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INDOLE ACETIC ACID AND EXOPOLYSACCHARIDE PRODUCTION OF NITROGEN FIXING BACTERIA ISOLATED FROM NUTMEG TREE

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ABSTRACT

The constraint of plant cultivation in the tropics is low nitrogen (N) content in soil. The application of nitrogen fixing bacteria (NFB) is not only fulfill the N demand but also provide the phytohormones Indole acetic acid (IAA) and exopolysaccharides (EPS). The objective of the experiment was to was to isolate the NFB from the rhizosphere of nutmeg tree and characterized their IAA and EPS production in a liquid culture. The NFB was isolated by serial dilution plate method on Tryptic Soy Agar. In order to test the ability of various NFB isolates to produce IAA and EPS, all isolates were grown in Tryptic Soy Broth for 72 hours at room temperature. The results showed that at the end of experiment the media acidity was shift from neutral to alkaline. The population of five isolates of NFB was approximately 8-9 log10 (of CFU/mL). The IAA production in liquid culture of all isolates was similar but the EPS production was varied and depend on the isolates, The highest EPS content was in PL1 culture and the lowest was in the culture of PL5. This experiment verified that the NFB isolated from nutmeg rhizosphere enable to produce IAA and EPS.

Keywords: Acidity, Bacterial Count, N-free Media, Rhizosphere.

1. INTRODUCTION

Nutmeg plant (*Myristica fragrans* Houtt) is a tree spices originated of Maluku Island include Ambon Island. Indonesia contributes to 50% world nutmeg production but the top nutmeg exporter is India. Indonesian nutmeg production has enhanced 9.4% each from between 2010-2019 (Purba et al., 2021). The potency of nutmeg in Maluku may reached 3000 kg/ha (Basir et al. 2018) but the productivity of nutmeg plantation in Ambon Island is only 0.39–0.77 t/ha (Leatemia et al. 2017). Low nutmeg yield in Ambon may be caused by limited nutrient availability in soil and the plant age. In general, soil in tropics contained low essential nutrient nitrogen (Sellan et al., 2020). Traditionally, farmers didn't apply fertilizer other than organic fertilizer during the transplanting.

In common, urea and compound NPK fertilizer provide available nitrogen and are suggested to increase plant growth and yield of nutmeg elsewhere. In order to use more environmentally friendly fertilizer, application of biofertilizer is nowadays common in seedling of plantation crops i.e palm oil and tea, for supporting plant growth and performane and reducing chemical fertilizer (Gebrewold and Yildiz, 2018; Ajeng et al., 2020). Nonetheless, the study of biofertilizer on nutmeg is focused for their effect on seedling performance (Nair et al., 2001; Kalay et al., 2020; Hindersah et al., 2021). Biofertilizer contain beneficial and nonpathogenic

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microbes that enable to proliferate in plant rhizosphere or phyllosphere in order to improve plant growth by increasing certain nutrient availability. Group of rhizosphere beneficial microbes which is potentially used as biofertilizer are free-living heterotrophic nitrogen fixing bacteria (NFB) including Bacillus, Bulkholderia and Azotobacter (Islam et al., 2012; Hindersah et al., 2020).

The mechanisms by which NFB improve plant growth and then yield are nitrogen fixation, as well as phytohormones and exopolysaccharides (EPS) production. The nitrogen gas (N_2) is reduced to NH_3 catalyzed by nitrogenase composed of Fe- and FeMo-protein (Sivasakthi et al., 2017). A significant amount of important plant growth regulator IAA was found in liquid culture of NFB Azotobacter and rhizosphere bacteria (Zulaika et al., 2017; Mohite, 2013). The EPS that composed of sugar and organic acid has been detected in NFB Azotobacter culture (Hindersah, 2015; Ventorino et al., 2019). The Bacillus and Bulkholderia has been reported to synthesize the EPS in liquid culture (Ding et al., 2018; Herasimenka et al., 2007). The bacterial EPSs are prominent to improve soil porosity and aggregation of soil (Cheng et al., 2020).

The used of NFB to substitute chemical fertilizer is essential. It is reported that the NFB *A. chroococcum* applied with reduced dose of N fertilizer levels produced highest wheat yield and enable to replace 47.6 kg N/ha (Mohamed & Almaroai, 2016). In nutmeg nursery, mixed biofertilizer that also contained NFB increased seedlings growth (Hindersah et al., 2021). The overuse of agrochemicals such as chemical fertilizer can be decreased by NFB and in the same time sustain the agricultural (Soumare et al., 2020). The NFB is easily isolated from the bulk soil as well as rhizosphere of plant. Nonetheless, the population of bacteria is greater in rhizosphere due to abundance of root exudates.

Nutmeg tree cultivated for generations in Maluku Province; in Ambon, the trees are cultivated in local agroforestry system mixed with another economic trees and shurbs (Leatemia et al. 2017). In some area, the farmers establish nutmeg plantation supported by local governments by using seedling from seed or grafting-based seedling. In order to develop biofertilizer for nutmeg seedling as well as young nutmeg tree, The exploration of beneficial microbes living in lower part of nutmeg is needed. To date, isolates of indigenous NFB have not yet been isolated and screened from the rhizosphere of nutmeg tree. The objective of this preliminary experiment was to isolate the NFB from the rhizosphere of nutmeg tree and characterized their properties in IAA and EPS production in a liquid culture.

2. MATERIAL AND METHOD

The nutmeg rhizosphere soil was collected from 15-years old nutmeg tree grown in tropical mixed forest in Liliboi Village, Maluku Tengah Regency of Maluku Province (Figure 1). The sampling point in Liliboi located at 20 m above sea level with geographical position of -3.743887 and 128.029364. The bulk soil surrounding five nutmeg tree trunk (included tree sample) was collected and mixed for further chemical soil properties analysis. The rhizosphere soil was taken up from the roots at 20 cm-deep from the soil surface; the soil then put in sealed plastic and stored in the cold box.

The NFBs were isolated by serial dilution plate method with Tryptic Soy broth (TSB) and Tryptic Soy agar (TSA) composed of Casein Peptone 15.0 g, Soy Peptone 5.0 g, Sodium Chloride 5.0 g, demineralized water 1 L; and Agar 15.0 g for the TSA, in neutral acidity. A total of 1 g of rhizosphere soil was poured into 50 mL TSA and inoculated at 30^{o}C for 5 days untuk

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pellicle growing in the surface of TSB. A full loop of pellicle then streaked on the TBS and stored for 3 days at 30°C. The five colonies that distinctly separated from other colonies (Figure 2) were passed the Gram stained and checked for the purity under the light microscopes before transferred to TSA slant. The culture incubated for 3 days before storing at 4°C.

All NFB pure cultures were inoculated to 100 mL TSA in 250 mL Erlenmeyer flask; incubated for 72 hours on gyratory shaker of 115 rpm at room temperature. The media acidity before incubation were 7.2 while the bacterial count was approximately 10^4 CFU/mL. The bacterial density was determined at 48 and 72 h by serial dilution plate method in TSA. The IAA and EPS concentration in the liquid culture were analyzed at the end of experiment. The IAA was determined by spectrophotometry at wavelength of 350 m after 1 mL supernatant of bacterial cultured was mixed with 2 mL of Salkowski solution. Meanwhile the EPS content was weighing at 35°C followed the method of Hindersah et al. (2017). At the end of experiment, the pH and EC of liquid culture were measured. All raw data were collected from triplicate measurement. The mean and standard deviation of data were calculated and data were presented in the table or histogram.

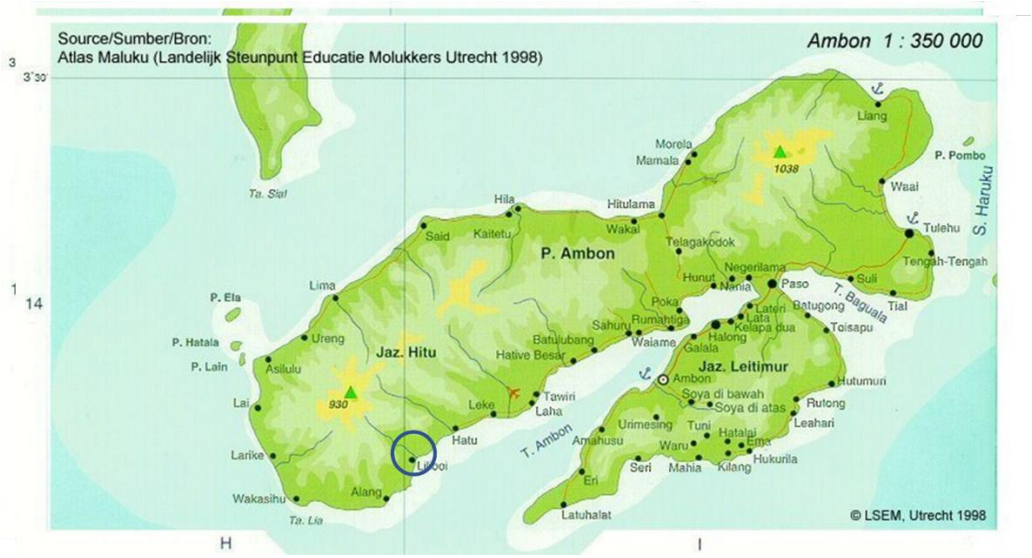

Figure 1. Sampling location of rhizosphere soil in mixed anthropogenic forest in Liliboi Village of Maluku Tengah Regency, Maluku. Photograph provided by Souisa PB (2012).

3.RESULTS

Base in proximate analysis the soil in sampling location, the soil were silty clay loam with low acidity (pH pf 4.77). The soil as 1.27% of organic carbon, 0.12% of total Nitrogen, 4.14 mg/kg of soluble P 10.53 of C/N and 22.19 mg/kg of total K_2O. In general, the soil contained low N, P and K. The NFB isolation revealed that the morphology of colonies was almost uniform. The colonies were round, smooth, white, opaque, and mucoid (Figure 2a). The edge of colony was entire and all colonies raise above the agar. A total of five colony was isolated included PL1, PL2, PL3, PL4 and PL5; and purified by several strike on TSA, and transferred to

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TSA slang (Figure 2b). After 72 h incubation, the pH of all NFB culture in TSA was raised compared to the pH before experiment (7.2).

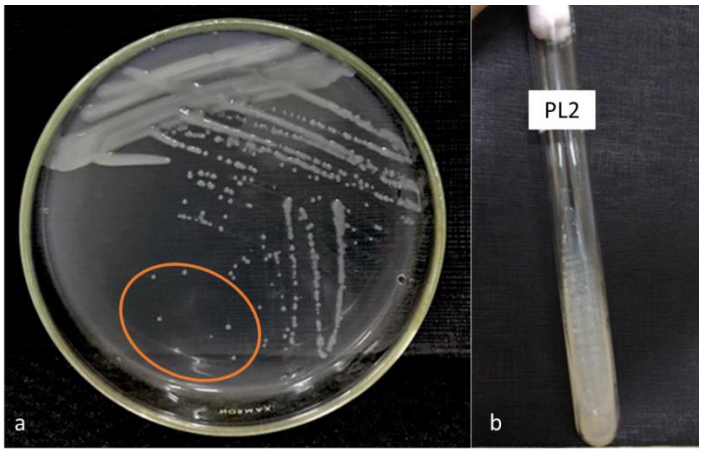

Figure 2. The free colonies of Nitrogen fixing bacteria in the circle (a) were purified and maintained in slant (b)

In current study, the pH of culture was varied between 7.79-8.33 but mostly around 8 (Table 1). The lower pH showed by PL1 liquid culture but this value was higher compared to uninoculated media (pH – 7.24). Before experiment, the EC has not been determined; at 72 h the EC of all isolates culture was approximately 0.01 (Table 1) which is the EC of media before experiment.

Table 1. The acidity (pH) and electrical conductivity (EC) of five Nitrogen Fixing Bacteria liquid culture in TSA after 72 h incubation

NFB Isolates	pH	EC (ds/cm)
PL1	7.96 ± 0.58	0.011 ± 0.0004
PL2	8.24 ± 0.05	0.011 ± 0.0001
PL3	8.33 ± 0.03	0.011 ± 0.0000
PL4	8.29 ± 0.02	0.011 ± 0.0002
PL5	8.30 ± 0.02	0.010 ± 0.0002

Each value is the mean of three replications

The population of NFB before incubation was 10^4 CFU/mL equal to 4 $\log_{10}$ CFU/mL; the bacterial count was increase up to approximately 7 $\log_{10}$ and 9 $\log_{10}$ at 48 h and 72 h respectively (Table 2). The highest population at the end of experiment was found in the culture of PL3 even though their NFB population at 48 h was lowest.

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Table 2. Population of NFB in N-free broth of five different Nitrogen fixing bacteria isolates at 48-h and 72-h incubation

NFB Isolates	NFB Population	
	48 h	72 h
PL1	6.99 ± 0.42	8.96 ± 0.31
PL2	7.24 ± 0.33	9.19 ± 0.23
PL3	7.05 ± 0.45	9.23 ± 0.43
PL4	7.59 ± 0.32	8.66 ± 0.14
PL5	6.93 ± 0.09	8.41 ± 0.13

Each value is the mean of three replicates

The IAA and EPS were detected in the N-free liquid culture inoculated with five isolates of NFB (Figure 3). The content of IAA was equal in all five NFB cultures but the EPS concentration was varied among the isolates. The highest capacity to synthesize EPS demonstrated by PL1 isolates and the lowest one was found in the culture of PL5 which is 41% lower than PL1. In average, the EPS content in the liquid culture of PL2, PL3, PL4 and PL6 were approximately 50% lower than PL1.

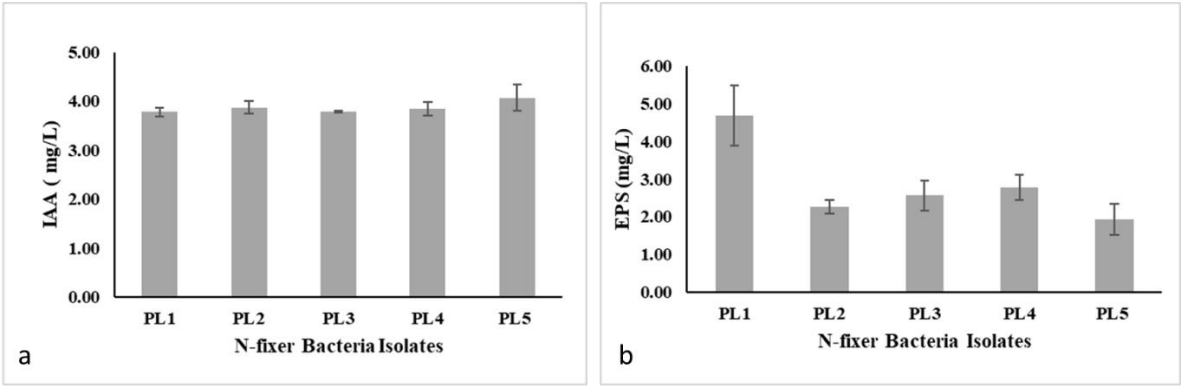

Figure 3. The concentration of IAA (a) and EPS (b) in the 72-hours olds liquid culture of Nitrogen fixing bacteria. IAA: Indole Acetic Acid; EPS: exopolysaccharide. Each value is the mean of three replicates

4. DISCUSSION

` In current study, five isolates of NFB have been isolated by specific N-free medium from the rhizosphere of nutmeg tree. The NFB has an important role in the nitrogen cycle in soil particularly in the rhizosphere. The five isolates of NFB were non-symbiotic microbes which is living free in the soil without form the specific mutualistic relationship with the plants. The species of all isolates have not yet been identified since the biochemistry as well as molecular characterization have not been performed

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All isolates enable to grow in N-free liquid culture which is the main character of NFB. The culture of all bacteria changes from neutral to alkaline after 72-hours incubation at room temperature. At the beginning of experiment, the bacteria used carbohydrate as a source of energy, at this time the pH of culture might shift to acid. In the following hours (72 h) the pH was increased due to the utilization of protein. The bacteria were grown in TSB that composed of casein peptone and soy peptone; a water-soluble substance of partial hydrolysis of proteins. Certain bacteria excrete a proteolytic enzyme proteases to hydrolyze the polypeptide chain of amino acids (Das and Prasad, 2010). In current study, the rise of liquid culture acidity might be due to hydrolysis of proteins (peptone) by bacterial protease that released amino acids and increase the acidity (Ciurko et al., 2021). Meanwhile, the pH of uninoculated media remained stable (pH = 7.24).

Among five isolates, the growth of PL2 and PL3 in the TSB were better than other isolates. The population of bacteria in the artificial media was depend on the chemical characteristic as well as nutrient availability. It is likely that both isolates adapt well to the nutrient composition of TSB. The utilization of TSB for proliferating the NFB was widely reported included for Micrococcus Roseus (Herehera et al., 2018), Bacillus (Ciurko et al., 2021), and Azotobacter (Zhang et al., 2022).

All isolates synthesized IAA approximately 3-4 mg/L growth media. Zulaika et al., (2017) reported that free-living NFB Azotobacter A10 produced IAA up to 4.59 mg/L. Meanwhile, various isolates of NFB enable to synthesize IAA 0.7-0.18 g/L (Mohite, 2013); he conlude that the IAA production in general depend on Carbon and Nitrogen Sources, the presence of L-Tryptophan, pH and temperature of substrate. It is likely that the isolates have similar adaptability degree to TSB to synthesize the IAA. The PL1 isolate showed higher EPS secretion; the synthesis of EPS largely depends on the available Carbon and Nitrogen in the medium. It is reported that Bacillus utilize sucrose and glucose for EPS synthesis while the Pseudomonas prefer sucrose (Herehera et al., 2018). In this current research, the species of each isolate have not yet been characterized so the profile of their IAA and EPS production cannot be compared with certain species.

This experiment verified that the NFB colonized the nutmeg rhizosphere. Moreover, the bacteria enable to synthesize the phytohormones IAA and EPS that prominent for plant growth. The IAA synthesized by PGPR in small amount but have crucial role to modulate plant growth (Kalimuthu et al., 2019). Bacterial exopolysaccharides are known as binding agent for aggregate formation (Cheng et al., 2020); such physical properties of soil is essential to ensure plant growth and then nutrient uptake. The isolates were Plant Growth Promoting Rhizobacteria (PGPR) that has a potency to be formulated as biofertilizer. Therefore, optimizing the N-fixer capacity as well as production of both metabolites in liquid culture is suggested to formulate biofertilizer for nutmeg.

5. CONCLUSION

Five isolates of NFB have been isolated from the nutmeg rhizosphere. The bacteria enabled to proliferate in Tryptic Soy Broth (TSB) media with the population approximately 8-9 $\log_{10}$ (of CFU/mL) after 72 h incubation. The phytohormone IAA and EPS have been detected in the liquid culture of Tryptic Soy Broth media. The IAA production in liquid culture of all isolates was comparable but the EPS production was varied and depend on the isolates. The highest EPS

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content was in PL1 culture and the lowest was in the culture of PL5. In average, the EPS content in the liquid culture of PL2, PL3, PL4 and PL6 were approximately 50% lower than PL1.

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