ENDOPHYTIC MICROBES OF NEEM PLANT AS NATURAL SOURCES OF IAA GROWTH HORMONE POTENTIAL FOR AGRICULTURAL INDUSTRIES

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Abstract

Indonesia with its tropical climate is rich in diversity of microorganisms with potential functions and essential values that can be developed for industrial, health, and agricultural products. Neem (*Azadirachta indica* A. Juss) is a plant commonly used as natural insecticide for pest control. Biotechnology can be assigned to exploit the unique and interesting potential of endophytic microbes of neem plant to obtain beneficial bioactive compounds without taking a large amount of the plant biomass. This study aims to explore potential endophytic microbes of neem plant as producer of Indole-3-acetic acid (IAA) growth hormone to support plant growth. Through isolation and screening, we obtained three isolates of IAA hormone-producing bacteria from 19 isolates of endophytic bacteria tested. Thirty-six isolates of endophytic fungi were also tested, of which 18 isolates were positive for IAA hormone production. One endophytic fungus isolate produced the highest IAA hormone (1,676 ppm) and has been identified molecularly as *Colletotrichum gloeosporioides*. Endophytic microbes of neem plant are potential to be developed into biotechnology products to support plant growth with high commercial value by utilizing promising genes.

Keywords: *neem, IAA hormone, endophytic microbes*

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Introduction

Indonesia is a tropical country with the most abundant flora and fauna and microorganisms diversity of the world. They play an essential role in human lif e, including in agriculture.

Plant maintenance is done not only by controlling pests, the availability of nutrients is also crucial for plant's survival. Neem (*Azadirachta indica* A. Juss) is a source of plant-based insecticides, which is often used for pest control. The leaf and the seed are essential parts of neem plant because they can be applied as insecticides ingredient for vegetables. They also contain azadirachtin as the main bioactive compound. Neem plant, which is usually used as a source of insecticides for vegetables, has some other economic potentials, including its endophytic microbes. Currently, research on microbial resources in plant tissues is gettingmore attention (Strobel *et al*., 2004, Gunatilaka, 2006).

Endophytic microbes are microbes associated with plant tissue. Some endophytic microbes are useful and have high economic value, hence potential to be utilized for industrial purposes. Indole-3-acetic acid (IAA) growth hormone is usually added to insecticides to help plant recovery so that yield production remains high (Shaharoona *et al*., 2006; Joshi and Bath, 2011). Some endophytic microbes are able produce natural growth hormones, including IAA. The growth hormones produced by endophytic microbes will help stimulate root growth so that water and nutrients can be easily absorbed to support plant growth. This research activity aims to obtain endophytic microbes from neem plant, which have another potential in producing plant

growth hormone especially IAA to stimulate plant growth.

Materials and Methods

Neem Plant Isolation and Isolation Media.

CMM (*Corn meal malt extract*) isolation media with an addition of 50 ppm chloramphenicol antibiotic, was used to isolate endophytic fungi from neem plant. The composition of CMM media was: cornmeal agar (Difco) 1.7 gr/100mL; malt extract 2 gr/100mL; yeast extract 0.2 g/100mL. NA (*Nutrient Agar*) isolation media was used to isolate endophytic bacteria from neem plant. The materials used were nutrient agar (Difco) 2.3 gr/100mL, and nystatin 10 ppm. The isolation media was poured into petri dish for aseptic isolation of endophytic bacteria and fungi.

Endophytic Isolation from Neem Plants.

The samples of neem plants were washed under running water. The plant samples used in this study were stems and leaves, including young and old stems and old leaves. The plant samples were surface-sterilized by immersing them in 75% alcohol solution for 1 minute and then in 5.3% sodium hypochlorite solution for 5 minutes. Soft tissues were only sterilized using 75% alcohol solution for 30 seconds. Samples were grown on aseptic isolation media. The samples were incubated, and the isolates were observed, then the endophytic bacteria and fungi were purified. Pure endophytic bacteria colonies were grown on NA slants whereas endophytic fungi were grown on Potato Dextrose Agar (PDA) slants.

Screening of Potential Endophytic IAA-Producing Microbes from Neem Plants.

One percent of tryptone broth medium was prepared. Bacterial isolates were cultured in tryptone and incubated for 24 hours, while fungal isolates were incubated for six days. All cultures were harvested and then centrifuged at 4000 rpm for 20 minutes at 4^0C. The supernatant obtained was taken as much as 0.6 mL and then was added with 2.4 mL of Salkowski reagent (100 mL distilled water; H_2SO_4 60 mL, $FeCl_3.6H_2O$ 3 mL) then the observation and absorbance were measured by using spectrophotometer (Tanaka *et al.*, 2003).

Molecular Identification of NBT 41 DNA from Fungal Isolates.

The identification of isolates was carried out at the Genetics Science Laboratory, Jakarta. Fungal DNA extraction was carried out using Quick-DNA Fungal / Bacterial Miniprep Kit (Zymo Research, D6005). Internal Transcribed Spacer (ITS) gene was used as molecular marker to identify fungal isolates. PCR amplification was carried out using MyTaq HS Red Mix (Bioline, BIO-25048) and the PCR product was purified using the ZymoBIOMICS ™ DNA Miniprep Kit (D4300). To clarify the ITS fragments from isolates, the purified fragment was sent to Malaysia 1st BASE sequencing service provider. Sequencing results were then processed following the BLAST (Basic Search Alignment Search Tool) procedure from NCBI (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/), and BOLD (Barcode of Life Data System http: // www.boldsystems.org/), and MycoBank (http://mycobank.org) for species identification.

Results

Isolates of Endophytic bacteria from Neem Plant.

A total of 19 isolates of endophytic bacteria from neem plant were obtained and screened for their IAA-producing potential. Table 1 shows the growth rate of the endophytic bacteria which, on average is relatively fast. In this study, the endophytic bacteria grew rapidly within 24 hours. However, some slow-growing isolates, namely NBM 11, NBM 12 (4 days), and NBT 11, NBT 12, took four days to grow. The isolation results of endophytic bacteria are listed in Table 1. Based on the data from the isolation results in table 1, if the food availability is sufficient and the environment is suitable, endophytic bacteria will grow relatively quickly.

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Table 1. List of endophytic bacteria isolate from neem plants.

No.	Isolate code	Colony color + size	Growth rate
1	NTBD 111	milky white + small	fast
2	NTBD 112	milky white + small	fast
3	NTBD 211	milky white + small	fast
4	NTBD 212	milky white + medium	fast
5	NBM 11	milky white + small	slow
6	NBM 12	milky white + medium	slow
7	NBM 21	milky white + small	fast
8	NBT 11	milky white + small	slow
9	NBT 12	milky white + big	slow
10	NBT 21	milky white + small	fast
11	NDT 111	milky white + small	fast
12	NDT 112	milky white + small	fast
13	NDT 113	yellow + small	fast
14	NDT 211	milky white + big	fast
15	NDT 212	milky white + small	fast
16	NDT 311	milky white + big	fast
17	NDT 312	milky white + small	fast
18	NDT 313	milky white + big	fast
19	NDT 314	milky white + big	fast

Note: NTBD: stem buds, NBM: young stems, NBT: old stems, NDT: old leaves

Endophytic bacteria from neem plants have different colony colors, such as milky white and milky yellow. Another difference was found in colony size, which can be categorized as large, medium, and small.

The next results are the isolation of endophytic fungi as shown in Table 2. Based on the data from Table 2, the growth of endophytic fungal spores showed a unique character from each isolate obtained. A difference was seen in the colony size, the largest diameter were NDT 92 and NDT 51 (8.0 cm), while the smallest was NTBD 21 (3.8 cm). On average, it took about four days for endophytic fungi to form spores.

However, when compared to endophytic bacteria, the time for endophytic fungi to form spores was relatively longer than that of endophytic bacteria. There were some endophytic bacteria that grow fast within 24 hours. The difference in growth time is because endophytic microbes have a unique cell

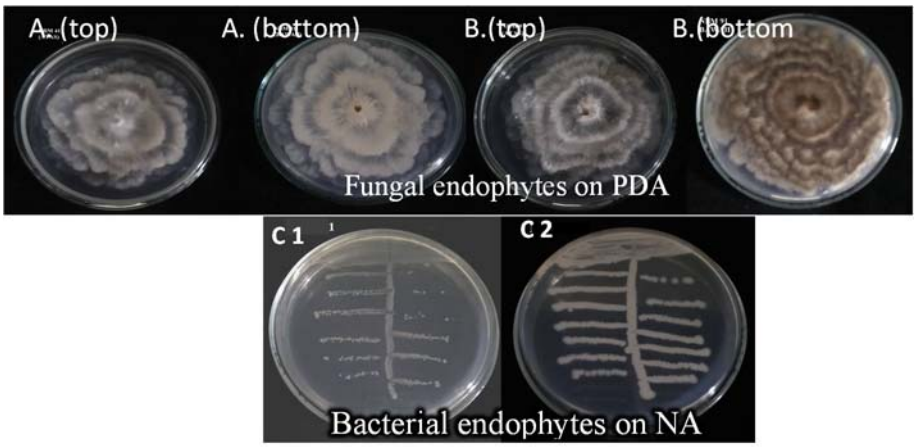

Figure 1. Growth of endophytic microbes from neem. (A) NBM 41 (B) NBM 91 (C1) NBT 21 (C2) NDT 211.

Table 2. List of isolates of endophytic fungi from neem plant.

No.	Isolate code	Spore color (top)	Spore color (bottom)	Diameter (cm)	Growth rate
1	NTBD 11	white	greenish white	5,0	slow
2	NTBD 21	white	white	3,8	slow
3	NBM 11	white	greenish white	6,8	fast
4	NBM 21	white	brownish white	6,1	fast
5	NBM 31	white	greenish white	4,8	slow
6	NBM 32	white	brownish white	6,4	fast
7	NBM 41	white	yellowish white	7,2	fast
8	NBM 51	white	brownish white	6,8	fast
9	NBM 52	white	orange white	6,5	fast
10	NBM 61	white	greenish white	5,8	slow
11	NBM 62	white	greenish white	6,8	fast
12	NBM 71	white	greenish white	6,4	fast
13	NBM 81	white	greenish white	6,8	fast
14	NBM 91	white	greenish white	7,6	fast
15	NBT 11	white	orange white	6,5	fast
16	NBT 12	white	white	5,9	slow
17	NBT 21	white	white	6,2	fast
18	NBT 31	white	brownish white	6,2	fast
19	NBT 41	white	white	6,3	fast
20	NBT 51	white	brownish white	5,0	slow
21	NBT 61	white	brownish white	5,4	slow
22	NDT 11	white	blackish white	6,1	fast
23	NDT 21	white	greenish white	6,7	fast
24	NDT 311	white	white	5,0	slow
25	NDT 32	white	greenish white	6,0	fast
26	NDT 33	white	white	6,9	fast
27	NDT 41	white	white	6,9	fast
28	NDT 51	white	yellowish white	8,0	fast
29	NDT 61	white	greenish white	7,1	fast
30	NDT 71	white	greenish white	5,1	slow
31	NDT 81	white	white	6,2	fast
32	NDT 82	white	white	5,4	slow
33	NDT 911	white	white	5,0	slow
34	NDT 92	white	brownish white	8,0	fast
35	NDT 10.1	white	white	4,5	slow
36	NDT 11.	white	greenish white	6,5	fast

Note: NTBD: stem buds, NBM: young stems, NBT: old stems, NDT: old leaves

structure and metabolism for their growth. The endophytic fungi had different colors and characteristics, for example, some have white spores (as seen from the top) and greenish white (as seen from the bottom) or their spores were white at the top and brownish at the bottom.

Figure 1. shows the obtained fungal isolates grown on agar media. The growth pattern of endophytic fungi observed from the top, shows a regular pattern of spore with a distinctive and unique spore color. The top appearance of the fungi is neatly arranged and the pattern also looks similar from the bottom. If there is contamination, it will form a different patch of contaminant fungi. Endophytic bacteria derived from neem plants as shown in Figure 1. have been purified, then the bacterial colonies will be transferred to a slanted tube and stored as culture stock. Unique and interesting endophytic microbes from neem plants can potentially be exploited and developed so they have high commercial value.

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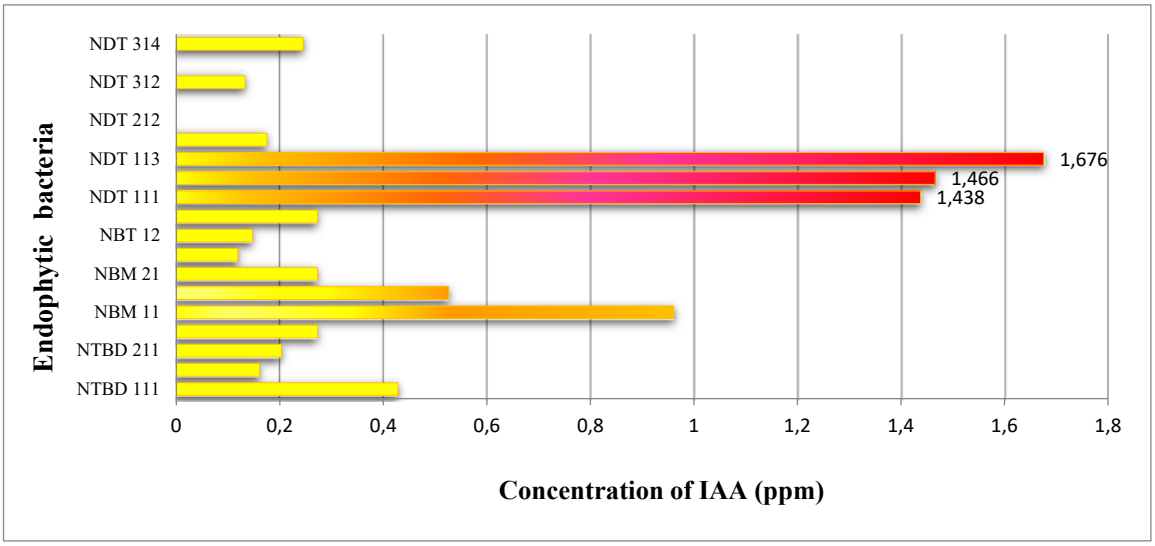

Figure 2. IAA concentrations produced by endophytic bacteria from the neem plant

Screening of Potential Endophytic IAA-Producing Microbes from Neem Plants.

Endophytic fungi that can produce certain secondary metabolites can sometimes change the agar medium (where to grow) the fungus. The changing media color occurs at the bottom of the media. The color of the media formed can be brown, black or other colors. According to Collemare et al. (2008), secondary metabolites produced by fungi play an important role in agriculture to inhibit infection mechanisms and pathogenesis (plant resistance). The secondary metabolites produced are the same as those produced in their original habitat. We obtained a total of 36 pure isolates and tests were carried out on potential endophytic fungi and bacteria. The results of bacteria capable of producing IAA are listed in Table 3 below.

Table 3. IAA test results on endophytic bacteria from neem plants

No.	IAA Code	Isolate Code	Optical density	Color	IAA(ppm)
1	37	NTBD 111	0.027	yellow	0.428
2	38	NTBD 112	0.008	yellow	0.161
3	39	NTBD 211	0.011	yellow	0.203
4	40	NTBD 212	0.016	yellow	0.273
5	41	NBM 11	0.065	yellow	0.961
6	42	NBM 12	0.034	yellow	0.526
7	43	NBM 21	0.016	yellow	0.273
8	44	NBT 11	0.005	yellow	0.119
9	45	NBT 12	0.007	yellow	0.147
10	46	NBT 21	0.016	yellow	0.273
11	47	NDT 111	0.099	light pink	1.438
12	48	NDT 112	0.101	light pink	1.466
13	49	NDT 113	0.116	deep pink	1.676
14	50	NDT 211	0.009	yellow	0.175
15	51	NDT 212	-0.008	yellow	0
16	52	NDT 311	-0.007	yellow	0
17	53	NDT 312	0.006	yellow	0.133
18	54	NDT 313	-0.008	yellow	0
19	55	NDT 314	0.014	yellow	0.245

Note: NTBD: stem buds, NBM: young stems, NBT: old stems, NDT: old leaves

Figure 2 shows that bacteria from neem plant with the code NDT 113 produced the highest IAA of 1,676 ppm. The IAA produced by bacteria will help plants to stimulate root growth, enabling wider nutrients absorption and increase plant resistance to disease. According to Kutschera (2007), bacteria enter plant tissue and eventually live as endophytic bacteria. Some endophytic microbes are known to produce phytohormones, especially the growth hormone IAA. Host plants with endophytic microbes have many advantages, such as faster growth, increased resistance to drought and pest attacks. Nester and Liu (2006) also added that the biosynthetic mechanism of IAA occurs due to bacterial infection in plants. These bacteria have Ti-plasmid in which there is a T-DNA containing the Tms 1 and Tms 2 genes. At the time of infection, the T-DNA is transferred to the host plant cell and fuses with the genome in the plant cell nucleus. Both genes go to the cytosol and synthesize two types of enzymes. The tms-I gene synthesizes the enzyme tryptophan-mono-oxygenase which converts tryptophan to Indole-3acetamide, while the tms-II gene synthesizes the enzyme Indole acetamide hydrolase, which converts Indole-3-Acetamide to Indole-3-Acetic Acid (IAA). These two genes are called Root-inducing genes (Roi-genes), known as genes that stimulate root growth.

IAA is a growth hormone that can promote plant growth. Plants have limitations in synthesizing IAA in supporting optimal growth, so additional growth-stimulating hormones from external are needed which can be given through fertilizers or microorganism symbiosis. One of which is through the help of endophytic bacteria. Endophytic bacteria that can produce IAA are used for plant growth and biocontrol. Neem is commonly used as a biocontrol agent to kill insect, pests and inhibit the development of plant diseases (Wang *et al*., 2010; Krishnamurthy and Shashikala, 2006). According to Torres-Rubio *et al.* (2000), the addition of endophytic microbes might induce soil microbes to produce IAA hormone including *Azospirillum* sp., *Enterobacter* sp., *Azotobacter* sp., *Klebsiella* sp., *Bacillus* sp., *Cyanobacteria* sp., and sulfur bacteria which can promote plant growth.

Based on the IAA test results on endophytic bacteria from the neem plant, three isolates produced the highest IAA growth hormone. The three isolates are NDT 111, NDT 112, and NDT 113 (Figure 2). These isolates can produce IAA hormone characterized by a change in color to pink caused by a reaction between the Salkowski reagent and IAA. NDT 113 was the isolate with the highest absorbance of 0.116. Meanwhile, NDT 212, NDT 311 and NDT 313 were the isolates with the lowest absorbance (no IAA production activity), and 13 isolates produced IAA on a low scale.

Isolates showing the most pronounced red color produced greater IAA. The reddish discoloration of the isolates after being given Salkowski's reagent occurred because of the reaction between Salkowski's reagent and IAA or with several IAA-forming compounds. IAA binds with $FeCl_3$ and $HClO_4$, which are the constituent compounds of the Salkoswki reagent, to form a complex tris- (indole-3-aceto) iron (III) which gives a pink to red color. The color change to red in Salkowski test of endophytic microbial isolates from neem plants, indicates the endophytic microbes' ability to metabolize L-tryptophan to IAA (Rahman *et al.*, 2010). Endophytic microbial isolates produce higher IAA when L-tryptophan precursor is added and will synthesize IAA through the Trp-pathways. Under natural conditions, plants will secrete organic matter, including L-tryptophan, which bacteria can use for IAA biosynthesis (Chaiharn & Lumyong, 2011; Ahmad *et al.*, 2005).

The results of the potential test of endophytic fungi in producing IAA can be seen in Table 4. According to Dewi *et al.* (2015), the IAA hormone produced by microbes in the stationary phase shows high IAA production. IAA effects are not limited to cell division and elongation, but also on initiation of root, leaf, and flower systems, fruit development, and aging. IAA also reduce ethylene levels in plants, allowing greater IAA signal transduction. Endophytic microbes are able to adapt and interact more quickly in plant tissues compared to the rhizosphere (Rashid *et al*., 2012).

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Table 4. IAA test results on the endophytic fungi from neem plant

No.	Isolate Code	Optical Density	Color	IAA (ppm)
1	NTBD 11	0.022	yellow	0.358
2	NTBD 21	0.042	yellow	0.638
3	NBT 11	0.147	light pink	2.111
4	NBT 12	0.426	deep pink	6.024
5	NBT 21	0.361	deep pink	5.112
6	NBT 31	0.024	yellow	0.386
7	NBT 41	0.592	deep pink	8.352
8	NBT 51	-0.002	yellow	0.021
9	NBT 61	0.010	yellow	0.189
10	NBM 11	0.332	deep pink	4.705
11	NBM 21	-0.006	yellow	-0.035
12	NBM 31	0.001	yellow	0.063
13	NBM 32	-0/005	yellow	0.049
14	NBM 41	0.051	yellow	0.764
15	NBM 51	0.024	yellow	0.386
16	NBM 52	0.368	deep pink	5.210
17	NBM 61	0.352	deep pink	4.986
18	NBM 62	0.143	light pink	2.055
19	NBM 71	0.387	deep pink	5.477
20	NBM 81	0.239	deep pink	3.401
21	NBM 91	0.024	yellow	0.386
22	NDT 11	-0.010	yellow	-0.091
23	NDT 21	0.348	deep pink	4.930
24	NDT 311	0.457	deep pink	6.459
25	NDT 32	0.304	deep pink	4.313
26	NDT 33	0.348	deep pink	4.930
27	NDT 41	0.174	light pink	2.489
28	NDT 51	0.009	yellow	0.175
29	NDT 61	0.218	deep pink	3.107
30	NDT 71	0.001	yellow	0.063
31	NDT 81	-0.020	yellow	-0.231
32	NDT 82	-0.006	yellow	-0.035
33	NDT 911	0.403	deep pink	5.701
34	NDT 92	-0.006	yellow	-0.035
35	NDT 10.1	0.026	yellow	0.414
36	NDT 11.	0.207	light pink	2.952

Note: NTBD: stem buds, NBM: young stems, NBT: old stems, NDT: old leaves

Figure 3 shows the ability of the endophytic fungi to produce IAA, although several fungi do not produce IAA. The fungus that produces the highest IAA is NBT 41 isolate which is around 8,352 ppm. These results provide information that not only bacteria have the potential to produce IAA growth hormone, the endophytic fungi from neem plants can also have high IAA production in the range of 8,352-0.021ppm.

IAA growth hormone has numerous roles on plant growth. According to Astriani *et al.* (2014), *Colletotrichum gloeosprorioides* and other fungi such as *Phanero chaetechrysosproium* and *Aeschynomene* are able to produce IAA.

Figure 4 shows the results of IAA production on endophytic microbes using the Salkowski method. According to Tanaka *et al.* (2003), IAA production is tested qualitatively using Salkowski method, given the addition of tryptophan. The use of tryptophan is needed because tryptophan is a primary precursor in the biosynthesis of IAA.

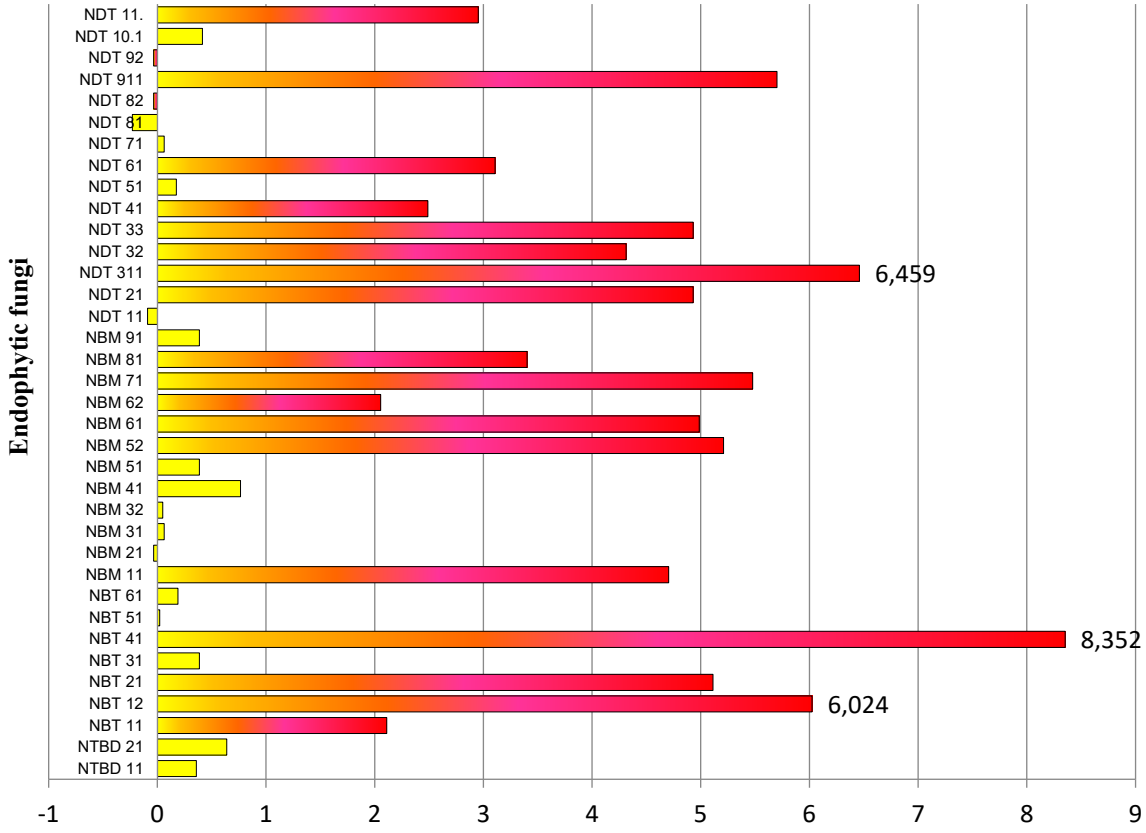

Figure 3. IAA concentrations produced by various endophytic fungi from neem plant.

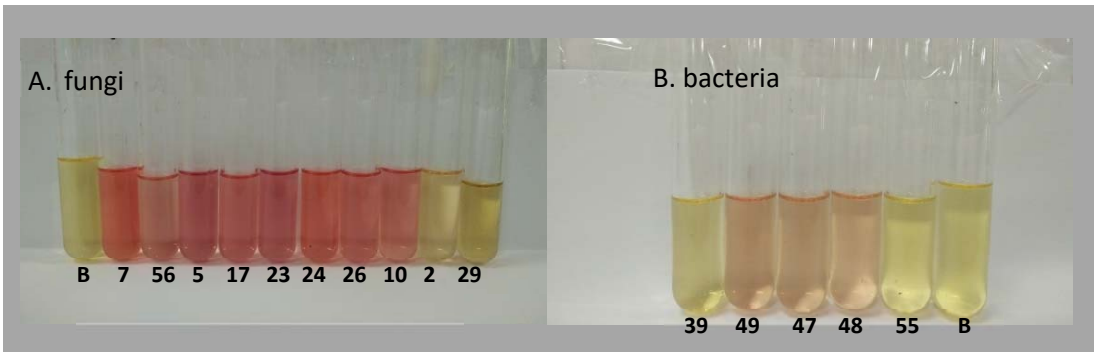

Figure 4. IAA test results using Salkowski method. (A) Ten fungal endophytic isolates samples and the negative control. (B) Five bacterial endophytic isolates samples and the negative control. B = Blanko (negative control).

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Based on the endophytic fungi test data from the neem plant, 18 isolates were positive for the IAA hormone. These isolates were NBT 11, NBT 12, NBT 21, NBT 41, NBM 11, NBM 52, NBM 61, NBM 62, NBM 71, NBM 81, NDT 21, NDT 311, NDT 32, NDT 33, NDT 41, NDT 61, NDT 911, and NDT 11. Each of these isolates indicated IAA content from the change in color of the solution to light pink until deep pink. NDT 311 was the isolate with the highest absorbance of 0.457, while the isolate with the lowest absorbance was NDT 81 of -0.020. The production of the IAA hormone by microbes is strongly influenced by supernatant culture, tryptophan concentration, carbon sources, agitation, dissolved oxygen concentration, growth rate and incubation time (Bose *et al.*, 2013).

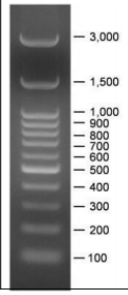
Molecular Identification of DNA Isolate of endophytic fungi (NBT 41) from neem

The results of DNA identification of the NBT 41 fungal isolates. To determine the type, the NBT 41 isolate was continued with DNA analysis. Figure 3 shows the PCR amplification result of the NBT 41 strain with a fragment length of 554 bp. From BLAST search results via NCBI GenBank, the strains showed 100% identical to *Colletotrichum gloeosporioides* https://www.ncbi.nlm.nih.gov/nuccore/ MT043778.1, KJ676455.1, KF192821.1, KF1776 85.1, GU066671.1, KM111484.1, KJ676454.1, KJ632415.1, KJ632405.1, KF177684.1).

A.

1 µL PCR Products were assessed by electrophoresis with 1% TBE agarose

M, 100bp ladder (loaded 2,5 µL); samples ranged in the order of upper table



No	Sample Name	Sequences
B	NBT 41	**Sequence Assembly 554bp** 1 ACCTGCGGAG GGATCATTAC TGAGTTTACG CTCTACAACC CTTTGTGAAC ATACCTGTAA 61 CTGTTGCTTC GGCGGGTAGG GTCTCCGTGA CCCTCCCGGC CTCCCGCCCC CGGGCGGGTC 121 GGCGCCCGCC GGAGGATAAC CAAACTCTGA TTTAACGACG TTTCTTCTGA GTGGTACAAG 181 CAAATAATCA AAACTTTTAA CAACGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC 241 GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC 301 ATTGCGCCCG CCAGCATTCT GGCGGGCATG CCTGTTCGAG CGTCATTTCA ACCCTCAAGC 361 TCTGCTTGGT GTTGGGGCCC TACAGCTGAT GTAGGCCCTC AAAGGTAGTG GCGGACCCTC 421 CCGGAGCCTC CTTTGCGTAG TAACTTTACG TCTCGCACTG GGATCCGGAG GGACTCTTGC 481 CGTAAAACCC CCCAATTTTC CAAAGGTTGA CCTCGGATCA GGTAGGAATA CCCGCTGAAC 541 TTAAGCATAT CAAT

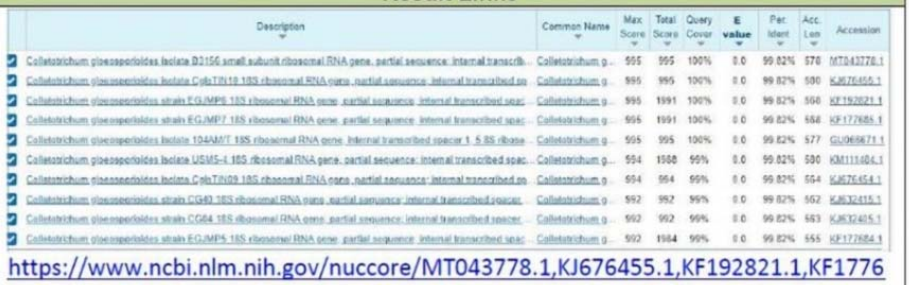
No	Sample Name	Result Links
C	NBT 41	 https://www.ncbi.nlm.nih.gov/nuccore/MT043778.1,KJ676455.1,KF192821.1,KF177685.1,GU066671.1,KM111484.1,KJ676454.1,KJ632415.1,KJ632405.1,KF177684.1

Figure 5. Identification of NBT 41 endophytic fungus isolate. (A) PCR electrophoresis result of NBT 41 isolate using ITS primer. The 554bp PCR product aligned to 1 kb DNA

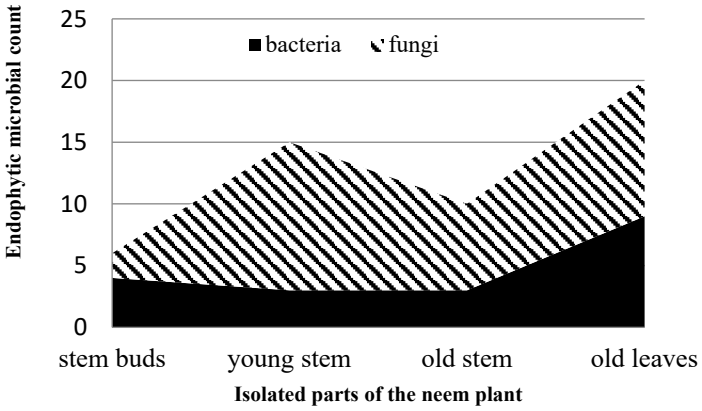


Figure 6. Microbial diversity patterns from neem plants

In Figure 6. the pattern of abundance and diversity of endophytic microbes from neem plants is dominated by endophytic fungi. According to Hata and Sone (2008), endophytic microbes can colonize stems, roots, petioles, leaf segments, flowers, fruits, shoots, seeds, and even on dead plant cells.The main factors in the occurrence of endophytic colonization in a plant are including plant genotype, plant growth patterns, plant physiology, type of plant tissue, soil environmental conditions where the plant grows, the season when the sampling is being conducted, surface sterility, selective media and cultural conditions as well as different agricultural practices (Gaiero *et al.*, 2013; Golinska *et al.*, 2015). Endophytic microbes that live in plant tissue do not cause disease symptoms (Bacon and White, 2000). Figure 6 shows the neem plant to be a suitable host or habitat for endophytic fungi. Fungi have a strategy to find host plant as a place to live and have a symbiotic relationship with the plant.

Discussion

According to Strobel and Daisy (2003); Jalgaonwala *et al.* (2011); Godstime *et al*. (2014); Shukla *et al.* (2014), bioactive compounds with potential applications are opportunities for agricultural, medical, food and cosmetic industries. From the results of this study, interesting and unique fungal endophytes were found, with high population and showed ability to produce bioactive compounds higher than endophytic bacteria. The potential of the IAA hormoneobtained from the endophytic fungi is therefore higher than the endophytic bacteria.

Many factors have the potential to stimulate IAA production by fungi or endophytic bacteria and are also involved in plant resistance. Some evidence shows that IAA produced by endophytic microbes stimulates secondary metabolites so that endophytic microbes such as fungi will differentiate into invasive forms and increase filaments if the environment is unfavorable. According to Prusty *et al*. (2004) the role of IAA in interacting with plant pathogens is by utilizing exogenous tryptamine for IAA synthesis. The production of IAA by fungi increases in biotrophic and necrotrophic phases of infection. According to Somers *et al.* (2005), IAA production can also be stimulated by ipdC gene which is the key gene of IAA production through the IPA (indole-3-pyruvate) pathway.

Endophytic microbes can increase the ability of plants to tolerate various types of abiotic and biotic stresses, increase plant resistance to insects and pests, produce phytohormones and other bioactive compounds that are of interest to biotechnology. Endophytic microbes play an essential role in producing natural bioactive compounds, the potential of which can be exploited in the health sector, agricultural sector, and industry to obtain the discovery of new medicinal substances/compounds.

According to Suzuki *et al.* (2003); Marathe *et al.* (2017), Indole-3-acetic acid is a natural hormone produced by plants but some microbes present in plant tissues show the ability to produce IAA and help the plant's

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growth. IAA produced by endophytic bacteria, for example, *Pseudomonas fluorescens* HP72 acts as a stimulator of cell proliferation and elongation and increases the absorption of minerals and nutrients from the soil. IAA produced by endophytic microbes is stimulated when plants grow under stress conditions, such as stress due to water deficit, temperature, salt, pollutants, and so on (Husen *et al.*, 2016; Ma *et al.*, 2016). According to Chen *et al.* (2017), plant growth and chlorophyll synthesis can be parameters to indicate plant conditions in a stressful environment. Stress will be greater in plants that do not receive additional IAA treatment, while plants given exogenous IAA from IAA-producing microbes can increase chlorophyll production in leaves under stress conditions.

According to Ali *et al*. (2014), the increased production of IAA is then transported to plant tissues by endophytes, helps plant growth, and also triggers the synthesis of ACC (1-aminocyclopropane-1-carboxylic acid). This happens because IAA induces mRNA transcription to make ACC synthase enzymes, that convert S-adenosyl methionine (SAM) into ACC (Glick, 2014). Increased level of ACC induces the synthesis of ACC deaminase enzyme in endophytes, which then will break down ACC (but not all) into ammonia and Alpha-ketobutyrate. IAA production induces ACC, and since endophytes produce ACC deaminase, the presence of endophytes can protect plants from damaging levels of ethylene (Ali *et al.*, 2014)

Genetic modification of endophytes fungi can be employed to increase IAA production which hopefully correlate to enhanced resistance in plants. Naturally, endophytic microbes originating from neem plants that produce the IAA hormone and helping plant growth can also protect plants by increasing plant immunity against invading pathogens (Kulkarni *et al*., 2011). The resistance is formed not only from endophytic fungi but from endophytic bacteria present in plant tissues. Interesting findings from this research are to be continued using molecular approaches so that the results can be more detailed and can be utilized.

The identification results showed that the endophytic fungi of the neem plant were obtained from old stem part. One particular fungus was chosen to be identified because it had the highest IAA hormone-producing activity. The endophytic fungus was identified as *Colletotrichum gloeosporioides*. According to Sudirga (2016), *Colletotrichum* fungus is the main pathogen that causes anthracnose. The fungus has an oval to elongated body, slightly curved and in large quantities reddish in color. This fungus attacks all plant's parts, such as leaves, flowers, fruit, twigs, and seedlings. *Colletotrichum* is a facultative parasitic fungus from the ordo melanconiales, its spores are arranged in acervules, which are asexual structures. *Colletotrichum* fungus belong to the class deuteromycetes, where the anamorphic phase is asexual, while the telemorphic phase is sexual. Based on the research results, endophytic fungi grow on host plants and produce secondary metabolites for plant resistance.

According to Keller and Hohn (1997), secondary metabolite profiles are produced by endophytic fungi to differentiate between pathogenic and non-pathogenic. *Colletotrichum gloeosporioides* is able to perform IAA biosynthesis through the indole-3-acetamide (IAM) pathway. Another metabolite, the presence of thiazole derivative compounds is widely used as a therapy with various properties. These compounds can be synthesized and produced by the agrochemical industry into alcohol and urea (Robinson *et al.*, 1998; Lu *et al.*, 2012). *Colletotrichum gloeosporioides* from the results of this study is derived from neem (*Azadirachta indica* A. Juss) which may have azadirachtin, a secondary metabolite produced similar to its host plant. This metabolite belongs to the triterpenoid group which is used as an active ingredient of vegetable insecticides. The results of this research on endophytic microbes may lead to discovery of high hormone-producing genes that can be explored further to be engineered so that they can be used to create products with economic value to help plant growth without causing disease in other plants. There will be a lot of information if we identify the activity of the isolate, however, careful

precaution must be taken into consideration if we are using certain *Colletotrichum* fungus so as not to cause disease in other plants.

Conclusion

The isolation of endophytic microbes from Neem plant (*Azadirachta indica* A. Juss) samples results in 19 pure endophytic bacterial isolates and 36 pure endophytic fungal isolates. Microbial activity test results show that three endophytic bacterial isolates have the ability to produce IAA growth hormone, namely NDT 111, NDT 112, NDT 113. Moreover, 18 endophytic fungi have IAA hormone activity which are NBT 11, NBT 12, NBT 21, NBT 41, NBM 11, NBM 52, NBM 61, NBM 62, NBM 71, NBM 81, NDT 21, NDT 311, NDT 32, NDT 33, NDT 41, NDT 61, NDT 911, and NDT 11. Based on molecular identification analysis, the endophytic fungus NBT 41, which has the highest production activity of IAA hormone, is identified as *Colletotrichum gloeosporioides*. The results of this study indicate that the neem plant's endophytic microbes have a potential as IAA growth hormone producers, which can be used as materials in genetic engineering to improve plant growth and perhaps, increase plant resistance to biotic/abiotic stresses.

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