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The Effects of Nitrogen Fixation Activity and Phytohormone Production of Diazotroph in Promoting Growth of Rice Seedlings

F. Keyeo, O. Noor Ai'shah and H.G. Amir
School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

Abstract: The aim of this study was to observe the influence of diazotroph inoculation on growth of paddy plants under greenhouse conditions. Diazotroph are the most studied and well known Plant Growth-Promoting Rhizobacteria (PGPR) that are able to promote plant growth *via* Biological Nitrogen Fixation (BNF) and phytohormone production. A total of four diazotrophic bacteria inocula were used for the experiment which include *Azospirillum brasilense* (Sp7), *Herbaspirillum seropedicae* (Z78), *Enterobacter* sp. (L2) and *Gluconacetobacter* sp. (L15). All four strains were able to fix nitrogen with the same ability but produces phytohormone indole-3-acetic acid (IAA) in different concentration. The inoculation effects were tested on paddy plants (*Oryza sativa*) variety MR220. The rice seedlings were planted in non-sterilized soil and grown under aerobic conditions. Growth of the host plants was measured on parameters such as plants dry weight, plants height, root elongation and total leaf chlorophyll and protein content. Results showed that following inoculation, overall plant growth observation for plants inoculated with L2 and Control positive (+100% N fertilizer) showed better plant growth compared to plants inoculated with L15, Sp7, Z78 and Control negative (+Sp7K). Plants inoculated with Sp7 and Z78 both showed inferior growth compared to Control negative due to excess amount IAA supplied by the inoculants that suppresses the plant growth. Results also showed promising effect of L2 inoculants, to promote rice growth through optimal phtyhormone (IAA) production activity plus the added benefit through successful fixed nitrogen intake by the plant that was provided by L2 *via* BNF.

Key words: Diazotroph, biological N_2 fixation (BNF), optimal phytohormone indole-3-acetic acid (IAA), biofertilizer, paddy (*Oryza sativa*), *Enterobacter* sp.

INTRODUCTION

Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development *via* direct or indirect mechanisms have been defined as Plant Growth Promoting Rhizobacteria (PGPR). PGPR are soil bacteria that are able to colonize the roots or rhizosphere of plants following inoculation onto seedlings or rhizosphere and have beneficial effects whereby it enhanced plant growth (Ashrafuzzaman *et al*., 2009; Zahir *et al*., 2004; Kloepper *et al*., 1986). PGPR mainly can promote plant growth by two different mechanisms, first is by direct mechanisms and second by indirect mechanisms (Verma *et al*., 2010). Direct mechanisms include nitrogen fixation, production of phytohormone, enzymes and mobilization of nutrients (Gray and Smith, 2005; Lucy *et al*., 2004; Khalid *et al*., 2004). Indirect mechanisms include increasing efficiency of fertilizers uptake, increasing the plant's tolerance towards stress, inducing host resistance or producing pathogen-suppressing substances (Van Loon, 2007; Raj *et al*., 2003). These mechanisms can work independently or simultaneously with each other.

One of the most important mechanisms of PGPR in promoting plant growth is through biological nitrogen fixation (BNF) activity. The process changes inert N_2 to useful NH_3 and is only mediated in nature by bacteria and certain species of actinomycetes (Baldani *et al*., 2002). Therefore, there is a strong interest in finding and isolating nitrogen fixing PGPR (also known as diazotrophs) to be utilized as alternative to N fertilizers or microbial inoculants (Woyessa and Assefa, 2011). This is especially true in cultivation of rice. Cultivated rice (*Oryza sativa*) is an important crop, whereby it feeds almost half of world's populations. Therefore, with the utilization of diazotroph as biofertilizer, diazotrophs will provide a major source of nitrogen for plants and lead to less dependency on inorganic nitrogen fertilizer (Sofi and Wani, 2007; Meunchang *et al*., 2004). This will also ultimately lead to environmentally friendly and

Corresponding Author: Amir Hamzah Ahmad Ghazali, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia Tel: 604-653-4008 Fax: 604-656-5125

sustainable agricultural practices (Anthony *et al*., 2009; Kennedy and Tchan, 1992).

Other than directly enhancing plant growth *via* fixation of atmospheric nitrogen, the diazotrophs also affect plant growth through synthesizing phytohormones such as indole-3-acetic acid (IAA) (Malhotra and Srivastava, 2009; Asghar *et al*., 2002; Zimmer and Bothe, 1989). IAA is a type of phytohormone auxin, known to influence a number of plant functions such as promotion of cell elongation and cell division, apical dominance, root initiation, differentiation of vascular tissue, ethylene biosynthesis, mediation of tropistic responses and the alteration of the expression of specific genes (Davies, 2010; Malhotra and Srivastava, 2009; Chasan, 1993; Sachs, 1993). Although IAA have been proven to have stimulatory effect, it is important to note that IAA inhibition has also been reported (Yaqub Chaudhry, 2005). This substance performs optimally at low concentration compared to the nutrients and vitamins that normally affect plant processes. Different bacterial strains produce different concentration of IAA (Yasmin *et al*., 2009). The production and concentration of IAA could also be influenced by other factors besides species or strains of rhizobacteria, such as culture and medium conditions, growth stage and availability of substrates (Frankenberger and Arshad, 1995).

Thus, through this study several diazotrophs are tested on their ability to influence growth of the host plant (rice) through BNF and IAA production activity. This research was aimed to: (1) assess nitrogen fixation capability and phytohormone production of free living diazotrophic bacteria and (2) observe the effects of diazotrophs inoculation on growth of rice plants.

MATERIALS AND METHODS

The study was conducted from July 2009 to May 2010 at School of Biological Sciences, Universiti Sains Malaysia. From the total of four strains examined, two were isolated from leaf tissues of oil palm tree which is *Enterobacter* sp. (L2) and *Gluconacetobacter* sp. (L15) by a previous study in the year 2007. Identification was done *via* molecular identification by sequencing the 16S rRNA gene. The other strains tested were *Azospirillum brasilense* ATCC 29729 (Sp7) and *Herbaspirillum seropedicae* ATCC 870153 (Z78).

Acetylene Reduction Assay (ARA): The assay for nitrogenase enzyme was conducted for both free-living and associated conditions of the diazotrophs. For free living condition, the method of Somasegaran and Hoben (1985) were used. In associated condition, the nitrogen fixation was defined based on ethylene concentration (μmol C_2H_4) and sample fresh weight (g) of the inoculated host plants (μmol C_2H_4 fresh weight g^{-1} h^{-1}) (Azlin *et al*., 2009; Baldani and Baldani, 2005).

Indole Acetic Acid Assay (IAA) : An estimation of IAA was assayed using Salkowski's colorimetric technique for the inocula under free-living conditions (Asghar *et al*., 2002; Patten and Glick, 2002).

Plant growth experiment: A pot experiment was conducted in the greenhouse using soil and sand at 3:1 ratio as the growth medium (1.5 kg^{-1}polythene bag). Before planting, soil was ensured to be moist and wet enough for paddy seed planting. Three-day-old rice seedlings (*Oryza sativa* MR220) were transplanted with one seedling per polythene bag. During the growth period, the plants were maintained under greenhouse conditions and watered twice daily. Complete fertilization of N (urea), P (Triple Super Phosphate) and K (Muriate of Potash) was applied to paddy plant (Ai'shah and Amir, 2005). However, for the plants treated with microbial inoculation, the concentrations of N supplied was reduced to 25% of the total N requirement (1.14 g $seedling^{-1}$) but without any reduction of P and K concentrations. A total of four diazotrophic bacteria inocula were used for the experiment which include *Azospirillum brasilense* ATCC 29729 (Sp7), *Herbaspirillum seropedicae* ATCC 870153 (Z78), *Enterobacter* sp. (L2) and *Gluconacetobacter* sp. (L15). The bacterial culture at the exponential growth stage (ranging from 1.7×10^9 to 2.0×10^9 cfu mL^{-1}) was used as the inoculum (50 mL inocula per plant at D_0 and D_{35}). The inoculation treatments involved in the experiment were as follows: 1) + *Herbaspirillum seropedicae* (Z78), + 25% N; 2) +*Azospirillum brasilense* (Sp7),+25% N; and locally isolated diazotrophs; 3) +*Enterobacter* sp. (L2),+25% N; 4) +*Gluconacetobacter* sp. (L15),+25% N; 5) Positive control (- Inoculation,+100% N) and 6) Negative control (+Sp7 Killed,+25% N). The experiment was laid out in a Completely Randomized Design (CRD) with five replications for each treatment and was harvested after 70 days of growth (D_{70}). At day harvest (D_{70}), the inoculated plants were monitored for plant dry weight (g), root elongation (cm), plant height (cm), N_2 fixation rates *via* Acetylene Reduction Assay (ARA) (μmol C_2H_4 g^{-1} fresh weight h^{-1}) and total leaf chlorophyll and protein content.

Total leaf chlorophyll and protein content: Leaf greenness of each plant was recorded using portable chlorophyll meter (MINOLTA™ SPAD-502) (Neufeld *et al*., 2006). The leaf SPAD values were compared to the standard

curve for actual values of total leaf chlorophyll content (mg chlorophyll mg^{-1} leaf fresh weight) (Azlin *et al*., 2009). Total leaf protein content was determined using Lowry's colorimetric assay (Lowry *et al*., 1951). The actual protein content of each sample was determined based on the protein assay standard curve (mg BSA mL^{-1} protein).

Statistical analysis: The statistical evaluations of data, differences in results between treatments were evaluated using one-way analysis of variance (ANOVA) and multiple group comparison using Tukey-Kramer HSD. All statistical analyses were carried out by using JMP 8.0.2 program at $p<0.05$.

RESULTS AND DISCUSSION

Estimation of acetylene reduction assay and indole-3 acetic acid (IAA) production of free-living bacteria: There are various studies that have been conducted on Plant Growth-Promoting Rhizobacteria (PGPR), since it can be used as a biofertilizer to promote sustainable agricultural practices. As diazotrophic PGPR colonize the plant roots, they are able to promote plant growth based on the ability to fix nitrogen and to excrete plant growth regulator such as indole-3-acetic acid (IAA) (Martinez-Viveros *et al*., 2010; Park *et al*., 2005; Baldani and Baldani, 2005; Ryu *et al*., 2005; Hoque *et al*., 2001). Diazotrophs are mostly known as bacteria that are able to fix atmospheric nitrogen. This ability is one of the reasons for the introduction of diazotrophs to host plants as an effort to reduce inorganic nitrogen fertilizer usage. All of the isolates tested have the ability to fix nitrogen that ranges from 0.2×10^{-09} to 4.1×10^{-09} μmol C_2H_4 cfu h^{-1} with all of the isolates confirmed as N_2 fixers and are able to fix nitrogen with the same capabilities (Fig. 1). The assessment of nitrogen fixation in free-living condition is among the very first step to screen for the possible beneficial bacteria that can successfully transfer the fixed nitrogen to the plant. It acts as the preliminary screening by assessing the acetylene reduction capabilities of the diazotroph (Gough *et al*., 1997). The diazotrophs also produced IAA 0.02 to 0.12 μg mL^{-1} with isolate Z78 as the highest producer (0.12 μg mL^{-1}) followed by Sp7 (0.10 μg mL^{-1}), L2 (0.08 μg mL^{-1}) and lastly L15 which has the lowest IAA production (0.02 μg mL^{-1}) (Fig. 1). The concentration of IAA secreted by each isolate was significantly different from one another. IAA is actually a type of natural auxin that has been studied extensively and it was reported that PGPR were auxin mediated. This type of phytohormone stimulates root growth and elongation and also induces strong modification of lateral roots which are thickened and prolific in root hairs. With stimulated root growth, subsequently nutrients and water uptake will increase, thus promoting the whole plant development (Davies, 2010; Vessey, 2003; Barazani and Friedman, 1999). Positive responses had been garnered from bacterial inoculation of PGPR and this was attributed by phytohormones excreted by bacteria.

Plant growth observation and acetylene reduction assay for associative diazotroph with the host plants: A plant is better able to achieve its optimized physical growth when it receives enough nutrients (e.g., fixed N) and this can be influenced by the presence of diazotrophic bacteria in association with the host plants. Ai'shah *et al*. (2010) and Nguyen *et al*. (2003) both emphasized on reducing chemical fertilizer through application of nitrogen-fixing diazotrophs to the host plants. The diazotrophs provide nutrients and phytohormones to the

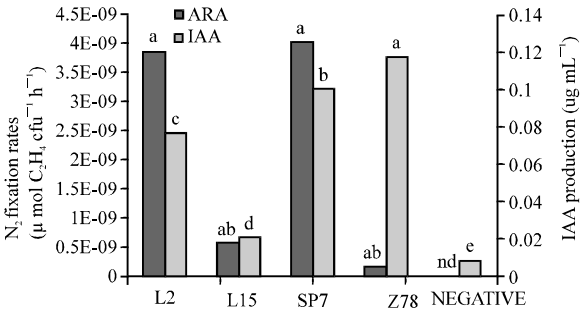

Fig. 1: N_2 fixation activity and phytohormone (indole-3-acetic acid) production potentials of diazotrophs under free living conditions. Means with same letter is not significantly differ at $p<0.05$. nd: Not detected, ARA: Acetylene reduction assay, IAA: Indole acetic acid assay

Table 1: Plant growth observation and nitrogen-fixing capabilities of diazotrophs in association with the host plants after 70 days of growth (D_{70})

Treatments	Plant dry weight	Root elongation	Leaf chlorophyll content	Leaf protein content	N_2 fixation rates (acetylene reduction assy)
Enterobacter sp. (L2)	1.11^{ab}	30.7^{a}	0.12^{a}	1.3×10^{-3a}	0.065^{a}
Gluconacetobacter sp. (L15)	1.12^{ab}	31.3^{a}	0.11^{ab}	1.1×10^{-3ab}	0.069^{a}
Azospirillum brasilensis (Sp7)	0.37^{d}	18.1^{bc}	0.08^{b}	0.8×10^{-3c}	0.031^{ab}
Herbaspirillum seropedicae (Z78)	0.63^{cd}	21.6^{b}	0.09^{b}	0.9×10^{-3bc}	0.040^{ab}
Positive control (+100% N)	1.31^{a}	29.9^{ab}	0.12^{ab}	1.3×10^{-3a}	nd
Negative control(+Sp7 Killed)	0.75^{cd}	24.7^{b}	0.10^{b}	0.9×10^{-3bc}	0.033^{ab}

Means with the same letter is not significantly different at $p<0.05$, nd: Not detected

host plants, a practice which was proven to have significant economic benefit to farmers. Phytohormone production of the bacteria will stimulate root growth, thus increasing the root area available for diazotroph colonization, consequently increases the probability of the plant in getting fixed nitrogen. The beneficial effects of Plant Growth Promoting Rhizobacteria (PGPR) have been attributed to both BNF (Mia and Shamsuddin, 2010; Meunchang *et al*., 2004; Boddey, 1995) and production of phytohormones that promote root development and proliferation, thus resulting in more efficient uptake of water and nutrients (Ashrafuzzaman *et al*., 2009; Jacoud *et al*., 1999). The results have shown that inoculation of L15 and L2 significantly promoted higher plant dry weight (1.12 and 1.11 g consecutively) compared to Sp7 and Z78 (0.37 and 0.63 g consecutively) (Table 1). Response of the inoculation treatments (L2 and L15) was similar to the control plants with complete N fertilization (+100%N) that have the highest plant dry weight. The results can be associated with the success of the diazotrophic bacteria in colonizing the roots, promoting root development, *via* phytohormone IAA secretion, added with the abilities of the isolates (L2 and L15) in fixing atmospheric nitrogen and subsequently increasing the uptake of nutrients by the plants. Watanabe *et al*. (1987) and Zhu (1989), suggested that BNF process could provide significant amount of nutrient (up to 20-25% of N) needed by paddy plant. The results also showed that plants inoculated with L2 and L15 had the highest roots elongation amongst all of the treatments. The response is proportional to IAA concentration, but overproduction may affect growth stimulation in plants (Davies, 2010; Malhotra and Srivastava, 2006, 2008a, b; Patten and Glick, 1996). The phytohormones enhance cell division and root elongation of host plants; however, it will only benefit the host when produced in a minimal amount. Inoculation of Sp7 and Z78 showed the lowest root elongation and growth due to higher levels of IAA produced that incapacitate its roots development. Excess amount of IAA produced by both Sp7 and Z78 had interfered with the root development, thus slowing the growth of the plant (Table 1). Sp7 and Z78 also recorded the lowest for leaf chlorophyll and protein content similar with the results of root elongation. Both isolates Sp7 and Z78 in IAA free-living assessment have the highest IAA production. However, the root development was suppressed by the over-production of IAA. When the root is suppressed, there is less colonization site for the attachments of diazotroph. It is known that the effectiveness of PGPR in the field has often been attributed to their ability to colonize plant roots. It is reasonable to assume that PGPR must colonize the rhizosphere of the host plant to be most beneficial (Malhotra and Srivastava, 2009; De Weger *et al*., 1995). If colonization failed to occur then the beneficial effect of diazotroph such as providing fixed nitrogen *via* BNF cannot be exerted to plants. With this, the intake of water, minerals and nutrients from the soil become slow, thus affecting the overall growth of plants. So, even if the diazotrophic bacteria readily fix nitrogen in the vicinity of the rhizosphere, since there is a deficiency in attachment site for colonization, the plants ability to absorb nutrients and grow to its optimal level is incapacitated. Minimal concentration of IAA produced by L2 (0.08 μg mL^{-1}) and L15 (0.02 μg mL^{-1}) could be the most suitable amount of IAA to improve root growth of paddy plants. Introduction of suitable amount of IAA to the host plant stimulates root development through increasing root length and density, enhanced nutrient and water absorption and thus promoted good plant growth (Baca and Elmerich, 2007). It was reported that plant root growth will be enhanced when auxin is at its optimal level which is in low concentration of 10^{-7} and 10^{-13} M and any higher than that it will inhibit root development (Fassler *et al*., 2010; Gaspar *et al*., 2002).

Well developed root systems and longer roots can influence better growth of paddy plants. The diazotrophs supposedly will be able to increase root growth and function and this will increase yield of crops (De-Bashan *et al*., 2008; Kennedy *et al*., 2004). The plants inoculated with L2 showed higher values for leaf chlorophyll and protein content (highest amongst the 4 strains – L2, L15, Sp7 and Z78) which is as good as the fully fertilized treatment of Control Positive (+100%N). This can be related to the optimal concentration of IAA secreted by isolate L2 that managed to develop extensive root system of the plant and with the addition of N_2 fixation of the isolate that provide fixed nitrogen for plants excellent growth (Table 1). All of this is indicates that L2 can successfully promote plant growth even with minimal N fertilizer (25%). This is in accordance to the study by Yasmin *et al*. (2007) that PGPR can be used as biofertilizer for plants at reduced rate of N fertilization.

CONCLUSION

In overall, the inoculation of paddy plants with diazotrophic bacteria, such as *Enterobacter* sp. (L2) may increase plant dry weight, chlorophyll content, root elongation and protein content of the host plants. Combination of both N_2 fixation activity and optimal phytohormones production of L2 may increase nutrient uptake of the plant itself, making it thrive compared to other isolates tested. However, it is acknowledged that the results cannot be explained only through nitrogen

fixation and the production of phytohormone IAA. Further understanding of the potential value of these bacteria as biofertilizers, especially L2, would help in the studies conducted towards future studies on sustainable agriculture practices for paddy plants. However, in order to reach the maximum potential of the diazotrophs as biofertilizers, strategies to fully manipulate the bacterial factors should continue to be emphasized.

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