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Artificial symbiotic association and growth induction of embryogenic calli (*Elaeis guineensis* Jacq.) inoculated with *Herbaspirillum seropedicae* (Z78)

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ABSTRACT

Aims: The purpose of this experiment was to determine the artificial symbiosis interaction of *Herbaspirillum seropedicae* (Z78) on oil palm embryogenic calli.
Methodology and results: For this purpose, symbiotic associations were established between Z78 and embryogenic calli of oil palm tissue cultured. A total of five treatments involved, in particular: i) + 3.0 mg/L 2,4-D + 100% N MS medium (control), ii) + Z78 pellet cells (1 mL) + 25% N MS medium, iii) + Z78 supernatant (1 mL) + 25% N MS medium, iv) + Z78 broth culture (1 mL) + 25% N MS medium, and v) + Z78 sonicated cells (1 mL) + 25% N MS medium. All treatments were supplied with minimal N sources (25% N), ammonium nitrate and potassium nitrate, while the control was treated with 100% N sources. Treated samples were harvested on D_{80} and observed for biomass and diameter increment (%), formation of embryoids, and Z78 colonization. The results showed embryogenic calli in the inoculated treatments that contained depleted N produced similar result to the control treatment which contained 100% N nutrients. Positive interactions occurred between the diazotroph and host plant tissues as viewed under FESEM and EFTEM. Among the treatments, Z78 sonicated cell showed better growth of embryogenic calli compared to others.
Conclusion, significance and impact study: The *in vitro* nitrogen-depleted artificial symbiosis environment allowed the diazotroph (Z78) to be expressed and provide the nitrogen sources and indole-3-acetic acid for cell growth. This study represents beneficial co-culture interaction effects of different inocula of diazotrophic bacterial cells with *in vitro* embryogenic calli of oil palm.

Keywords: beneficial interaction, embryogenic calli**,** *Herbaspirillum seropedicae* Z78, *in vitro* oil palm

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a perennial monocot plant, which is different from most other plant, somatic embryogenesis is the only path of its vegetative propagation. The micropropagation is unique, undergoing callusing and embryogenesis processes (Kushairi *et al.*, 2010). However, the process is less productive, with only 3% to 6% success rate. This is the main concerned for the oil palm micropropagation industry today (Wooi, 1995; Chan *et al.*, 2014). Due to low success of somatic embryogenesis rate, researchers try to discover an alternative way or protocol to accelerate and enhance the process (Chan *et al.*, 2014; Marbun *et al.*, 2015). One of the alternative idea to promote higher proportion of successful somatic embryogenesis process could be achieved through the application of diazotrophic rhizobacteria to plant tissue culture materials.

Diazotrophic bacteria, *Herbaspirillum seropedicae* (Z78), is a plant endophytic diazotroph that is capable of colonizing roots, stems and leaves of its hosts without causing disease (Baldani *et al.*, 1992; Olivares *et al.*, 1996; James and Olivares, 1998; Chubatsu *et al.*, 2012). Full benefits of the diazotrophs with the host plant could be achieved if the diazotrophs could interact with the plant host at very initial growth stage. It was believe that, the interactions would improve the micropropagation process due to the ability of the diazotrophs in colonizing the plant tissues (Azlin *et al.*, 2007), by supplying phytohormones and fixing N_2 (Kefalogianni and Anggelis, 2002; Azlin *et al.*, 2007). Earlier reports by Parray *et al.* (2015) showed that co-culture of rhizobacteria with *in vitro Crocus sativus* L. illustrated a significant effect in morphogenetic responses towards plant growth. However, the understanding of the interaction is still limited. The interactions of the diazotrophic bacteria to promote somatic embryogenesis of the host plant is necessary to be understand more thoroughly. Thus, the aim of this experiment was to determine the potential benefits and the interactions of the co-culture of endophytic diazotrophs (*Herbaspirillum seropedicae* Z78) on *in vitro* oil palm embryogenic calli. Successful colonization of *H.*

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seropedicae (Z78) on the cell surfaces and inner parts of the embryogenic callus was also viewed under Field Emission Scanning Electron Microscope (FESEM) and Energy-Filtered Transmission Electron Microscope (EFTEM).

MATERIALS AND METHODS

Bacterial culture

Diazotrophic bacteria *H. seropedicae* (Z78) (ATCC 35893) was tested and involved in four different inocula preparation, in particular; i) broth culture, ii) supernatant, iii) pellet cells and iv) sonicated cells. The bacterial culture (Z78) was subcultured into N-limited medium (100 mL per flask) and incubated on a rotary shaker at 180 rpm for 48 h at room temperature (30-32 °C) until it reached an inoculum size of 10^9 CFU/mL. The broth culture inoculum was directly used for inoculation. While for the supernatant inoculum, 1 mL of broth culture was centrifuged at 10000 rpm for 10 min and the supernatant was transferred to another microcentrifuge tube and ready for inoculating. The pellet was resuspended in 1mL distilled water and re-centrifuged at 10000 rpm for 10 min (for pellet cells inoculum). On the other hand, the bacterial cells were sonicated for 2 min in a sonicator JAC Ultrasonic 2010p to provide the sonicated cell inoculum. All inocula preparations were tested on embryogenic calli cultures of the oil palm (*Elaeis guineensis* Jacq.) Tenera fruit form clone LN912.

Plant materials

Embryogenic calli of the oil palm (*Elaeis guineensis* Jacq.) fruit form Tenera clone LN912 was provided by Advanced Biotechnology and Breeding Centre of Malaysian Palm Oil Board (MPOB), Bangi, Selangor, Malaysia. The entire procedure of culturing the embryogenic calli (0.5-1.0 cm clumps) were performed on N-limited Murashige Skoog (MS) medium (25% N). Details of the inoculation treatments on the embryogenic calli cultures were as follows; i) + 3.0 mg/L 2,4-D + 100% N MS medium (control), ii) + Z78 pellet cells (1 mL) + 25% N MS medium, iii) + Z78 supernatant (1 mL) + 25% N MS medium, iv) + Z78 broth culture (1 mL) + 25% N MS medium, and v) + Z78 sonicated cells (1 mL) + 25% N MS medium. The experiment was conducted in a completely randomized design (CRD) with 10 replicates and harvested after 80 days of incubation (D_{80}). The observations involved were as follows; 1) increment of embryogenic callus biomass (g), 2) formation of embryoids, 3) increment in embryogenic callus diameter and 4) bacterial colonization on embryogenic callus at D_{80} viewed under Scanning Electron Microscope (Leo Supra 50 VP) and Transmission Electron Microcope (Libra 120).

Acetylene Reduction Assay (ARA)

The ability of bacteria to fix the nitrogen from environment can be tested by using Acetylene Reduction Assay (ARA). Z78 was cultured in amber brown glass bottle with N-free semisolid medium (Nfb) and was closed tightly with rubber cap. Then, 5% of air inside the incubation bottle was removed from the headspace of amber brown glass bottle by using syringe. Same amount of acetylene gas (99.8% purity) was injected into the vessels. The vessels were then incubated for 24 h at room temperature. Upon completion of the incubation period, a total of 5 mL gas was sampled from the incubation bottle and was injected into the vacuumtainer for storage. Then, 1 mL of gas was injected into Shimadzu Gas chromatography (GC-2014) to assay the presence of ethylene gas. The GC-2014 was fitted with a Supelco Carboxen 1004 stainless steel micropacked column, 2 m × 0.76 mm ID, and equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. Temperature of column, injection and FID were fixed at 80 °C, 180 °C and 180 °C, respectively. The concentration of ethylene reading was recorded. Nitrogenase activity of the associative bacteria was calculated by using the formula below:

$$\text{Nitrogenase activity } (\mu\text{mol } C_2H_4/\text{g/h}) = \mu\text{mol } C_2H_4 \times \frac{1}{\text{sample fresh weight}} \times \frac{1}{\text{incubation hours}}$$

Indole-3-acetic acid (IAA) assay

The IAA production of free living Z78 was assayed based on ultra-performance liquid chromatography (UPLC). The bacteria Z78 was cultured in a 250 mL Erlenmeyer flask containing 100 mL of minimal N medium (Okon *et al.*, 1977) and supplied with 0.5 g/L L-tryptophan as a precursor for auxin biosynthesis (Glickmann and Dessaux, 1995; Ai'shah *et al.,* 2013; Tan *et al.*, 2015). The presence of auxin in broth culture was detected through the filtrate of bacteria supernatant, filtered by membrane filter (Acrodisc® Sterile Syringe Filter, 0.2 µm). The filtrates were injected to Acquity UPLC with PDA Chi 280 nm @ 1.2 nm detector and C-18 UPLC column was used, Acquity UPLC @ BEH (181.7 µm, 2.1 × 100 mm) at ambient temperature. The buffer solvents were set, in specific, 1% (v/v) acetic acid as solvent A and 100% acetonitrile as solvent B. The elution gradient was adjusted as: 2 min of 50% solvent A and 3 min of 99% solvent A while 2 min of 50% solvent B and 3 min of 1% solvent B. The running time was 3 min at a flow rate of 0.25 mL/min and 280 nm wavelength. The peak retention time and area were compared with IAA standard curve.

Field scanning electron microscope (FESEM) and Energy-filtered transmission electron microscope (EFTEM)

For both FESEM and EFTEM processing, the untreated and treated embryogenic calli samples were fixed in McDowell fixatives, containing 0.1 M phosphate buffer pH

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7.2 at 4 °C (McDowell and Trump, 1976) and washed in the same buffer (3 × 10 min) followed by post fixing in 1% osmium tetraoxide (prepared in 0.1 M phosphate buffer pH 7.2) for 2 h, washed with distilled water for 2 times. The tissues were undergone a series of dehydration processes; 50% ethanol for 15 min; 75% ethanol for 15 min; 90% ethanol for 15 min (two times) and 100% ethanol for 20 min (two times) (Glauert and Lewis, 1998).

For FESEM, the dehydrated samples were ready and immersed in 2 mL of hexamethyldisilazane (HMDS) for 10 min (Nation, 1983). HMDS solution was decanted and the samples were air dried in the desiccators. Next, the specimens were mounted on SEM specimen stubs and covered with aurum (20 nm) by Sputter Coater (Polaron SC515). Finally, the samples were ready for viewing under Field Scanning Electron Microscope (Leo Supra, 50 VP). While for EFTEM, the dehydrated samples were immersed in Acetone: Spurr's resin mix (1:1) overnight in a rotator. The samples were infiltrated in a new change Spurr's mix for the following 3 days before fully molded by using the embed cube at 60 °C for 24 h. Next, the samples were prepared for specimen block by performing the ultramicrotomy started with rough trimming; fine trimming; semi-thin trimming and followed by ultra-trimming. Then, samples were collected on copper grips and stained with Uranyl Acetate and Lead Citrate. The samples were then ready for viewing under Energy-Filtered Transmission Electron Microscope (Libra 120).

Statistical analysis

One-way ANOVA was performed to test the significance of treatment effects. For all characteristics studied, the statistical significance differences between means were determined by Tukey HSD at $P < 0.05$**.** Statistical procedures as in SPSS V20.0 were used for data analysis.

RESULTS AND DISCUSSION

In general, the percentage of biomass increment of embryoid from embryogenic calli treated with bacterial inoculation under N-depleted condition was higher than the uninoculated embryogenic calli (control). The highest embryoid biomass increment was recorded for treatments with inoculants of Z78 sonicated cells (323.30%), supernatant (284.67%) and broth culture (272.96%) compared to the control (196.07%) (Figure 1). The increment in the diameter of the embryogenic calli were almost similar for the control and inoculants treatments of Z78 sonicated cells, supernatant and broth culture. These results clearly highlighted that embryogenic calli treated with Z78 under N-depleted condition was successfully developed into embryoid, similar to that observed for the control treatment (+ 2,4-D, + 100% N) (Figure 2). In addition, the result was also clearly demonstrated that treated embryogenic calli, in particular, Z78 sonicated cell showed a significant biomass and diameter increment under minimal nitrogen sources condition (Figure 1). The cellular content for sonicated bacteria was expected to release due to the disruption of bacterial cell membrane and also phytohormone can be released from the rupture bacterial cell during the sonication process and initiated the embryogenic calli to proliferate within the shortest period of incubation. In our study, the nitrogenase enzyme activities of free living *H. seropedicae* (Z78) was detected as 6.477 µmol C_2H_4/CFU·h under microaerophilic condition. While, the IAA phytohormone production ability was also recorded at 0.121 µg/mL·h (supplied with L-tryptophan as a precursor). Hence, these metabolites production can potentially improve the embryogenic calli development. Furthermore, Tan *et al.* (2015) also highlighted that rhizobacteria Z78 has the ability to produce indole-3-acetic acid (IAA) and fix atmospheric

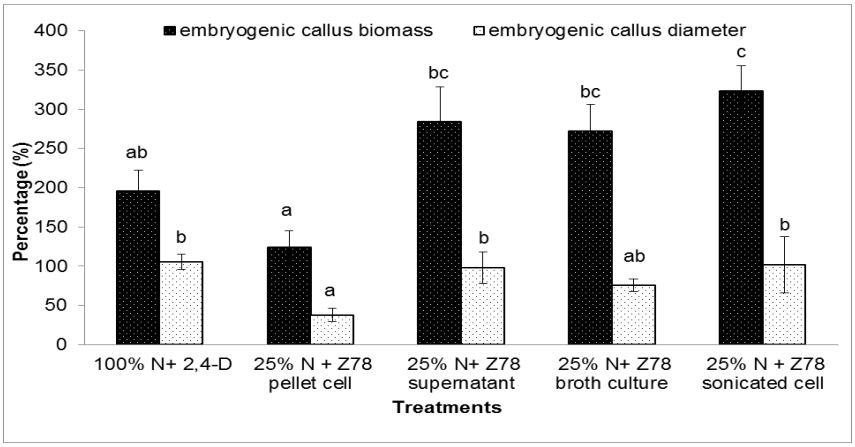

Figure 1: Percentage of embryogenic calli biomass and diameter increment at D_{80}. Note: means followed by the same letter indicate no significant differences among treatments and analyzed by Tukey HSD at $P < 0.05$.

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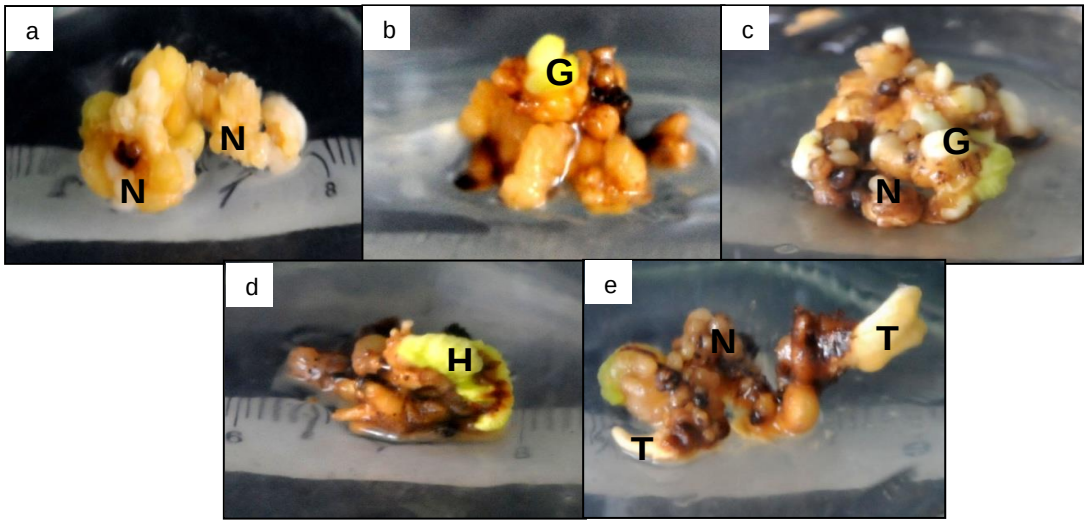

Figure 2: Formation of various types of embryoids from embryogenic calli at D_{80}. a) T1: 2,4-D + 100% N medium (control), (b) T2: Z78 pellet cells + 25% N medium, (c) T3: Z78 supernatant + 25% N medium, (d) T4: Z78 broth culture + 25% N medium, and (e) T5: Z78 sonicated cells + 25% N medium. N, nodular; G, globular; T, torpedo; H, haustorium.

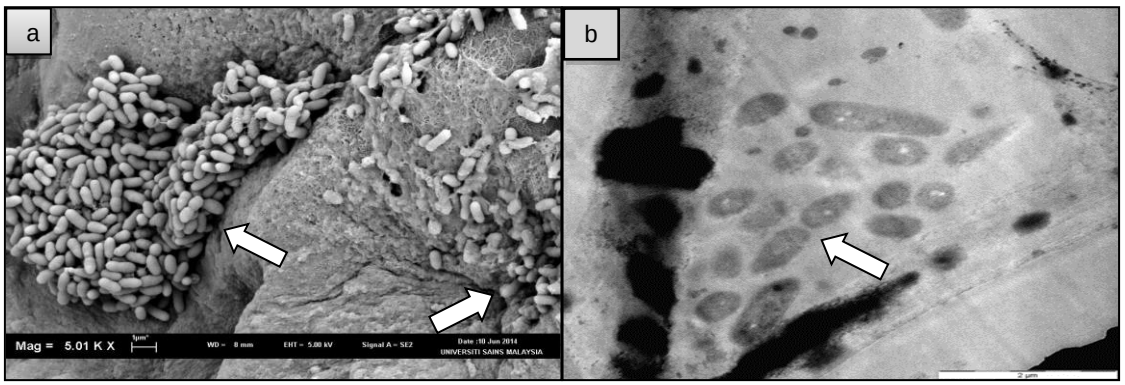

Figure 3: Scanning electron micrograph showing (a) close up of oil palm embryogenic callus inoculated with Z78. Bacterial attachment and colonization on the surface of embryogenic callus (arrow). Bar = 1 µm (b) Transmission electron micrograph of the symbiotic associated by Z78 and embryogenic callus oil palm. Bacteria are shown in the intercellular spaces of the tissue (arrow). CW, cell wall; ICS, intercellular spaces. Bar = 2 µm.

nitrogen in the free-living condition. As reported by Lim *et al*. (2016), nitrogenase enzyme activities and also phytohormone production can be detected for associated *H. seroepdicae* with plant tissue materials. Hence, we are presumed that the respond may due to the fixed nitrogen and phytohormone produced by the inocula (Z78) that improved embryogenic calli development. It was also reported that rhizobacteria can interfere with plant development by distributing auxin during the interaction with the host plants (Spaepen and Vanderleyden, 2011; Do Amaral *et al.*, 2014). IAA production by *H. seropedicae* is one of the mechanisms that could promote growth of the host plant (Bastian *et al.*, 1998). Nevertheless, earlier studies were also revealed that rhizobacteria with the ability to produce phytohormone and fix atmospheric nitrogen can lead to a better growth of oil palm plantlets (Azlin *et al.*, 2005) and rice seedlings (Keyeo *et al.*, 2011).

Our observations also showed that *H. seropedicae* (Z78) was exclusively colonised the cell and influenced the embryoid growth. The microscopy observation is essential for better understanding of successful *in vitro* plant-microbes interaction and also the bacterial colonisation. The microscopy observation showed inner and outer cell attachment and colonisation (arrow) (Figure 3a, 3b). Based on the results, the bacterial cells colonised the embryoid surfaces and this may due to the high chemo-attractant concentrations produced by the host plant cells. It is speculated that these embryoid surfaces could provide a specific attachment site for the bacterial cell. Moreover, the colonisation of the bacteria not only can be observed on the surface of the treated embryogenic calli but also in the intercellular spaces (Figure 3b). These observations suggested that the inoculated Z78 conferring no pathogenesis to the plant cell, in fact, it can perform a symbiotic relationship and

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promote better cell growth under nitrogen-depleted condition.

In somatic embryogenesis, the proembryo is the initial structure which forms during embryogenic calli proliferation. In our observation, the embryogenic calli treated with bacterial inoculation under N-depleted condition has more opaque white embryoid formed compared to the uninoculated embryogenic calli (control). The opaque white embryoid was observed in the Z78 sonicated cells inoculant treatments in N-depleted condition, with the highest percentage of 35.40%, followed by broth culture (29.60%) and supernatant (21.23%) treatments compared to the control (14.85%) (Table 1). Some studies have shown that the opaque white structures tend to produce shoots. Most of the time, a shoot apex formation can be observed within the opaque white structures (Tarmizi *et al.*, 2011). With this, we can postulate that the embryoid formation was faster for embryogenic calli treated with Z78 compared to the control. Also, the formations of small, green and globular shape of green brown embryoids were observed in the treatment with Z78 pellet with 25% N (Figure 2b). Most of the somatic embryos were yellow-greenish in colour with smooth surface (Sumaryono *et al.*, 2008). These globular-shaped embryos can be developed at the second stage of embryogenesis after the formation of the proembryos. On the other hand, the formation of white opaque and hard green embryoids with globular shape was detected for embryogenic calli treated with Z78 supernatant + 25% N (Figure 2c). Sumaryono *et al.* (2008) and Termizi *et al.* (2014) reported that at the early developmental stage of oil palm somatic embryogenesis, nodular, globular and heart-shape structures were observed to develop into somatic embryos and advanced develop into structure stages of torpedo and cotyledonary before the shoot formation. Through this, we can presume that the inoculated embryogenic calli tend to develop into a more advance stage of embryo after the artificial symbiosis formation.

Table 1: Formation of different type of embryoids (%) from embryogenic calli proliferation at D_{80}.

		Percentage embryoids (%)		
	Treatments	Yellowish white	Opaque white	Yellowish brown
1	2,4-D + 100% N	85.15	14.85	0
2	Z78 pellet cell + 25% N	0	0	100
3	Z78 supernatant + 25% N	0	21.23	78.77
4	Z78 broth culture + 25% N	0	29.60	70.40
5	Z78 sonicated cell + 25% N	0	35.40	64.60

Generally, *in vitro* plant culture requires an axenic condition, and any outer infection from microorganisms could be considered as contamination (Bhojwani and Razdan, 1986). However, our study revealed that inocula Z78 was able to establish beneficial interactions with *in vitro* oil palm tissues. Our results support the hypothesis that artificial associative relationship was potentially achieved between bacterial cells (Z78) and *in vitro* plant cultures, this can eventually enhance plant cell growth under the minimal nitrogen supplied. Through this, a mutual relationship was artificially created in the association of oil palm embryogenic calli and Z78. In most cases, plant tissue culture has unique malleable to alter their developmental program in order to adapt to the changes from the culture conditions either through the biotic or abiotic manners (Neelakandan and Wang, 2012). Due to this ability, we speculated that introduction of Z78 into the plant tissue culture system may benefit the host cells by accelerating and promote better embryoid formation. Several studies were also reported for *in vitro Dancus-Azotobacter* association (Varga *et al.*, 1994), *in vitro* association of Strawberry-*Azomonas* (Preininger *et al.*, 1997), and the most recent study of *Crocus sativus* L-plant growth promoting rhizobacteria (PGPR) interactions (Parray *et al.*, 2015). However, no observation showed the role of bacteria and the interaction with tissue culture materials of oil palm in the early stage. Our study has provided a better understanding for the *in vitro* plant-microbes interaction under N-depleted condition and without any phytohormone supplied during the cultivation. Not only morphological observation but also the bacterial colonisation was studied in our experiment. Based on the presented results, we found that Z78 has a high potential to accelerate embryoid formation under limited nitrogen supplied by fixing the atmospheric nitrogen and with the capability to produce indole-3-acetic acid.

CONCLUSION

In conclusion, this study revealed that rhizobacterial (Z78) inoculation could establish growth of *in vitro* oil palm cultures under nitrogen-depleted condition. This may be due to the excretion of phytohormones and nitrogen fixing abilities of inocula tested. This study also demonstrates the symbiotic co-culture of different inocula conditions for Z78 with embryogenic calli of oil palm. We believe that this experiment can become a new paradigm for today plant tissue culture industry whereby the diazotrophic bacteria can be introduced to the plant tissue culture materials in a more early stage and not only for plantlets. This eventually suggests a novel contribution by the artificial symbiosis of bacterium for *in vitro* oil palm somatic embryogenesis.

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