Nitrogenase Activity and IAA Production of Indigenous Diazotroph and Its Effect on Rice Seedling Growth

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ABSTRACT
The diazotroph bacteria as ecofriendly biofertilizers play an important role in improving the N status and availability of paddy soil. Laboratory experiment to study nitrogenase activity and IAA production of diazotroph from rice rhizosphere and to assess its effect on the growth of rice seedling has been conducted from September to October 2014 in Agronomy and Horticulture Laboratory Faculty of Agriculture Jenderal Soedirman University, Purwokerto. The experiment was arranged as a completely randomized design and consisted of seven treatments and provided with 4 replications. The treatments were the isolates of indigenous diazotroph (T₁ = A11003, T₂ = A230041, T₃ = A24001, T₄ = A230022, T₅ = A230021, T₆ = A230042 and T₇ = without inoculation). The nitrogenase activity measured by acetylene reduction assay method and IAA production was measured by HPLC method. Plant height, leaf greenness, leaf area, total dry weight and total root length were determined on 21 days after sowing. The experimental results showed that the isolat A230021 was identified as Rhizobium sp. LM-5 and have the highest nitrogenase activity at 0.07 μM C₂H₂ ml⁻¹ h⁻¹ and IAA production reached 19.01 ppm. Inoculation with strains of diazotroph enhanced chlorophyl content, total root length, and biomass production.

Keywords: diazotroph; IAA; N fixation; nitrogenase; plant growth

INTRODUCTION
Nitrogen play a significant role and become the limiting factor in rice based production system. The high dosage of N to meet the nutrient requirement increase the rice productivity (Choudhury & Kennedy, 2004). Sularso & Ismangil (2008) report that the use of nitrogen fertilizers in Banyumas Regency has been very high, above 300 kg ha⁻¹ but the yield is not comparable to the increase of fertilizer used. Intensive use of inorganic nitrogen has accelerated the mineralization of soil organic matter and has caused the levelling off. Consequently, the crop becomes less responsive to the inorganic fertilizer application (Nungkat, Kusuma, & Handayanto, 2015). Currently, about 70% of paddy soils in Indonesia have a low organic content (< 2 % of org-C), low nutrients availability and categorized as sick soils (Turmuktini, Simarmata, Joy, & Resmini, 2012; Simarmata, Joy, Sofyan, Turmuktini, & Sudjana, 2015).

The indigenous diazotroph is an alternative source of nitrogen for rice plant and to bio-remediation of agricultural environment as the impact of long-term nitrogen fertilizer application (Shrestha & Maskey, 2005; Widowati, Nursyamsi, Rochayati, & Sarwani, 2011). The free-living biological nitrogen fixation can contribute to the availability of N in soil about 50-150 kg ha⁻¹ N (Chowdhury & Mukherjee, 2006). Application of diazotroph can be used to substitute urea fertilizer, like Azotobacter (Kennedy & Tchan, 1992), Azospirillum (Saikia & Jain, 2007), Enterobacter spp., Klebsiella spp., Bacillus sp. (Khan, Mohiuddin, & Rahman, 2008), and promoting growth of the rice plant as plant growth promoting rhizobacteria (PGPR) (Choudhury & Kennedy, 2004).

The inoculated rice with diazotroph has shown a significant improvement in vigorous seedling development, plant growth and grain production (Hardoim, 2015). Applications of Azotobacter inoculant to maize plant has increased the plant height, shoot and seeds dry weight, nutrient uptake (N, P, K, Fe and Cu),


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total biomass, chlorophyll content and plant height of rice (Biari, Gholami, & Rahmani, 2008; Roy, Deb, & Sharma, 2009).

This research aimed to study the ability of nitrogen fixation of indigenous diazotroph from rice rhizosphere and to assess the effect of these strains on rice seedling growth.

MATERIALS AND METHODS

Diazotroph Strains

Diazotroph strain was isolated from rice rhizosphere in three sub-districs (Baturaden, Kembaran, and Sumbang) of Banyumas Regency, Central Java. The isolates consisted of A11003 from Baturaden paddy soil, A24001 from Sumbang paddy soil, A230041, A230022, A230021 and A230042 from Kembaran paddy soil. The bacteria were cultivated in Ashby medium.

Acetylene Reduction Assay

The Acetylene Reduction Assay (ARA) method was used to measure the nitrogenase activity (Zechmeister-Boltenstern, 1996). All Diazotroph strains were cultured in nutrient broth. 0.5 ml culture of each isolates were added to vials individually and incubated for 48 h at 22°C in thermostatic incubator. The cotton plugs were replaced with rubber stopper. Using a gas-tight syringe to exchange 10% of the air volume of the respective assay vessel with acetylene. Five minutes after the addition of acetylene, inject a 500 µl gas sample into the gas chromatography, and analyze it for the initial ethylene concentration. Determination of calibration curve has been done by injecting 100, 200, 300, 400 and 500 µl of ethylene standard into the gas chromatography. The concentration of ethylene was measured with calibration curve.

Indol Acetic Acid Assay

IAA production was measured by High Performance Liquid Chromatography (HPLC) (Shahab, Ahmed, & Khan, 2009). Diazotroph strains were cultured in nutrient broth at 28°C. 20 ml of cultures was centrifugated at 8,000 x g for 30 minutes at 4°C. 1 ml of supernatant was extracted with 100 µl ether for three times. Extract samples was added with methanol 60-65%. The extract (10 µl) was injected on to column in a chromatograph equipped with a differential ultraviolet detector (280 nm). Quantification was done by comparison of peak heights.

Determination of 16s rRNA

The extraction of DNA from each isolates was performed by GES method as described by Pitcher, Saunders, & Owen (1989) and used for DNA template. Amplification of 16s rRNA gene was performed with primers 9F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1541R (5'-AAG GAG GTG ATC CAG CC-3'). PCR product was purified with PEG precipitation method and was continued with sequencing cyclus. The DNA sequence was purified with ethanol purification method. The DNA sequence was read with automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The data from DNA sequencing was trimming with MEGA 4 software and assembly with BioEdit to convert in FASTA format. These sequences were compared with others found by BLAST searching in data base of national Center fo Biotechnology Information (http://www.ncbi.nlm.nih.gov/) or DDBJ (http://www.ddbj.nig.ac.jp).

In Vitro Test of Diazotroph Isolates to Rice Seedling Growth

The study was carried out from 19 September - 21 October 2014 in the Laboratory of Agronomy and Horticulture Jenderal Soedirman University at Purwokerto. This research was arranged by a completely randomized design and the treatment was diazotroph isolates consisted of T1 = A11003, T2 = A230041, T3 = A24001, T4 = A230022, T5 = A230021, T6 = A230042 and T7 = without inoculation. Each unit consisted of two tubes and fourth time replicates.

Culture medium used Yoshida medium (Yoshida, Forno, Cock, & Gomez, 1976) without N. The nutrient concentrations of Yoshida medium were P 10 ppm, K 40 ppm, Ca 40 ppm and added with micronutrient. Each tube was filled with 5 ml medium and sterilized at 121°C and 15 Psi with an autoclave. The medium was inoculated with 1 ml of Diazotroph culture (10⁶ cfu). Rice seeds were sterilized with HgCl₂ 0.02 % for 1 minute and then was washed with aquadest. Rice seeds were germinated in petridish for 5 days and then were tranferred in to Yoshida medium. The plants were harvested on 21 days after sowing. The observed data consisted of plant height (cm), leaf greeness (measured with portable leaf chlorophyll meter
MINOLTA SPAD-502), leaf area (cm²), total dry weight and total root length. Total root length was measured with intersection methods (Bohm, 1979). The wet roots were poured into the dish with some water and they are spreaded randomized over the grid and did not overlap. Counts were made from the intersection of the roots with vertical and horizontal grid lines. Intersection could be converted to centimeter measurement using the formula:

\[ \text{Root length} = \frac{11}{12} \times \text{number of intersection} \times \text{grid unit} \]

**Statistical Analysis**

The observed data were analyzed by using F test, when significant then continued with Duncan Multiple Range Test at P < 0.05.

**RESULTS AND DISCUSSION**

**Nitrogenase Activity and IAA Production of Azotobacter sp**

Based on the acetylene reduction assay (ARA) all the indigenous diazotroph isolates from Banyumas paddy land has the ability to fix \( \text{N}_2 \) (Table 1).

Table 1. Nitrogenase activity and IAA production of Diazotroph

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Nitrogenase activity (µM ml⁻¹ h⁻¹)</th>
<th>IAA (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A11003</td>
<td>0.04</td>
<td>18.21</td>
</tr>
<tr>
<td>A230041</td>
<td>0.06</td>
<td>19.01</td>
</tr>
<tr>
<td>A24001</td>
<td>0.05</td>
<td>21.01</td>
</tr>
<tr>
<td>A230022</td>
<td>0.06</td>
<td>18.12</td>
</tr>
<tr>
<td>A230021</td>
<td>0.07</td>
<td>19.10</td>
</tr>
<tr>
<td>A230042</td>
<td>0.04</td>
<td>18.04</td>
</tr>
</tbody>
</table>

Clearly, the isolate A230021 has the highest nitrogenase activity at 0.07 µM C₂H₄ ml⁻¹ h⁻¹ followed by A230041 and A230022 isolates respectively 0.06 µM C₂H₄ ml⁻¹ h⁻¹, and the lowest nitrogenase activity is resulted by isolate A230042 and A11003 respectively 0.04 µM C₂H₄ ml⁻¹ h⁻¹. In addition, as shown in Table 1, all of the Diazotroph strains were capable to produce IAA. There were variabilities on the Capacity of IAA production among the isolates. The isolate A24001 has the highest capacity to produce IAA at 21.01 ppm, followed by A230021 and A230041 isolates at 19.01 ppm, while the A230022 isolate has the lowest IAA production capacity reached 18.12 ppm.

Diazotroph isolated from rice rhizosphere had a positive contribution to fix the dinitrogen as indicated by the nitrogenase activity. Nitrogenase is a key indicator of the capacity of diazotroph which capable to fix dinitrogen and release the nitrogen to soil and become available for the plant (Keyeo, Ai’shah, & Amir, 2011). Shrestha & Maskey (2005) stated that presentation of biological nitrogen fixation bacteria more dominant in the rice rhizosphere to total micro-flora and flooded soil giving favourable environment to nitrogen fixation activity. The biodiversity and diazotrophic population growth are highly correlated with sugars and amino acids from the root exudates (Naher, Radziah, Halimi, Shamsuddin, & Mohd Razi, 2009). Nitrogenase enzyme catalyzes the reduction of \( \text{N}_2 \) to \( \text{NH}_3 \) and its reaction is very sensitive to the presence of O₂ (Sharifi & Khavazi, 2011). The diazotroph are able to fix dinitrogen with high O₂ condition due to the respiratory, conformation protection and nitrogenase enzyme located in the cells, so the nitrogenase activity remains high (Dighe et al., 2010).

All the six pure isolates are able to synthetize IAA (Table 1). Consequently, they can act as plant growth promoting rhizobacteria (PGPR). A24001 isolate was able to produce the highest IAA at 21.01 ppm, followed by A230022 and A230041 at 18.12 and 19.01 ppm respectively. This result in line with Torres-Rubio, Valencia-Plata, Bernal-Castillo, & Martinez-Nieto (2000) that stated Azotobacter chroococcum was able to produce IAA in the range 16.1-32.22 ppm, and Azotobacter vinelandii was able to produce IAA in the range of 21.2-32.2 ppm. IAA production capacity among the six pure cultures of diazotroph was not in line with the nitrogenase activity. This result showed that isolate with highest IAA production capacity, but low in the nitrogenase activity. Therefore, the character of nitrogenase activity is chosen as a trait to select the best isolate to be used as biofertilizer.

**Determination of 16s rRNA of Diazotroph**

The 16s rRNA was used to identification of the diazotroph isolates. Isolate A230021 was identified to have the highest nitrogenase activity reached 0.07 µM ml⁻¹ h⁻¹ (Figure 1).
The 16s rRNA gene sequence was used to describe the species of diazotroph. This shared 100% sequence identity with *Rhizobium* sp. LM-5. The draft genome sequence of *Rhizobium* sp. LM-5 consisted of 1333 bp. *Rhizobium* sp. LM-5 was isolated from rice rhizosphere. Traditionally, genus *Rhizobium* sp. was classified into symbiotic organism with legume plant, but *Rhizobium* can act as non-symbiotic diazotroph in plant rhizosphere (de Souza et al., 2015). Naher, Radziah, Halimi, Shamsuddin, & Mohd Razi (2009) found that *Rhizobium* strains from rice rhizosphere were able to colonize endophytically in cortex and vascular system in the lateral roots. Cortex region give favourable condition to diazotroph for dinitrogen fixation with low of oxygen concentration and available nutrients.

**Response of Rice Seedling to Diazotroph Inoculation**

Diazotroph inoculation on rice seedling gave the significant effect on leaf greenness, total root length, and total biomass, while leaf area and plant height were not affected significantly. Diazotroph inoculation increased plant height up to 37.60 % than uninoculated. The highest plant was obtained 17.063 cm under *Rhizobium* sp. strain LM-5 application, but not significant among the isolates (Table 2). Diazotroph inoculation was significant to increase the leaf greenness than uninoculated. The higher leaf greenness of 20.188 was obtained under inoculation with *Rhizobium* sp. strain LM-5. This result was 1.9 times higher than uninoculated. All of isolates give positive effect on leaf greens. However, leaf area was not affected by inoculation with Diazotroph.

Rice seed inoculation with diazotroph has a positive effect on root growth. The increase of total root growth occurred with the inoculation of diazotroph strains. The highest root length of 14.42 cm was obtained under inoculation with *Rhizobium* sp. strain LM-5 than among isolates. The increase of total root length will effect on the broad area of contact between root and medium, so that nutrient uptake, bacterial colonization on root and activity of N\textsubscript{2} fixation will increase. The improvement of root colonization can increase nutrient uptake and mineral for plant growth (Ai’shah, Amir, Keng, & Othman, 2009). The similar result revealed that inoculation of bacteria increases the shoot dry weight. In addition, the improving the plant growth also related to the ability of bacteria to produce IAA (Table 1).

**Table 2. Effect of The diazotroph of rice rhizosphere on in vitro growth of rice seedlings**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Plant Height (cm)</th>
<th>Leaf Greenness (SPAD Units)</th>
<th>Leaf Area (cm\textsuperscript{2})</th>
<th>Total Root length (cm)</th>
<th>Plant Biomass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A230021</td>
<td>17.063 a</td>
<td>20.188 a</td>
<td>1.983 a</td>
<td>15.420 a</td>
<td>22.375 a</td>
</tr>
<tr>
<td>A230022</td>
<td>16.138 a</td>
<td>16.150 ab</td>
<td>2.413 a</td>
<td>12.080 ab</td>
<td>20.500 ab</td>
</tr>
<tr>
<td>A24001</td>
<td>16.875 a</td>
<td>14.931 b</td>
<td>1.913 a</td>
<td>11.540 b</td>
<td>20.250 ab</td>
</tr>
<tr>
<td>A230041</td>
<td>16.550 a</td>
<td>14.875 b</td>
<td>2.215 a</td>
<td>12.278 ab</td>
<td>18.125 bc</td>
</tr>
<tr>
<td>A230042</td>
<td>16.275 a</td>
<td>15.850 ab</td>
<td>1.963 a</td>
<td>13.260 ab</td>
<td>20.375 ab</td>
</tr>
<tr>
<td>A11003</td>
<td>14.863 a</td>
<td>15.625 b</td>
<td>1.815 a</td>
<td>11.835 b</td>
<td>17.875 c</td>
</tr>
<tr>
<td>Control</td>
<td>12.400 b</td>
<td>6.850 c</td>
<td>1.680 a</td>
<td>8.250 c</td>
<td>16.250 c</td>
</tr>
</tbody>
</table>

Remarks: Data in each column followed by the same letter show insignificantly different at DMRT of 5%.
IAA enhances the cell division and root elongation on host plant (Keyeo, Al’ishah, & Amir, 2011). Inoculation with A24001, which performed high capacity of IAA production at 21.01 ppm depressed the root growth. The lower root length of 11.835 cm was obtained with A24001 inoculation than among the isolates. Under low root growth the bacteria will have a little chance to colonize the root due the lower nutrient from root exudate and nitrogenase activity will decrease. The use of sugar and amino acid from root exudate are correlated with population growth of diazotrophic in rhizosphere. Root exudate provides favorable environment to grow and to colonize the roots due to the availability of nutrient for diazotroph growth (Naher, Radziah, Halimi, Shamsuddin, & Mohd Razi, 2009).

Rice seedling inoculation with *Rhizobium* sp. strain LM-5 provides better leaf greenness when compared to the other isolates. *Rhizobium* sp. strain LM-5 was able to increase the leaf greenness up to 1.94 times greater than uninoculation. The value of leaf greenness (SPAD unit) has a close correlation with the content of chlorophyll a and b (Purwanto, 2009). The increase of leaf greenness may be affected by the capacity of root to uptake nitrogen from the medium. Roots colonized by diazotrophic will increase the capacity of nitrogen fixation and available N in the medium.

The inoculation with Diazotroph strain increased the plant biomass up to 37.69 % significantly, as compare to uninoculated (Table 2). Higher plant biomass of 22.375 g was obtained under inoculation with A230021 strain. A230021 acted as PGPR increasing the root growth by producing the IAA up to 19.01 ppm, and it may optimize concentration of phytohormones in the medium. Increase of plant biomass has a close correlation with leaf greenness (chlorophyll a and b content) and root length. Good root growth will effect on water, mineral and nutrient and lead to increase the chlorophyll content in leaf and photosynthetic rate resulting in accumulation of biomass.

The enhancement of plant growth by application of diazotrophic has been reported by many researchers. Mutalib, Radziah, Shukor, & Naher (2012) reported that rice plant inoculated with PGPR revealed an increase in N uptake, chlorophyll content, photosynthetic rate and plant biomass. The Diazotroph strain used in this research had positive effect on rice growth under in vitro condition. A230021 strain is the best strain to lead the plant growth with the capacity of nitrogen fixation.

CONCLUSION

The Diazotroph strain A230021 was identified as *Rhizobium* sp. strain LM-5 and had the highest nitrogenase activity at 0.7 µM C2H4 ml⁻¹ h⁻¹ and also acts as PGPR to increase the rice plant growth, root length, leaf greenness (total chlorophyll content) and biomass. *Rhizobium* sp. strain LM-5 is potentially to be developed as be biofertilizer and a source of PGPR.

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