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ISOLATION AND CHARACTERIZATION OF NITROGEN AND INDOLE ACETIC ACID PRODUCING RHIZOBACTERIA FROM LOCAL UPLAND RICE PLANTS IN SOUTH SULAWESI, INDONESIA

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Abstract

This study aimed to determine the type of isolate and the characteristics of nitrogen fixing bacteria in the rhizosphere of local organic upland rice in the highlands. This research was conducted in North Luwu Regency, South Sulawesi, Indonesia and the Pest Science Laboratory, Department of Plant Pests and Diseases, Hasanuddin University. The research method was a descriptive study using morphological and physiological characterization (Gram reaction test using 3% KOH), nitrogen fixation ability test of bacteria and Indole Acetic Acid (IAA) production test. Morphological characterization of nitrogen fixing bacterial isolates from local organic rice rhizospheres showed 5 of the tarone variety and 7 of the local rice tarone hoyane variety from Seko Subdistrict. The results of the morphological characterization showed different results in terms of color, size, shape and level. Nitrogen fixation ability test of the isolate from the rhizosphere of the local rice tarone hoyane variety achieved the highest nitrogen binding ability (2.082), Seko Subdistrict (962), Rongkong Subdistrict (996), and the lowest was the isolate from the banjara rice variety (707). The ability test of IAA production of the local upland rice rhizosphere bacterial isolates of the tarone hoyane rice variety produced a more intense pink color and had the highest concentration of IAA (1.835 mg L^{-1}), Seko Subdistrict (1.630 mg L^{-1}), and Rongkong Subdistrict (1,566 mg L^{-1}). Meanwhile, the bacterial isolate of the local rice banjara variety from Rongkong Subdistrict obtained the lowest IAA (0.316 mg L^{-1}).

Keywords: Indole Acetic Acid; Nitrogen; Rhizobacteria; Rice

INTRODUCTION

Paddy produces rice which is estimated to be a staple food for half of the world's population (Chen *et al.* 2019). The increase in the world's population is targeted to reach 45% by 2050, so that the demand for staple foods, especially rice, is experiencing a soaring demand and giving pressure to improve the agricultural system where production yields began to decline (Medina-Cordoba *et al.* 2021). Infertile land requires increased processing intensity. Climate change and environmental challenges, continuous land use, especially for rice cultivation, have an impact on decreasing land quality and productivity. In addition, land management through inorganic fertilization causes a decrease in organic matter and soil fertility (Hadija *et al.* 2021).

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Agricultural practices needed to increase productivity that are environmentally friendly and sustainable (Ghaffari *et al.* 2018; Ulloa-muñoz *et al.* 2020). The development of environmentally friendly agricultural productivity can minimize energy use and apply modern agriculture using alternative resources such as biological fertilizers (Abd-Alla *et al*. 2019). Bio-fertilizers consist of microbial inoculants that promote plant growth, thus providing an alternative or complementary approach to increasing crop yields, that are more sustainable and environmentally friendly (Medina-Cordoba *et al.* 2021).

Nitrogen is a basic requirement for plants because it can affect the growth period and plant production in synthesizing complex protein molecules. Therefore, nitrogen is the main nutrient for plants because it is the most required component of all fertilizer components. Plants cannot grow normally if there is a nitrogen deficiency. Excessive use of inorganic fertilizers can cause a decrease in the quality of agricultural land (Jimenez-Salgado *et al*. 1997). One solution that can be applied is the use of non-symbiotic nitrogen fixing bacteria as a biological fertilizer agent (Pereg *et al*. 2018). The use of bacteria for the natural fixation of nitrogen from the atmosphere can benefit plants (Li *et al*. 2021). Rhizobacteria in plants are microorganisms that live in plant roots (rhizosphere) (Khatoon *et al.* 2020). The relationship between plants and microorganisms consists of the interaction of fungi and bacteria (Farrar *et al*. 2014), which have various trophic levels and living habits whose saprophytic or symbiotic relationship with plants can be detrimental or beneficial (Barea 2015). These microorganisms can be grouped as the rhizosphere, which lives in the soil adjacent to roots; or endophytes, when they pass all or part of their life cycle inside their host without causing obvious symptoms of some diseases (Ochoa-Velasco *et al.* 2016). Rhizobacteria were reported as plant growth promoters by fixing atmospheric nitrogen and producing hormones (Shridhar 2012).

Free-living bacteria on plant roots and tissues are *Pseudomonas*, *Bacillus*, *Burkholderia* (Yan *et al.* 2018), *Enterobacter cloacae* (Takada *et al.* 2019), *Pantoea* sp. (Loiret *et al.* 2004), *Azospirillum melinis* spnovember (Peng *et al.* 2006). Nitrogen fixing bacteria can fix nitrogen from the atmosphere because this type of bacteria has a specific enzyme in its cells known as Nitrogenase. This enzyme consist of two components that support each other, i.e., Fe and Mo-Fe proteins. The main strategy used to isolate and enumerate bacteria from this genus is to use N-free semi-solid media.

Research on the isolation and characterization of nitrogen fixing bacteria of rice plants has been mostly carried out on mine soil (Navarro-Noya *et al.* 2012), on paddy soil with two different agro-ecosystems (Haerani *et al.* 2021), and paddy soil with organic fertilizer application (Hadija *et al*. 2021). Research on associative nitrogen fixation has been reported on rice (Navarro-Noya *et al*. 2012), corn (Arsita *et al.* 2020), and cassava (Zhang *et al.* 2022) cultivation plants which have high fertilizer requirements. However, there was still little research on the nitrogen fixing characteristics of local upland rice plant rhizospheres that grow on nitrogen deficient substrates. This study aimed to determine the type of isolate and the characteristics of nitrogen fixing bacteria in the rhizosphere of local organic upland rice in the highlands areas.

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MATERIALS AND METHODS

Research Location

This research was conducted from August to October 2022 at the Pest Science Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, Hasanuddin University, Makassar, Indonesia. Samples of soil and roots of upland rice plants were taken from two highland areas in North Luwu Regency, South Sulawesi. Six plant samples were collected from Seko Subdistrict with an altitude of 1,485 above sea level (asl), soil type of regosol yellowish red, pH of 5 – 7, and rainfall of 2,301 mm including two varieties of rice plants, i.e., tarone and tarone hoyane. Six plant samples were also collected from Rongkong Subdistrict with and altitude of 900 asl, average temperature of 20 – 25 °C, soil type of regosol yellowish red, pH of 5 – 7, and rainfall of 2,301 mm consisting of two local rice varieties, i.e., bandarata and banjara varieties. Soil and plant samples were taken and then put in paper envelopes labeled according to the location and local rice varieties. Plant samples were transported to the laboratory using a cool box for immediate analysis (Haerani *et al.* 2021; Kesaulya *et al.* 2015).

Isolation and characterization of nitrogen fixing bacteria

Nitrogen fixing bacteria were isolated using Burk's N-free media (Park et al. 2005). Burk's N-free solid media materials consisted of 20 g sucrose, 0.64 g K_2HPO_4, 0.16 g KH_2PO_4, 0.20 g $MgSO_4.7H_2O$, 0.20 g NaCl, 0.05 g $CaSO_4.2H2O$, 5 mL Na_2 $_4.2H_2O$ (0.05%), 5 mL $FeSO_4.7H_2O$ (0.3%), 21 g agar, and 1,000 mL aquadest (Hartono *et al.* 2014). Dilution of soil and plant samples was performed by grinding 1 gram of soil using a mortar until finely ground then 10 mL aquadest was added while stirring until it became a homogeneous solution. Furthermore, multilevel dilution from 10-1 to 10-6 was carried out by taking 1 mL of the mixture using a pipette. Then it was put into the first test tube containing 9 mL aquadest, and shaken until the composition was consistent using a vortex. This was called the first dilution (10-1). This procedure was repeated for 10-6 (6 test tubes). Diluted 0.1 mL of soil sample (10-3, 10-4, 10-5, and 10-6) was cultured on Burk's media using a triangular spreader rod and was incubated for 24 hours at room temperature. Single colonies growing on growth media were then cultured by streaking them on Nutrient Agar (NA) medium and were purified 3 times. Pure colonies that grow with different appearances were characterized morphologically according to the method proposed by (Cappuccino 2018).

Gram test

The gram test was performed according to Pambudi *et al*. (2017). A full ose of bacterial isolate was taken using an ose needle and placed on a glass object that had been dripped with 10 µL of 3% KOH and then stirred repeatedly. If the mixture of bacterial suspension and KOH became very viscous or formed a gel within 5 to 60 seconds, the isolate was classified as Gram-negative (-). If there was no gel formation, the isolate was classified as Gram-positive (+). The rhizobacteria IAA production test was carried out to test the ability of the rhizobacteria to produce IAA hormones using a spectrophotometer. Microorganisms were cultured on NA medium containing 200 ppm L tryptophan. Then 10 mL of bacterial isolates were taken and put into a 15 mL centrifuge tube and were centrifuged at 8000 rpm for 10 minutes. Two mL of

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supernatant was taken and put into a test tube, then 2 drops of orthophosphoric acid and 4 mL of Salkowski reagent were added (Glickmann and Dessaux 1995). The samples were then incubated in a dark room for 24 h. A change in color to pink indicated the ability of microbes to produce auxin. The pink color was caused by a reaction between the Salkowski reagent and the hormone. Optical density of the sample was measured at a wavelength of 535 nm using a UV-VIS spectrophotometer (Geddes *et al.* 2015). The catalase test was carried out according to the modified method of (Suhartanti *et al.* 2010) to determine the ability of microbes to degrade hydrogen peroxide (H_2HAI_2). A full loop of pure single colony culture was smeared on a slide previously dripped with two drops of 3% H_2HAI_2. A positive reaction was indicated by the appearance of gas bubbles from the free oxygen.

Nitrogen fixation ability test

The ability of bacteria to fix nitrogen qualitatively was tested on Burk's N-free liquid media according to the method described by (Park *et al.*2005). Bacterial isolates were grown on Burk's N-free media in glass bottles and placed in an orbital shaker at 28 °C for 24 hours. The total N content of bacterial cultures was measured quantitatively using the Kjeldahl method (Ramadhan *et al*. 2017). 5 mL of the supernatant was put into a digestion tube and 1 g of a selenium mixture and 3 mL of concentrated sulfuric acid were added. The sample was digested for 3-4 hours until a white vapor appeared. The digestion tube was removed and cooled. Then, the extract was diluted with aquadest up to 50 mL and was shaken until homogeneous. The extract was allowed to stand for 24 hours, so that the particles settled. The extract was transferred into an Erlenmeyer flask as a boiling flask containing 10 mL of 1% boric acid and 3 drops of red Conway indicator and was connected to the distillation apparatus. Ten mL of 40% NaOH was added to the boiling flask and was immediately closed. The distillation process was carried out until the container volume reached 50-75 mL (green). The nitrogen content (%) was calculated using the formula:

IAA ability test

Rhizobacterial IAA production test was conducted to determine the ability of rhizobacteria to produce IAA hormones using a spectrophotometer. Microorganisms were cultured on NA media containing 200 ppm L tryptophan. 10 mL of bacterial isolates were taken and put into a 15 mL centrifuge tube and were centrifuged at 8000 rpm for 10 minutes. Two mL of supernatant was taken and put into a test tube, then 2 drops of orthophosphoric acid and 4 mL of Salkowski reagent were added (Glickman and Dessaux 1995). The samples were then incubated in a dark room for 24 hours. A change in color to pink indicated the ability of microbes to produce auxin. The pink color was caused by a reaction between the Salkowski reagent and the hormone. Optical density of the sample was measured at a wavelength of 535 nm using a UV-VIS spectrophotometer (Geddes *et al*. 2015).

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RESULTS AND DISCUSSION

Isolation and characterization of nitrogen fixing bacteria from rice rhizosphere

Based on the isolation results of nitrogen fixing bacteria from organic upland rice rhizosphere in two highland areas, there were 24 samples of bacterial isolates that had the potential to fix nitrogen. Twenty four isolated samples of nitrogen fixing bacteria from two locations in the highlands showed different morphological and biochemical characteristics (Table 1).

Table 1: Morphological and biochemical characteristics of bacteria isolated from the local upland rice rhizospheres in the highlands of North Luwu Regency, South Sulawesi, Indonesia.

No	Sample Code	Colony Morphology					Gram	Catalase
		Shape	Form	Colony edge	Ketinggian	Colour		
1	PTHKN01	small	Round	All	Raised	Yellow	-	+
2	PTHKN02	small	Round	All	Flat	Cream	+	+
3	PTHKN03	small	Round	All	Raised n	Cream	+	+
4	PTHKR04	small	irregular	Wavy	Flat	Yellow	+	+
5	PTHKR02	small	Round	All	Flat	Cream	+	+
6	PTAKN01	small	Round	All	Flat	Cream	-	-
7	PTAKN02	Medium	Round	All	Flat	Transparent	-	-
8	PTAKN03	Medium	irregular	Wavy	Flat	Yellow	-	+
9	PTAKN04	Small	Round	All	Raised	Cream	-	+
10	PTAKR01	Small	Round	All	Flat	Cream	-	+
11	PTAKR02	Small	Round	All	Flat	Yellow	-	+
12	PTAKR03	Medium	Round	All	Flat	Cream	-	+
13	PBU101	Small	Round	All	Flat	Yellow	-	+
14	PBU102	Medium	Round	All	Flat	Cream	-	+
15	PBU103	Medium	Round	All	Raised	Cream	-	+
16	PBU104	Medium	Round	All	Flat	Transparent	+	+
17	PBU105	Medium	Round	All	Flat	White	-	+
18	PBU201	Medium	Round	All	Flat	Cream	+	+
19	PBU202	Small	irregular	Wavy	Flat	Cream	-	+
20	PBU203	Small	Round	All	Flat	White	-	+
21	PBU204	Small	Round	All	Flat	Cream	-	-
22	PBU205	Small	Round	All	Flat	Cream	-	-
23	PBU206	Small	Round	All	Flat	Yellow	-	+
24	PBU207	Small	Round	All	Flat	Cream	-	-

Based on Table 1, the morphological characterization of nitrogen fixing bacterial isolates from local organic upland rice rhizospheres showed that there were 5 isolates from samples of the tarone variety and seven from the local rice tarone hoyane variety from Seko Subdistrict. Meanwhile, from Rongkong Subdistrict, 5 samples from the bandarata variety and 7 from the banjara variety were identified. The nitrogen fixing bacterial isolates generally showed different morphological characteristics. The isolates were grouped based on colony size consisting of small and medium size, i.e., 16 small and 8 medium size colonies, respectively. The classification based on colony shape was dominated by 21 round/spherical colonies and then 3 irregular shaped colonies. The colony edges varied in shape from flat, wavy, curved and

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jagged. The colony elevation from the macroscopic characterization results showed 20 colonies with flat elevation and 4 convex colonies.

Nitrogen fixation ability

The ability of nitrogen fixing bacterial isolates was tested qualitatively using Burk's N-free media. All isolated samples were able to produce nitrogen in various amounts (Figure 1). The test results showed that of the 24 nitrogen fixing bacteria isolates, the isolate from the local rice tarone hoyane rhizosphere obtained the highest nitrogen fixing ability (2082.57), followed by the isolate from the rhizosphere of tarone variety from Seko Subdistrict (962.25), the isolate from the rhizosphere of bandarata variety from Rongkong Subdistrict (996.25), and the isolate with the lowest ability was the isolate from the banjara variety (707.28). The variation in the bacterial ability to fix nitrogen was due to the different bacterial species and their capacity to perform this function.

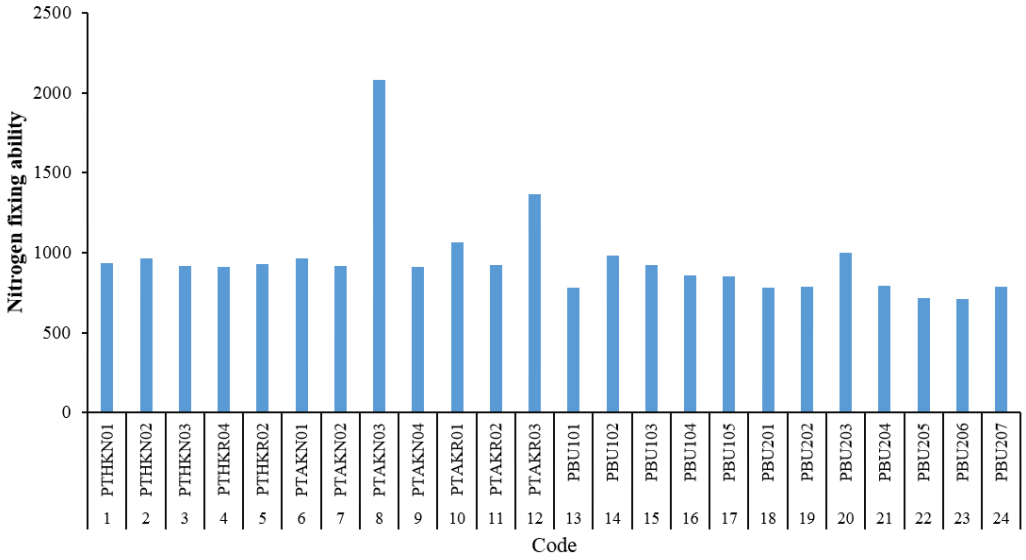

Fig.1: Nitrogen fixation ability of bacterial isolates from local rice rhizosphere

The rhizobacterial ability to produce IAA

The ability of bacterial isolates to produce IAA varied based on the type of local rice rhizosphere isolates, the sampling location and altitude. The isolates were tested qualitatively and quantitatively to produce IAA. The results showed that all isolates can produce IAA. The qualitative test results (Fig 2) showed that there were variations in the color change of the suspension to pink in the IAA production test for all local upland rice nitrogen fixing bacterial isolates after application of the Salkowski reagent compared to the control. The results of the qualitative analysis were directly proportional to the quantitative test. Local upland rice rhizosphere bacterial isolates producing IAA from Seko Subdistrict, i.e., the tarone hoyane variety produced a more intense pink color and achieved the highest concentration of IAA (1,835 mg L^{-1}), followed by the isolate of the local rice tarone variety from Seko Subdistrict

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(1.630 mg L^{-1}), and the isolate of the local rice rhizosphere bandarata variety from Rongkong Subdistrict (1.566 mg L-1). Meanwhile, the bacterial isolate of local rice banjara variety from the Rongkong Subdistrict had the lowest value (0.316 mg L^{-1}) (Figure 3). This difference was presumably because the types varied based on the plant sample locations and the types of bacteria.

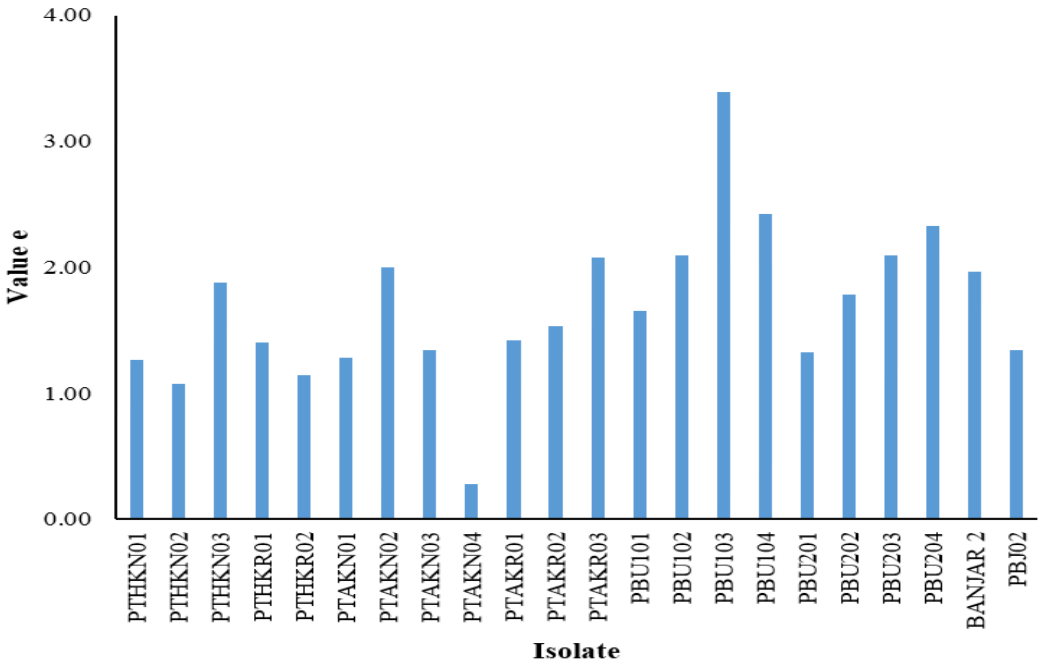

Fig. 2: IAA-producing ability of bacterial isolates from local rice rhizosphere

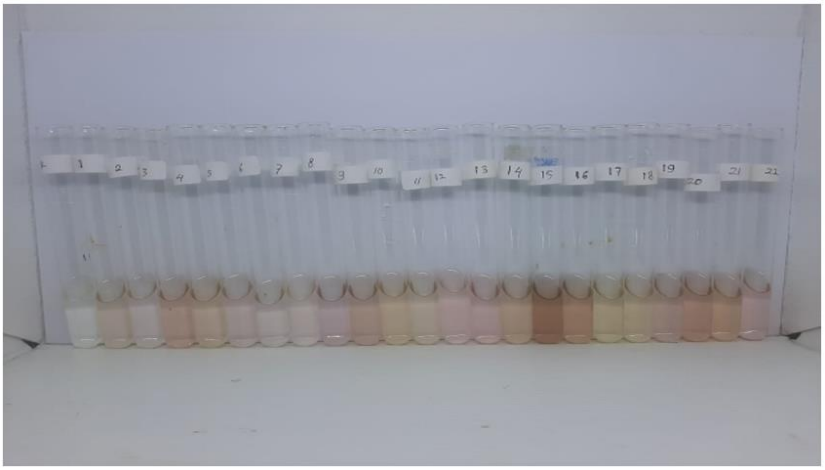

Fig. 3: Qualitative analysis of IAA production by bacterial isolates with Salkowski reagent. The pink color change was determined in the supernatant.

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DISCUSSION

The addition of Hydrogen Peroxide solution produced bubbles indicating that the 19 samples were positive for producing the catalase enzyme, while the remaining 5 isolates were negative catalase bacteria that did not have the catalase enzyme to break down Hydrogen Peroxide into water and oxygen (Pulungan and Tumangger 2018). Hydrogen peroxide (H_2HAI_2) is toxic to bacterial metabolic systems when they cannot degrade this chemical into other harmless compounds (Prathama *et al*. 2018). However, this can also be achieved when the catalase enzyme is available without bacteria (Cappuccino 2018).

Table 1 showed the color of the bacterial colonies varied greatly, i.e., 6 yellow, 15 cream, 2 clear, and 1 white. Colony color differences were due to the presence of intracellular pigments produced by bacteria (Mitra *et al*. 2014; Sudewi *et al.* 2020). The Gram test results using 3% KOH showed that 19 bacterial isolates were Gram negative (-) and 6 isolates were Gram positive (+) bacteria. The Gram test method using Potassium Hydroxide provides more accurate and practical analytical results than the Gram staining procedure (Dash and Payyappilli, 2016). The catalase test results showed that the nitrogen fixing bacteria were dominated by catalase positive of 19 isolates, while 5 isolates were catalase negative.

Isolation and characterization were carried out by examining the morphological characteristics of the colonies which include size, shape and color. The diversity of nitrogen fixing characteristics can be produced from a variety of bacterial isolates. Table 1 shows a total of 24 isolates of nitrogen fixing bacteria from the rhizospheres of local upland rice plants grown in two highlands areas and four different varieties of rice plants. The isolated bacteria showed different morphological characteristics of the colonies. This is in agreement with previous research by (HartonoJumadi 2014; Sukmawati *et al.* 2020; Tarigan *et al.* 2014) that different locations and agro-ecosystem environments can affect the diversity of the nitrogen fixing bacterial characters. According to David *et al.* (2016), differences in sampling locations, rainfall, and cultivation aspects are the factors that affect the character diversity of various bacteria. This study showed that colony size was classified in small and medium size of 16 small size, and 8 medium size, respectively. Meanwhile, the colony shape was predominantly round or spherical. Colony edges varied in shape from flat, wavy, curved, and jagged. Colony elevation from the macroscopic characterization showed 20 colonies with flat elevation and 4 convex colonies. The color of the bacterial colonies varied greatly and were dominated by cream, i.e., 6 yellow, 15 cream, 2 clear, and 1 white.

Different nitrogen fixing bacterial isolates can have different morphological characteristics (Hadija *et al*. 2021). According to Arsita *et al.* (2020), the identification of nitrogen fixing bacteria in the rhizosphere of plants showed diverse morphologically and physiologically bacterial cells with various shapes. In the temperature, pH and salinity tolerance tests, the presence of nitrogen fixing bacteria was proven by the presence of a pellicle and a change in the color of the media containing the isolates. The results of this study showed that 19 bacterial isolates were Gram negative (-), while 6 isolates were Gram positive (+) bacteria. The Gram test method using Potassium Hydroxide provides more accurate and practical analytical results compared to the Gram staining procedure (Dash and Payyappilli 2016). The catalase test

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results showed that the nitrogen fixing bacteria from the local upland rice rhizospheres were dominated by catalase positive of 19 isolates, while 5 isolates were catalase negative. The difference between positive and negative catalase was due to the structure of the bacterial cell wall consisting of peptidoglycan in Gram-positive (+) bacteria and lipids in Gram-negative (-) bacteria (Sudewi *et al.* 2020). According Amaria *et al.* (2019), non-pathogenic bacteria, such as Gram-positive and Gram-negative, play an important role as biocontrol agents in controlling plant diseases.

The ability of bacterial isolates taken from local upland rice rhizospheres to produce the IAA hormone quantitatively showed various results. The lowest of 0.28 mg L-1 was from the organic local rice rhizosphere from Seko Subdistrict, while the highest was from the local rice rhizosphere of the bandarata variety from Rongkong Subdistrict. The difference occurred because there were two different locations of isolated samples. Differences in IAA hormones occur due to types and strains of bacteria, environmental factors, growth rates and availability of substrates such as amino acids and other sources of nitrogen (Susilowati *et al.* 2018). Hormone concentrations varied due to differences in agro-ecosystem conditions and cultivation techniques at the sampling sites (Haerani *et al.* 2021). Israwan *et al.* (2015) stated that the surrounding environment greatly influences the ability of bacteria to survive and produce IAA. Munif *et al.* (2012) reported that soil properties, organic matter, cultivation techniques, fertilization, and pesticide applications affect the presence of bacteria. Auxin is the most abundant phytohormone secreted by bacteria in plants which plays a role in stimulating the formation of lateral roots, adventitious roots and root hairs, providing nutrition for roots so as to increase root growth and development (Sahu 2019).

Figure 3 Variations in types and strains within the same genus can produce varying IAA due to environmental factors, growth rates, and availability of substrates such as amino acids and other nitrogen sources (de Souza *et al.* 2015; Susilowati *et al*. 2018). The production of IAA by bacteria plays a role in promoting plant growth, so that synthesis by certain bacteria causes an increase in plant growth rate (Herlina *et al.* 2016).

CONCLUSIONS

This research concluded that there were 24 rhizobacterial isolates. Morphological characterization showed high diversity and differences of isolates in terms of color, size, and shape. The results of nitrogen fixation ability and IAA production tests showed that bacterial isolates from the rhizosphere of the local rice tarone hoyane variety from Seko Subdistrict achieved the highest nitrogen fixing ability (2.082) and the highest IAA concentration (1.835 mg L^{-1}).

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Author Contributions

Sitti Maryam Yasin contributed to the study conception and design, material preparation, data collection and analysis. Sitti Maryam Yasin, Elkawakib Syam'um, Burhanuddin Rasyid, and Amir Yassi commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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