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SHORT COMMUNICATION

Estimation of Nitrogenase Enzyme Activities and Plant Growth of Legume and Non-legume Inoculated with Diazotrophic Bacteria

Salwani Shaffie, Amir Hamzah Ghazali* and Nazalan Najimudin

School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
E.mail: amirhq@usm.my

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ABSTRACT

Biological Nitrogen Fixation (BNF) process benefits the agriculture sector especially for reducing cost of nitrogen fertilizer. In the process, the diazotrophs convert N_2 into ammonia (NH_3) which is useable by plants. The BNF process is catalysed by nitrogenase enzyme that involved protons and electrons together with evolution of H_2 therefore, the assessment of N_2 fixation is also available *via* H_2 production and electron allocation analysis. Thus, the aims of this experiment were to estimate the nitrogenase enzyme activities and observe the influence of diazothrophs on growth of legume (soybean) and non legume (rice) plants. Host plants were inoculated with respective inocula; *Bradyrhizobium japonicum* (strain 532C) for soybean while *Azospirillum brasilense* (Sp7) and locally isolated diazotroph (isolate 5) for rice. At harvest, the plants were observed for plant growth parameters, H_2 evolution, N_2 fixation and electron allocation coefficient (EAC) values. The experiment recorded N_2 fixation activities of inoculated soybean plants at 141.2 µmol N_2 h^{-1} g^{-1} dry weight nodule, and the evolution of H_2 at 144.4 µmol H_2 h^{-1} g^{-1} dry weight nodule. The electron allocation coefficient (EAC) of soybean was recorded at 0.982. For inoculated rice plants, none of the observations was successfully recorded. However, results for chlorophyll contents and plant dry weight of both plants inoculated with respective inocula were similar to the control treatments supplied with full nitrogen fertilization (+N). The experiment clearly showed that inoculation of diazotrophic bacteria could enhance growth of the host plants similar to plants treated with nitrogenous fertilizer due to efficient N_2 fixation process

Key words: nitrogenase, electron allocation coefficient (EAC), H_2 evolution, N_2 fixation, diazotroph

INTRODUCTION

Diazotrophic microorganisms are able to fix atmospheric N through Biological Nitrogen Fixation (BNF) process (Alam *et al.,* 2003; Madigan *et al*., 2003; Dixon and Kahn, 2004; Raja *et al.,* 2006; Mia and Shamsuddin, 2010). This process benefits the agriculture sector especially for reducing cost of inorganic nitrogen fertilizer. It is due to the ability of the microorganisms to convert atmospheric N into ammonia (NH_3) which is useable by plants. Thus, most of the studies in this field of research were conducted to optimize the N_2 fixation activities (Rebah *et al.,* 2007). The most important factor for effective BNF process is the occurrence of nitrogenase enzyme that catalyse the fixation process (Sur *et al.,* 2010). The reaction involved is as follows (Giller, 2001):

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

Nitrogenases enzyme catalyze the reduction of N_2 into NH_3 that involved protons and electrons together with evolution of H_2. Thus, assessment of N_2 fixation activities *via* H_2 production is easy and with minimal disturbance to the plant during the analysis. Besides, determination of electron allocation involved in N_2 fixation process known as Electron Allocation Coefficient (EAC) is a convenient way to study the portioning of electrons between ammonia (NH_3) production and H_2 evolution (Curtis *et al.,* 2004; Schulze *et al.,* 2011). Six electrons are involved in producing NH_3, while two electrons are allocated for H_2 evolution (Hunt, 1997; Giller, 2001; Curtis *et al.,* 2004; Mia and Shamsuddin, 2010; Schulze *et al.,* 2011). Earlier findings by Schubert and Evans (1976) and Feijtel *et al.,* (1985) reported that 40-60 % of electron flow in nitrogenase was used for ammonia production in plant. Thus, the objectives of this experiment were; 1) to estimate the nitrogenase enzyme activities of diazotrophs in relationships with soybean (legume) and rice (non-legume) plants and 2) to observe influence of inoculation on growth of soybean and rice plants.

MATERIALS AND METHODS

The study was conducted from July 2005 to May 2006 at the School of Biological Sciences, Universiti Sains Malaysia. Soybean and rice seeds were surface sterilized with 95 % ethanol, 0.1 % $HgCl_2$ solution and washed with

*Corresponding author

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5 changes of sterile distilled water. The seeds were germinated aseptically for 3-5 days in the dark (Somasegaran and Hoben, 1985). The seeds were planted in pots (15.5 x 10 cm) containing sterilized sand (0.5 mm particle diameter; 1 kg pot^{-1}) as the planting medium for both crops. Sand was chosen as the planting medium which allows maximum gas exchange from the root system during H_2 evolution analysis by using Qubit Systems™, Logger Pro v. 3.2. The plants were maintained under glasshouse conditions for 28 days for soybean and 35 days for rice. The inoculation treatments involved for soybean plants were as follows: T1) +*Bradyrhizobium japonicum* (0.25 g of commercial inoculum), T2) + N (+ 0.5 mM KNO_3, -inoculum) and T3) – N (-nitrogen, -inoculum). For rice plants, four treatments were applied to each pot which included T1) + *Azospirillum brasilense* (Sp7), T2) + Isolate 5 (locally isolated diazotroph), T3) + N (+0.5 mM KNO_3, -inoculum) and T4) -N, -inoculum. The experiment was laid out in a Completely Randomized Design (CRD) with five replications for each treatment. The pots for both crops were randomly arranged in glasshouse and watered daily with nutrient solution (30 ml pot^{-1}, pH 6.8) which consisted of the followings: (a) 257 µmol KH_2PO_4, b) 57 µmol K_2HPO_4, c) 502 µmol K_2SO_4, d) 243 µmol $MgSO_4.7H_2O$, e) 246 µmol $MgCl_2.6H_2O$, f) 748 µmol $CaCl_2.2H_2O$, g) 10 µmol $MnSO_4.H_2O$, h) 1.0 µmol $CuSO_4.5H_2O$, i) 1.0 µmol $ZnSO_4.7H_2O$, j) 31 µmol H_3BO_3, k) 0.5 µmol $Na_2MoO_4.2H_2O$, l) 0.2 µmol $CoSO_4.6.5H_2O$ and m) 38 µmol Fe from Fe- Sequestrine. Peat-based inoculant of *B. japonicum* (strain 532C) while *A. brasilense* (Sp7) and locally isolated diazotroph (isolate 5) were used in the study.

The Peat-based inoculant was provided by Qubit Systems, Kingston, Ontario. While, *A. brasilense* (Sp7) and isolate 5 were cultured in N-free Media and shaken at 160 rpm, room temperature for 3 days (Eskew *et al*., 1977; Enrique, 1985). A total of 0.25 g of peat-based inoculant of *B. japonicum* (strain 532C) was applied to each soybean plants (Goss and de Varennes, 2002). For rice plants a total of 1 ml of *A. brasilense* and Isolate 5 (10^7-10^8 cfu ml^{-1}) were inoculated to each pot with respective inoculation treatments. At harvest (D_{28} for soybean and D_{35} for rice), the H_2 evolution, N_2 fixation and EAC values of the host plants were estimated by using the gas analysis system (Qubit Systems™, Logger Pro v. 3.2). Other observations involved were chlorophyll content (mg chlorophyll content/mg leaf fresh weight) and plant dry weight (Goss and de Varennes 2002).

The Leaf Chlorophyll Content of each plant was recorded using a portable leaf-chlorophyll meter (MINOLTA™ SPAD-502) (Neufeld *et al.,* 2006). The youngest fully expanded leaves of soybean and rice plants were selected to measure chlorophyll content at day harvest. The actual leaf chlorophyll content was determined based on the standard curve of SPAD values and total leaf chlorophyll content (mg chlorophyll mg^{-1} leaf fresh weight) as modified by Amir *et al.* (2001).

The inoculated plants were attached to the gas analysis set up for nitrogenase enzyme analysis and electron allocation coefficient (EAC) (Qubit Systems™, Ontario). Pots with inoculated plants were sealed properly and attached to the gas exchange system. Diazotroph in root system of intact plant would fix N_2 from the air supplied and released H_2. This system provided corrected H_2 evolution values in voltage output data. The N_2 fixation rate was calculated based upon measurement from the rate of H_2 evolution as described by Layzell *et al.* (1984). The data were analyzed statistically *via* one way Analysis of Variance (ANOVA) using SPSS V 12.0 software. The Tukey procedure, $P<0.05$ was chosen to test the significant differences between means of the treatment (Colman and Pulford, 2006). Mean values for growth parameters with the same letter(s) were not statistically significant at the Tukey probability of $P<0.05$.

RESULTS AND DISCUSSION

In nature, N_2 is commonly fixed, reduced to form NH_3 and contribute an important input to agriculture (Dixon and Kahn 2004; Rebah *et al.,* 2007; Jolly *et al.,* 2010; Mia and Shamsuddin, 2010). Nitrogenases enzyme catalyze the reduction of N_2 into NH_3 and evolution of H_2. Since the nitrogenase enzyme can reduce N_2 and H^+, it is necessary to calculate the electron allocation coefficient (EAC) of nitrogenase for both reactions (Hunt, 1997; Giller, 2001; Curtis *et al.,* 2004; Lopez *et al.,* 2008; Schulze *et al.,* 2011). Our experiment recorded N_2 fixation activities of inoculated soybean plants at 141.2 µmol N_2 h^{-1} g^{-1} dry weight nodule (Table 1). The evolution of H_2 of inoculated soybean was also recorded at 144.4 µmol H_2 h^{-1} g^{-1} dry weight nodule (Table 1). The inocula tested (*B. japonicum*) had infected the root cells and initiated formation of 11 effective nodules for soybean plants (Table 1). The total dry weight of the nodules was recorded at 60 mg (Table 1).

A bright red colour was observed inside the root nodules which indicated the presence of O_2-scavenging molecule (leghemoglobin) and proved that the nodules were effectively fixed N_2. The function of this molecule in nodules is to reduce the amount of free O_2, and thereby to protect the nitrogenase enzyme (Lopez *et al.,* 2008). The leghemoglobin function similarly as the O_2-carrying haemoglobin in blood. In addition, the outer and inner cortexes in the nodule also react as an O_2 barrier, which have a mechanism for delivering a high flux of O_2 to optimal concentration. The electron allocation coefficient (EAC) of soybean for N_2 reduction was recorded at 0.982 (98.2 % of electrons were used for ammonia production). While, the electron allocated for H^+ reduction was only 1.8 %. Higher EAC values of inoculated soybean indicated that most of electron were used for N_2 reduction to ammonia (*via* N_2 fixation activities) and not for H_2 evolution (Hunt and Layzell, 1993; Hunt, 1997; Schulze *et al.,* 2011). In nature, N_2 is commonly fixed, reduced to form NH_3 and contribute an important input into agriculture (Dixon and Kahn, 2004; Rebah *et al.,* 2007).

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Table 1: Influence of bacterial inoculation on growth of soybean plants at harvest (D_{28}); H_2 evolution (µmol H_2 h^{-1} g^{-1} dry weight nodule), N_2 fixation (µmol N_2 h^{-1} g^{-1} dry weight nodule), Electron Allocation Coefficient (EAC), number of nodule and nodule dry weight.

Treatment	H_2 evolution	N_2 fixation	EAC	No. of nodule	Nodule Dry weight (mg)
+*B. japonicum*	144.4	141.2	0.982	11	60

Note: None of the observations above was successfully recorded for soybean plants treated with uninoculated treatments: T2) +N (+0.5 mM KNO_3, -inoculum) and T3) -N (- nitrogen, -inoculum).

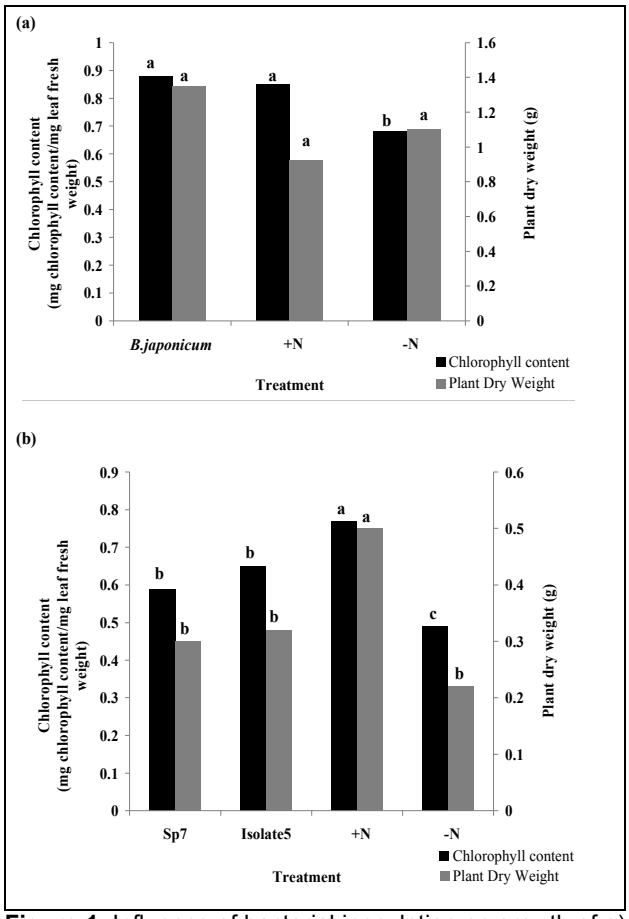

Figure 1: Influence of bacterial inoculation on growth of a) soybean plants at harvest (D_{28}) and b) rice plant growth at D_{35} for chlorophyll content (mg chlorophyll content/mg leaf fresh weight) and plant dry weight (g).
Note: Means with the same letters are not statistically significant at $P<0.05$.

Earlier report by Curtis *et al.* (2004) have shown that N_2 fixation activity of soybean was recorded at 36-38 µmol N_2 h^{-1} g^{-1} dry weight nodule. Earlier reports have shownthat up to 40-60 % of electron flow in nitrogenase was use for nitrogen (ammonia) production in plant (Schubert and Evans, 1976; Feijtel *et al.,* 1985). For inoculated rice plants, none of the observations, which involve N_2 fixation, H_2 evolution and EAC, was successfully recorded. It is true, since in any associative relationships, the dizotroph only colonized the root surface of the host plants and the nitrogenase enzyme were subjected to high exposure of O_2 under the gas analysis set up (Qubit Systems^{TM}). Nevertheless, the diazotroph require microaerophilic conditions for N_2 fixation and takes place at optimal rate of O_2 only between 0.507 and 0.709 kPa (Dobereiner, 1995). This complex nitrogenase enzyme is inactivated when exposed to O_2 (Serraj, 2003; Dixon and Khan, 2004; Ladrera *et al.,* 2007; Fischinger and Schulze, 2010).The chlorophyll contents and plant dry weight of soybean plants inoculated with *B. japonicum* were similar to the control treatments supplied with full nitrogen fertilization (+N). The results clearly proven the potential of inocula tested in fixing N_2 in association with the host plants (**Figure 1a**) (Jolly *et al.,* 2010). Rice plant inoculated with diazotrophic bacteria produced higher plant growth compared to uninoculated plant without N (-N). It proved that these bacteria successfully colonized the roots, fixed the N_2 and later enhanced growth of rice plant (Kannan and Ponmurugan, 2010) even though N_2 fixation activities could not be detected by using the gas analysis system. Nevertheless, higher leaf chlorophyll contents were observed for rice plants inoculated with Sp7 and Isolate 5 compared to the control (- Nitrogen) (Figure 1b). Similarly, plant inoculated with Sp7 and Isolate 5 also showed higher host plant biomass (g) (Figure 1b).

CONCLUSION

Soybean plants inoculated with *B. japonicum* had recorded superior plant growth and higher N_2 fixation activities. Additionally, rice plants inoculated with Sp7 and Isolate5 had shown influenced growth even though no N_2 fixation activities were recorded. In consequence, the results clearly showed that inoculation of diazotrophic bacteria enhanced plant growth similar to that treated with nitrogenous fertilizer.

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