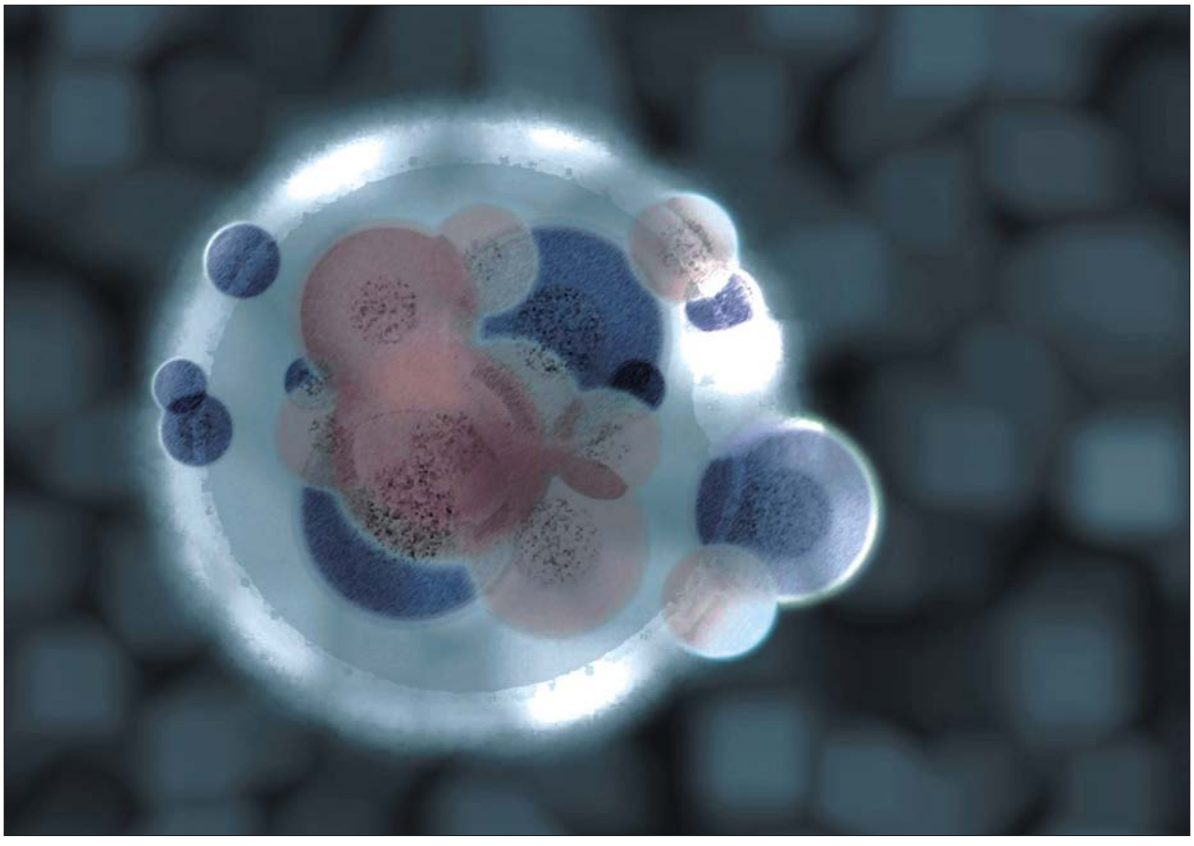

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An Easy Method for *Agrobacterium tumefaciens*-Mediated Gene Transfer to *Nicotiana tabacum* cv. TAPM26

[1]P.C. Kutty, [2]G.K.A. Parveez and [1]F. Huyop
[1]Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering,
Universiti Teknologi Malaysia, 81300 Skudai, Johor, Malaysia
[2]Advanced Biotechnology and Breeding Centre, Biological Research Division,
Malaysian Palm Oil Board (MPOB), P.O. Box 10620, 50720 Kuala Lumpur, Malaysia

Abstract: Research in *Agrobacterium tumefaciens* mediated gene transfer has advanced and opened new avenue for plant improvement via introduction of various beneficial genes. Current study investigates transformation parameters during co-cultivation to improve T-DNA delivery into *Nicotiana tabacum* cv. TAPM 26 by monitoring GUS expression level. The techniques involved basic plant tissue culture and establishment of plant transformation systems. Conditions assessed were bacterial inoculum concentration, infection period, wounding and pre-culture of explants, acetosyringone (AS) concentration and co-cultivation temperature. Optimized conditions resulted in high transformation efficiency at transient level were as follows; *Agrobacterium tumefaciens* growth phase of A_{600nm} 0.8, infection period of 30 min, pre-culture of wounded explants prior to infection, addition of acetosyringone (AS) in bacterial growth culture (100 μM) and in co-cultivation medium (200 μM) and co-cultivation temperature of 22°C. Although higher density bacterial culture used for the infection process gave higher transformation rate, however, it compromised the viability of the explants. On the other hand, dilution of bacterial suspension reduced necrosis in explants and improved transformation events greatly. The transformation efficiency was increased 9 fold when the infection process was carried out at low temperature of 22°C. Current study has proven among the parameters assessed, temperature was the critical factor during co-cultivation process in *Agrobacterium tumefaciens* mediated gene transfer.

Key words: Plant transformation, *bar* gene, *gus* gene, Basta, *Agrobacterium tumefaciens*, phosphinothricin

INTRODUCTION

Agrobacterium tumefaciens mediated transformation has been widely used for research in plant molecular biology and for genetic improvement of crops since the first successful transformation of tobacco (De Block *et al*., 1987). The advantage of this method is the wide host-range including major crops such as soybean, cotton, rice, maize, sugarcane and wheat. Other merits include integration of the small copy number of T-DNA into plant chromosomes and stable expression of transferred genes. However, even with those superior attributes, it is still difficult to achieve high reproducibility and consistency of stable transformation and transformed cells may or may not give rise to gametes that pass genetic material on to subsequent generations. Transformation is a simple task with *E. coli*, but is usually more difficult with multicellular eukaryotes and can be challenging with some important plant species (Bent, 2000). Many physical conditions can be manipulated in order to increase plant's competency towards *Agrobacterium* infection and subsequently to increase *vir* gene expression by improving transformation efficiency.

Some of the practical limitations in plant transformation system arise from gene silencing phenomena as described by Vaucheret (2006) and Matzke *et al.* (2009). The reason was to protect against viruses attack and transposons (Baulcombe and English, 1996). Another possibility associated with the remarkable exploitation of polyploidy in plant evolutionary and developmental processes (Leitch and Bennett, 1997). However, Birch *et al.* (2010) suggested that patchy and progressive transgene silencing maybe associated with increasing endoploidy during maturation of differentiated tissues.

A widely used method to evaluate transformation factors is to measure transient expression of the reporter genes. Theoretically, an increase in transient expression

Corresponding Author: Dr. Fahrul Huyop, Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia Tel: +60 7 5534556 Fax: +60 7 553 1112

of transferred gene indicates higher chances of stable transformation events. β-glucoronidase (GUS) reporter gene system was used as screenable marker in plant transformation studies (Jefferson, 1987). It is advantage to use GUS reporter system since transformed plant cells are easily detected at an early stage of the transformation process. In such effort, parameters of co-cultivation conditions in *Agrobacterium tumefaciens*-mediated transformation were evaluated to determine the most effective conditions to increase transient expression level by monitoring GUS expression in co-cultivated explants. Therefore, combined and modified several existing methods were used to produce an easy to perform and reproducible protocol.

Current study aimed at evaluating an easy and reproducible *Nicotiana tabacum* transformation method using *Agrobacterium tumefaciens* by identifying physical factors affecting co-cultivation procedures. The co-cultivation parameters tested were *Agrobacterium tumefaciens* growth phase for inoculum preparation, infection period, wounding of the explants, pre-culture of explants prior to infection process, addition of acetosyringone (AS) in inoculum and co-cultivation medium and the less explored parameter of co-cultivation temperature. The effect of AS was evaluated on both *Agrobacterium* growth culture and also in co-cultivation medium. Optimal conditions of these parameters differ for different species or even different cultivars of plants and *Agrobacterium* strains. Therefore, it is highly essential to standardize co-cultivation conditions for each protocol of different type of plants, explants and bacterial strains used.

MATERIALS AND METHODS

Bacterial strain and plasmid: Bacterial stock cultures of *Agrobacterium tumefaciens* strain LBA 4404 harbouring pCambar was kindly provided by Dr. Norwati Adnan, Forest Research Institute of Malaysia (FRIM), Kepong, Malaysia. pCambar, originally derived from pCambia 1301 (CAMBIA, Canberra, Australia), contains *nptII* gene encoding resistance to kanamycin and *bar* gene encoding resistance to phosphinothricin (PPT), which were used for bacterial selection and plant selection respectively (Fig. 1). Both selection genes are driven by a double enhancer version of the CaMV35S promoter. The construct also contains *E. coli gus*A reporter gene as a visual marker in regenerated plants, detectable by GUS assays. An intron (cloned from castor bean catalase gene) present in the coding sequence of *gusA* gene to ensure expression of β-glucoronidase activity is derived from

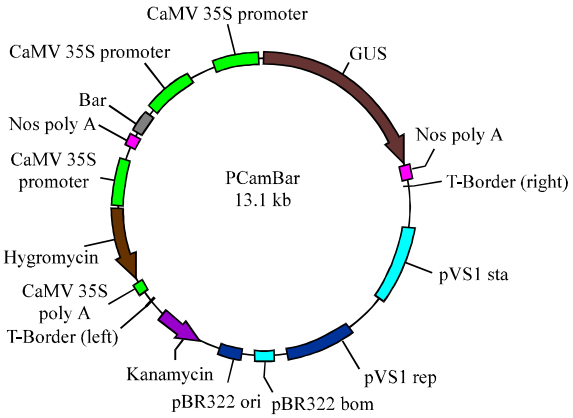

Fig. 1: The plasmid used in this study (pCamBar)

eukaryotic cells and not from expression by residual *Agrobacterium tumefaciens* cells left on infected explants.

Bacterial growth conditions: The *Agrobacterium* strain LBA4404 harbouring pCamBar was grown in liquid YEP medium as described by Sambrook *et al*. (1989) containing 10 g yeast extract, 10 g peptone and 5 g NaCl L^{-1}. The medium was also supplied with 100 mg L^{-1} kanamycin (SIGMA) and 100 mg L^{-1} streptomycin (SIGMA). The culture was incubated at 28°C on rotary shaker at 200 rpm. Bacto agar (1.5% (w/v)) was used for solid medium. pH of the medium was adjusted to 7.0 prior to sterilization via autoclaving.

Plant material: Seeds of *Nicotiana tabacum* cv. TAPM 26 were obtained from the National Tobacco Board, Kota Bharu, Kelantan, Malaysia. Seeds were surface sterilized by immersing in 20% (v/v) of CLOROX® (commercial bleach with 5.25% of sodium hypochlorite as active ingredient) for 20 min with gentle agitation. The seeds were then thoroughly rinsed several times with sterile double distilled water. Sterilized seeds were placed on MS (Murashige and Skoog, 1962) solid medium containing 0.1 g myo-inositol, 30 g sucrose and 2.7 g phytagel per liter for germination process. pH of the medium was adjusted to 5.7-5.8. Petri dishes (Brandon™) with 10 seeds per plate (90×15 mm) were incubated on tissue culture chamber at 25°C under a 16/8 h light/dark photoperiod with light intensity of 12.16 μmol/m^2/sec. After an incubation period of 5 to 6 weeks, young, green and expanded leaves were harvested from *in vitro* grown seedlings to be used as explants for all transformation experiments.

Preparation of bacterial inoculum: *Agrobacterium tumefaciens* strain LBA 4404 from glycerol stock culture

was streaked onto solid YEP medium containing 100 mg L^{-1} kanamycin and 100 mg L^{-1} streptomycin and incubated at 28°C for 2 days. A single colony was inoculated into 2 mL of YEP culture medium overnight at 28°C with agitation of 200 rpm. Overnight culture was then added into 30 mL of liquid YEP medium. Bacterial culture from this medium, were harvested by centrifugation (Zentrifugen) for 15 min at 5000 g. Pellet obtained was re-suspended in liquid MS medium. The whole bacterial pellet was not used to infect leaf explants due to extreme loss of viability of explants and bacterial overgrowth. A modified method involved using 1 mL of bacterial pellet re-suspended in 10 mL liquid MS medium (10x dilution) as inoculum for infection of explants was used to overcome the above viability and overgrown problems.

Evaluation of transformation factors

The effects of bacterial growth phase and infection period: In order to assess the effect of *Agrobacterium tumefaciens* growth phase on the transient expression, bacteria cells were harvested at different growth phases, which ranged from A_{600nm} 0.6, 0.8, 1.0 and 1.5. A total volume of 16 mL of liquid culture was used to infect *Nicotiana tabacum* leaf explants from 4-6 weeks old seedlings. The explants were co-incubated with bacterial suspension at room temperature. The infection period was allowed to last for 15, 30 or 45 min separately in order to evaluate the effects of different infection periods on the transformation process.

After the infection process, explants were retrieved from infection medium and blotted on sterile filter papers (Advantec) to remove excess bacterial suspension. The explants were then placed on co-cultivation medium (solid MS medium with 4 mg L^{-1} BAP) and maintained in a tissue culture chambers at 25°C in dark for two days. Two days later, the explants were gently rinsed using sterile distilled water to remove excess agar and subjected to GUS histochemical analysis. The most effective treatments/parameters were used in subsequent evaluation experiments.

The effects of wounding procedure: The effects of wounding the explants on transformation efficiency were evaluated by comparing the wounded explants to the explants which were left intact (unwounded). The wounding procedure involved cutting the margins of the leaves without removing the petiole and making small pricks (approximately 10 to 15 times) on the surface of the leaves using sterile scalpel blade. Unwounded leaves were used as control explants. The wounded and unwounded explants were co-incubated with *Agrobacterium tumefaciens* inoculum from A_{600nm} 0.8 culture, over 30 min exposure and co-cultivated for two days in dark at 25°C. After co-cultivation process, the explants were rinsed using sterile distilled water and subjected to GUS histochemical analysis. The most effective treatments/parameters were used in subsequent evaluation experiments.

The effects of explants pre-culture: Pre-culture process consisted of incubating wounded leaf explants in MS medium for 2 days (Sunilkumar *et al*., 1999) at room temperature before the infection process, whereas freshly wounded leaves were used as explants in control experiments. Pre-cultured explants and the control explants were co-incubated with bacterial suspension and later co-cultivated as in the conditions mentioned earlier. GUS histochemical analysis was conducted on explants after 48 h to determine the most effective treatments/parameters.

The effect of acetosyringone: Two separate experiments were conducted to evaluate the effects of adding acetosyringone (AS) in bacterial inoculum (for the infection process) and in co-cultivation medium, on transformation efficiency.

- **The effects of inclusion of AS in bacterial inoculum:** Overnight culture (1 mL) of *Agrobacterium tumefaciens* LBA 4404 was used to inoculate 30 mL of YEP medium supplemented with 100 mg L^{-1} kanamycin and 100 mg L^{-1} streptomycin and different concentration of AS at 0, 100, 200, 300 and 400 μM. Cultures were grown at 28°C for 2 h until A_{600} of 1. Cells were pelleted by centrifugation and were re-suspended in 10 mL YEP liquid medium. Then, 1 mL of the suspension was later used for the infection of leaf explants. After co-cultivation period, the explants were subjected to GUS histochemical analysis to determine the effects of AS on transformation efficiency
- **The effects of inclusion of AS in co-cultivation medium:** The explants were placed on co-cultivation medium for 2 days in the dark at 25°C. The co-cultivation medium made of solid MS medium consisted of 4 mg L^{-1} BAP, 0.1 mg L^{-1} IBA and various concentration of AS at 1, 100, 200, 300 and 400 μM. Co-cultivation medium without AS was used as control. GUS histochemical analysis was conducted to determine the most effective treatments/parameters

The effects of co-cultivation temperatures: The infected leaves were co-cultivated in the dark at different

co-cultivation temperatures of 22, 25 and 28°C. The most effective co-cultivation temperatures were determined using GUS histochemical analysis.

GUS histochemical analysis: Histochemical assay of the *gus* gene expression was carried out according to Jefferson (1987). Histochemical analysis was performed on explants infected after 2 days of co-cultivation period. The analysis was carried out by incubating the explants in GUS substrate buffer at 37°C for 18-24 h. Explants were immersed in ethanol 70% (v/v) to remove chlorophyll for ease of visualization. Each blue spot indicating GUS activity was counted using stereomicroscope (Leica, model S6D), irrespective of its size.

Data analysis: Each treatment in this study consisted of three replicates and each replicate consisted of at least 10 explants. All data were subjected to Analysis of Variance (ANOVA) statistical test using SPSS software version 15.0. The means were compared for significant differences at $p<0.05$ level. All experiments were repeated at least thrice.

RESULTS

Bacterial growth phase and infection period: *Agrobacterium tumefaciens* LBA 4404 inoculums prepared from various stages of growth and concentrations, ranged from A_{600nm} 0.6-1.5, were assessed at different infection periods of 15, 30 and 60 min. Results were presented in Table 1. The highest number of blue spots were detected using A_{600nm} 1.0 culture (0.63±0.09), followed by A_{600nm} 0.8 (0.43±0.10), whereas at A_{600nm} 0.6, showed the lowest GUS activity. It was also evident that as the bacterial concentration increased to A_{600nm} 1.5, the transient activity and transformation frequency decreased, especially with prolonged infection period. Even though higher number of spots were found at A_{600nm} 1.5 compared to A_{600nm} 0.6, the outcome was statistically insignificant at $p<0.05$ level.

As for infection periods, the level of transient activity was found to increase when the explants have been infected for more than 15 min. However, 60 min of infection period was proven to be detrimental as demonstrated by the increased frequency of necrotic explants during 60 min of infection period, especially at higher bacterial concentrations. Prolonged infection period also resulted in reduced transformation efficiency. This indicated the explant viability has a direct effect on transformation efficiency. Consequently, A_{600nm} 1.0, which gave highest transformation frequency (63.3%) was regarded as not suitable for subsequent experiments as it had higher necrotic events than that of A_{600nm} 0.8. Moreover, the mean difference for transformation frequency between A_{600nm} 1.0 with 30 min of infection period and A_{600nm} 0.8 with 30 min of infection period were found to be statistically insignificant.

On the other hand, A_{600nm} 0.8 with an infection period of 30 min had little chance (1.1%) of inducing necrotic symptoms in leaf explants, in addition to high transformation frequency (43.3%), which was significantly higher than that of A_{600nm} 0.6 and 1.5. Therefore, A_{600nm} 0.8 with an infection period of 30 min was chosen to be used in subsequent experiments.

Wounding and pre-culture of explants: The effect of wounding explants prior to infection with *Agrobacterium tumefaciens* cells were evaluated by comparing wounded explants with intact (non-wounded) explants for transient GUS activity. Wounded explants showed significantly higher transient GUS expression (spots) than that of non-wounded explants. Sixty percent of GUS positive explants were obtained by wounding the explants prior to the infection process. In another experiment, the effect of pre-

Table 1: Effects of bacterial concentrations and infection periods on transient GUS expression and explants' viability

Bacterial density (A_{600nm})	Infection period (min)	No. of spots (Mean±SE)	Percentage of GUS positive explants	Necrotic explants (Mean±SE)	Percentage of necrotic explants
0.6	15	0.00 ± 0.00^{a}	0.0	0.00 ± 0.00^{a}	0.0
	30	0.03 ± 0.03^{a}	3.3	0.00 ± 0.00^{a}	0.0
	60	0.03 ± 0.03^{a}	6.7	0.67 ± 0.33^{a}	2.2
0.8	15	0.17 ± 0.07^{abc}	16.7	0.00 ± 0.00^{a}	0.0
	30	0.43 ± 0.10^{bcd}	43.3	0.33 ± 0.33^{a}	1.1
	60	0.43 ± 0.09^{bcd}	43.3	1.67 ± 0.33^{ab}	5.6
1.0	15	0.53 ± 0.12^{cd}	53.3	5.67 ± 0.88^{bc}	18.9
	30	0.63 ± 0.09^{d}	63.3	9.67 ± 0.88^{cd}	32.2
	60	0.57 ± 0.08^{d}	56.7	16.33 ± 1.45^{e}	54.4
1.5	15	0.27 ± 0.07^{abcd}	30.0	12.67 ± 1.20^{de}	42.2
	30	0.17 ± 0.06^{abc}	23.3	23.67 ± 1.45^{f}	78.9
	60	0.19 ± 0.25^{ab}	13.3	29.67 ± 0.33^{g}	98.9

Data within the same column followed by the same letter indicated no significance at 5% level

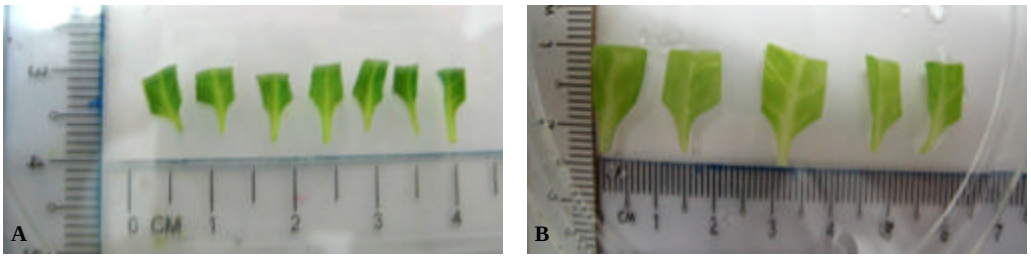

Fig. 2: The appearance of explants (A) before and (B) after 24 h of pre-culture. B shows 2 fold expansion of explants in size after 2 days of pre-culture

Table 2: The effects of acetosyringone on transient GUS expression

	Bacterial inoculum		Co-cultivation medium	
Concentration of acetosyringone (μM)	No. of spots (Mean±SE)	GUS positive explants (%)	No. of spots (Mean±SE)	GUS positive explants (%)
0	2.93±0.30[a]	90.0	18.53±0.53[a]	100.0
100	19.27±0.46[b]	100.0	37.63±1.32[b]	100.0
200	18.23±0.48[b]	100.0	89.00±1.38[c]	100.0
300	18.53±0.77[b]	100.0	84.63±2.64[c]	100.0
400	18.10±0.72[b]	100.0	40.67±2.75[b]	100.0

Data within the same column followed by the same letter indicated no significance at 5% level

Table 3: The effects of different co-cultivation temperatures on transient GUS expression

Co-cultivation temperature (°C)	No. of spots (Mean±SE)	GUS positive explants (%)
22	951.30±46.64[a]	100.0
25	94.20±4.75[b]	100.0
28	32.30±3.29[b]	100.0

Data within same column followed by the same letter indicated no significance at 5% level

culture of wounded explants for 48 h was tested for GUS activity and was compared with explants that were infected directly after the wounding procedure. An expanded in size was observed in explants pre-cultured, which might be an indication of active cell division (Fig. 2A, B). Not only more pronounced GUS activity (larger spots) was found, the transformation frequency reached almost 100% for the pre-cultured explants. Therefore, all the explants used in subsequent experiments were wounded and pre-cultured for 2 days prior to the infection process with *Agrobacterium tumefaciens* culture.

Addition of acetosyringone in bacterial culture and co-cultivation medium: To investigate whether different level of sensitivity is exhibited by LBA 4404 toward different concentrations of AS (thereby yielding different level of transformation frequency), five different concentration of AS were included in bacterial inoculum for infection process. Table 2. showed that, addition of AS into bacterial inoculum yielded significantly higher transient GUS expression as compared to the control. A hundred percent of transformation frequency was observed for all the experiments, except for the control. However, increasing concentrations of AS from 100 to 400 μM showed no effect on GUS activity as supported by statistical analysis. Therefore, AS concentration of 100 μM was chosen for all subsequent experiments.

The influence of AS on transient transformation efficiency was also evaluated by adding AS in varying concentration to the co-cultivation medium. Different concentrations of AS produced various effects on transient GUS activity, as shown in Table 2. The control medium, without AS, gave significantly lower level of GUS expression (18.53±0.53) as expected, whereas, highest level of transient GUS expression was obtained at AS concentration of 200 μM, followed by 300 μM. However, at 400 μM, AS induced low level of GUS expression and found to have no significant difference in mean number of GUS expression as compared to 100 μM AS concentration. This observation could be considered as an indicator of adverse effect of high concentration of AS on the transformation process. Based on the above results, AS concentration of 200 μM was used in all experiments.

Co-cultivation temperature: Evaluation of different co-cultivation temperatures on transient expression of GUS revealed that co-cultivation at 22°C gave the highest level of GUS expression as compared to the other two co-cultivation temperatures. In Table 3, explants co-cultivated at 22°C had 951.30±46.64 blue spots per explant, which is 9 times higher than the transient GUS expression level obtained in previous experiment on the effect of AS (89.00±1.38). As compared to co-cultivation temperature

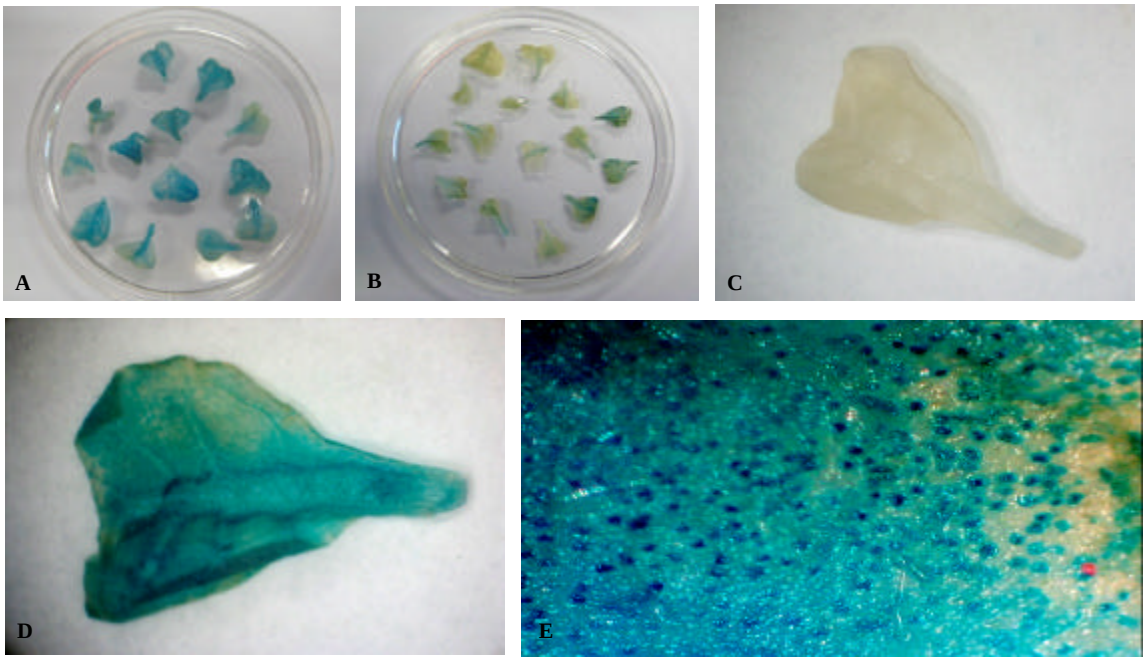

Fig. 3: A set of representative explants used for evaluation of temperatures are shown. (A) Explants co-cultivated at 22°C expressing high level of transient expression. (B) Explants co-cultivated at 25°C expressing moderate level of transient GUS activity. (C) Uninfected control explant. (D) Infected explant co-cultivated at 22°C. (E) A close up image of blue spots found in the explant in D

of 22°C, the other two temperatures produced significantly lower level of GUS expression. Although numerical differences were observed between the two temperatures of 25 and 28°C, in which transient expression of GUS at 28°C was almost three times lower than at 25°C, these were not found to be significantly different. Apart from that, explants co-cultivated at 28°C showed necrotic symptoms which may have been responsible for the level of GUS expression observed. Based on the results obtained, co-cultivation temperature at 22°C was determined to be the most effective and was used for all transformation processes. A representative explants used in co-cultivation temperature evaluation was shown in Fig. 3A-E.

DISCUSSION

Reporter genes such as GUS, plays an utmost important role in developing and optimizing transformation protocols for plants. Efficiency of DNA delivery into intact plant explants can be conveniently measured by recording number of cells which transiently expresses an incoming reporter gene (Batra and Kumar, 2003; Dundar, 2008). Various factors with possible influence on the efficiency of T-DNA delivery and its expression were evaluated via the transient GUS expression to explore the ways by which genetic transformation mediated by *Agrobacterium tumefaciens* strain LBA 4404 can be improved further for example a simple protocol was developed in ripe fleshy fruit (Spolaore *et al*., 2001). Transformation factors evaluated consisted of bacterial concentration, infection period, wounding of explants, explant pre-culture prior to infection, addition of acetosyringone (AS) and co-cultivation temperature.

It has been considered in many studies that *Agrobacterium tumefaciens* growth phase or density at different level may affect transformation efficiency. Different concentrations of *Agrobacterium tumefaciens* have been used in many transformation studies for different plant materials. In addition, different strains of *Agrobacterium* used in transformation study and type of cultivars also could influence the regeneration and transformation of plants (Surekha *et al*., 2007; Shahriari *et al*., 2006). High concentrations of *Agrobacterium tumefaciens* have proven to be more efficient in transforming plants which were previously considered recalcitrant to *Agrobacterium* mediated

transformation such as in rice (Chan *et al*., 1992), sweet potato (Gonzalez *et al*., 2008) and pepper (Ismail *et al*., 2006).

For other plant species, low concentration of bacterial cells from log or mid phase have been more favourable such as in Brocolli (Metz *et al*., 1995) and wheat (Cheng *et al*., 1997). Clough and Bent (1998), claimed that different bacterial density resulted in different transformation efficiency of *Arabidopsis* and the best responses were obtained at A_{600nm} 0.8. Similar findings have been reported by Anuradha *et al*. (2006) in peanut transformation, which coincide with the findings of the current study.

Bacterial cells collected at A_{600nm} 0.8 growth phase was found to be most effective in producing high transient GUS activity; however, bacterial cells harvested at this stage, were diluted to 1:10 before infection of explants, as undiluted culture caused problems such as induction of necrotic symptoms in explants and bacterial overgrowth during co-cultivation period. Similar approached was used in a study reported by Chakrabarty *et al*. (2002), in which bacterial inoculum was diluted before the infection process. Although higher densities of cells could increase transient expression, it may damage the explants severely which in turn resulted in lower cell recovery and lower stable transformation. Nevertheless, if high density bacterial cells are necessary, especially for recalcitrant plant species, transformation frequency along with viability of explants can be improved via methods such as short infection period, diluting inoculum for infection process or antinecrotic pre-treatments of explants could be adapted. Effect of infection duration of explants with *Agrobacterium* suspension was another parameter investigated in this study. Extending the infection period increased the transformation efficiency in terms of GUS expressing spots, however bacterial overgrowth greatly decreased the explants' viability. Infection period of 30 min was found to be most favorable for transformation efficiency and explants viability.

In this study, wounded explants were found to have significantly higher transient GUS expression than the non-wounded control explants. This shows that wounded cells were more susceptible to infection by *Agrobacterium tumefaciens* strain LBA 4404 rather than intact cells (Song and Sink, 2005; Sumilkumar *et al*., 1999). The actively dividing cells resulting from wound induced cell division were found to be more prone to transformation, whereas newly synthesized cell wall was found to be essential for the productive attachment of *Agrobacterium* transformation (Binns, 1991).

Apart from the initiation of rapid cell division, another significance of wounding was, releasing *vir* inducing phenolic compounds (Stachel *et al*., 1985; Joubert *et al*., 2002). These *vir* genes are located on tumour inducing plasmid (Ti) of *Agrobacterium* and activation of these *vir* genes is required for the T-DNA transfer (Hoekema *et al*., 1983; Zupan *et al*., 2000; Ziemienowicz *et al*., 2001). According to Stachel *et al*. (1986), wounded tobacco cells had 10 fold more acetosyringone, a phenolic compound, than the uninjured cells.

Transient GUS expression of tobacco leaf explants were further enhanced by the procedure of pre-culture for 48 hours. Explants which were pre-cultured after the wounding procedure had higher GUS expression than the explants which were subjected to the infection process directly after wounding. This finding was comparable to those of reported for sour cherry (Song and Sink, 2005) and tobacco (Sumilkumar *et al*., 1999). According to Sumilkumar *et al*. (1999), production of *vir* gene inducers by the wounded tobacco cells during pre-culture period is an important factor that had contributed to the increased transformation efficiency. Pre-culturing wounded explants prior to infection is an effective method to induce rapid cellular dedifferentiation and also a way of rejuvenating the explants. Newly formed juvenile plant cells are more susceptible to *Agrobacterium* infection due to the weakened pathogen recognition ability which is attributed to the perturbation of membrane structure (Sangwan *et al*., 1992). By delaying or reducing the plant defense level, necrotic events in explants could be prevented to achieve higher transformation efficiency.

Addition of AS (100 μM) into bacterial inoculum gave higher GUS expression than the control bacterial inoculum without AS. However, increased concentration of AS had no significant effect on the transient expression than the expression level found at 100 μM. It is likely that *Agrobacterium* cells may have been induced maximally towards virulent stages at 100 μM concentration of AS. In contrary, addition of AS into co-cultivation medium gave varying responses in terms of transient expression. Concentration at 200 μM was proven most effective while higher concentration of AS resulted in reduced transient expression. This result indicates that at high concentration, AS may have a detrimental effect on the explants' responses to the transformation process. According to Gonzalez *et al*. (2008), the optimal concentration of AS varied according to genotype and cultivar of plant and therefore it is recommended to take into consideration the chosen cultivar and its responses and sensitivity towards different concentration of AS to achieve high transformation efficiency.

Apart from its ability to induce virulence in *Agrobacterium*, AS has been reported to have bioactive properties that influence early events in plant pathogen pathogenesis (Baker *et al*., 2005), which reduce the plant's defense mechanism. Plants attacked by pathogens, have been found to activate defense mechanism. The defense responses included either hypersensitive response or the production of antimicrobial secondary metabolites, phytoalexins and pathogenesis related proteins or wound healing or repairs of the damage (Bowles, 1990). Some of the defense responsive genes are active in the site of wound and in non-damaged parts prevent the pest from spreading. Events such as hypersensitive response with cells around the infected area died, could lead to lower efficiency of *Agrobacterium* transformation. Therefore, it is essential that optimal concentration of AS is included in the transformation protocols to moderate the detrimental effect of hypersensitive response.

The effect of temperature during co-culture on T-DNA delivery was first reported in dicot plants (Opabode, 2006). In this study, three different co-cultivation temperatures were investigated. At co-cultivated temperature of 22°C, showed higher number of blue spots. Similar finding was also reported by Dillen *et al*. (1997), where optimal T-DNA delivery for tobacco transformation was found at 22°C. However, another study on tobacco transformation revealed that co-cultivation temperature at 25°C led to highest number of transformed plants of tobacco, even though 19°C was found optimal for T-DNA delivery (Salas *et al*., 2001). Efficient DNA delivery into plant cells at 22°C, has also been found in sweet potato (Gonzalez *et al*., 2008), cotton (Sunilkumar and Rathore (2001) and cauliflower (Chakrabarty *et al*., 2002). Early studies on *Agrobacterium* infection mechanism by Braun (1947) revealed that crown gall tumour which is induced by wild type *Agrobacterium*, increased in size with decreased temperature during infection. Another study found that Ti plasmids were lost in *Agrobacterium tumefaciens* when the culture was grown over 36 h at elevated temperatures (Watson *et al*., 1975). Low temperatures from 20 to 22°C, were found to promote pilus assembly in *Agrobacterium* cells, which leads to an increased number of pili on the cell surface. This is probably influenced by enhanced functioning of the *VirB* gene at low temperature which is required for conjugal transfer of T-DNA into plant cells (Fullner *et al*., 1996). From this studies the result obtained revealed that the co-culture temperature of 22°C can positively influence transformation efficiency.

In conclusion, an easy and useful *Agrobacterium tumefaciens* transformation efficiency was optimized by evaluating of various important parameters. The evaluated factors consist of 1:10 dilution of A_{600nm} 0.8 bacterial density as infection inoculum, infection period of 30 min, pre-culturing of wounded explants for 48 h, addition of AS into bacterial inoculum at 100 μM, addition of AS into co-cultivation medium at 200 μM and co-cultivation temperature of 22°C. However, current transient studies may not always directly lead to stable transformation but they were proven to be critical factors in increasing the transformation efficiency. Factors like appropriate concentration of selection agents, effective regeneration system and proper elimination of *Agrobacterium* cells after co-cultivation also plays complementary important role in recovery of transgenic plant.

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