

## ISOLATION OF NODULE-ASSOCIATED BACTERIA FROM *Indigofera zollingeriana* AND ITS CROSS INOCULATION TO MUNGBEAN

Isolasi *nodule-associated bacteria* dari *Indigofera zollingeriana* dan kemampuan nodulasi silang pada kacang hijau

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### ABSTRAK

HUTAPEA PS, ABDULLAH L, KARTI PDMH, and ANAS I. 2017. Isolasi bakteri dari nodul *Indigofera zollingeriana* dan inokulasi silangnya pada kacang hijau.

*Indigofera zollingeriana* merupakan legum yang dipertimbangkan sebagai hijauan pakan ternak oleh karena kandungan protein yang tinggi. Sebagai legum, tanaman ini dapat memenuhi nitrogen bagi dirinya sendiri karena dapat bersimbiosis dengan rhizobium. Selain rhizobium, terdapat bakteri pendukung tanaman lain yang dapat diisolasi dari nodul akar. Penelitian ini bertujuan untuk menemukan rhizobium atau bakteri pendukung pertumbuhan tanaman dari *I. zollingeriana* dan aktivitas nodulasinya pada tanaman lain, yaitu kacang hijau (*Vigna radiata*). Sampel akar diperoleh dari tiga daerah yang berbeda. Sebanyak sembilan isolat dikultur pada media *Yeast Extract Mannitol Agar* (YEMA). Kultur cair berumur lima hari diinokulasi pada biji *I. zollingeriana* dan kacang hijau selama 30 menit, lalu ditanam di pasir steril. Eksperimen ini menggunakan Rancangan Acak Lengkap (RAL) dengan tiga ulangan. Morfologi koloni, pewarnaan Gram, *nitrogenase activity* dari kultur cair, dan jumlah nodul diamati dan diukur. Seluruh isolat memiliki koloni sirkular, permukaan cembung, dan warna putih mengilat. Sebagian besar isolat adalah gram negatif, namun isolat JP<sub>1</sub> dan KF merupakan gram positif. Seluruh isolat memiliki aktivitas enzim nitrogenase. Seluruh isolat tercatat dapat menodulasi *I. zollingeriana* lebih baik dari kontrol. Isolat BM, yang memiliki berat segar nodul terkecil pada *I. zollingeriana*, dapat meningkatkan nodulasi 10.73% lebih baik dari kontrol. Kasus berbeda ditemukan pada kacang hijau. Isolat yang dapat menodulasi tanaman lebih baik dari kontrol hanya JM<sub>1</sub> dan BM.

**Kata Kunci:** *I. zollingeriana*, Bakteri Pendukung Pertumbuhan Tanaman, Aktivitas Nitrogenase

### ABSTRACT

HUTAPEA PS, ABDULLAH L, KARTI PDMH, and ANAS I. 2017. Isolation of nodule-associated bacteria from *Indigofera zollingeriana* and its cross inoculation to mungbean.

*Indigofera zollingeriana* is a shrubby legume which highly accepted as fodder to animal ration because of its high protein content. As a legume, this plant could provide itself a nitrogen because of its symbiotic relationship with rhizobia. Moreover, there are some other bacteria could be found in root nodule which also supports plant growth. This experiment was aimed to find rhizobia or Nodule-Associated Bacteria from *Indigofera zollingeriana* and its cross-nodulation capability on mungbean (*Vigna radiata*). *Indigofera zollingeriana* root nodules were collected from three different locations. Total of 9 isolates were collected and cultured on *Yeast Extract Mannitol Agar* (YEMA). Isolates were also tested for nodulation on *I. zollingeriana* and mungbean. Five days old isolate were inoculated to *I. zollingeriana* and

mungbean seed for approximately 30 minutes and then sown into sterile sand. This experiment was designed in completely randomized design with three replications. Colonies morphology, Gram's nature, nitrogenase activity of asymbiotic isolates, nodules number, nodules fresh weight, were observed. Isolates showed a raised glistening white colonies. Most of isolates showed a gram negative, but JP<sub>1</sub> and KF isolate were found to be a gram positive. All isolates possessed a nitrogenase activity. Nodulation test showed that all isolates could renodulate *I. zollingeriana* better than control. BM isolate, which had the lightest nodule fresh weight on *I. zollingeriana*, could increased nodulation 13.62% better than the uninoculated treatment. Different case was found on mungbean, only JM<sub>1</sub> and BM isolate that could nodulate better than control.

**Key Words:** *I. zollingeriana*, Nodule-Associated Bacteria, Nitrogenase Activity

## INTRODUCTION

Availability of feed raw material plays an important role in animal husbandry sector. Grain concentrate, fish meal, meat bone meal are mostly used to fulfill protein need in ration. But, this material is not locally available, therefore feed industries in Indonesia often import some feed raw material to meet the demands. Nowadays, green concentrate has become an interest to substitute or complement grain concentrate. Not only contains a slightly high protein, green concentrate also contributes a vitamin and mineral in ration.

*Indigofera zollingeriana* is a potential legume plants because of its high protein content (29.76% up to 29.83%) and low total tannin content (0.09%-0.65%) (Abdullah 2010). It contains high utilisable fibre (NDF 49.41%-59.97%; ADF 26.23%-37.82%) and dry matter digestibility (67.39%-81.80%) (Abdullah and Suharlina 2010). Latter experiment also found that this plant could reduce methane emission and improve feed conversion values (Suharlina et al. 2016). Due to its protein content, this legume is highly recommended as a green concentrate for animal feed. The use of *I. zollingeriana* as feed for dairy goat could increase feed dry matter digestibility from 17% to 73%, protein use efficiency (1% to 2.5%), feed conversion values, and average milk production (121 to 383 mL/day). This legume also decreased feed cost up to 0.39 USD (Abdullah et al. 2012). The powder of *I. zollingeriana* shoot which was added into layer quail ration could replace 50% uses of soybean meal, decreased malondhyaldehyde (5.40%-3.02%), and increased quail consumption, cholesterol properties, egg weight as well as yolk colour score (Faradillah et al. 2015).

The best cultivation method must be studied furthermore to meet a better biomass production and quality. Biological fertilizer became a focus on this experiment to get a sustainable enviroment. Nodule-Associated Bacteria are bacteria which are often used as biofertilizer for legume plants. There are five distinct genera found in *Indigofera tinctoria* such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Pseudoalteromonas*, and *Cupriavidus*. It was reported that all five different genera could nodulate *I. tinctoria* (Leelahawonge et al. 2010). Other isolates except rhizobia also found within *Phaseolus vulgaris*'s nodules and found to be potential as rhizobia co-worker (Korir et al. 2017). The objection of this experiment was to isolate nodule-associated bacteria, observe its colony morphology, and study the nodulation capability on *I. zollingeriana* and also mungbean (*Vigna radiata*).

## MATERIALS AND METHODS

### Collection of *Indigofera zollingeriana* root nodules

Sample of nodules were collected from *I. zollingeriana* grown in three different locations i.e. Education and Research Garden, Jonggol; Goat breeding farm, Cilumbang; and Agrostology Garden, Bogor Agriculture University. Nodules were detached from three months old *I. zollingeriana*, then cleaned from adhering soil. Clean intact nodules were preserved in plastic tube filled with silica gel and cotton.

### Isolation and maintenance of bacteria

Bacteria isolation was performed at Soil Biotechnology Laboratory, Agriculture faculty Bogor Agriculture University. Clean intact nodules were surface sterilized by soaking it in sterilized distilled water and 5.25% NaClO for approximately 10 and 20 seconds, respectively. Then, nodules were thoroughly rinsed using sterilized distilled water as many as three times for 10 seconds each. Thereafter, nodules were sterilized using alcohol 96% by dipping it for 10 seconds. Lastly, nodules were rinsed two times using sterilized distilled water and crushed in a tube filled with sterile sodium chloride solution. Nodule extract was streaked on Yeast Extract Manitol (YEM) agar and incubated for 3-5 days under room temperature (Somasegaran and Hoben 1985) (Graham and Parker 1964).

Isolate were morphologically observed for its colonies shape and colour. Colonies purification were done by subculturing single colony into a new YEM agar. Different shape and colour of colony were also subcultured on a different YEM agar. Isolates were restreaked on YEM agar slant and preserved in the refrigerator.

### Bacteria gram stain and nitrogenase activity

Gram staining was performed at Animal Husbandry Faculty, Bogor Agriculture University, while nitrogenase activity were done at Balai Pascapanen Cimanggu, Bogor. Single colony of isolate was used for gram staining. A heat-fixed thin smear of bacterial culture were stained by crystal violet for a minute then rinsed under running water. Next, slide was flooded by iodine for a minute and rinsed. Decolorization was done by flooding the slide using 95% alcohol for 30 minutes and thoroughly rinsed under a running water. Lastly, safranin was added into slide and sit for a minute before rinsed. Nitrogenase activity was performed using a five days old isolate.

### Nodulation assay

Nodulation assay was done at Agrostology Greenhouse, Bogor Agriculture University. This assay were performed using *I. zollingeriana* and mungbean (*Vigna radiata*) seed. Seed were inoculated by soaking it into bacteria culture. One loopful single colony isolate was grown in YEM broth for 5 days on the shaker. Seeds were chosen by soaking it inside water. Seeds were scarified to facilitate a better water imbibition. Seeds were sterilized before sowing by soaking it into 96% alcohol for 10 seconds followed by soaking it into 5.25% NaClO for about 5 minutes. Thereafter seeds were thoroughly rinsed using sterilized distilled water for at least six times. Sterilized seeds were soaked into 70 °C sterilized distilled water for two hours. Lastly, seeds were soaked into bacteria culture for 30 minutes.

Sand was used as a planting media. Sand were sterilized for 30 minutes using autoclave before use. Sterility was check using Natrium Agar (NA) media. Sand was wetted by sterilized Jensen solution before planting. Jensen solution composition are a gram of dipotassium fosfat, 0.5 gram of magnesium sulphate, 0.5 gram of natrium chloride, 0.1 gram of ferrous sulphate, 0.005 gram of sodium molybdate, and 2 gram of calcium carbonate which were diluted into a liter of aquadest.

As many as 5 inoculated seeds of mungbean and 10 inoculated seeds of *I. zollingeriana* were sown into the sterilized sand. After germination, only 2 healthy seedling were maintained for each glass pot. Plant were watered using sterilized distilled water once every two days and watered using Jensen solution once a week. Plant growth and nodulation activity were observed. Harvesting were conducted after 37 days of planting.

### Experimental design and nodule observation

All 9 different isolates obtained from nodules and control treatment were used for nodulation assay. This experiment was designed in completely randomized design with 3 replication then analyse using analysis of variance (ANOVA) while significant different means were tested using Duncan. After 37 days of planting, *I. zollingeriana* and mungbean were uprooted. Roots were rinsed using running tap water to detach adhering sand. Nodules were counted and dried in oven 60 °C.

## RESULT AND DISCUSSION

### Isolate characteristic and nitrogenase activity of *Rhizobium*

As many as nine isolates were isolated from *Indigofera zollingeriana* root nodules. Isolate of JL, JM<sub>1</sub>, JM<sub>2</sub>, JM<sub>3</sub>, JP<sub>1</sub>, and JP<sub>2</sub> were obtained from *I. zollingeriana* which were grown in Jonggol. Isolate of BM and BP were isolated from *I. zollingeriana* grown in Cilumbang while KF isolate was obtained from *I. zollingeriana* in Dramaga. All isolates could grow well on YEM media which indicated the probability of nitrogen fixing activity. Most of all colonies showed a glistening white opaque colour and circular convex/raised shape (Table 1). Some of bacteria were positive gram while the other negative gram under microscope observation. The gram-negative isolate might be nitrogen-fixing bacteria. Therefore, ARA (Acetylene Reduction Assay) was conducted to examine nitrogenase activity. Glasshouse experiment was also conducted to screen all isolates nodulation capability.

**Table 1.** Isolate characteristic

Isolates	Sample site Location	Growth on YEM	Colour	Colonies Morphology	Gram Staining	Bacteria shape
JL	Jonggol	Yes	Glistening white opaque	Circular convex	Negative	Bacilli
JM <sub>1</sub>	Jonggol	Yes	Glistening white semitranslucent	Circular raised	Negative	Bacilli
JM <sub>2</sub>	Jonggol	Yes	Glistening white opaque	Circular flat	Negative	Bacilli
JM <sub>3</sub>	Jonggol	Yes	Glistening white semitranslucent	Circular raised	Negative	Short rods
BM	Cilumbang	Yes	Glistening pinkish white	Circular convex	Negative	Coccus
BP	Cilumbang	Yes	Glistening white opaque	Circular convex	Negative	Short rods
KF	Dramaga	Yes	Glistening white translucent	Circular convex	Positive	Short rods
JP <sub>1</sub>	Jonggol	Yes	Glistening, gummy white opaque	Circular convex	Positive	Bacilli
JP <sub>2</sub>	Jonggol	Yes	Glistening white opaque	Circular raised	Negative	Short rods

There are several bacteria strain which are found inside root nodule, including nitrogen-fixing bacteria. *Rhizobium* was well known as a rod-shape gram-negative bacteria and has a circular, raised and mucoid colonies (Gachande and Khansole 2011) (Rai and Sen 2015) (Singha et al. 2015). It also had been found before, a non-rhizobial nodule forming negative

gram bacteria that belonged to order *Rhizobiales*. *Methylobacterium nodulans* could nodulate *Crotalaria glaucoides*, *Crotalaria perrottetii*, and *Crotalaria podocarpa* (Jourand et al. 2004). *Phyllobacterium trifolii* was found to be able to nodulate *Trifolium* and *Lupinus* (Valverde et al. 2005). There also were *Devosia neptuniae* (nodulated *Neptunia natans*), *Blastobacter denitrificans* (nodulated *Aschynomene indica*), *Ochrobactrum lupini* (nodulated *Lupinus albus*), *Agrobacterium* like strains (nodulated *Phaseolus vulgaris*), *Burkholderia tuberum* (nodulated *Mimosa*), and *Cupriavidus taiwanensis* (also nodulated *Mimosa*), *Herbaspirillum lusitanum* (nodulated *Phaseolus vulgaris*) (Balanchadar et al. 2007).

Gram-positive bacteria were also found to be able to fix nitrogen. *Frankia* could nodulate *Alnus glutinosa* and *Casuarinaceae*. Moreover it could fix nitrogen in free living and also symbiotic state (Gtari et al. 2002). Not all nodule-associated bacteria could nodulate its host. Some of those bacteria worked as plant growth promotor. *Micromonospora* were able to increase N uptake and enhance *Alfalfa* nodulation when inoculated along with *Ensifer meliloti* (Martínez-Hidalgo et al. 2014). It had also been reported that dual inoculation of common bean with *Rhizobium* and Plant growth-promoting Rhizobacteria (PGPR) significantly increase higher yield (Wekesa et al. 2016). Moreover, isolates belong to PGPR could supply available soil nutrient such as phosphorus which was reported in other experiment. *Exiguobacterium sp* obtained from *Fenugreek* root nodules showed an IAA (Indol Acetic Acid) production, protease production, phosphate solubilization, and antifungal activity which supported *Rhizobium* nodulation activity (Rajendran et al. 2012).

All isolates could fix nitrogen in free living state which was shown by their nitrogenase activity. *Frankia* were also able to fix nitrogen in free living state (Gtari et al. 2002). After five days incubation, JP<sub>1</sub> isolate showed the highest nitrogenase activity. Symbiotic relationship between bacteria and plant host were performed because of heterotrof trait of bacteria. For nitrogen fixing bacteria, plant needs ammonium while bacteria needs carbon source for life survival which is supported from macrosymbiont (Haag et al. 2013). Some experiment showed that before the bacteria possessed a symbiotic relationship with plant host, bacteria still could fix N<sub>2</sub> for example *Rhizobium sp.* 32H1 that was grown at low oxygen tension, stationary culture and with a presence of yeast extract as a nitrogen source (Evans and Keister, 1976) (Tjepkema and Evans 1975). Another experiment also found that *Rhizobium* could reduce acetylene in agar culture (Bender et al. 1986). This experiment found that isolates JP<sub>1</sub> possessed the highest nitrogenase activity while isolate KF showed the lowest.

**Table 2.** Nitrogenase activity and nodulation activity of *I. zollingeriana*

Isolates	Nitrogenase activity ( $\mu\text{mol mL}^{-1}$ )	Nodules number (nodules plant <sup>-1</sup> )	Nodules fresh weight (mg plant <sup>-1</sup> )
KTRL		8.33 ± 2.72ab	14.83 ± 3.24
JL	13.94	12.83 ± 1.55a	28.28 ± 3.60
JM <sub>1</sub>	4.10	6.83 ± 2.05b	37.92 ± 9.74
JM <sub>2</sub>	7.50	9.17 ± 3.47ab	19.48 ± 7.92
JM <sub>3</sub>	4.33	6.00 ± 1.47b	20.00 ± 9.60
BM	5.94	7.17 ± 3.70b	16.85 ± 6.25
BP	5.01	4.00 ± 1.08b	24.48 ± 8.43
KF	3.10	9.33 ± 3.70ab	24.35 ± 5.51
JP <sub>1</sub>	37.05	6.00 ± 1.08b	30.52 ± 13.92
JP <sub>2</sub>	3.96	3.67 ± 1.31b	23.93 ± 2.41

KTRL was treatment without inoculation. Nitrogenase activities were obtained from 5-d-old isolate which was cultured in 5.5 mL YEM broth. Seed were inoculated with 5 d old isolates and planted for 37 d. Superscript within column followed by the same letter are not significantly different according to Duncan (P<0.05)

All isolates could nodulate *I. zollingeriana* better than control proven by a higher nodule fresh weight (Table 2). Nodules number were significantly different each treatment. JL isolate which was obtained from Jonggol gave a highest number of nodule compared to other

treatments. Different with nodules number, the highest nodules weight was obtained from JP<sub>1</sub> isolate followed by JM<sub>1</sub> isolate. The lightest nodule fresh weight which was formed by isolate BM still got a heavier nodules (13.62%) compared to control. The result showed that there was no correlation between nodules number and nodules fresh weight. A great number of nodules had a lighter nodules fresh weight because it had a smaller size. In the other hand, there were strains that formed a small amount yet big nodules.

Nodulation occurred because of signal exchange between the host and its symbiont. Under nitrogen starvation, plants secrete flavonoids that were recognized by rhizobia. Then, rhizobia synthesize lipochitooligosaccharides that triggers nodulation to plants. Lipochitooligosaccharides are the nod factor that could be synthesized differently by each rhizobia. It explains that rhizobia strain could not nodulate every legume plants (Guilhem et al. 2011) (Madsen et al. 2011). Ardley et al. (2013) found that genus *Listia* could be nodulated exclusively only by *Methylobacterium* or *Microvirga* while *Leobordea* and *Lotononis s.s.* interacted with rhizobia of diverse chromosomal and symbiotic lineage.

All isolate also showed a nodulation activity on mungbean. Surprisingly, JM<sub>1</sub> isolate could nodulate mungbean as effective as on *I. zollingeriana*. JM<sub>1</sub> isolate also produced a bigger size of nodules. Conversely, JP<sub>1</sub> isolate which nodulated *I. zollingeriana* effectively did not show a good nodulation on mungbean (Table 3).

**Table 3.** Nitrogenase activity and nodulation activity of mungbean

Isolates	Nitrogenase activity ( $\mu\text{mol mL}^{-1}$ )	Nodules number (nodules plant <sup>-1</sup> )	Nodules fresh weight (mg plant <sup>-1</sup> )
KTRL		4.00 $\pm$ 1.47	46.68 $\pm$ 11.68
JL	13.94	8.83 $\pm$ 1.18	46.50 $\pm$ 14.53
JM <sub>1</sub>	4.10	7.50 $\pm$ 2.55	52.82 $\pm$ 10.90
JM <sub>2</sub>	7.50	7.67 $\pm$ 1.93	51.93 $\pm$ 3.34
JM <sub>3</sub>	4.33	4.83 $\pm$ 1.55	45.77 $\pm$ 13.85
BM	5.94	8.83 $\pm$ 1.65	65.12 $\pm$ 7.10
BP	5.01	6.33 $\pm$ 1.25	41.87 $\pm$ 3.45
KF	3.10	10.17 $\pm$ 1.43	52.67 $\pm$ 8.17
JP <sub>1</sub>	37.05	2.25 $\pm$ 1.75	27.80 $\pm$ 20.75
JP <sub>2</sub>	3.96	8.50 $\pm$ 4.64	40.08 $\pm$ 8.89

KTRL was treatment without inoculation. Nitrogenase activities were obtained from 5-d-old isolate which was cultured in 5.5 mL YEM broth. Seed were inoculated with 5 d old isolates and planted for 37 d.

It produced few number and small size of nodule contrary to its high asymbiotic nitrogenase activity. Surprisingly, BM isolate which gave the poorest nodulation activity on *I. zollingeriana* exhibited the best nodulation on mungbean. Effectivity depends strain specificity with the host plants (Jia et al. 2013).

The best nodulation capability were influenced by effective strain. The effectiveness of strain must be proven by screening test with nitrogen deprivation. Moreover, strain effectivity is not determined by nodule number but it might be seen by its big size of nodule. *Rhizobium* inoculation experiment on bean in Cerrado soil stated that the great number of nodules did not correspond to a better plant production (Raposeiras et al. 2006). An ineffective strain would form an ineffective nodule which was marked by a small size of nodules. Ineffective strain also could be determined by the absence of leghaemoglobin (Kukkamalla and Vardhan 2016). This study showed that JM<sub>1</sub> isolate worked effectively on both *I. zollingeriana* and mungbean. JP<sub>1</sub> isolate worked better only on *I. zollingeriana* while BM isolate worked specifically only with mungbean. Nodulation effectivity actually depends on compatibility between bacteria and its plant host. The most compatible and effective strain would nodulate the plant better (Zang et al. 2014).

Low nitrogenase activity of free living state JM<sub>1</sub> isolate seemed to have no correlation with its nodulation capability. It could nodulate both plants effectively. Differently, JP<sub>1</sub> and JL isolate that had the highest pure isolate nitrogenase activity only gave a high nodules production on *I. zollingeriana*. The inconsistency and unpredictable result explained that there is no certain correlation between nitrogenase activity of an asymbiotic isolate and nodulation activity. Mishra and Pandey (2009) found that nodules number were correlated with nitrogenase activity of symbiotic rhizobia. From the study, plant diseases affected nodules number as well as the nitrogenase activity. High nodulation would only be directly correlated with nitrogenase activity of the plant which certainly possessed a symbiotic relationship with the isolate.

### CONCLUSION

All of the isolates were able to fix nitrogen in a free living state. JM<sub>1</sub> isolate from Jonggol that possessed a low nitrogenase activity turned out to be the most effective strain for both *I. zollingeriana* and also mungbean while JP<sub>1</sub> isolate worked effectively only on *I. zollingeriana* and BM isolate only work better with mungbean.

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