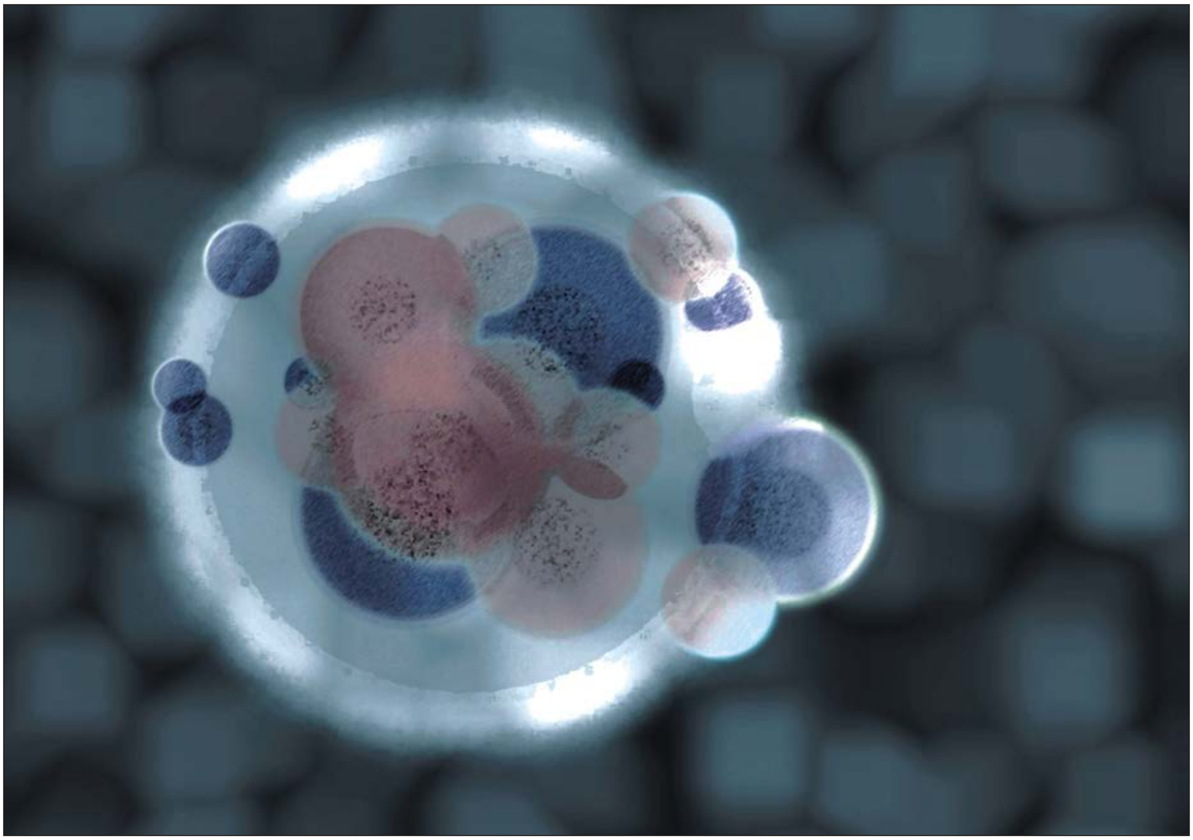

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Investigation of Factors in Optimizing *Agrobacterium*-Mediated Gene Transfer in *Citrullus lanatus* cv. Round Dragon

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Abstract: *Agrobacterium tumefaciens* strain LBA 4404 harboring a binary plasmid pCambar (containing Basta resistance (*bar*) gene, neomycin phosphotransferase (*npt II*) gene and β-Glucuronidase (GUS) gene) was used to optimize the transformation efficiency in *Citrullus lanatus* cv. Round Dragon. In this study, the ability of *Agrobacterium tumefaciens* to mediate a gene transfer in *Citrullus lanatus* was highly dependent on various transformation factors. In current investigation, we have established factors influencing gene expression including explant ages of cotyledon- (5 days old cotyledon), pre-culture condition (2 days), wounding technique (multi wired with massive wounding technique), *Agrobacterium* concentration ($A_{600\ nm}$ 0.8), co-incubation period of explants with *Agrobacterium* (30 min) and acetosyringone concentration (200 μM) in co-cultivation medium. These factors gave maximized transformation efficiency. The expression of the foreign functional gene in the plant genome was confirmed by histochemical GUS assay activity after 3 days of co-cultivation period. By combining the best conditions from each evaluated factors, we successfully established an efficient and reproducible *Agrobacterium*-mediated transformation protocol for *Citrullus lanatus* cv. Round Dragon which yielded 100% of transgene expression with 181.18±0.57 blue spots per responding explant. In conclusion, the transformation system for *Citrullus lanatus* cv. Round Dragon was optimized. The transformation procedure was proved to influence the transformation efficiency in *Citrullus lanatus* at 100% transient expression. Present study also suggested that different plant varieties may affect the transformation rate. The optimized conditions will then be used in future research to make a transgenic plant.

Key words: *Agrobacterium tumefaciens*, *Citrullus lanatus*, transformation factors, transgene expression, GUS gene

INTRODUCTION

Genetic transformation has become a powerful tool for crop improvement in introducing desirable foreign genes into the plant genome. Among techniques used for the introduction of foreign genes in plants, *Agrobacterium tumefaciens*-mediated transfer remains the most popular and efficient in compatible plant species. Successful transfer of foreign genes by *Agrobacterium tumefaciens* have been achieved with banana (Ganapathi *et al*., 2001), rice (Chern *et al*., 2001) and white pine (Levee *et al*., 1999). Other findings has also been devoted to the genetic transformation of *Citrullus lanatus* via *Agrobacterium tumefaciens* using cotyledon as explant (Choi *et al*., 1994; Akashi *et al*., 2005; Park *et al*., 2005). However, Cho *et al*. (2008) suggested that establishment of genetically-engineered herbicide resistant *Citrullus lanatus* has been met with only limited success at present, since *Citrullus* species is known as one of the most recalcitrant plants and highly depends on the efficiency of *Agrobacterium*-mediated transformation protocol.

Previous studies on genetic improvement of crops clearly demonstrated the importance of various factors such as *Agrobacterium* strain (Wenck *et al*., 1999; Saharan *et al*., 2004), type and age of explant (Song and Sink, 2005), pre-culture period (Barcelo *et al*., 1998; Agarwal *et al*., 2004), wounding techniques (Velde *et al*., 2003), bacterial concentration (Wenck *et al*., 1999), pre-incubation period (Yong *et al*., 2006), effect of acetosyringone (Wenck *et al*., 1999; Lievrea *et al*., 2005) and co-cultivation period (Steffen *et al*., 1986; Cardoza and Stewart, 2003). With respect to *Citrullus lanatus* transformation via *Agrobacterium*-mediated transformation, evaluation and optimization of transformation factors plays a crucial role in order to yield high transformation efficiency. However, the systematic optimization of *Agrobacterium*

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tumefaciens-mediated transformation factors has been least reported in *Citrullus lanatus*. At present, only Akashi *et al.* (2005) focused on optimization of transformation factors in *Citrullus lanatus* with regard to *Agrobacterium* strain, bacterial concentration, pre-incubation period and effect of acetosyringone. Due to the limitation of transformation factors in *Citrullus lanatus*, more attention is required to optimize transformation factors in order to yield high *Agrobacterium*-mediated transformation efficiency.

Therefore, in this study, various factors affecting *Agrobacterium*-mediated genetic transformation will be evaluated to improve the transformation frequencies. As a result, successful T-DNA integration into the plant genome via *Agrobacterium*-mediated transformation will be detected via transient expression of *gusA* gene and led to an easy, stable and effective protocol of *Agrobacterium tumefaciens*-mediated transformation of *Citrullus lanatus* using cotyledon as explants.

MATERIALS AND METHODS

Explant preparation for transformation: *Citrullus lanatus* seeds imported from Taiwan were purchased from local supplier ACE Seed Trading (Malaysia) Sdn. Bhd. Decoated seeds were surface sterilized with 5% (v/v) sodium hypochlorite followed by five successive rinses with distilled water. Then, the sterile seeds were subjected for germination on basal MS medium supplemented with 20 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 3.2 g L^{-1} phytagel for 5 days in a tissue culture chamber at 25±1°C with 16 h photoperiod under 12.16 μmol/m^2/sec/ from cool white fluorescent lamps. At day 5, *in vitro* germinated cotyledon explants were excised into two halves and the distal portions were discarded. The proximal region of 5-day-old seedlings was used for the subsequent *Agrobacterium tumefaciens*-mediated transformation experiments.

Bacterial strains and plasmids: *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary plasmid pCambar was used in the transformation experiments. The binary vector pCambar carries the Basta resistance gene (*bar*) as a selectable marker that confers resistance to herbicide Basta, *uid* A gene encoding for β-glucuronidase (*gus*) as a reporter gene (located at the right border of T-DNA region) with a castor bean catalase intron and *npt II* gene coding for neomycin phosphotransferase (located outside the T-DNA region) confers resistance to kanamycin, each driven by the cauliflower mosaic virus (CaMV 35S) promoter and NOS (nopaline synthase) terminator which provides polyadenylation signal. The binary vector pCambar is originated from plasmid pCambia 1301 but has been modified with the presence of the *bar* gene at the multiple cloning site.

Preparation of *Agrobacterium* suspension: Preparation of *Agrobacterium tumefaciens* strain LBA 4404 suspension for transformation was carried out according to Acereto-Escoffie *et al.* (2005) and Qiu *et al.* (2007) with some modifications. *Agrobacterium tumefaciens* strain LBA 4404 was inoculated into 10 mL of YEP broth containing 100 mg L^{-1} of streptomycin and 100 mg L^{-1} kanamycin. The cultures were allowed to grow with agitation at 200 rpm, 28°C for 16 h. Subsequently, the 10 mL of bacterial culture was added to 190 mL of fresh YEP broth supplemented with 100 mg L^{-1} of streptomycin and 100 mg L^{-1} kanamycin and grown at 28°C on a shaking incubator until $A_{600\ nm}$ reached 0.6. The cells were then harvested by centrifugation at 4°C (1398 g, 20 min) and further resuspended in 20 mL of liquid co-incubation medium containing MS basal medium supplemented with 2.3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 100 μM acetosyringone. This *Agrobacterium* suspension was used for transformation.

***Agrobacterium tumefaciens*-mediated transformation:** The preliminary *Agrobacterium tumefaciens*-mediated transformation protocol was based on the standard protocols with some modifications (Dabauza *et al.*, 1997; Saharan *et al.*, 2004; Wang *et al.*, 2008). The proximal region of the cotyledon explants were wounded once with multi-wire points of electric cord and immersed in 30 mL of liquid pre-culture medium in 50 mL centrifuge tube containing MS basal medium supplemented with 2.3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 100 μM acetosyringone for two days. After two days pre-culture period, the cotyledon explants were inoculated with 10 mL of *Agrobacterium* suspension strain LBA 4404 at $A_{600\ nm}$ 0.6 in the 50 mL centrifuge tube for 10 min at room temperature.

Then the cotyledon explants were removed from the *Agrobacterium* suspension and blotted dried before transferred to solid co-cultivation medium in 90×15 mm plastic petri dishes containing MS basal medium supplemented with 2.3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 100 μM acetosyringone. The petri plates were sealed with parafilm and placed in the dark for three days. After three days of co-cultivation period, transient expression of a *gus* reporter gene in the explants was determined through histochemical GUS assay.

Evaluation of transformation influencing factors: Several factors affecting the *Agrobacterium*-mediated transformation frequency in *Citrullus lanatus* were evaluated. The factors are age of cotyledon explants (5, 7 and 9-day-old seedlings), pre-culture conditions (2 days pre-culture period and without pre-culture period), wounding technique (mild wounding, massive wounding and without wounding creation), *Agrobacterium* concentration ($A_{600\ nm}$ 0.5, 0.6, 0.8, 1.0 and 1.2), co-incubation period of explants with *Agrobacterium* (1, 10, 30 and 60 min) and acetosyringone concentration in co-cultivation medium (0, 100, 200, 300 and 400 μM).

Optimized *Agrobacterium tumefaciens*-mediated transformation protocol: The optimized *Agrobacterium*-mediated transformation protocol was applied to the proximal region of 5-day-old seedlings. The cotyledon explant was gently wounded once with multi-wire points of electric cord. Wounded cotyledons were pre-cultured for 2 days in 30 mL of liquid MS medium supplemented with 2.3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 100 μM acetosyringone. After the pre-culture period, the explants were submerged in 10 mL of *Agrobacterium tumefaciens* suspension at $A_{600\ nm}$ 0.8 for 30 min. Following this, the cotyledon explants were blotted dry on sterile filter paper and subsequently cultured on co-cultivation medium in 90×15 mm plastic Petri dishes containing MS medium supplemented with 2.3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 200 μM acetosyringone. For the negative control, the proximal region of the cotyledon explants was cultured on co-cultivation medium without immersion in *Agrobacterium* suspension. The co-cultivation medium was maintained in dark for 3 days prior to the histochemical GUS assay to determine transient *gus* expression in cotyledon explants.

Statistical analysis: All experiments were studied through statistical analysis (SPSS for Windows software, Version 15). Each evaluation experiment was replicated three times and each replicate consisted of 30 explants. Data were analyzed using one-way ANOVA (analysis of variance) and Tukey's Honestly Significant Difference Test (HSD value) at $p<0.05$ level (Jackson and McLean, 1998).

RESULTS

Evaluation of transformation factors: In this study, several factors affecting transformation efficiency were evaluated. The efficiency of foreign gene delivery into the plant genome was determined through histochemical GUS assay to detect the activity of the *uid* A gene. The expressions of this gene appeared as blue spots and were visualized under stereoscopic microscope after 3 days of co-cultivation period.

Effect of explant ages and pre-culture conditions: Among different explants ages evaluated, 5-day-old seedlings showed the highest frequency of transformation in both without pre-cultured condition (52%) and 2-days pre-cultured condition (71%) compared to the 7-day-old seedlings and 9-day-old seedlings (Table 1). However, cotyledon explants of 5-day-old seedlings produced significantly higher number of spots per explant than 7-day-old seedlings and 9-day-old seedlings. On the other hand, 2 days of pre-culture period of cotyledon explants gave high frequency of transformation compared to the obtained without pre-culture condition. Thus, explants of 5-day-old seedlings with pre-culture period of 2 days in MS liquid pre-culture medium prior the transformation with *Agrobacterium* were recommended so far.

Effect of wounding technique: High transient GUS expression were observed in cotyledon explants with massive wounding (stabbed four to five times with multi wire points of electric cord) followed by mild wounding (stabbed once with multi wire points of electric cord) of the cotyledon explants. In contrast, unwounded cotyledon explants yielded low transformation frequency with low rate of blue spots per explant after 3 days of co-cultivation period (Table 2). The result showed that, there was a significant difference between wounding

Table 1: Effects of explants ages and pre-culture conditions on transformation efficiency

	Pre-culture period			
	Without pre-culture		2 days pre-culture	
Explant ages (days)	Frequencies (%)	No. of spots explant^{-1}±SE	Frequencies (%)	No. of spots explant^{-1}±SE
5	52	22.16±0.25^{a}	71	62.19±0.20^{a}
7	26	5.32±0.95^{b}	44	20.22±1.09^{b}
9	9	0.37±0.30^{c}	19	3.84±0.77^{c}

Values within a column followed by different letters are significantly different at the p<0.05 level procedures studied in this experiment ($p<0.05$). Yet, massive wounding completely bleached the cotyledon explants, turned flaccid and led to necrosis in the further analysis. Based on this result, it was proposed that mild wounding of the cotyledon explants maybe the best for the *Agrobacterium* to transfer their DNA efficiently.

Effect of *Agrobacterium* concentration: Significant increase of the transient *gus* expression were observed at the *Agrobacterium* concentration up to $A_{600\ nm}$ 0.8. However, *Agrobacterium* concentration at $A_{600\ nm}$ 0.5 had the lowest transient GUS frequency (Table 3). Blue-stained area per responding explant dropped dramatically when treated with *Agrobacterium* concentration at $A_{600\ nm}$ 1.0 and $A_{600\ nm}$ 1.2 compared to explant infected with bacterial concentration at $A_{600\ nm}$ 0.8. Therefore, *Agrobacterium* concentration at $A_{600\ nm}$ 0.8 was suggested to be used in transformation study.

Effect of co-incubation period: The numbers of cotyledon explants induced transient *gus* expression after infection with *Agrobacterium* varied significantly depending on the co-incubation period. The cotyledon explants produced high frequency of transformation when co-incubated in *Agrobacterium* suspension for 30 min (Table 4). In contrast, the frequency of transformation on cotyledon explants infected with *Agrobacterium* for 10 min decreased to 43%. Furthermore, significant decrease was observed in the transformation efficiency at the co-incubation period of 60 minutes. In short, co-incubation period of cotyledon explants for 30 min in *Agrobacterium* suspension was the best to increase the transformation efficiency.

Table 2: Effects of wounding procedures on transformation efficiency

Wounding procedures	Frequencies (%)	No. of spots explant^{-1}±SE
Non wounding	26	0.81±0.16^{a}
Mild wounding	57	38.2±0.54^{b}
Massive wounding	67	55.0±1.13^{c}

Values within a column followed by different letters are significantly different at the $p<0.05$ level

Table 3: Effects of *Agrobacterium tumefaciens* concentrations on transformation efficiency

Agrobacterium concentrations	Frequencies (%)	No. of spots explant^{-1}±SE
$A_{600\ nm}$ 0.5	16	2.66±0.49^{a}
$A_{600\ nm}$ 0.6	47	31.90±0.87^{b}
$A_{600\ nm}$ 0.8	69	43.50±0.32^{c}
$A_{600\ nm}$ 1.0	41	24.06±1.77^{d}
$A_{600\ nm}$ 1.2	25	19.83±1.85^{d}

Values within a column followed by different letters are significantly different at the $p<0.05$ level

Effect of acetosyringone: Among different concentrations of acetosyringone tested, 200 μM of acetosyringone was found to be comparatively better responsive than other concentrations (Table 5). Cotyledon explants cultured on co-cultivation medium supplemented with 200 μM of acetosyringone yielded significantly higher number of spots per explant compared to other concentrations. The transient *gus* expression in cotyledon explants after co-cultivation for 3 days was extremely low when acetosyringone was omitted. Further analysis of data showed addition of acetosyringone to 300 and 400 μM did not lead to an increase in transient expression and the blue-stained zone per responding explant drops gradually. Moreover, no significance differences were observed for these concentrations. In this experiment, co-cultivation medium supplemented with 200 μM acetosyringone was maintained.

Effect of optimized transformation protocol on transient expression of *gus* A gene: Optimized *Agrobacterium* transformation protocol led to high frequency of transformation (up to 100 % frequency) and yielded high blue spots per explant (181.2±0.57) after 3 days of co-cultivation in dark. Microscopic observation of the blue region on the explants revealed blue spots located on the cotyledon explants as a transient expression of *gusA* gene incorporated into the plant genome (Fig. 1a-e). However, no blue-stained tissue was observed in control explants (without *Agrobacterium* infection) after 3 days of co-cultivation. Thus, this optimized *Agrobacterium tumefaciens*-mediated transformation protocol that gave the highest transformation efficiency in *Citrullus lanatus*.

Table 4: Effects of co-incubation periods on transformation efficiency

Co-incubation periods (min)	Frequencies (%)	No. of spots explant^{-1}±SE
1	24	3.01±0.97^{a}
10	43	38.20±0.55^{b}
30	72	59.53±0.22^{c}
60	56	23.57±1.11^{d}

Values within a column followed by different letters are significantly different at the $p<0.05$ level

Table 5: Effects of acetosyringone concentrations on transformation efficiency

Acetosyringone concentrations (μM)	Frequencies (%)	No. of spots explant^{-1}±SE
0	13	1.70±0.34^{a}
100	46	21.61±0.61^{b}
200	71	62.18±0.20^{c}
300	59	42.67±1.21^{d}
400	52	32.61±1.33^{bd}

Values within a column followed by different letters are significantly different at the $p<0.05$ level

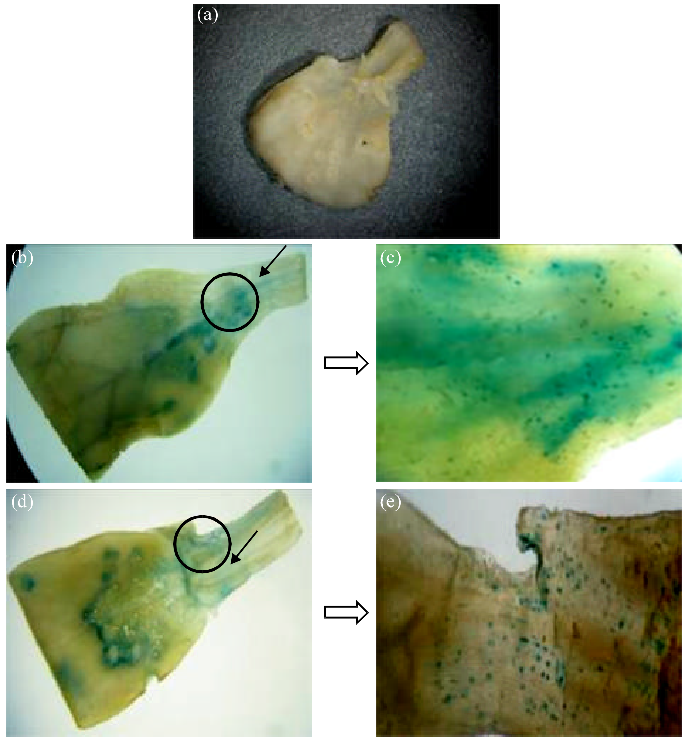

Fig. 1: Transient *gus* expression in cotyledon explants of *Citrullus lanatus*. (a) control cotyledon explants (no blue region observed). (b, c), transient *gus* expression in cotyledon explants (stained in blue). (d, e), microscopic observation revealed blue spots on cotyledon explants. An arrow shows blue patches in cotyledon explants which subsequently lead to visualization of blue spots under microscopic observation

DISCUSSION

The results show that we have established conditions in *Agrobacterium*-mediated gene transfer in *Citrullus lanatus* cv. Round Dragon. This is the first reported study on transformation of the mentioned cultivar. Generally, different plant varieties may affect the rate of transformation. The presence of T-DNA in the explants transformed with *Agrobacterium* was detected by transient expression of *gus* A gene appeared as a blue region on the explants.

The choice of the explant ages is very important to ensure good transformation efficiency because cotyledon explants from younger seedlings (4 or 5-day old) produced more shoots than those from older seedlings. Current study suggested that cotyledon explants derived from 5-day-old seedlings showed higher *gus* expression than 7-day-old seedlings and 9-day-old seedlings. These results are consistent with other studies suggesting cotyledon from 5-day-old seedlings has been used as explants for transformation in hybrid watermelon (Choi *et al*., 1994), tomato (Qiu *et al*., 2007) and loblolly pine (Wei, 2001). According to Wei (2001), among different tissue that has been tested, cotyledon explants yield high *gus* expression compared to hypocotyls and radicles. A possible explanation for this might be that cotyledon explants retained high competence cells which enable to respond to the endogenous or exogenous growth regulator to induce shoot regeneration in plants. Other reported study indicated that 4-day-old seedlings has been used for *Agrobacterium* transformation in bottle gourd (Han *et al*., 2005) and in wild watermelon (Akashi *et al*., 2005). It seems possible that these are due to the small and delicate explants, perhaps capable to receive more T-DNA strands incorporated into the plant genome (Sreeramanan *et al*., 2005).

Pre-culture period defined as the condition between where the explants were excised and infected with *Agrobacterium*. Explants pre-cultured for 2 days produced the highest frequency of transformation in *Citrullus lanatus* than the explants transformed with *Agrobacterium* without pre-culture condition. These findings are consistent with Han *et al*. (2005) that 2 days of pre-culture period had marked an effect on transformation frequency. Similar transformation efficiency was achieved by 48 h of pre-culture in agroforestry tree (Agarwal *et al*., 2004). In addition, pre-culturing explants prior the transformation was efficient for barley (Shrawat *et al*., 2007), canola (Cardoza and Stewart, 2003) and in carnations. Sreeramanan *et al*. (2005) and Yong *et al*. (2006) also reported that explants without pre-culture condition gave low frequency of transformation and less gus spots were observed on the explants. This may be due to the fact that pre-culture condition could be attributing to the initiation of active cell division and greatly increase the number of competent cells as potential targets for transformation.

Wounding of the explants prior transformation is necessary to induce high transient *gus* expression in *Citrullus lanatus*. In this study, cotyledon explants were stabbed with multi-wire points of electric cord to create a wound. A positive effect of wounding prior the *Agrobacterium* transformation has been demonstrated in many plant species. Yong *et al*. (2006) had suggested that wounding of the explants provides the released of phenolic compound to enhance transformation frequency.

Cotyledon explants of *Citrullus lanatus* treated with massive wounding enable to induce high transient *gus* expression compared to the cotyledon explants with mild wounding. But this finding did not seem to be consistent with Yong *et al*. (2006), who claimed explants with massive wounding produced low transient *gus* expression compared to the explants with mild wounding. However, explants with massive wounding became flaccid and totally bleached after three days of co-cultivation and unable to survive. The results of current study seemed to confirm the findings of Araujo *et al*. (2004). Thus, mild wounding of the cotyledon explants might be an optimal condition at which the plant cell is most accessible to *Agrobacterium* infection.

To verify the ability of *Agrobacterium tumefaciens* to transform the cotyledon explants of *Citrullus lanatus*, various bacterial concentration were tested. The highest transient gus expression produced on cotyledon explants infected with $A_{600\ nm}$ 0.8 bacterial concentration. This finding was similar to Lee *et al*. (2006) on orchard grass and Yong *et al*. (2006) on *Melastomaceae* transformation. However, higher bacterial concentrations above $A_{600\ nm}$ 0.8 or less $A_{600\ nm}$ 0.8 decreased the transformation efficiency of cotyledon explants. Higher bacterial concentrations may caused competitive inhibition which decreased the potential of the bacteria to attach at the explants for the T-DNA transfer into the plant genome (Yong *et al*., 2006). On the other hand low bacterial concentration resulted in low availability for transforming plant cells since least cells would attach on the explants resulted with low transient *gus* expression. In other cases, at higher bacterial concentration of $A_{600\ nm}$ 1.0 allowed the successful transformation in Indian cowpea (Chaudhury *et al*., 2007) and sunflower (Mohamed *et al*., 2006). In contrast, lower bacterial concentrations at $A_{600\ nm}$ 0.2 and $A_{600\ nm}$ 0.35 permitted transformation in paddy straw mushroom (Wang *et al*., 2008) and in *Medicago* and *Trifolium* species (Ding *et al*., 2003). The differences of bacterial concentration required for the *Agrobacterium* transformation highly depends on the plant species in order to yield high transient *gus* expression.

Optimizing the infection time of cotyledon explants with *Agrobacterium* is essential to allow the maximum transient *gus* expression in plant genome. The result of this study showed that co-incubation period for 30 min gave the highest transient *gus* expression in *Citrullus lanatus*. However, transformation frequency and transient *gus* expression decreased sharply with incubation period for 60 min compared to the 10 min and 30 min. According to Tao and Li (2006), the reason could be due to the *Agrobacterium* could give negatively transformation efficiency by reducing bacterial affinity for competitive attachment on the cotyledon explants. In contrast, other finding suggested that 60 min was an optimum co-incubation period in *Melastomataceae* sp. to achieve high transformation level (Yong *et al*., 2006). However, very low transformation frequency observed in cotyledon explants co-incubated for 1 minute might be that too short for the infection time of *Agrobacterium* to allow the attachment of the bacteria on the cotyledon.

Acetosyringone plays a major role in the natural infection of plants by *Agrobacterium tumefaciens* and is known to induce the virulence gene of the Ti-plasmid that initiates the transfer of the T-DNA region into the plant genome (Wei, 2001). A co-cultivation medium supplemented with 200 μM of acetosyringone was found to produce higher transformation rate on cotyledon explants after 3 days of co-cultivation period in dark. The effect of the co-cultivation medium supplemented with the 200 μM of acetosyringone on transformation efficiency was well documented in orchardgrass (Lee *et al*., 2006) and paddy straw mushroom (Wang *et al*., 2008).

The transient *gus* expression was extremely low when acetosyringone was omitted from the co-cultivation medium. This was confirmed in previous research that low frequency of explants showed *gus* positive was obtained due to the absence of the acetosyringone in co-cultivation medium (Shrawat *et al*., 2007). Furthermore, addition of acetosyringone to 300 μM and 400 μM in co-cultivation medium did not lead to an increase in transient expression. However, supra-optimal acetosyringone concentration is toxic and can cause harmful effect (Sreeramanan *et al*., 2005). In any transformation study, acetosyringone has been routinely used because it is a potent *vir* gene inducer and can enhance the transformation efficiency (Saharan *et al*., 2004; Wenck *et al*., 1999).

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