate-

solubilizing microorganisms is influenced by soil acidity (Fatmala *et al*., 2015). Besides, the fungus *A. terreus* tends to also increase the growth of Ceriops tagal seedlings (Egbuta *et al*., 2016). Furthermore, Mehmood *et al.* (2018) and Radhakrishnan *et al.* (2015) reported that fungi in the genus *Fusarium* have great potential to dissolve phosphate and produce IAA. Both species are endophytic fungi found in soil and are associated with plants. *A. terreus* and *F. oxysporum* belong to the same division namely Ascomycota. Egbuta *et al.* (2016), stated that the most researched and utilized kingdom fungus is Ascomycota.

CONCLUSION

Endophytic fungi of nutgrass (*Cyperus rotundus* L.) have great potential to dissolve phosphate and produce IAA. The highest phosphate and IAA levels were produced by URT4 and URT1 isolates. Furthermore, URT4 has similarities with *Aspergillus terreus*, while URT1 has similarities with *Fusarium oxysporum*, meanwhile, both isolates are applicable as biofertilizers.

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