



Micromorphological analysis of *Aranda Broga Blue* orchid PLBs for *Agrobacterium*-mediated transformation system

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Aims: The aim of this work was to analyse the morphological aspects of ABB's PLBs and interaction between ABB's PLBs and *Agrobacterium*.

Methodology and results: *Aranda Broga Blue*'s (ABB) protocorm-like bodies (PLB) proliferated on Vacin and Went medium by incubating at $25 \pm 2^\circ\text{C}$ under 16 h semi-photoperiod was used to study the micromorphology of PLB, bacterial attachment and chemotaxis studies. The anatomy and micromorphology study indicates that ABB's PLBs provide the platform for attachment of *Agrobacterium tumefaciens* strain LBA 4404. Successful propagation of ABB's PLBs was achieved on modified Vacin and Went solid media supplemented with organic homogenates. Histological analysis of the development of ABB's PLBs shows it is a meristematic tissue and contains actively dividing cells. Transmission electron microscope (TEM) studies signify the presence of specialized organelles to indicate PLB is similar to a mature plant. Scanning electron microscope (SEM) observation on ABB's PLBs and bacterial attachment studies proves that trichomes and stomata on the surface facilitate the interaction with *A. tumefaciens*. Quantification of the attachment of *Agrobacterium* to ABB's PLBs using spectrophotometric system displayed higher affinity compared to the attachment mutant *Escherichia coli* DH5 α . The interaction of *Agrobacterium* to ABB's PLBs was also evaluated based on chemotaxis studies. Highest motility respond of *Agrobacterium* was recorded (1.6 unit) towards severely wounded PLBs indicating wounding is necessary for the interaction between bacterium and PLB.

Conclusion, significance and impact study: Micromorphology of protocorm-like bodies (PLBs) enhances the physical interaction with bacteria proved that PLB is a suitable explant for the *Agrobacterium*-mediated genetic transformation.

Keywords: *Aranda* orchid, *Agrobacterium tumefaciens*, micromorphology, protocorm-like-bodies

INTRODUCTION

Malaysia is the most likely cradle of 'orchidhood' of the over 25,000 species found worldwide, and 850 were found in peninsular region (Shahril, 1999). The pulchritudinous of orchids lead to development of orchids industry upon high demands and viable commercialized market. Breeding orchids through conventional means is a lengthy process. Thus, it would have been beneficial if transgenic technologies could be applied for orchids to improve essential traits such as novel flower colour, delicate scent and shape, cut-flower longevity and many more. *Aranda Broga Blue* (ABB) is subjected to become one of the most important orchids grown for commercial production; therefore genetic engineering approaches are widely used to transfer potential genes into orchids (Liau *et al.*, 2003; Sreeramanan *et al.*, 2009). Currently, *Agrobacterium*-mediated transformation system has been productively and consistently used in orchids such as *Cymbidium* (Chen *et al.*, 2002; Chin *et al.*, 2007), *Phalaenopsis* (Belarmino and Mii, 2000; Mishiba *et al.*, 2005; Sreeramanan *et al.*, 2008), *Dendrobium* (Bunnag and Pilahome, 2012; Zheng *et al.*, 2012), *Cattleya* (Zhang

et al., 2010) and *Oncidium* (Liau *et al.*, 2003). However, monocots including orchids are not the natural hosts of *Agrobacterium* strains, which made the initial trials difficult on the transformation of orchids with *Agrobacterium*-mediated method. It is important to optimize the transformation system, because plants demonstrate wide variation in susceptibility to *Agrobacterium* (Tsay *et al.*, 2012).

The accomplishment of genetic engineering in orchids depends on the totipotency of plant cells; for example the inherent ability of plant tissue to produce cells that can be regenerated into a completely viable plant (Teixeira da Silva *et al.*, 2011). The success of orchid genetic transformation lies in the ability of putatively transformed tissue to regenerate. Protocorm-like bodies (PLBs) are the most commonly used target tissue for successful genetic transformation in orchids. Therefore *Agrobacterium tumefaciens* is a reliable genetic transformation tool in order to enhance the orchid features especially *Aranda Broga Blue* (ABB).

In this present study, we report that micromorphology of ABB's PLBs supports the attachment of *A. tumefaciens* to enhance transformation efficiency. *A. tumefaciens*-

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mediated transformation of ABB orchid is possible since *A. tumefaciens* chemically attracted towards exudates from ABB PLBs.

MATERIALS AND METHODS

Plant materials

In vitro culture of protocorm-like bodies (PLBs) of ABB was obtained from Plant Biotechnology Laboratory, 310, School of Biological Sciences, USM. The culture was incubated at 25±2 °C under 16 h semi-photoperiod under cool white fluorescent lamps (supplied by Philips TLD fluorescent light tubes of 36 W, 150 µmol/m².s¹). PLBs were proliferated on modified Vacin and Went medium supplemented with 15% coconut water, 20 g/L banana and 20 g/L potato extracts to produce large quantities of explants for experiments. PLBs were used as the explants for its anatomy, micromorphology, bacterial attachment and chemotaxis studies.

Bacterial strain

Agrobacterium tumefaciens (strain LBA4404 harbouring pCAMBIA 1304 plasmid containing β-glucuronidase (*gusA*) gene and antibiotic (*nptII*) resistance genes) was maintained at -80 °C for long term storage in 70% (v/v) glycerol.

Histology analysis

Ten (10) Protocorm-like bodies (PLBs) were fixed in formaldehyde (FAA) containing scintillation vial (2 PLBs in each vial) at room temperature for 1 week. The PLBs were then placed under running tap water for 24 h. PLBs were then dehydrated by immersing in tertiary-butyl (TBA) alcohol of varying concentration. Then, PLBs were immersed in xylene until sample became transparent. This was followed by treatment with xylene and wax for 30 min to acclimatize PLBs to wax. Later, PLBs were treated with wax I, II, and III (Shandon HistoplastPelletised Paraffin Wax, Thermo Scientific) for 1 h respectively at 60 °C in an oven (Mettler, Germany). PLBs were then placed in metal mold containing melted wax to form wax blocks. Wax blocks were then sliced using 6 Micron Microtome (Leica RM 2135) and placed on clean glass slides. Samples were then stained with safranin and fast green. Slides were observed using light microscope (Olympus BX41).

Preparation of *Agrobacterium tumefaciens* culture

Three (3) mL cultures from the glycerol stock were transferred into Luria-Bertani (LB) broth (Miller Hi Media, India) containing 50 mg/L kanamycin (Duchefa, Netherlands) and incubated at 28 °C and 120 rpm overnight. Bacterial suspension were then streaked on solid LB medium containing 50 mg/L kanamycin using inoculation loop and incubated at 28 °C for 2-3 days until a visible single colony forms. Single colony were then

inoculated to LB broth containing 50 mg/L kanamycin and incubated at 28 °C and 120 rpm for 16 h to reach an optimal density (OD) 0.9 units at OD 600 nm. The OD of the *A. tumefaciens* suspension culture at 600 nm were adjusted to 0.9 using spectrophotometer (Hitachi U-1900 UV/VIS, Japan) before used for the bacterial attachment and chemotaxis studies. *Escherichia coli* strain DH5α harbouring pMRC 1301 plasmid consists of *gusA* and *nptII* genes were prepared in the same manner as the *A. tumefaciens* and used as a control for attachment and chemotaxis studies.

Microscopical studies of bacterial attachment

Microscopical analysis was carried out to confirm the capability of *A. tumefaciens* strain LBA4404 harbouring pCAMBIA 1304 plasmid to adhere onto PLBs (Liau *et al.*, 2003). Attachment mutant *Escherichia coli* strain DHα (pMRC 1301) was included as negative control in all the conducted experiments. Overnight grown bacterial cultures were pelleted (3400xg for 10 min) and resuspended in 25 mM phosphate buffer (pH 7.5) to infect PLBs. PLBs kept in 1.5 mL Eppendorf tubes were then co-cultivated with 250 µL of bacterial suspension and incubated in a rotary shaker at 28 °C at 25 rpm for 2 h. A total of 10 PLBs were immersed in bacterial suspension by placing 2 PLBs in each Eppendorf tubes. After 2 h, unbound bacteria were removed by washing the PLB once with 1 mL fresh buffer and vortexed 30 sec each time to discard unattached bacteria. Explants were then directly observed using optical microscopy or freeze dried for observation using scanning electron microscope (SEM) and transmission electron microscope (TEM) (Phillips CM12 with Docu Version 3.2 image analysis).

Scanning Electron Microscope tissue preparation freeze drying technique

Two (2) PLBs were placed on a planchette placed on filter paper lined Petri dish. The filter paper was damped with few drops of distilled water. The Petri dish was brought into fume hood and again damped with few drops of 2% osmium tetroxide (O₃O₄). The petri dish was closed immediately and kept in the fume hood for 2 h. This is known as vapour fixation. Once the sample has been vapour fixed, the planchette was plunged into liquid nitrogen and transferred on to the peltier-cooled stage of the freeze dryer (Emitech k750, England) for about 10 h. The planchette then kept in a dessicator if it's not to be viewed immediately. The specimen was then sputtered with 5-10 nm gold particles using SEM coating system (Polaron SC515, England) before viewing it under Leo Supra 50VP Field Emission SEM (Carl Zeiss SMT, Germany) equipped with Oxford INCA 400 energy dispersive microanalysis system.

Transmission Electron Microscope tissue preparation

Two (2) PLBs were fixed in McDowell-Trump fixative prepared in 0.1 M phosphate buffer at 4 °C for 24 h. The

following day, the PLBs were washed in the 0.1 M phosphate buffer for three times at 10 min interval. The PLB were then post fixed in 1% osmium tetroxide prepared in the same buffer as above at room temperature for 2 h. Then the PLBs were washed in distilled water for two times at 10 min interval. The PLBs were then dehydrated in a graded series of ethanol (50% ethanol (15 min, 75% ethanol (15 min), 95% ethanol (15 min, twice), 100% ethanol (30 min, twice), and 100% acetone (10 min, twice). Resin containing a mixture of acetone and Spurr's resin was prepared at a ratio of 1:1. The resin was infiltrated into the samples in a rotator for 15-30 min. The samples were infiltrated in Spurr's mix overnight in the rotator. Samples were infiltrated in a new change of Spurr's mix for another 5 h in the rotator. Samples were embedded in resin beads formed in the mold. Samples were cured at 60 °C for 12 to 48 days. Samples were then sliced using ultramicrotome cutter and gathered on carbon planchette. Samples were stained with lead citrate and uranyl acetate and viewed under TEM.

Quantification of bacterial attachment to PLBs

Quantification of bacterial attachment assays were carried out according to method of (Pérez Hernández *et al.*, 1999) in order to confirm the binding aptitude of *A. tumefaciens* strain LBA4404 harbouring pCAMBIA 1304 plasmid. Attachment mutant *Escherichia coli* strain DH α (pMRC 1301) was included as negative control in all the conducted experiments. Overnight grown bacteria suspension was centrifuged (Universal 320, Hettich Zentrifugen) at 3400 rpm for 10 min at 4 °C for the quantification of *Agrobacterium* attachment onto PLBs. The bacterial pellet was suspended in 25 mM phosphate buffer (pH 7.5). A total of 18 PLBs divided to 2 in each 1.5 mL Eppendorf tubes were maintained in 1 mL of 25 mM phosphate buffer (pH 7.5). For attachment, the Eppendorf tubes were loaded with 50 μ L aliquots of buffer suspended bacteria. Tubes were then incubated in a rotary shaker at 28 °C at 25 rpm for 2 h. After 2 h, unbound bacteria were removed by washing the PLB once with 1 mL fresh buffer and vortexed 30 sec each time to discard unattached bacteria. β -glucuronidase activity in the samples was measured following the pre-established method (Wilson *et al.*, 1992). Washed explants were transferred to 1 mL of extraction buffer (50 mM sodium phosphate (pH7), 10 mM dithiothreitol, 1 mM sodium EDTA, 0.1% (v/v) sodium lauryl sarcosine, 0.1% (v/v) triton X-100), vortexed and incubated at 37 °C for 10 min. The GUS enzyme substrate p-nitrophenyl β -D-glucuronide was added at a final concentration of 1mM. After incubation at 37 °C for 30 min reactions were stopped by the addition of 400 μ L of 400 mM Na₂CO₃ solution. GUS activity was quantified by measuring light absorbance at 415 nm in a spectrophotometer (Hitachi U-1900 UV/VIS, Japan) (A_{415nm}) as described previously (Remans *et al.*, 1999).

Chemotaxis Assay

Chemotaxis assays were carried out according to Shaw's protocol for the modified swarm agar plate method (Shaw, 1995). Three types of wounding were used in this assay such as Intact PLBs (positive control) (W1), PLBs subjected to mild wounding with sterile needles (W2) and PLBs subjected to severe wounding with scalpels (W3). Each set of experiment were repeated twice in triplicates. The data were analyzed using a one-way ANOVA and different between means were compared using Tukey test.

RESULTS AND DISCUSSION

Micropropagation of *Aranda*

Protocorm-like bodies (PLBs) are used as the target material for genetic transformation studies in orchids due to the rapid proliferation within short period of time and high capabilities to regenerate into complete plantlets (Sreeramanan *et al.*, 2008). PLBs are somatic embryos that are meristematic in nature and can be induced to regenerate into whole plant. This is because PLB is a bipolar organ that develops shoot and root. Proliferation of PLBs could be established by providing sufficient nutrients required for growth (Vacin and Went, 1949). Therefore, modifications were made in the current study by including organic substances such as coconut water, potato homogenate and banana pulp.

Banana pulp was added to the modified Vacin and Went media because it contains higher level of iron, potassium, vitamin B6 and B12 and also tryptophan to promote PLBs growth. The stimulatory effect of banana pulp could be owed to its ability to stabilize the pH of the medium and the potent to act as antacid to neutralize acidity condition (Gnasekaraan *et al.*, 2010). Higher percentage of sucrose, fructose and glucose concentrations in banana pulp extract makes it a better choice to be included in the proliferation media (Aktar *et al.*, 2008). Banana has added benefit for ethylene production, which can induce germination and maturation. However, in large amount, it can also cause cell death. Ethylene reacts differently in plants.

Usually coconut water is added to tissue culture medium because it contains diphenyl urea, a growth factor which exhibits cytokinin-like responses (Gnasekaran *et al.*, 2010). A balanced level of plant growth regulating hormones such as cytokinins and auxins in coconut water (Vyas *et al.*, 2005) is expected to enhance the rapid growth of ABB's PLBs. Some compounds in the coconut water were reported to prevent the blackening of the plant tissues caused by the oxidation of phenolic compounds (Dix and Van Staden, 1982). After 2 weeks of subculture, PLBs grow rapidly; and absence of browning effect was noticed as well.

Histology of PLBs

Histology is the study of the structure of plant or animal tissues using special staining techniques combined with observation under light microscopy. Histology was done to study the anatomy of ABB's PLB. The apical part of protocorm, consist of smaller cells, is the shoot apex while the basal part which functions as a 'storage organ', consist of larger parenchymatous cells (Figure 1A). Apical part of embryo is clearly shown in (Figure 1B).

Actively dividing cells were detected in ABB's PLBs. Figure 2A shows the dividing nucleus which indicates the ongoing mitotic division beneath the epidermis for the continual growth of ABB's PLBs. Active cell division in the ABB's PLB compensate for the thickening of the somatic embryo. Histology studies also proved the presence of raphides (Figure 2B). Raphides are also known as crystal oxalate (Nakata, 2003) found abundantly in ABB's PLBs. Calcium oxalate crystal involves in calcium regulation, defense, and metal detoxification (Franceschi and Horner, 1980). In all cases, the crystals are formed from environmentally derived calcium and from biologically synthesized oxalate as part of calcium regulation (Nakata, 2003). Thus, it can be concluded that ABB's PLBs are either able to utilize the medium content to accumulate the calcium or it could be due to abundance of calcium that was not metabolized by the PLB itself, hence forming raphides. Calcium has long been recognized as one of the most important signalling components in plants (Trewavas and Knight, 1994; Bickerton and Pittman, 2012). Many abiotic and biotic stimuli are transduced into a cellular response by temporal and spatial changes in cellular calcium concentration. Therefore raphides will likely to be one of the reasons to signal the *A. tumefaciens* towards the PLBs, justifying the structure of raphide will be providing excellent plant-bacterium recognition. The crucial step of this interaction can be achieved. Vascular system starts differentiating basipetally in the center of the PLB during the development of shoot and root apex. Development of vascular bundles in ABB's PLBs was proven by the presence of coil shaped xylem (Figure 2C). Presence of xylem in ABB's PLBs ensures the efficient uptake of water. Anatomy of ABB's PLBs contribute understanding in fundamentals of establishment *A. tumefaciens* onto ABB's PLBs, the presence of actively dividing cells, raphides and xylem will be enhancing the interaction and attachment of *A. tumefaciens* on ABB's PLBs.

SEM and TEM analysis of PLBs

Scanning electron microscope (SEM) provides the information about the external morphology of the sample. Surface of PLBs are expected to have trichomes. Trichomes are single or multicellular outgrowths of the plant epidermis (Cherdantsev and Grigorieva, 2012) and collectively constitute the pubescence (hairiness) of the plant surface (Nawab *et al.*, 2011). These epidermal hairs are specialized for plant defence against herbivory by

physical and chemical deterrents (Tian *et al.*, 2012). Trichome was observed almost all over the surface of ABB's PLBs (Figure 3A). Multi branched trichomes which is hollow in nature was identified on the ABB's PLBs (Figure 3A). Trichome is a hollow structured projection (Redha *et al.*, 2011), thus it may contain chemical attractant that attract the *A. tumefaciens* to attach to the ABB's PLBs. Furthermore, the morphology of trichomes facilitate in successful attachment. ABB's PLBs trichomes are branched which increase the surface area for bacterial attachment and results in efficient bacterial attachment.

Random distribution of stomata on the epidermis of PLB was identified through SEM studies. Figure 3B shows round shaped stomata possessing two guard cells. Stomata are microscopic pores on the surface (epidermis) of plants (Withers *et al.*, 2011). Presence of stomata indicates that ABB's PLB undergoes respiration. Transpiration via stomata supplies water and minerals to the entire plant system (Withers *et al.*, 2011). Stomata provide a natural opening on the surface of PLB for the access and attachment of the *Agrobacterium* cells (Underwood *et al.*, 2007). It allows the in depth colonization by *Agrobacterium* cells into the plant cells. Stomata is the best place for *A. tumefaciens* to avoid being wash or food sanitization, it does not have active mechanism to prevent bacterial infection, thus provide the easiest place to be accessed by *A. tumefaciens*.

TEM analysis was determined to identify various organelles on the PLB cells. The observations indicate the presence of specialized organelles such as mitochondria (Figure 4A) and chloroplast (Figure 4B). Chloroplasts are specialized organelles found in all higher plant cells. Various shapes and sizes of mitochondria were observed in ABB's PLBs (Figure 4A). Mitochondria are membrane-bound organelles, forming folds called cristae as clearly seen in Figure 4A. The presence of specialized organelles such as mitochondria and chloroplast in ABB's PLB shows it's no different from an intact mature plant. Furthermore, it can be concluded that ABB's PLB will play self recovery role to prevent cell death in case of excessive colonization by *A. tumefaciens* since it's a photosynthesizing and respiring tissue.

Since ABB's PLB is a meristematic tissue and contains actively dividing cells it can also recover from the damages caused by colonization by *Agrobacterium* cells or wounds caused during handling process. Attributes such as the occurrence of specialized organelles, vascular system, actively dividing cells and ability to regulate calcium content prove ABB's PLB as the suitable explant for *Agrobacterium*-mediated transformation. Occurrence of trichomes and stomata on the surface of ABB's PLB further enhance *Agrobacterium*-mediated transformation by providing the area for successful attachment of *Agrobacterium* cells. Penetration and aggregation of bacteria via stomata and trichome has been recorded in previous years (Takeuchi and Frank, 2000; Burnett and Beuchat, 2001; Melotto *et al.*, 2008).

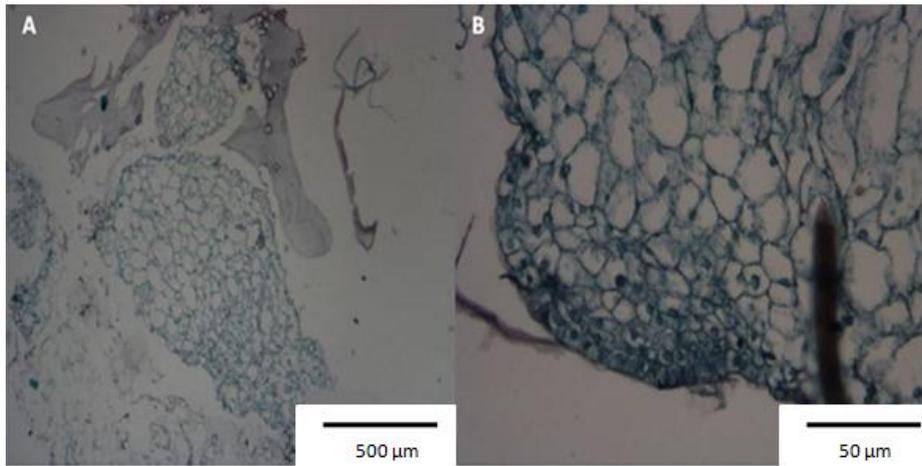


Figure 1: Well developed PLB contained (A) different sizes of cells, and (B) Apical part of PLB.

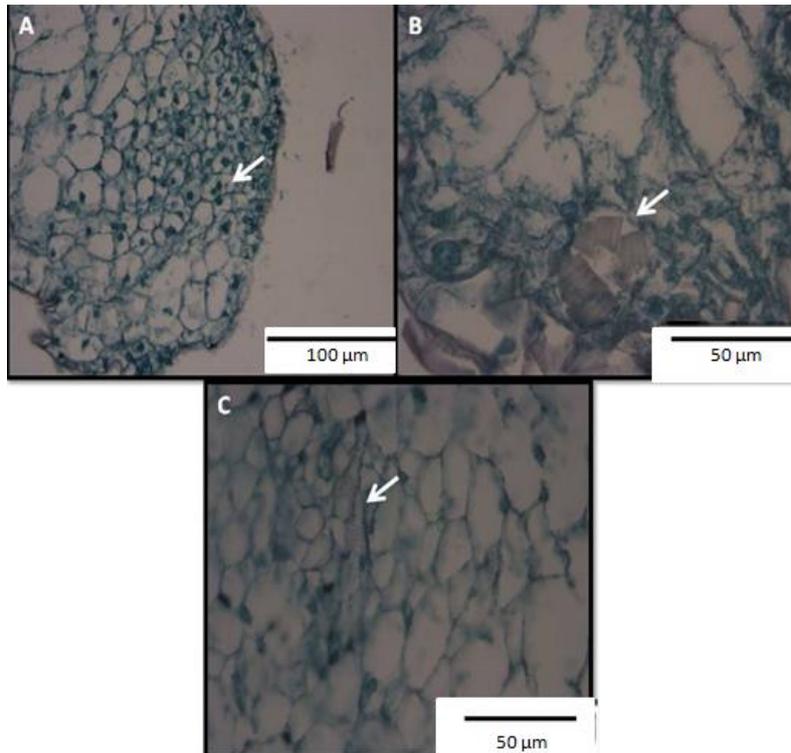


Figure 2:(A) Two nuclei cells suggest an event of active mitosis beneath the epidermis. Arrow indicate actively dividing cell;(B) Arrow indicates a stack of raphids;(C) Arrow indicates vascular bundle which is coil shaped xylem.

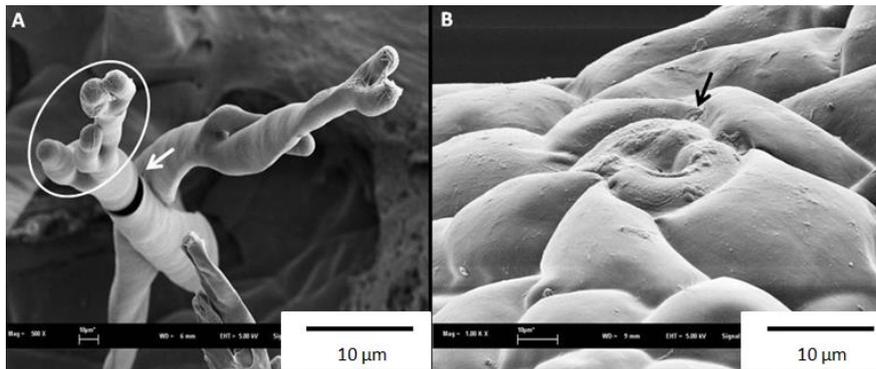


Figure 3: Elongated projections of a cluster of branched trichomes from the surface of the PLB. (A) Arrow indicates trichomes are hollow in cavity. Circle indicates the branched portion of PLB;(B) Arrow indicates the presence of stomata on the surface of PLB.

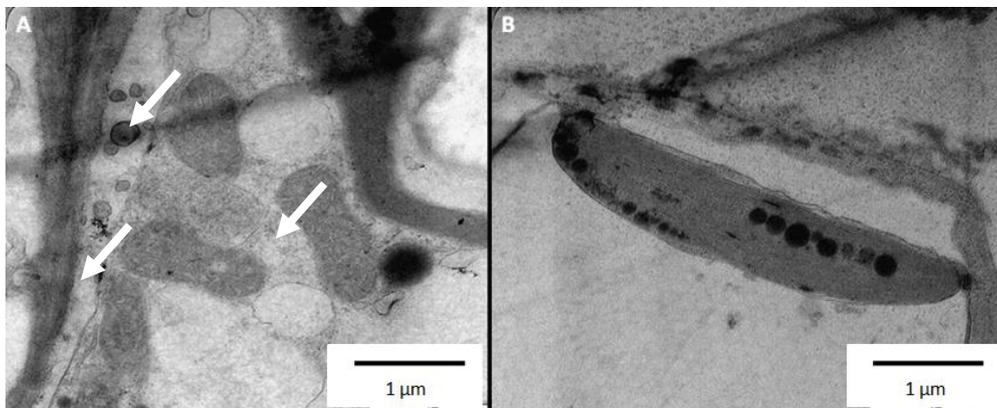


Figure 4:(A) Arrows indicate various shapes of mitochondria with highly folded cristae;(B) Chloroplast lines along the height of the cell wall.

Quantification of *Agrobacterium* attachment on ABB's PLBs

Bacterial attachment to PLB was quantified through spectrophotometric measurement (A_{415}) of GUS expression in *A. tumefaciens* strain LBA 4404. Based on the results, the mean value of attachment of *A. tumefaciens* was higher (83%) compared to the negative control (*Escherichia coli* strain DH5 α) (20%) which is an attachment mutant (Figure 5). Therefore, the spectrophotometric GUS assay used for quantification of bacterial attachment shows the enhanced binding capacity for the attachment-efficient *A. tumefaciens* strain LBA 4404 over *E. coli* strain DH5 α which is attachment mutant. Quantification is a study to measure the attachment of *A. tumefaciens*, by 83% attachment indicates a very positive result which means *A. tumefaciens* attached on ABB's PLBs due to facilitation of ABB's PLBs anatomies and *A. tumefaciens* behaviour as well. This shows that bacteria adhesion onto ABB's PLBs will aid the further studies of genetic modification event.

Microscopical studies of bacterial attachment

SEM observation shows a significant attachment of *A. tumefaciens* onto the surface of ABB's PLBs. The early step was the lateral (Figure 6A) or polar mode (Figure 6B) attachment of the single bacteria to the ABB's PLB surface in increasing number. This is followed by the initiation of formation of fibrils (Figure 6C) to anchor freely moving *A. tumefaciens* to the surface of PLB. Hence a larger number of *A. tumefaciens* will be indirectly bound to the PLB. Aggregates of bacteria could be seen to be held together by extensive networks of fibril (Figure 6D).

TEM analysis also proved the occurrence of *A. tumefaciens* along the cell wall of the cells (Figure 7). This indicates that *A. tumefaciens* have successfully penetrated and colonized internal area of the ABB PLB. There are many factors involved in successful attachment of bacteria on PLBs; such as bacterial density (Underwood *et al.*, 2007), bacterial suspension at log phase and the structure of the plant tissue itself to make a suitable area for the attachment of the bacteria and wounding of explants (Wells and Butterfield, 1997; Dingman, 2000). For example, external structures such

as trichome and stomata will enhance the attachment of *A. tumefaciens* onto the ABB's PLB.

Actively moving *Agrobacterium* cells around the trichomes were detected under the phase contrast microscopy; indicating colonization happens very fast and efficiently. The bacteria absorb certain wavelength that allows them to glow under phase contrast microscope. Hence, the glowing light (Figure 8A) proves the successful colonization of bacteria onto the PLBs. Phase contrast studies of trichome confirmed that this area was extensively colonized by bacteria whereby a cap-like formation was produced by massive aggregation of *A. tumefaciens* strain LBA 4404 on the tip of trichomes of ABB's PLBs (Figure 8B). As stated above, *A. tumefaciens* cells have successfully colonized the crevices at the opening of the stomata as well. The rough surface inside the opening of stomata provides a strong grip for *Agrobacterium* cells to make a strong contact. Same goes to wounding which produce phenolic exudates and also provide an open area for the bacteria to attach. Naturally *A. tumefaciens* infects plants through an open wound. It seeks for phenolic compounds which released from wound and chemotactically moves toward its source (Harighi, 2008; Bhattacharya *et al.*, 2010). The T-DNA is then integrated into the plant's genome and directs the plant cell to synthesize opines (usually nopaline or agropine) which *A. tumefaciens* use as the energy source (Harighi, 2008). In *Agrobacterium*-mediated transformation studies, T-DNA is replaced by a novel gene expressing beneficial effect on the plant such as resistance to diseases, tolerance to environmental challenges or enhancing the aesthetic value.

Chemotaxis Response of *Agrobacterium tumefaciens* LBA 4404

Generally, wounded PLBs show higher bacterial chemotaxis response compared to the unwounded PLBs (Figure 9). After 24 h, bacteria chemotaxis response was hasten towards severely wounded PLBs scoring a ratio of 1.6 unit (positive chemotaxis response since ratio is ≥ 1) showing higher bacteria motility. This proves that *A. tumefaciens* LBA 4404 virulence characteristics to facilitate gene transfer for transformation purpose. Lower chemotaxis response of 1.3 and 1.0 unit (positive chemotaxis response since ratio is ≥ 1) (Figure 9) was observed in mildly wounded and intact PLBs respectively. This shows that there is a significant different between the wounding level intended on the PLBs. The overall chemotactic response of *A. tumefaciens* LBA 4404 was positive to the ABB's PLBs although *Agrobacterium* was noticed to swarm backward away from the ABB's PLBs.

Chemotaxis assay is the study of affinity of *A. tumefaciens* towards plant exudates whether it moves toward or away from chemical attractant or repellent. The capability of bacteria to drive themselves through their surroundings, often in response to chemical and physical gradients, is a significant adaptive mechanism that promotes optimal positioning of cells at microscopic spatial scales. Commonly, motile bacteria move through fluids and along surfaces by using external appendages, including pili and flagella. The biofilm-forming plant pathogen *Agrobacterium tumefaciens* drives swimming motility by utilizing a small group of flagella localized to a single pole or the subpolar region of the cell (Merritt *et al.*, 2007).

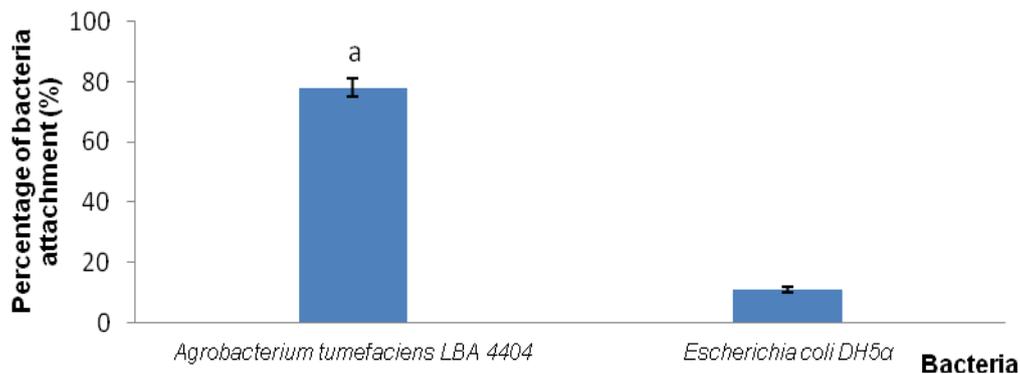


Figure 5: Quantification of *Agrobacterium tumefaciens* LBA 4404 and attachment mutant *Escherichia coli* strain DH5α onto ABB's PLBs. Data were analysed using one-way ANOVA and the differences contrasted using Tukey's multiple comparison test. Different letter represents significant difference at 0.05%.

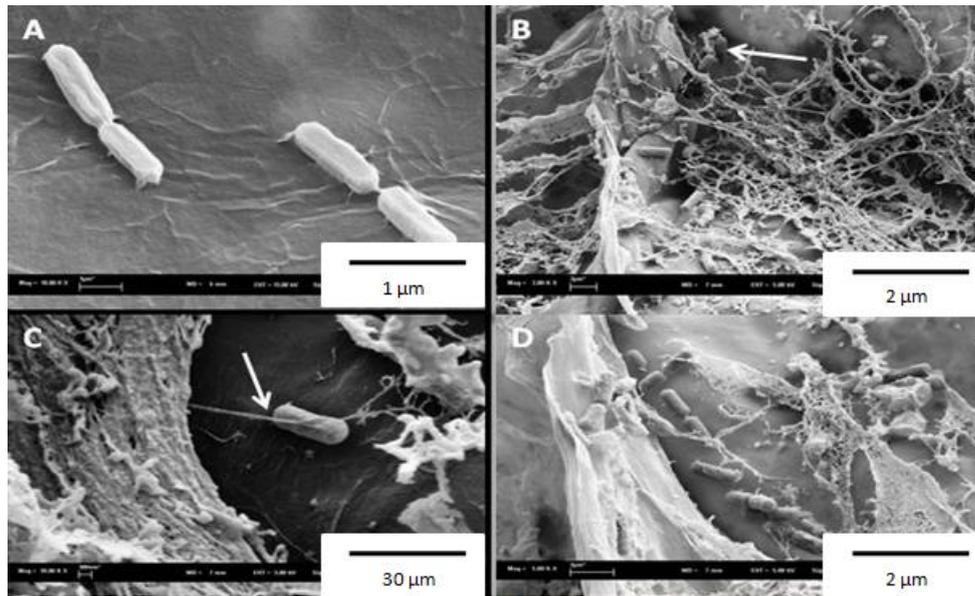


Figure 6: (A) Initial colonization of bacteria includes laterally bound bacteria;(B) Polarly bound bacteria. Arrow indicates *Agrobacterium* bound via polar mode attachment;(C) Initiation of formation of fibrillar net during the massive bacterial attachment;(D) Extensive massive network to form an *Agrobacterium* cluster.

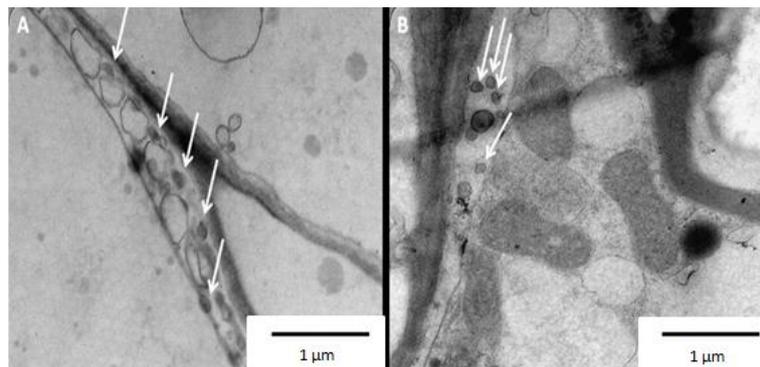


Figure 7: (A, B) *A. tumefaciens* strain LBA4404 successfully colonized the internal area of ABB PLB. Arrows indicate the round shaped structures which are actually *A. tumefaciens* found along the cell wall of the cells.

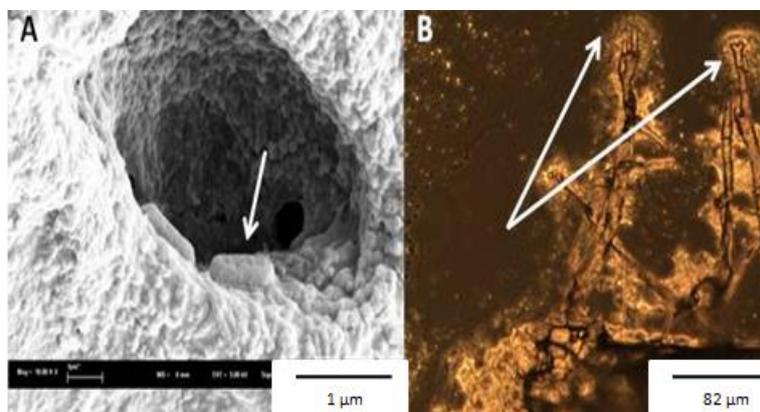


Figure 8: (A) *Agrobacterium* cells laterally bound to the inner surface of stomata;(B) Massive cap-like formation of *Agrobacterium* cells on the tip of trichome of the ABB PLB.

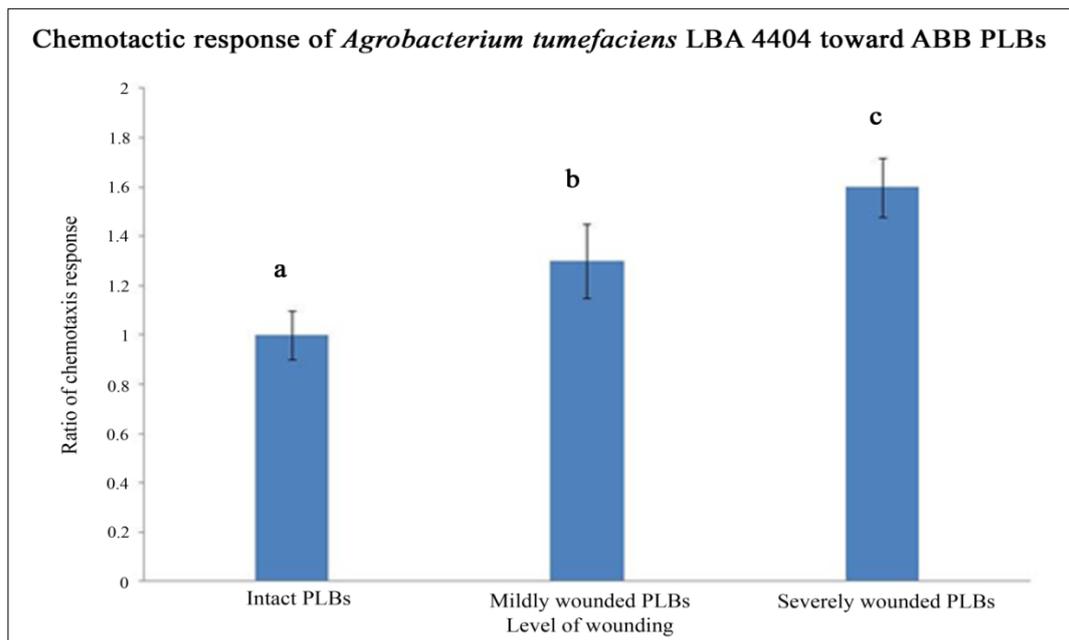


Figure 9: Chemotaxis of *Agrobacterium tumefaciens* LBA 4404 towards different levels of wounding on ABB's PLBs after 24 h. Data were analysed using one-way ANOVA and the differences contrasted using Tukey's multiple comparison test. Different letter represents significant difference at 0.05%.

CONCLUSION

Histology and microscopy recognizes the structures of protocorm-like bodies (PLBs), meanwhile the SEM and TEM are important aspect of this study in order to view the ABB and interaction with *A. tumefaciens*. GUS expression indicates successful attachment and viewed under phase contrast microscope. Quantification of attachment and chemotaxis assay are established according to the standard protocols, supplying enough information on the interaction of *A. tumefaciens* and the host.

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