

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

Purwanto ¹⁾, Yuyun Yuwariah ²⁾, Sumadi ²⁾ and Tualar Simarmata ²⁾

¹⁾ Faculty of Agriculture, Jenderal Soedirman University
Jl. Dr. Suparno PO BOX 125 Purwokerto, Central Java 53122, Indonesia

²⁾ Faculty of Agriculture, Padjadjaran University
Jl. Raya Jatinangor KM 21 Sumedang, West Java 45363, Indonesia

^{*}) Corresponding author E-mail: purwanto.unsoed@gmail.com

Received: September 11, 2015/ Accepted: June 16, 2016

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 $\mu\text{M C}_2\text{H}_4 \text{ ml}^{-1} \text{ h}^{-1}$ and IAA production reached 19.01 ppm. Inoculation with strains of diazotroph enhanced chlorophyll 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),

Cite this as: Purwanto, Yuwariah, Y., Sumadi, & Simarmata, T. (2017). Nitrogenase activity and IAA production of indigenous diazotroph and its effect on rice seedling growth. *AGRIVITA Journal of Agricultural Science*, 39(1), 31-37. <http://doi.org/10.17503/agrivita.v39i1.653>

Accredited: SK No. 81/DIKTI/Kep/2011

Permalink/DOI: <http://dx.doi.org/10.17503/agrivita.v39i1.653>

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-districts (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 were 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 cycle. 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 for 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 four 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, Mg 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 (10⁹ 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 transferred in to Yoshida medium. The plants were harvested on 21 days after sowing. The observed data consisted of plant height (cm), leaf greenness (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 :

$$\text{Root length (R)} = \left(\frac{11}{14}\right) \times \text{number of intersection (N)} \times \text{grid unit}$$

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 N₂ (Table 1).

Table 1. Nitrogenase activity and IAA production of Diazotroph

Isolates	Nitrogenase activity ($\mu\text{M C}_2\text{H}_4 \text{ ml}^{-1} \text{ h}^{-1}$)	IAA (ppm)
A11003	0.04	18.21
A230041	0.06	19.01
A24001	0.05	21.01
A230022	0.06	18.12
A230021	0.07	19.10
A230042	0.04	18.04

Clearly, the isolate A230021 has the highest nitrogenase activity at 0.07 $\mu\text{M C}_2\text{H}_4 \text{ ml}^{-1} \text{ h}^{-1}$ followed by A230041 and A230022 isolates respectively 0.06 $\mu\text{M C}_2\text{H}_4 \text{ ml}^{-1} \text{ h}^{-1}$, and the lowest nitrogenase activity is resulted by isolate A230042 and A11003 respectively 0.04 $\mu\text{M C}_2\text{H}_4 \text{ ml}^{-1} \text{ h}^{-1}$. 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 microflora 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 N₂ to NH₃ 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 synthesize 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, & Martínez-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 $\mu\text{M ml}^{-1} \text{ h}^{-1}$ (Figure 1).

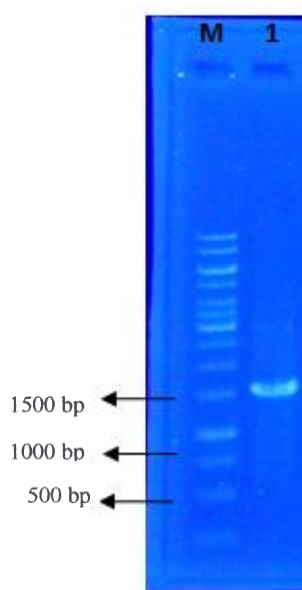


Figure 1. PCR Amplification of *Rhizobium* sp. strain LM-5

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 N₂ 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

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

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, Ai'shah, & 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 $\mu\text{M C}_2\text{H}_4 \text{ ml}^{-1} \text{ h}^{-1}$ 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.

ACKNOWLEDGMENTS

The authors are grateful to The Director of CV. Bintang Asri Arthaully Bandung and Head of Agronomy and Horticulture Laboratory Faculty of Agriculture Jenderal Soedirman University Purwokerto for Laboratory facilities to carry out this research.

REFERENCES

- Ai'shah, O. N., Amir, H. G., Keng, C. L., & Othman, A. R. (2009). Influence of various combinations of diazotrophs and chemical N fertilizer on plant growth and N₂ fixation capacity of oil palm seedlings (*Elaeis guineensis* Jacq.). *Thai Journal of Agricultural Science*, 42(3), 139–149. Retrieved from http://www.thaiagj.org/images/stories/Journal_online/2010/43-3/03-TJ-AGR-0410-18.pdf
- Biari, A., Gholami, A., & Rahmani, H. A. (2008). Growth promotion and enhanced nutrient uptake of maize (*Zea mays* L.) by application of growth promoting rhizobacteria in arid region of Iran. *Journal of Biological Sciences*, 8(6), 1015–1020. Retrieved from <http://docsdrive.com/pdfs/ansinet/jbs/2008/1015-1020.pdf>
- Böhm, W. (1979). *Methods of studying root systems*. Berlin: Springer-Verlag. <http://doi.org/10.1007/978-3-642-67282-8>
- Choudhury, A. T. M. A., & Kennedy, I. R. (2004). Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. *Biology and Fertility of Soils*, 39(4), 219–227. <http://doi.org/10.1007/s00374-003-0711-1>

- 1007/s00374-003-0706-2
- Chowdhury, A., & Mukherjee, P. (2006). Biofertilizers. *ENVIS Centre on Environmental Biotechnology*, 2–7. Retrieved from <http://deskuenvi.nic.in/pdf/Newslet9.pdf>
- de Souza, R., Sant'Anna, F. H., Ambrosini, A., Tadra-Sfeir, M., Faoro, H., Pedrosa, F. O., ... Passaglia, L. M. P. (2015). Genome of *Rhizobium* sp. UR51a, isolated from rice cropped in Southern Brazilian fields. *Genome Announcements*, 3(2), e00249-15. <http://doi.org/10.1128/genomeA.00249-15>
- Dighe, N. S., Shukla, D., Kalkotwar, R. S., Laware, R. B., Bhawar, S. B., & Gaikwad, R. W. (2010). Nitrogenase enzyme: A review. *Der Pharmacia Sinica*, 1(2), 77–84. Retrieved from <http://pelagiaresearchlibrary.com/der-pharmacia-sinica/vol1-iss2/DPS-2010-1-2-77-84.pdf>
- Hardoim, P. R. (2015). Heading to the origins - Rice microbiome as functional extension of the host. *Journal of Rice Research*, 3(2), 1–3. Retrieved from <http://www.esciencecentral.org/journals/heading-to-the-origins-rice-microbiome-as-functional-extension-of-the-host-2375-4338.1000e133.pdf>
- Kennedy, I. R., & Tchan, Y. T. (1992). Biological nitrogen fixation in non-leguminous field crops: Recent advances. *Plant and Soil*, 141(1), 93–118. <http://doi.org/10.1007/BF00011312>
- Keyeo, F., Ai'shah, O. N., & Amir, H. G. (2011). The effects of nitrogen fixation activity and phytohormone production of diazotroph in promoting growth of rice seedlings. *Biotechnology*, 10(3), 267–273. <http://doi.org/10.3923/biotech.2011.267.273>
- Khan, M. H. R., Mohiuddin, M., & Rahman, M. (2008). Enumeration, isolation and identification of nitrogen-fixing bacterial strains at seedling stage in rhizosphere of rice grown in non-calcareous grey flood plain soil of Bangladesh. *Journal of the Faculty of Environmental Science and Technology*, 13(1), 97–101. Retrieved from http://ousar.lib.okayama-u.ac.jp/files/public/1/12834/20160527192202548508/13_097_101.pdf
- Mutalib, A. A., Radziah, O., Shukor, Y., & Naher, U. A. (2012). Effect of nitrogen fertilizer on hydrolytic enzyme production, root colonisation, N metabolism, leaf physiology and growth of rice inoculated with *Bacillus* sp. (Sb42). *Australian Journal of Crop Science*, 6(9), 1383–1389. Retrieved from http://www.cropj.com/nahe6_9_2012_1383_1389.pdf
- Naher, U. A., Radziah, O., Halimi, M. S., Shamsuddin, Z. H., & Mohd Razi, I. (2009). Influence of root exudate carbon compounds of three rice genotypes on rhizosphere and endophytic diazotrophs. *Pertanika Journal of Tropical Agricultural Science*, 32(2), 209–223. Retrieved from <https://core.ac.uk/download/pdf/12224776.pdf>
- Nungkat, P., Kusuma, Z., & Handayanto, E. (2015). Effects of organic matter application on methane emission from paddy fields adopting organic farming. *Journal of Degraded and Mining Lands Management*, 2(2), 303–312. <http://doi.org/10.15243/jdmlm.2014.022.303>
- Pitcher, D. G., Saunders, N. A., & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in Applied Microbiology*, 8(4), 151–156. <http://doi.org/10.1111/j.1472-765X.1989.tb00262.x>
- Purwanto, (2009). *Pertumbuhan dan hasil empat varietas tanaman padi pada sistem pertanian organik, semiorganik, dan konvensional* [Growth and yield of four rice varieties at organic, semiorganic and conventional farming] (Unpublished master thesis). Gadjah Mada University, Yogyakarta.
- Roy, B. D., Deb, B., & Sharma, G. D. (2009). Dinitrogen nutrition and rice cultivation through biofertilizer technology. *Assam University Journal of Science & Technology: Biological Sciences*, 4(1), 20–28. Retrieved from <http://www.inflibnet.ac.in/ojs/index.php/AUJSAT/article/view/237/235>
- Saikia, S. P., & Jain, V. (2007). Biological nitrogen fixation with non-legumes: An achievable target or a dogma? *Current Science*, 92(3), 317-322. Retrieved from [http://14.139.56.90/bitstream/1/37888/1/S-Biological nitrogen.pdf](http://14.139.56.90/bitstream/1/37888/1/S-Biological%20nitrogen.pdf)
- Shahab, S., Ahmed, N., & Khan, N. S. (2009). Indole acetic acid production and

- enhanced plant growth promotion by indigenous PSBs. *African Journal of Agricultural Research*, 4(11), 1312–1316. Retrieved from [http://www.academicjournals.org/article/article1380959702_Shahab et al.pdf](http://www.academicjournals.org/article/article1380959702_Shahab%20et%20al.pdf)
- Sharifi, R. S., & Khavazi, K. (2011). Effects of seed priming with Plant Growth Promoting Rhizobacteria (PGPR) on yield and yield attribute of maize (*Zea mays* L.) hybrids. *Journal of Food, Agriculture and Environment*, 9(3–4), 496–500. Retrieved from <http://world-food.net/effects-of-seed-priming-with-plant-growth-promoting-rhizobacteria-pgpr-on-yield-and-yield-attributes-of-maize-zea-mays-l-hybrids/>
- Shrestha, R. K., & Maskey, S. L. (2005). Associative nitrogen fixation in lowland rice. *Nepal Agriculture Research Journal*, 6, 112–121. Retrieved from <http://www.nepjol.info/index.php/NARJ/article/download/3373/2921>
- Simarmata, T., Joy, B., Sofyan, E. T., Citraresmini, A., Turmuktini, T., & Sudjana, B. (2015). Innovation of biofertilisers - Organic based nutrients management and water saving technology to secure rice productivity. In *International Research on Food Security, Natural Resource Management and Rural Development* (pp. 1–4). Tropentag. Retrieved from <http://www.tropentag.de/2015/abstracts/full/250.pdf>
- Sularso, K. E., & Ismangil. (2008). *Identifikasi Kesesuaian Agroekosistem untuk Budi-daya Padi di Kabupaten Banyumas* [Identification of agroecosystem suitability for rice cultivation in Banyumas]. Purwokerto, ID: Faculty of Agriculture UNSOED.
- Torres-Rubio, M. G., Valencia-Plata, S. A., Bernal-Castillo, J., & Martínez-Nieto, P. (2000). Isolation of enterobacteria, *Azotobacter* sp. and *Pseudomonas* sp., producers of indole-3-acetic acid and siderophores, from Colombian rice rhizosphere. *Revista Latinoamericana de Microbiologia*, 42, 171–176. Retrieved from <http://www.medigraphic.com/pdfs/lamicro/mi-2000/mi004f.pdf>
- Turmuktini, T., Simarmata, T., Joy, B., & Resmini, A. C. (2012). Management of water saving and organic based fertilizers technology for remediation and maintaining the health of paddy soils and to increase the sustainability of rice productivity in Indonesia. *Asian Journal of Agriculture and Rural Development*, 2(4), 536–551. Retrieved from <http://search.proquest.com/docview/1416208080?pq-origsite=gscholar>
- Widowati, L. R., Nursyamsi, D., Rochayati, S., & Sarwani, M. (2011). Nitrogen management on agricultural land in Indonesia. In *International Seminar on Increased Agricultural Nitrogen Circulation in Asia: Technological Challenge to Mitigate Agricultural N Emissions* (pp. 181–195). Taipei. Retrieved from <http://balittanah.litbang.pertanian.go.id/ind/dokumentasi/ainnya/ladiyani.pdf>
- Yoshida, S., Forno, D. A., Cock, J. H., & Gomez, K. A. (1976). *Laboratory manual for physiological studies of rice* (3rd ed.). Los Banos: The International Rice Research Institute. Retrieved from http://books.google.com/books?id=e1w_7XJNlWIC&pgis=1
- Zechmeister-Boltenstern, S. (1996). Nitrogenase activity by acetylene reduction. In F. Schinner, R. Ohlinger, E. Kandeler, & R. Margesin (Eds.), *Methods in soil biology* (pp. 125-128). Berlin: Springer-Verlag.