Growth and biochemical profiling of artificially associated micropropagated oil palm plantlets with *Herbaspirillum seropedicae*

Shey-Li Lim*, Sreeramanan Subramaniam, Ishak Zamzuri and Hamzah Ghazali Amir

*School of Biological Sciences, Universiti Sains Malaysia, Minden, Malaysia; Advanced Biotechnology and Breeding Centre, Malaysian Palm Oil Board, Kajang, Malaysia*

**ABSTRACT**

The challenges of various biotic and abiotic stresses can imperil the growth of micropropagated plantlets either direct or indirectly. Hence, in this study, a mutual relationship was established between diazotrophs and micropropagated plantlets to enhance plant growth and development. Artificial symbiosis was created for different inoculums of *Herbaspirillum seropedicae* (Z78), namely sonicated cells, broth culture, and pellet cells with micropropagated oil palm plantlets *Elaeis guineensis* Jacq. Results reveal significant differences on root volume, total protein content, and Brix value for Z78 broth culture treatment compared with plantlets treated with 25% N. High nitrogenase enzyme activities (6.7 × 10^{-4} µmol C2H4 g^{-1} h^{-1}) and indole-3-acetic acid production (205.21 µmol (g FW)^{-1}) were also detected on roots of plantlets treated with Z78 broth culture. These beneficial traits reviewed that the application of diazotrophs (Z78) in associative manner for micropropagated plantlets hold vast potential for promoting plant growth and plant’s healthiness.

**Introduction**

Nitrogen is an essential nutrient taken up by plants for growth. In this huge ecosystem, limited natural N supplied in the soil could restrict plant yield and prevent healthy growth particularly for micropropagated plantlets. The plantlets are naturally more fragile compared to conventional breeding plant materials. In addition, roots of plants produced in vitro are very frangible and therefore could lead to inadequate absorption of water and nutrients from the soil and significantly caused unhealthy plant (Vidal et al. 2003; Sumaryono and Riyadi 2011). One of the alternatives to promote better plant growth can be achieved by the application of diazotrophic rhizobacteria, which is also known as plant growth enhancer (Vestberg et al. 2004). A current interest in agricultural field is to simulate the associations of selected diazotrophs with micropropagated plants to improve plant growth, as well as to minimize the need of chemical fertilizers. Apparently, plant-growth-promoting rhizobacteria is an option to increase nitrogen availability, without causing any pathogenicity, simultaneously reducing the environmental biotic and abiotic stress. With the inoculation of plant-growth-promoting rhizobacteria into rhizosphere system, it was able to optimize the functions of diazotrophs to enhance plant growth and development (Amir et al. 2001; Azlin et al. 2007; Al’shah et al. 2009; Al’shah et al. 2013).

*Herbaspirillum seropedicae* strain Z78 is a nitrogen-fixing and phytohormone-producing bacterium which has great potential to form symbiotic association with a wide range of monocotyledon plants (Al’shah et al. 2009; Keyeo et al. 2011; Taule et al. 2012; Lim et al. 2016). It benefits the hosts with nitrogen in the form of ammonium. However, not many studies were conducted to further understand the benefits and interactions of this particular diazotrophic bacteria with the host plant. In most cases, re-introduction of isolated endophytic bacteria onto its host plant can eventually lead to vast improvements of plant growth and yield (Janarthine and Eganathan 2012). Thus this suggested that *H. seropedicae* has great potential to act as plant-growth-promoting rhizobacteria (inoculant) in oil palm. *H. seropedicae* is also a plant endophytic diazotroph capable of colonizing roots, stems, and leaves of its hosts without causing disease symptoms (Chubatsu et al. 2012). According to Lim et al. (2016), Z78 has the ability to fix nitrogen (N₂) biologically and produce phytohormone indole-3-acetic acid (IAA) to promote proliferation of callus and embryogenic callus of oil palm. In most cases, bacteria with the ability to synthesize phytohormone can induce better morphological and physiological changes to the inoculated plant especially in increasing root surface area and formation of primary and secondary roots (Pedraza 2008; Malhotra and Srivastava 2009). Larger branches of plant roots are able to form during the association, thus providing larger root surface areas for more nutrient uptakes (Vessey 2003).

In general, the diazotroph is better off in expressing their nitrogen fixation potential inside plant tissues due to the protection against high levels of oxygen present in the environment (Boddey et al. 1995; Baldani et al. 2000). During the fixation process, nitrogen from the atmosphere will be reduced to ammonia (NH₃) and ammonium (NH₄⁺) by involving specific enzymes and adenosine triphosphate (ATP) (Azam and Farooq 2003). Then, ammonium will be reduced to nitrate through the process of nitrification by rhizobacteria. Subsequently, plants can utilize the nitrogen source and show intense growth. On the other hand, the cost of conventional nitrogen fertilizers and plant growth regulators to be used in plantations is high. Biofertilizer and bioenhancer are an alternative in replacing conventional fertilizers in the oil palm cultivation (Vestberg et al. 2004). Keeping in view with the present literature, artificial symbiosis of diazotrophs...
with micropropagated plantlet seems to be one of the most appropriate approaches for mass propagation of the desired plant. For that reason, the objectives of this study were: (1) to observe the effects of various inoculums conditions of *H. seropedicae* (Z78) on growth, nutrient content, and biochemical profiling of micropropagated oil palm plantlets and (2) to observe the ability of *H. seropedicae* (Z78) to fix N\textsubscript{2} and produce phytohormones (IAA) in association with the plantlets.

**Materials and methods**

**Plant materials**

Oil palm plantlets (Clone P614D) at 1 month old were obtained from Advanced Biotechnology & Breeding Centre (ABBC), Malaysian Palm Oil Board (MPOB), Bangi, Selangor, Malaysia. The plantlets were grown in polybags (22 cm × 15.5 cm) containing 1 kg of sieved planting medium (3:1, soil: sand) from day 1 to day 125. After 4 months of growth, the plantlets were transplanted into larger polybags (34.5 cm × 31.6 cm) containing 8 kg of same composition planting medium. The plantlets were watered twice a day and maintained under a standard glasshouse for another 6 months with natural light and natural fluctuations of temperature and humidity. Plantlets were treated with the following treatments: T\textsubscript{1}, 100% (w/v) N (positive control), T\textsubscript{2}, 25% (w/v) N (negative control), T\textsubscript{3}, Z78 sonicated cells + 25% (w/v) N, T\textsubscript{4}, Z78 broth culture + 25% (w/v) N, T\textsubscript{5}, Z78 pellet cells + 25% (w/v) N. All treatments were supplied with 100% potassium and phosphorus. During the first 2 months of cultivation, liquid fertilizer solution was applied with 10 mL per polybag and nutrient solution was applied with 25% (w/v) N, while (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was reduced to 1.24 mg mL\textsuperscript{−1} of KH\textsubscript{2}PO\textsubscript{4} and 6.09 mg mL\textsuperscript{−1} of KH\textsubscript{2}PO\textsubscript{4} were used as 100% (w/v) NPK, while (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was reduced to 1.24 mg mL\textsuperscript{−1} for 25% (w/v) N). In the following month, complete fertilization was applied with N (urea), P (Triple Super Phosphate), and K (Muriate of Potash) based on the recommendation fertilizer rate requirement of oil palm (Azlin et al. 2009). The amount of fertilizers supplied were equivalent to 7 g per 100 palm of commercial fertilizer (NPK yellow) 15:15:6:4 from day 30 to day 60, 7 g per palm of NPK yellow 15:15:6:4 from day 90 to day 210, and 14 g per palm of NPK yellow 15:15:6:4 from day 240 to day 300.

**Bacterial culture preparation**

*Herbaspirillum seropedicae* strain Z78 (ATCC 35893) was cultured and prepared as the bacterial inoculum for inoculation. This strain was cultured in N-limited broth (100 mL per flask) at room temperature for 48 h in shaker with 180 rpm (Okon et al. 1977). Three different bacterial inoculums were prepared: (a) Z78 sonicated cells, (b) Z78 broth culture inoculum (directly from the bacterial growth medium), and (c) Z78 pellet cells. For Z78 sonicated cells preparation, bacterial cultures were centrifuged at 10,000 rpm for 10 min. Pellet was suspended in sterile distilled water and then re-centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and pellet was resuspended with sterile distilled water and put in the sonicator (JAC Ultrasonic 180 rpm, Korea). The working frequency and power were fixed at 40 kHz and 200 W, with high ultrasonic output variability and sonicated for 30 min prior to inoculation. A total of 10 mL of inoculum was inoculated to each plantlet at monthly intervals from day 0 until day 125. The inoculum size was increased to 20 mL per plantlet starting from day 125 to day 300 of growth. Each inoculum was determined for colony-forming unit in the range of 10\textsuperscript{7} to 10\textsuperscript{8} and optical density (OD\textsubscript{600nm}) using a spectrophotometer (Lambda Bio, Perkin Elmer, USA).

**Nutrient uptake analyses**

At the day of harvest (day 300), total nitrogen (N), organic carbon (C), phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) contents of micropropagated oil palm plantlets were analyzed at Felda Agricultural Services Sdn Bhd (Bandar Jengka, Pahang, Malaysia) by using inductive coupled plasma (Varian Vista MPX ICP – OES) and segmented flow analyzer (Skalar Auto Analyzer San).

**Observation on plant growth phenotype**

The measurement of plant height and stem diameter was performed using measuring tape and caliper before harvest. The number of primary root was counted manually. For actual root volume displacement, the roots of harvested plantlets were detached from the shoot, rinsed with distilled water and dried. Next, the root was placed and fully submerged into the 1 L cylinder filled with 600 mL of distilled water. Initial and final water levels were recorded and the differences were recorded as root volume (Novoselov 1960). The detached shoots and roots were then placed into an oven to heat dry for 48 h until fully dried before the dry weight was measured.

**Indole-3-Acetic Acid (IAA) production**

Quantitative IAA assay for samples of treated oil palm plantlets was conducted using an ultra-performance liquid chromatography (UPLC) system. The phytohormone IAA was extracted using an extraction procedure according to the modified QuEChERS method (Flores et al. 2011; Lim et al. 2016). A total of 100 mg of liquid nitrogen homogenized samples were weighed in a microcentrifuge tube and 1 mL of 1% (v/v) acetic acid in acetonitrile was added. The mixture was vortexed for 1 min. Subsequently, 0.4 mL of 10% (w/v) anhydride magnesium sulfate, 0.1 mL of 10% (w/v) sodium chloride, 0.1 mL of 10% (w/v) sodium citrate monobasic anhydride, and 0.1 mL of 2.25% (w/v) disodium citrate were added into the microcentrifuge tube and samples were agitated for 1 min on a vortex mixer. The samples were incubated at 4°C overnight. On the next day, samples were centrifuged at 4500 rpm for 5 min. Then supernatant was filtered by PTFE membrane filter and stored in 2 mL brown micro vial before analysis. Filtrates were injected into Acquity UPLC system equipped with PDA Chi 280 nm @ 1.2 nm detector and C-18 UPLC column, with Acquity UPLC @ BEH (181.7 µm, 2.1 × 100 mm) at ambient temperature. The buffer solvents used were as follows: 1% (v/v) acetic acid as solvent A and 100% (v/v) acetonitrile as solvent B. The elution gradient was adjusted to 2 min of 90% solvent A and 10% solvent B. The run time was set in 2 min at a flow rate of 0.25 mL min\textsuperscript{−1} with wavelength of 280 nm. The quantitative IAA concentration was determined based on the IAA standard curve value.
**Estimation of nitrogenase enzyme activity**

Acetylene reduction assay was used for the nitrogenase enzyme activity determination of harvested plantlets (Hardy et al. 1968). The root samples were weighed and incubated in the dark at room temperature for 24 h in an airtight leonard jar (1 L) aseptically. A total of 5% air from each jar was removed from the headspace and replaced with purified acetylene gas (99.8%) and incubated 24 h for reduction of acetylene to ethylene. After 24 h of incubation, a total of 5 mL gas mixture was sampled and transferred into vacuum tube (Vacutainer™ 6 mL). The assay was conducted by injecting 1 mL of gas mixture into GC-2014 Shimadzu gas chromatography fitted with Supercox carbon box 1004 stainless steel micropacked column, 2 m × 0.76 mm ID and equipped with flame ionization detector (FID). Nitrogen was used as the carrier gas at flow rate of 30 mL min⁻¹, with the temperature maintained at 80°C (column) and 180°C (injector and detector).

**Leaf chlorophyll content**

Leaf greenness of each plantlet was recorded using a portable leaf-chlorophyll meter (MINOLTA™ SPAD-502) (Neufeld et al. 2006). The youngest and fully expanded leaves were selected and marked to measure its chlorophyll content. The quantitative leaf chlorophyll content was determined based on the standard curve of SPAD values and total leaf chlorophyll content of the plantlet (µmol chlorophyll per mg leaf fresh weight) (Coombs et al. 1985, Amir et al. 2001, Ai’shah et al. 2009).

**Leaf protein content**

Total leaf protein content was determined using Bradford’s colorimetric assay (Bradford 1976). A total of 100 mg homogenized leaf sample was washed three times in hexane and acetone for depigmentation. Then samples were immersed in 0.5 mL of 0.1 N NaOH and incubated at 4°C overnight. After the incubation, a total of 0.25 mL aliquots of NaOH were added, followed by 1 h of incubation. This process was carried out twice. Next, a total of 1 mL of 10% (v/v) trichloroacetic acid was added to the samples. The sample was centrifuged at 13,000 rpm for 10 min for protein precipitation. Subsequently, the supernatant was discarded and the protein samples were resuspended in 0.5 mL of 0.1 N NaOH. The quantification of protein content was performed according to the method described by Bradford (1976). A total volume of 100 µL sample was pipetted into the microcentrifuge tube, to which 700 µL of deionized water and 200 µL of 20% (v/v) Bradford solution were added. After 5 min of dark incubation, absorbance readings were taken in a spectrophotometer (Lambda Bio +, Perkin Elmer, USA) at 595 nm. Protein content of each sample was determined based on the protein assay standard curve (mg BSA (mL protein)⁻¹).

**Brix value—total soluble solid (TSS)**

Total soluble solid (Brix value) was measured using a hand refractometer. Brix is the summation of the grams of sucrose, glucose, fructose, vitamins, minerals, amino acids, proteins, hormones, and other soluble solids over 100 g of the particular sample (Echeverria and Ismail 1990). A total of 1 g of the most mature leaves was chosen and homogenized using a mortar and pestle. The sap from the leaves was taken and measured by a hand refractometer (PAL-1, Atago, Tokyo Tech). Distilled water was used to blank the refractometer and the tested result was recorded.

**Statistical analysis**

The experiment was arranged in a completely randomized design (CRD). The experiment involved five treatments consisting of five replicates. The collected data were analyzed using analysis of variance (ANOVA) and significant differences among the treatments were compared based on Tukey’s HSD at P < .05 using SPSS. Pearson correlation coefficient was also conducted for all the morphological and biochemical parameters at P < .01 using SPSS (Version 20, SPSS).

**Results and discussion**

**Growth performances of treated micropropagated oil palm plantlets at day 300**

From a more practical standpoint, study of plants’ physical parameters is always essential and often can provide key insights into the plant growth. In this study, the interactions of micropropagated oil palm plantlets and Z78 bacterial cells were observed for its physical parameters of plant growth at day 300 (D300). The results of shoot dry weight and number of leaf fronds at D300 were illustrated in Figure 1(a). A significant difference was observed for inoculated micropropagated oil palm plantlets treated with Z78 broth culture (T4), Z78 pellet cells (T5) in comparison to noninoculated plantlets supplied with limited N (T2). However, no significant responses were detected among the treatments with respect to the number of leaf frond formation. The highest shoot dry weight was recorded for micropropagated oil palm plantlets inoculated with 100% (w/v) N (T1), followed by Z78 broth culture (T4), Z78 pellet cells (T5), Z78 sonicated cells (T3), and 25% (w/v) N (T2) with significant observation in gram (g) of 27.80, 24.38, 22.93, 19.85, and 14.60, respectively (Figure 1a). While the highest root dry weight in gram (g) was recorded for 100% (w/v) N (T1), Z78 broth culture (T4), Z78 sonicated cells (T3), followed by Z78 pellet cell (T5) and 25% (w/v) N supplied (T2) (Figure 1b). Additionally, a number of primary roots obtained for inoculated plantlets (T3, T4) were significantly higher than primary root formation of noninoculated plantlets (T2). Through these presented data, H. seropedicae has demonstrated the ability to enhance plant growth.

It was also observed that the root volume of plantlets treated with Z78 broth culture (T4) showed significantly higher volume than plantlets supplied with limited N (T2) (Figure 1c). In most cases, the inoculation of plant-growth-promoting rhizobacteria can stimulate root growth and development (Mia et al. 2010). Higher root density and area provide the plant with better uptake of nutrients and water (Vessey 2003). Additionally, diazotrophic bacteria have some nutritional advantages on the plant relative to other bacteria in the soil (Vessey 2003). The primary root formation was also significantly positively correlated with plant height, stem diameter, and root volume with an r value of 0.702, 0.748, and 0.805, respectively. The plant height accompanied with bigger stem diameter was also observed for inoculated plantlets. All of the inoculated plantlets were significantly
higher in plant height and stem diameter than oil palm plantlets fertilized with 25% (w/v) N (T2) (Figure 1d). From the aforementioned data, a better inoculation effect for nutrient accumulation and physical parameter solely on plantlets treated with Z78 broth culture (T4) was observed in comparison to plantlets treated with limited N supplied (T2).

Our results reveal that the Z78 inoculant can promote better plant growth compared to non-inoculated plantlets treated with limited N (T2). Similar result was also observed by Gosal et al. (2010), biotization of micropropagated Chlorophytum sp. with the Pseudomonas fluorescens improved plantlet survival rate, growth parameters, field performance, protein contents, and micronutrient acquisition. It is also believed that Z78 has the ability to colonize the plant root system. In the previous report of Roncato-Maccari et al. (2003), H. seropedicae was used to inoculate maize, sorghum, and wheat, and bacteria were able to re-isolate from the surface-sterilized tissues. The inoculated H. seropedicae bacterial cell was detected in high amounts from inner roots, stems, and leaves of maize, sorghum, and wheat, which eventually indicated an internal colonization (Roncato-Maccari et al. 2003). Mainly, a key feature of all plant-growth-promoting rhizobacteria is to create a symbiont environment that is efficient for colonization. In general, H. seropedicae interacts with plant hosts by a specific chemotaxis system and use type IV pili and lipopolysaccharide to attach to the root surface (Balsanelli et al. 2015). According to Botta et al. (2013), through GUS-staining analysis, H. seropedicae was proved as an endophyte; it has the ability to colonize the stems, leaves, and inner roots via lateral root emergence sites and root tips.

Figure 1. Effects of H. seropedicae (Z78) on micropropagated oil palm plantlets after 300 days of growth. (a) Shoot dry weight and number of leaves fronds, (b) root dry weight and number of primary root, (c) root volume, (d) plants height and stem diameter.

Note: T3, T4 and T5 were also supplied with 25% (w/v) N and 10% (w/v) PK. Means followed by the different letter in a line indicate significant differences among treatments and analyzed separately by Tukey’s HSD at P < .05.

Table 1. Effects of Herbaspirillum seropedicae (Z78) on treated micropropagated oil palm plantlets’ nutrient uptake and soil pH at day 300.

| Treatments                  | Nutrient uptake (% on dry matter) |
|-----------------------------|-----------------------------------|
|                            | Nitrogen (N) | Phosphorus (P) | Potassium (K) | Calcium (Ca) | Magnesium (Mg) |
| T1: 100% (w/v) N           | 2.202*       | 0.150*         | 1.464*        | 0.581bc      | 0.116*         |
| T2: 25% (w/v) N + 100% (w/v) PK | 2.256*       | 0.151*         | 1.495*        | 0.545*       | 0.164bc        |
| T3: Z78 sonicated cells    | 2.200*       | 0.150*         | 1.535*        | 0.629*       | 0.128bc        |
| T4: Z78 broth culture      | 2.282ab      | 0.149*         | 1.682b        | 0.560bc      | 0.159bc        |
| T5: Z78 pellet cells       | 2.376bc      | 0.153*         | 1.514*        | 0.623bc      | 0.178c         |

T1 = treatment 1; T2 = treatment 2; T3 = treatment 3; T4 = treatment 4 and T5 = treatment 5.
Note: T3, T4, and T5 were also supplied with 25% (w/v) N and 10% (w/v) PK. Means followed by the different letter in a line indicate significant differences among treatment and analyzed separately by Tukey’s HSD at P < .05.
on *Lycopersicon esculentum* plantlets. Through the coloniza-
tion, it can increase nutrient uptake and mineral nutrition 
that directly contribute to the development of the plant 
growth (Aï’shah et al. 2009). On the basis of the aforemen-
tioned data, treated plantlets have shown better plant-
growth-promoting traits compared to plantlets supplied 
with only 25% N (T2). It is believed that *H. seropedicae*
have successfully colonized the inner part of plant root and 
eventually increase plant nutrient uptake.

**Soil analysis and effects of different inoculums on the 
plant’s nutrient accumulations (N, P, K, Ca, and Mg)**

Plant nutrient accumulations were recorded after 300 days of 
growth (Table 1). Inoculated plantlets had significantly accumu-
lated high nitrogen (N). The inoculated plantlets treated 
with Z78 pellet cells (T5) accumulated significantly higher 
N in the fronds compared to the treatments with 100% (w/
v) N (T1), 25% (w/v) N (T2), and Z78 sonicated cells (T3). 
The results of N accumulation showed positive effects of 
the interaction for inoculated plantlets (T5) in comparison 
ononinoculated plantlets (T1, T2). It is presumed that the 
significant N accumulation in the shoot of plantlets was 
due to the N₂ fixing activities of the Z78 inoculums. Our find-
ing was supported by the work of Wu et al. (2005), who elu-
cidated the positive effects of biofertilizers consisting of N 
fixer *Azotobacter chroococcum* on the growth of *Zea mays*.
Through that finding, microbial inoculant has the ability to 
increase the growth and nutritional assimilation (total N) of 
maize and at the same time also improved soil properties. 
Also, Shaharoono et al. (2008) reported that pot and field 
experiments of wheat with the inoculation of *P. fluorescens* 
(strains ACC50 and ACC73) showed the efficiency of N 
increment after 160 days of cultivation.

However, no significant interaction was detected between 
the inoculated and noninoculated plantlets when assessed for 
phosphorus (P) accumulation. It is presumed that this par-
ticular *Herbaspirillum* strain is not a phosphate solubilizing 
strain; thus, no phosphorus interaction can be observed 
throughout this study. Similar results were also obtained by 
Remans et al. (2007), whereby total P content in plant 
was not affected by co-inoculation of *Rhizobium* and com-
mon bean (*Phaseolus vulgaris*). However, the inoculation of 
Z78 broth culture showed statistically significant high potassium (K) accumulation in the plant tissues. The total K content 
was found to be positively correlated with the accumulation of 
IAA in plantlets’ roots with an r value of 0.601. It is 
known that IAA-producing bacteria has the ability to 
enhance root structures and increase plant nutrient uptake 
(Khalid et al. 2005). It has therefore been assumed that in 
the presence of Z78 broth, the treated plantlet can uptake 
accessible soluble form of K from soil. Highest calcium (Ca) 
accumulation was recorded for the plantlets treated with 
Z78 sonicated cells + 25% (w/v) N (T3) (Table 1). In addition, 
a significantly higher concentration of magnesium (Mg) 
accumulation was shown in the treatments of Z78 pellet cells 
+ 25% (w/v) N (T5) in comparison with noninoculated plant-
lets (T1) (Table 1). Practically, Mg is crucial in assimilate 
translocation from source to sink organs and partitioning 
among plant parts, which may eventually promote root 
growth and contribute for N uptake (Senbayram et al. 2016). According to Grzebiwicz (2013), Mg is one of the basic 
nutrient to induce nitrogen uptake in plant. High N uptake 
in plantlet treated with Z78 pellet cell (T5) may also, at 
least in part, explain why high Mg content of the same treat-
ment plantlet was observed in this study.

**Biochemical responses of treated micropropagated oil 
(palm plantlets at day 300)**

In this study, the ability of Z78 in producing IAA was 
observed during the symbiotic relationship by quantifying 
the concentration of IAA in shoots and roots of the plantlets. 
A distinctive result on IAA accumulations was observed for 
the shoots and roots. As illustrated in Figure 2(a), overall, 
leaves from plantlets obtained higher value of IAA concen-
tration compared with the roots of plantlets. The inoculation 
of Z78 broth culture supplemented with 25% (w/v) N (T4) 
showed significantly high level of IAA components pro-
duction in the root part with 205.21 μmol (g FW)⁻¹ compared 
with noninoculated plantlets (Figure 2a). With this 
finding, we propose that Z78 broth culture is the potential 
treatment for the micropropagation of oil palm plantlets. 
In general, IAA is a principal auxin in plant that contributes 
to polar transport, which is usually synthesized in meriste-
matic regions at the shoot apex and transported to the root 
tips. More importantly, in our result, the detection of signifi-
cantly high level of IAA in the roots of plantlets inoculated 
with Z78 broth culture also directly contributed to high 
root volume, number of primary roots, and high protein con-
tent. The roots are always important in providing adequate 
nutrient absorption and support for the plant. The presented 
result was relevant and supported by Khalid et al. (2005), 
whereby the biosynthesis of IAA by various plant-growth-
promoting bacteria has been demonstrated to enhance root 
proliferation and elongation. Thus it is believed that IAA pro-
duced by bacterial cells can eventually enhance root growth of 
oil palm plantlets. According to Davies (1995), the localized 
accumulation of auxin in epidermal cells of the root initiates 
the formation of lateral or secondary roots and release of sac-
charides from the plant cell wall during the elongation step. 
Plants usually grow one or more primary roots from which 
lateral roots emerge by division of specific pericycle cells, 
whereas lateral and adventitious roots are induced by high 
concentrations of exogenous IAA (Patten and Glick 2002).
In addition, IAA production in the root zone is most likely 
controlled by the genetic and physiological properties of the 
microorganism and plant (Khalid et al. 2005).

The diazotrophs’ stimulatory effect comes from a manipu-
lation of the complex and balanced network of plant hor-
mones that are directly involved in growth or stimulation of 
the root formation. Plantlets treated with Z78 pellet 
cells (T5) had the highest accumulation of IAA (318.12 μmol (g FW)⁻¹) in the leaves compared to other 
treatments. In general, auxin is unique among plant hor-
mones in being actively and directionally transported from 
young apical parts. It is mainly produced in apical meristems 
and move cell to cell in polar gradient (Davies 1995). IAA is 
also an important phytohormone in the initial processes of 
lateral and adventitious root formation and shoot elongation 
(Parray et al. 2015). As reported by Soto et al. (2006), this 
particular plant hormone has no apparent function in the bac-
terial cells, but it is essential in plant–microbes interaction. 
Based on Van Loon (2007), auxin synthesis and translocation 
is often stimulated by endophytic microorganisms. Hence, it 
is firmly believed that Z78 has the ability to synthesize IAA.
and enhance plant growth. Our study was also supported by Ai’shah et al. (2013), Tan et al. (2015), and Lim et al. (2016), wherein H. seropedicae strain Z78 can produce IAA phytohormone in an associative manner and under free living conditions. The phytohormone (IAA) is important for plant-microbe interactions which is part of bacterial colonization strategy, including phytostimulation and basal plant defense mechanisms. Moreover, it has been indicated that IAA can also be a bacterial signaling molecule and therefore can have a direct effect on bacterial physiology (Spaepen et al. 2007). It is also known that bacterial IAA can loosen plant cell walls, thus promote an increasing amount of root exudation that provides additional nutrients to support the growth of bacteria (James et al. 2002; Chi et al. 2005). According to Patten and Glick (2002), seed inoculation with wild-type Pseudomonas putida induced the formation of canola roots with 35–50% longer than the roots from the seed treated with IAA-deficient mutant and the noninoculated seed.

Additionally, nitrogenase enzyme activity was also analyzed for the roots of micropropagated oil palm plantlets at day 300 immediately after harvest while leaf chlorophyll content was recorded in day 300 before harvest. The mechanism triggered by Z78 is associated not only with IAA production but also with the ability to fix atmospheric N. However, in most cases, diazotrophic bacteria cannot completely substitute for inorganic N through biological nitrogen fixation; limited inorganic nitrogen sources still need to provide during the plantation as a starter for plant growth. Previous study has reported that diazotrophic rhizobacteria supplied with 25% limited N fertilizer had promoted better growth of the host plants (Ai’shah et al. 2009). Due to this consideration, in this experiment, all of the inoculant treatments were supplied with 25% N fertilizer as an N starter for plant growth before the inoculum applied (Z78) begins to perform nitrogenase enzyme activities. As illustrated in Figure 2(b), the interactions of inoculated plantlets with Z78 bacterial cells (T3, T4, T5) and noninoculated plantlets with limited N source supplied (T2) were statistically significant with respect to nitrogenase enzyme activities but no significant difference was determined for total chlorophyll content. The higher chlorophyll content was obtained for micropropagated oil palm plantlets treated with 100% (w/v) N (T1), followed by other treatments involved.

It is presumed that the release of proteins and organelles via sonication may provide additional nutrients to support the growth of plantlets due to the detection of nitrogenase enzyme activities in inoculated plantlets with Z78 sonicated cells (T3). According to Harrison (1991) and Peternal (2013), the disruption of bacterial cell can cause the release of proteins and intracellular recombination products. With
this, we speculate that the release of certain recombinant product from sonicated cells allowed treated plantlets to secrete more root exudate. Kandel et al. (2017) also highlighted that root exudate, including organic acids, amino acids, and proteins, can profoundly modify soil microbial community, particularly to initiate early communication between host plants and bacterial cell, recruit bacterial endophytes from the rhizosphere, and steer the colonization process. It is widely known that the population of bacteria is extensively distributed in the vicinity of plant root or the rhizosphere zone. Roots secrete an enormous range of compounds especially exudate into the surrounding soil. According to Badri and Vivanco (2009), although every plant produces exudates, the amount and composition of root exudates regulated are determined by plant species and external environmental factors. From a conceptual point of view, sonicated cells release components that likely have a direct influence on exudation. Hence, increment population of diazotroph species can be expected in the treated root, thus high nitrogenase enzyme activities were detected from root treated with Z78 sonicated cells (T3). Although no significant differences of nitrogenase enzyme activities were detected for the treatments of different inoculums. Based on the result showed in Figure 2(b), the highest nitrogenase enzyme activities were detected in roots of micropropagated oil palm plantlets treated with Z78 broth culture (T4) (6.7 × 10⁻⁴ mol C₂H₄ g⁻¹ h⁻¹) among the treatments. This result reveals that the plantlets inoculated with Z78 broth culture had contributed nitrogen source to the plant by fixing the atmospheric nitrogen. In addition, the nitrogenase enzyme activity of plantlets was found to be positively correlated with the accumulation of Brix value with r value of 0.756. Given that plantlets promote positive correlation for Brix value and nitrogenase enzyme activities, we hypothesize that Z78 broth culture have the ability to increase plant nutrient uptake in an associated manner. Biological conversion of N₂ to plant-available ammonium, carried out by diazotrophic bacteria (Z78) is the best replacement for the conventional nitrogen fertilizers. Studies have shown that H. seropedicae can fix significant amounts of atmospheric nitrogen and contribute to the growth of sugarcane, rice, and oil palm (Boddey et al. 1995; Elbeltagy et al. 2001; Gyaneshwar et al. 2002; James et al. 2002; Roncato-Maccari et al. 2003; Al’shah et al. 2009; Lim et al. 2016). Nitrogen accumulation in inoculated plant can be obtained through biological nitrogen fixation or the increment of nitrogen uptake from the soil (Boddey et al. 1995; Elbeltagy et al. 2001; Oliveira et al. 2002; Mitter et al. 2013; Pérez-Montaño et al. 2014). It is an undeniable fact that diazotrophs have high potential to establish and create a better growth condition for the host plant cell.

On the other hand, leaf protein content was also analyzed in this experiment. Results were clearly revealed that the positive interaction for plantlets treated with Z78 broth (T4). Interestingly, total protein level in plantlets resulted in positive correlated responses to plant height, number of primary root, stem diameter and Brix value of plantlets (r = 0.727, 0.663, 0.649, 0.627). With the significant correlations, these have suggested that inoculants played an important role in promoting growth of micropropagated oil palm plantlet. In addition, Brix value was also determined and is found to positively correlate with the number of primary root formation of the plantlets, threshold at r value of 0.568. Generally, Brix can be defined as 1 g of sucrose in 100 g of solution and also indicates the strength of the solution as percentage by mass. If the solution contains dissolved solids such as mineral elements and trace minerals, then the dissolved solid content will also be detected as Brix value (Echeverria and Ismail 1990). At day harvest (D₃₀₀), the highest protein content and Brix value were obtained for the inoculated micropropagated oil palm plantlets with Z78 broth culture (T4) with a total of 243.33 mg (g FW)⁻¹ and 10.12%, respectively, in comparison to plantlets treated with limited N (T2) (Figure 2c). A similar result was also revealed by Dalibard (1999), the fresh sap of the healthy African oil palm containing 9.6–10.6% of sucrose. Eze and Organ (1988) also reported that fresh oil palm sap collected in Nigeria, through base tapping of the inflorescence, contained sucrose as the main sugar (10%, w/v).

All of the presented results have proved that H. seropedicae strain Z78 is one of the potential plant-growth-promoting diazotrophic bacteria, which can be used as inoculants for nonleguminous plants, especially for oil palm in our study. Not merely that, in our study, H. seropedicae has proved as an efficient source of N that can partly substitute for nitrogen fertilizer in oil palm cultivation. According to Cocking (2003), microbes can be used as biofertilizer, which are able to promote plant growth via hormonal substances, to increase availability of nutrients uptake in plant, and disease defense. Prominently, Z78 bacterial cell (particularly, Z78 broth culture) has great potential to use as biofertilizer for micropropagated oil palm plantlets.

Conclusion
In conclusion, this study clearly demonstrates that broth culture inoculant of H. seropedicae (Z78) has the ability to increase IAA production, control nitrogenase activity, increase root volume, plant height and boost the growth of micropropagated oil palm plantlets. The result obtained showed that the consistent supplementation of Z78 broth culture inoculant with minimal amounts of N fertilizer can promote a better plant growth in comparison with plantlets without Z78 inoculation in minimal N fertilizer supplied. To the best of our knowledge, this is the first report studying on micropropagated plantlets—bacterium interactions by using different H. seropedicae inoculums. We believe that this study can provide a better understanding on plant–diazotroph/plant-growth-promoting rhizobacteria interactions trajectory.

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No potential conflict of interest was reported by the authors.

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