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# Transient Expression of *gusA* and *gfp* Gene in *Agrobacterium*-mediated Banana Transformation Using Single Tiny Meristematic Bud

[1, 3]S. Sreeramanan, [1]M. Maziah, [1]M.P. Abdullah, [2]M. Sariah and [3]R. Xavier
[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400, Serdang, Selangor DarulEhsan, Malaysia
[2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia
[3]Department of Biotechnology, Asian Institute of Medicine, Science and Technology,
Amanjaya, 08000, Sungai Petani, Kedah

**Abstract:** Several factors were investigated for the transfer of an intron-containing β-glucuronidase (*gusA*) and green fluorescent protein (*gfp*) gene into banana cultivar, Pisang Rastali (AAB) single bud using the *Agrobacterium*-mediated transformation system. Two disarmed *Agrobacterium tumefaciens* strains, EHA 101 (pIG121-Hm) and LBA 4404 (pCambia 1304) were evaluated as vector systems. A number of parameters such as pre-culture period of buds prior to inoculation, co-cultivation period, acetosyringone concentration used during co-cultivation in MS medium, methods of wounding, *Agrobacterium* strains, influence of different bud sizes and post cultivation period were evaluated to maximise transformation efficiency. *Agrobacterium tumefacien*, EHA101 (pIG121-Hm), a supervirulent strain proved to be significantly better than LBA 4404 (pCambia 1304) based on higher expression intensity and even spread of GUS staining (pIG121-Hm harbours *gusA* gene only and pCambia 1301 harbour both *gusA* and *gfp* gene). The GFP expression was higher than the GUS using *Agrobacterium tumefacien*, LBA 4404 (pCambia 1304). The results from transient transformation of single tiny meristematic bud suggested that *Agrobacterium*-mediated transfer of T-DNA to banana cells is highly efficient. By combining the best treatments, an efficient and reproducible transformation procedure has been established for the production of transgenic banana using this system.

**Key words:** *Agrobacterium tumefaciens*, single bud, transient gene expression

## INTRODUCTION

Banana industry in Malaysia has the potential to become one of the most profitable fruit commodities in the future, marked by the increase in demand and consumption, which is expected to generate an increase in production to over 400,00 tones by the year 2010 (Rohizad, 1998). Banana cultivar, Pisang Rastali is a local dessert banana which belongs to the AAB group and is popular in Malaysia. The application of genetic engineering for the improvement of banana is necessary for higher production and for introduction of disease resistance especially to fungal infections. With respect to banana transformation via *Agrobacterium*-mediated transformation, transgenic plants of other banana cultivars have been obtained through the use of infected corm slices (May *et al*., 1995) and scalps (Aceroto-Escoffie *et al*., 2005) and embryogenic cell suspensions (Ganapathi *et al*., 2001; Khanna *et al*., 2004).

Early detection of plant transformation events is necessary for the rapid establishment and optimization of banana transformation protocols. Reporter genes code for products which provide a clear indication of the expression, transient or stable, of transferred genes in transgenic cells (Jeoung *et al*., 2002). Transient expression is often used in the optimization of *Agrobacterium*-mediated transformation protocols as an indicator of gene transfer and expression efficiency (Eady *et al*., 1996; Kapila *et al*., 1997; Maximova *et al*., 1998; Wang *et al*., 2001). Widely used reporter genes in transgenic plants are *gusA* (*uidA*) gene coding for the enzyme β-glucuronidase (GUS) (Jefferson *et al*., 1987) and the *gfp* gene from the jelly fish *Aequorea victoria* (Elliot *et al*., 1999) coding for a green fluorescent protein (GFP).

The *gusA* gene has been the most extensively used reporter in studies of transgenic plants. The GUS enzyme cleaves a wide variety of β-glucuronides that

**Corresponding Author:** S. Sreeramanan, Department of Biotechnology, Asian Institute of Medicine, Science and Technology, Amanjaya, 08000, Sungai Petani, Kedah Tel : 604-4422884 Fax: 604-4422881

are used as substrates for spectrophotometric, fluorometric and histochemical assays (Jefferson, 1987). In order to avoid false positives, GUS expression in *Agrobacterium tumefaciens* should preferably be suppressed to avoid interference with GUS expression from transformed plant tissues. This was achieved by inserting an intron sequence, isolated from a potato gene, into the *gusA* coding sequence (Vancanneyt *et al*., 1990; Franklin and Lakshim, 2003).

GFP expression is detected by the emission of bright green fluorescence when excited with ultraviolet (360-400 nm) or blue (440-480 nm) light, without any need for additional substrates, because chromophobe formation and light emission are autocatalytic properties of this protein, which only require common components (Chalfie *et al*., 1994). In contrast to GUS, the detection of GFP expression is noninvasive and nondestructive, with a broad range of applications in transgenic plants (Leffel *et al*., 1997), which has made it a possible successor to *gusA* as a reporter gene.

The development of a robust *Agrobacterium*-mediated transformation protocol requires the identification and optimization of the factors affecting T-DNA delivery. Hiei *et al.* (1997) reported that numerous factors including genotype of plants, types and ages of tissues inoculated, kinds of vectors, strains of *Agrobacterium*, selection marker genes and selective agents and various conditions of tissue culture, are of critical importance in rice and other transformation protocols.

Therefore, in this study optimization of several factors affecting transient *gusA* and *gfp* gene expression such as preculture period, co-cultivation period, acetosyringone concentrations, types of wounding, strains of *Agrobacterium tumefaciens*, influence of single bud sizes and post cultivation period were evaluated to determine the efficiency of *Agrobacterium*-mediated transfer during the early stages of transformation in banana cultivar, Rastali (AAB).

## MATERIALS AND METHODS

**Plant material:** Corm slices of *in vitro* banana cultivar, Rastali (AAB) plantlets were cultured in MS (Murashige and Skoog, 1962) medium supplemented with 10 mg $L^{-1}$ of 6-Benzylaminopurine (BAP) to obtain multiple bud clumps. The cultures were incubated at 25±2°C and a 16 h photoperiod with cool white fluorescent light of 150 μmol $m^{-2}$ $s^{-1}$ (supplied by Philips TLD fluorescent light tubes). Single buds (3 mm), excised from multiple bud clumps, were used for this study.

**Bacterial strains and culture conditions:** *Agrobacterium tumefaciens* strains, super-virulent strains EHA 101 and LBA 4404 were used for transformation experiments. *Agrobacterium tumefacien* strain EHA 101 contained the disarmed plasmid pIG 121-Hm with the eukaryotic *npt 11* gene, which confers resistance to kanamycin and *hpt* genes for resistance to the antibiotic hygromycin, as well as an intron containing β-glucuronidase (*gusA*) gene. The LBA 4404 strain contained a modified disarmed plasmid pCambia 1304 differing from pIG 121-Hm by the presence of the Green Fluorescence Protein (*gfp*) gene. Bacteria cultures were grown from single colonies in LB medium at 28°C with shaking speed 120 rpm for 20 h to reach an optimal density of 0.7 units at 600 nm ($OD_{600}$). Just prior to co-cultivation with explants, acetosyringone was added to the *Agrobacterium* suspension at a final concentration of 100 μM.

**Co-cultivation of *Agrobacterium* and culture conditions:** Single buds were separated from multiple bud clumps. Each bud was immediately immersed in conical flasks containing 20 mL of *Agrobacterium* suspensions for 30 min. Different concentrations of acetosyringone were added to assess factors affecting the transformation frequency. The phenolic compound acetosyringone (Sigma-Aldrich) was dissolved in ethanol v/v (100%) and the stock volume made up in autoclaved distilled water. The appropriate aliquot of the filter sterilized stock solution was added in *Agrobacterium* suspension. The buds were blotted dry with sterile filter paper and transferred to a regeneration medium containing MS salts, MS vitamins, 10 mg $L^{-1}$ BAP, 100 μM acetosyringone, 3% (w/v) sucrose and 0.25% (w.v) gelrite agar at pH 5.7 (unless mentioned otherwise).

**Optimization of parameters:** To assess factors affecting the transformation frequency, different treatments were performed. A range of parameters were evaluated and each experiment included four replicates, each containing 10 single buds. The experiments were repeated three times and were carried out at room temperature throughout the experiments. The parameters included the length of the pre-culture period (0, 1, 2, 3 and 4 days), co-cultivation period (0, 1, 2, 3, 4, 5 and 6 days), acetosyringone concentrations (0, 50, 100, 150, 200 and 250 μM) used during co-cultivation in *Agrobacterium* suspensions, methods of explant wounding [Excised but otherwise intact explants (first level of wounding: $W_1$), explants injured with scalpel (second level of wounding: $W_2$) and explants bombarded using Bio-Rad biolistic gun (third level of wounding: $W_3$)], different *Agrobacterium* strains (EHA 101 and LBA 4404), single bud sizes

(3, 5 and 10 mm) and post cultivation period (0, 3, 6, 9, 12, 15 and 18 days). All of the parameters were evaluated and optimized based on percentage of single buds GUS and GFP expressing positive.

**Histochemical GUS staining and fluorescence microscopy:** Single buds were assayed for GUS expression according to Jefferson *et al.* (1987). Samples were overlaid with X-Gluc staining solutions and incubated at 37°C overnight before examination of GUS activity. A fluorescence microscope (Leica MZFL 111) equipped with GFP 2 filter set was used to monitor GFP expression of transient activity in single buds. An imaging system (Leica DC 200) was attached to the fluorescence microscope to capture the image in real time using Leica DC Viewer software. *Agrobacterium* cells emitted a dull, pale green fluorescence as observed under the fluorescence microscope. However, this was quite distinct from the bright green fluorescence of GFP-expressing banana cells.

**Statistical analysis:** Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. All statistical analyses were performed at 5% using SPSS 10.0 (SPSS Inc. USA).

## RESULTS AND DISCUSSION

**Preculture period:** The effect of preculture period (0, 1, 2, 3 and 4 days) of single buds for transformation efficiency was first evaluated. The basis of the promotion of *Agrobacterium*-mediated transformation by preculturing explants has not been completely established in *Musa* species but it has been proposed that the production of *vir*-inducing compounds by metabolically active cells plays an important role (Sunilkumar *et al.*, 1999) in planta. This preculture period of the explants before co-cultivation helped inhibitory and increased the transformation efficiency in banana cultivar, Rastali (AAB).

Explants precultured for 2 days produced the highest number of GUS and GFP positive buds (Fig. 1) followed by those precultured for 3 and 1 day. This result is consistent with the finding of Sunilkumar *et al.* (1999) using tobacco leaf discs which were precultured for 2 days and produced the highest of GUS positive explants. However, Tsukazi *et al.* (2002) found that hypocotyl explants of *Brassica oleracea* var. *capitata* precultured for 3 days produced the highest number of GUS positive explants and spots. Weir *et al.* (2001) also found that immature embryos required a minimum of 3 days of preculture in order to see transient GFP expression. The

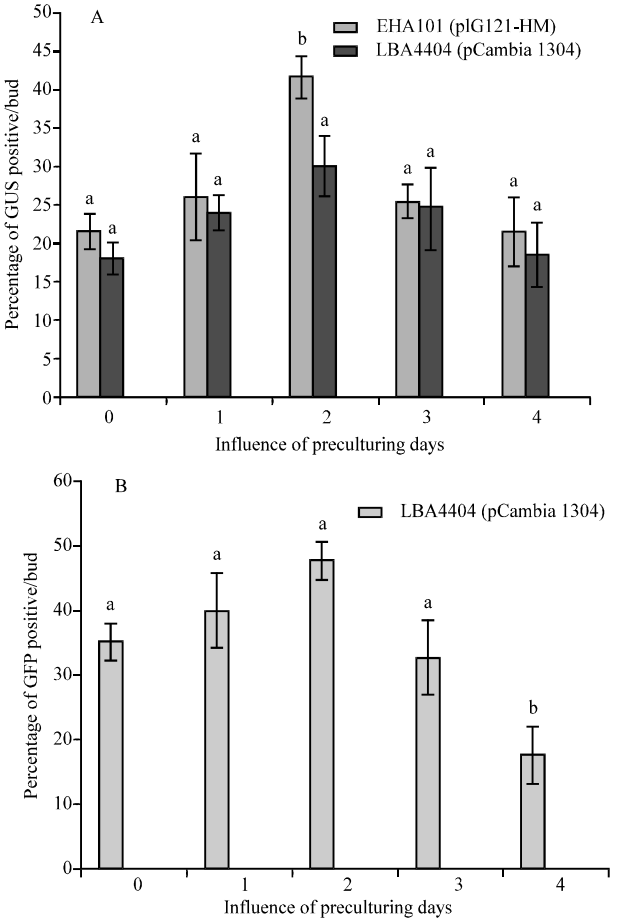


Fig. 1: Influence of preculturing explants on transient expression based on GUS (A) and GFP (B). For each parameter, four replicates were used containing ten single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

fewest GUS and GFP positive explants and spots were observed on 0-day preculture, followed by a 4 day preculture. Transient GUS expression obtained is higher obtained when using *Agrobacterium strain*, EHA 101 (pIG 121-Hm) compared to LBA 4404 (pCambia 1304) (Fig. 1).

Preculturing explants prior to inoculation and cocultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in some woody fruit plants, such as plum (Mante *et al.*, 1991). Sangwan *et al.* (1992) reported that the number of putatively competent of *Arabidopsis thaliana* cells for transformation was greatly increased by a preculture day treatment on a medium rich in auxins. It has been

suggested that stimulation of plant cell division and activation of the DNA replication machinery during the pre incubation period may play an important role in the integration of T-DNA leading to stable transformation (Sangwan *et al*., 1992).

However, preculture period had a negative effect on the induction of competent cells for transformation especially on the physiology of the explants in citrage (Cervera *et al*., 1998). Transformation efficiency based on screening for GUS expression in tea was 40- 44% without preculture, while it varied from 5 to 18% for one to four days of preculture period duration (Mondal *et al*., 2001). Elimination of the preculture condition was also helpful in producing transformants in kiwifruit and apple (Janssen and Gardner, 1993).

These results indicated that preculture period had influenced the frequency of banana cultivar, Rastali (AAB) by *Agrobacterium tumefaciens*. This improvement in transformation efficiency as the result of pre-culture condition could be attributed to the initiation of active cell division upon wounding with the improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites (Binns, 1991).

**Co-cultivation period:** Co-cultivation for 2-7 days was generally considered to be suitable for *Agrobacterium*-mediated transformation, as reported for many plant species. Two to three days of co-cultivation period have been used for general *Graminea* transformation (Hiei *et al*., 1994; Cheng *et al*., 1997) and *Brassica napus* (Khan *et al*., 2003). However, more than 5 days of co-cultivation encouraged an overgrowth of bacteria with a concomitant decrease in transformation efficiency in garden pea (De Kathen and Jacobsen, 1990) and flax (Dong and McHughen, 1993). In addition, longer co-cultivation periods in *Brassica napus* resulted in explants turning necrotic and remains devoid of shoot regeneration due to excessive growth of *Agrobacterium* strain, EHA 101 (pIG 121-Hm) (Khan *et al*., 2003).

The co-cultivation of single buds with the disarmed strains (EHA 101 and LBA 4404) for 3 days resulted in a high frequency of transient GUS expression (Fig. 2). Transient GFP expression shows higher frequency than GUS for 3 days co-cultivation using LBA 4404 (pCambia 1304). When the single buds were transferred to selective medium immediately after inoculation with *Agrobacterium* (no co-cultivation), no transformation was observed. The transformation frequency was very low after 1 day co- cultivation, but increased rapidly when the co-culture period was prolonged to 7 days, reaching maximum at day 3.

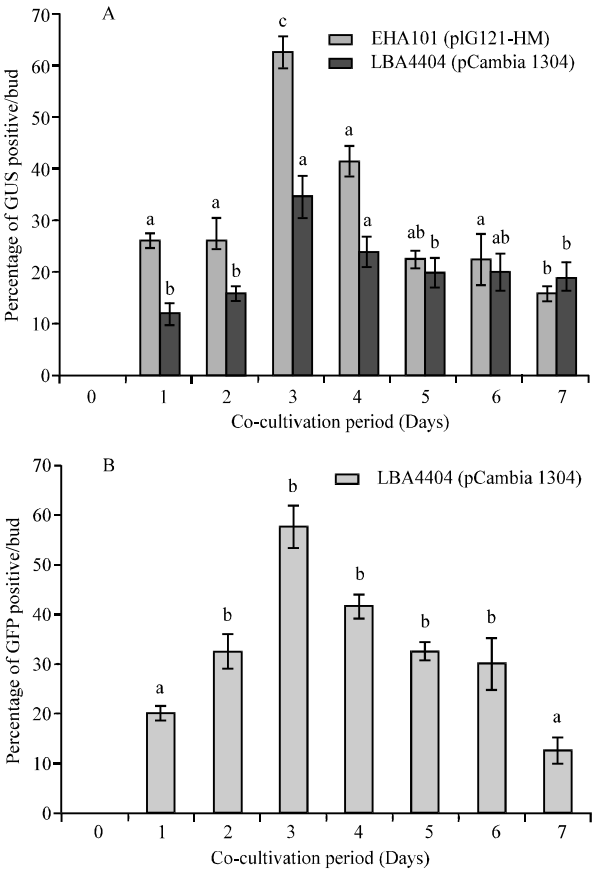


Fig. 2: Influence of co-cultivation period (days) on transient expressions based on GUS (A) and GFP (B) in single buds. For each parameter, four replicates were used containing ten single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

However, transient GUS expression did not vary significantly over 4 to 7 days co-cultivation. Similar results have been reported in broccoli (Metz *et al*., 1995a) and *Brassica napus* (Takasaki *et al*., 1997). Cervera *et al.* (1998) reported that 5 days co-cultivation period resulted in overgrow of *Agrobacterium* and subsequently decrease in the regeneration frequency of transformed shoots, although this period was the most effective for increasing the frequency of transient GUS expression in citrange explants Co-cultivation for more than 3 days led to a decrease in transformation frequency and about 50% explant loss because of bacterial overgrow in experiments to produce transgenic *Datura* (Ducrocq *et al*., 1994). The longer co-cultivation times in wheat, reduced the capacity of the immature embryos to form embryogenic callus and regeneration (Wu *et al*., 2003).

However, a prolonged co-cultivation period of 5 to 7 days has been shown to increase *Agrobacterium*-mediated transformation in *Lilium usitatissimum* and agapanthus (Dong and Mc Hughen, 1993; Suzuki *et al*., 2001). Seo *et al.* (2002) reported that GUS expression increased 2.3 times when co-cultivation period was prolonged 3 to 10 days.

Although prolonged co-cultivation period of more than 3 days have been successfully used for certain plants (Suzuki *et al*., 2001; Yu *et al*., 2001), 2-3 days co- cultivation has been routinely used in most reported transformation protocols. Mondal *et al.* (2001) reported that the differential requirement of co-cultivation period largely depends upon the *Agrobacterium* strain used or the medium for bacterial culture or co- cultivation.

**Acetosyringone treatment:** Phenolic inducers such as acetosyringone are known to enhance the ability of *Agrobacterium* to transform recalcitrant host plants and has been incorporated in various media used for the co-cultivation of bacteria and plant tissues (Boase *et al*., 1998).

In genetic transformation, this small phenolic compound is required for the transcriptional activation of the *Agrobacterium* virulence machinery (Nadolska-Orczyk and Orczyk, 2000). Acetosyringone as a phenolic signal is transduced through a receptor *virA* protein in the inner membrane of the bacterial cell. The expression of these genes triggers the transfer of a specific DNA segment, called transferred DNA (T-DNA), from the Ti plasmid to plant cells and its integration into their nuclear DNA (Koichi *et al*., 2002). Sunilkumar *et al.* (1999), demonstrated that the preculturing step in the transformation of tobacco leaf discs could be bypassed by providing acetosyringone to the freshly cut explants.

Six concentrations of acetosyringone (0-250 μM) in the co-cultivation medium were investigated. This experiment was repeated more than three times, giving consistent results where explants producing GUS and GFP positive results increased with increasing acetosyringone concentrations and the highest at 100 μM (Fig. 3). A high concentration of acetosyringone is toxic due to the harmful effect of supra-optimal concentration of acetosyringone and the alcohol solvent used in this experiment. However, in the absence of acetosyringone, some GUS and GFP expression was still evident in single buds.

When more than 100 μM acetosyringone was used, less GUS and GFP expression was observed and the single buds had necrotic zones. More than 20% of the explants did not survive this co-cultivation (Fig. 4 and 5). The single buds became brown at higher concentrations,

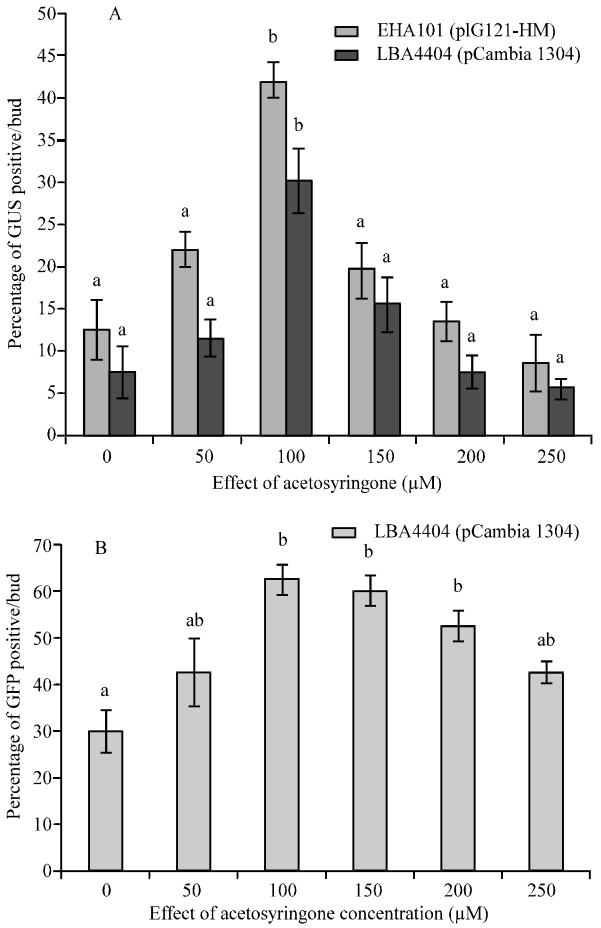


Fig. 3: Influence of acetosyringone concentration (μM) on transient expressions based on GUS (A) and GFP (B). For each parameter, four replicates were used containing ten single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

which might affect *Agrobacterium* inoculation. A positive effect of acetosyringone on *Agrobacterium*-mediated gene transfer to *Phaseolus* cells has been suggested in previous experiments (Becker *et al*., 1994), where increased tumour proliferation on cotyledonary node explants of *P. vulgaris* was observed after co-cultivation in the presence of 100 μM acetosyringone. Hiei *et al.* (1994) demonstrated that acetosyringone at 100 μM is a key to successful transformation of rice. The level of transient expression of β-glucoronidase (GUS), after co-cultivation was extremely low when acetosyyringone was omitted. However, Godwin *et al.* (1991) and Takasaki *et al.* (1997) obtained a higher frequency of *Agrobacterium* infection ability using

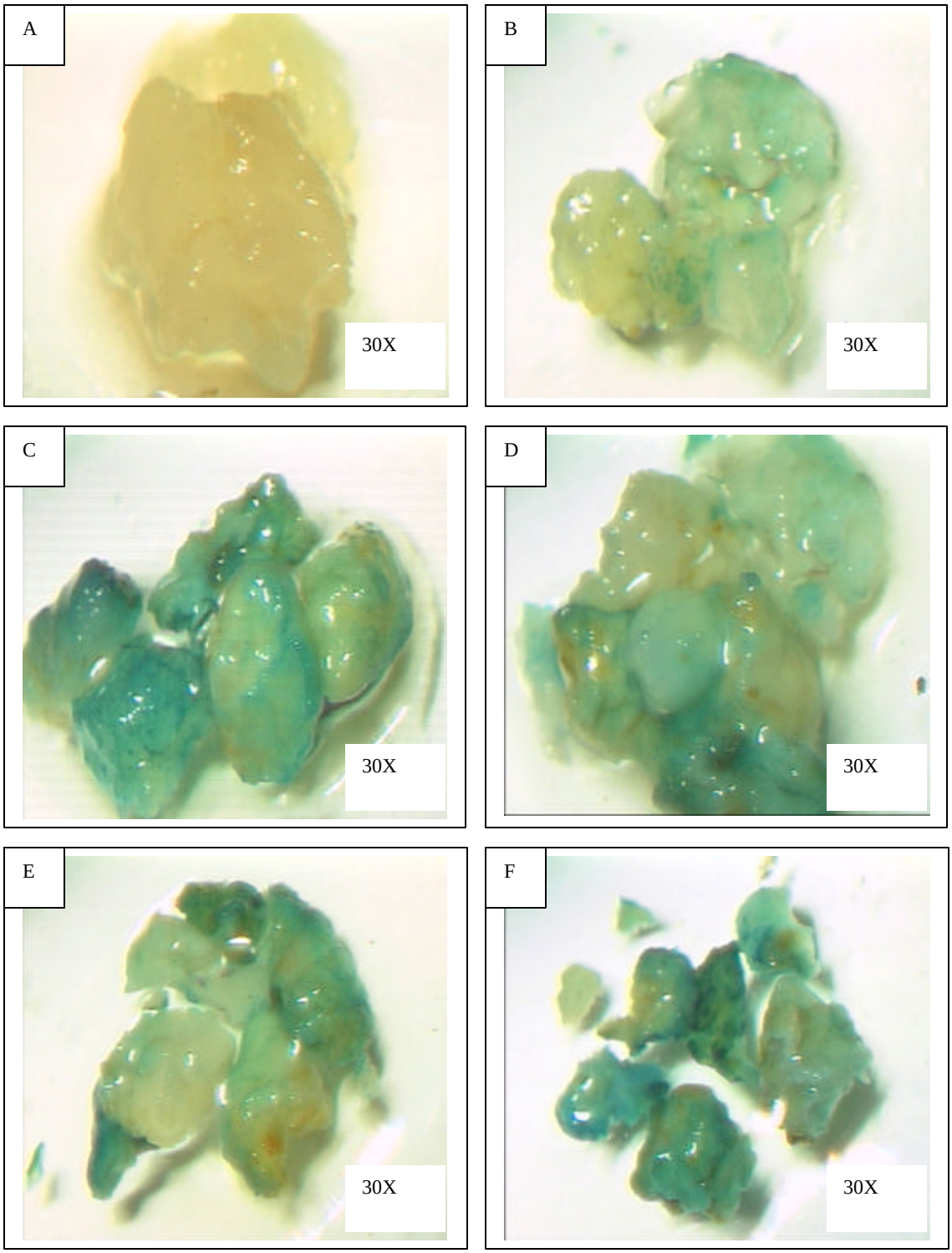


Fig. 4: Comparison of transient histochemical *gusA* gene expression in single buds with different concentration of acetosyringone (µM) obtained from *Agrobacterium* strain EHA 101 (pIG-121Hm). (A) 0 µM -control; (B) 50 µM; (C) 100 µM; (D) 150 µM; (E) 200 µM and (F) 250 µM. All pictures were taken under 30X magnification using a stereomicroscope

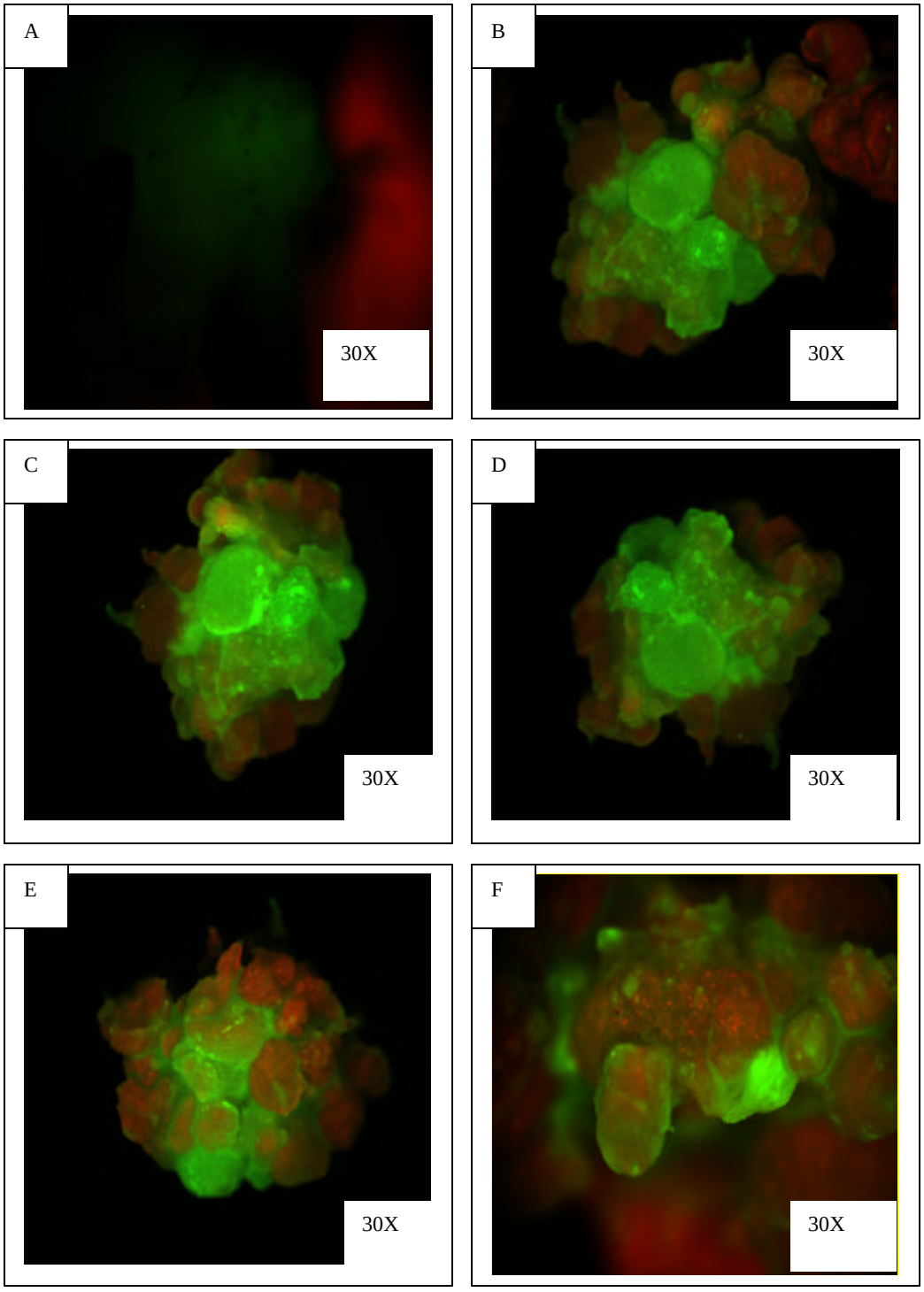


Fig. 5: Comparison of transient histochemical *gfp* gene expression in single buds with different concentration of acetosyringone (µM) obtained from *Agrobacterium* strain LBA 4404 (pCambia 1304). (A) 0 µM-control; (B) 50 µM; (C) 100 µM; (D) 150 µM; (E) 200 µM and (F) 250 µM. All pictures were taken under 30X magnification using a stereomicroscope

200 μM acetosyringone in *Brasicca rapa*. Henzi *et al.* (2000) obtained similar results in *Agrobacterium rhizogenes*-mediated transformation of broccoli with 200 μM acetosyringone.

The monocotyledonous plants have generally proven recalcitrant to *Agrobacterium* infections. In this background, the use of acetosyringone during infection and co-cultivation has been shown to be critical for *Agrobacterium*-mediated transformation in monocot plants such as japonica and *indica* rice (Hiei *et al.*, 1994; Seo *et al.*, 2002), *Phalaenopsis* orchid (Belarmino and Mii, 2000) and agapanthus (Suzuki *et al.*, 2001). Concentration of acetosyringone in the pre-culture and co-culture medium was found to influence the GFP transient expression in sorghum (Jeoung *et al.*, 2002). However, in several experiments on woody plants, particularly on poplars (Confalonieri *et al.*, 1997), acetosyringone did not help in increasing transformation efficiency. The inability of acetosyringone to improve upon the transformation efficiency could be due to the inherent prevalence of high amounts of phenolic in woody plant tissues.

***Agrobacterium* strains:** The choice of *Agrobacterium* strain can have a major effect on transformation efficiencies. *Agrobacterium tumefaciens* strain, EHA 101 (pIG 121-Hm), a super virulent strain was significantly more effective than LBA 4404 (pCambia 1304) based on higher intensity and even spread of GUS staining (Fig. 6 and 7).

Virulence of *Agrobacterium* strains varies widely among plant hosts (Davis *et al.*, 1991) and is particularly important for the transformation of recalcitrant species. Several studies have reported strain EHA 101 to be more effective for transformation than other strains of *Agrobacterium tumefaciens* (Tsukazaki *et al.*, 2002). Strains EHA 101 and EHA 105 are more effective than strain LBA 4404 since both are derived from supervirulent wild-type strain A281 (Hood *et al.*, 1993), whereas strain LBA 4404 was derived from less virulent strain Ach5 (Hoekema *et al.*, 1983).

However, it is not the same when the transformation efficiency of strain LBA 4404 was significantly than EHA 105 in the cotton transformation (Sunilkumar and Rathore, 2001). Less virulent strain LBA 4404 has been used for most plant transformation because it can be eliminated from plant tissues with low levels of antibiotics following co -cultivation (Maheswaran *et al.*, 1992).

**Wounding effect:** Highest GUS and GFP transient expressions were observed when explants were bombarded at the third level of wounding, $W_3$ compared to $W_1$ and $W_2$, the other wounding methods for *Agrobacterium*-mediated transformation (Fig. 8).

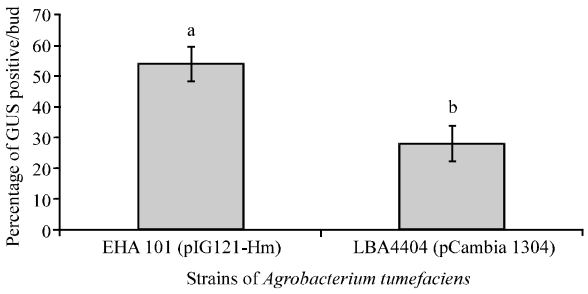


Fig. 6: Influence of strains of *Agrobacterium tumefaciens* on GUS expression. For each parameter, four replicates were used containing ten single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

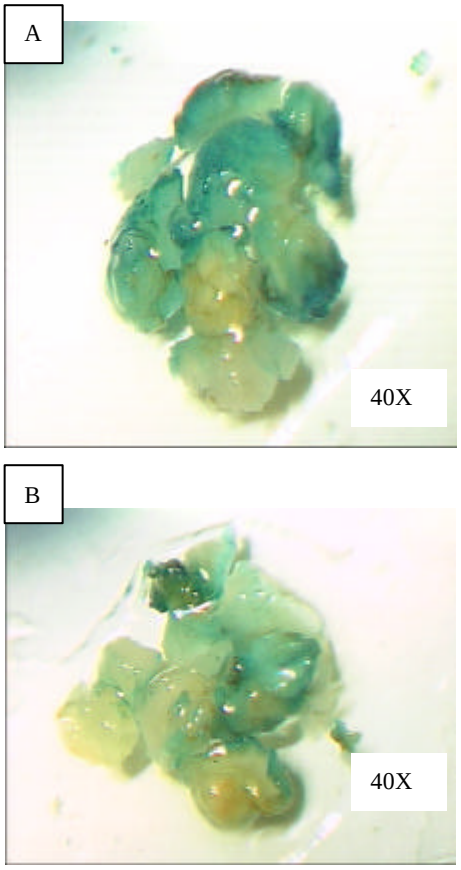


Fig. 7: Comparison of transient histochemical *gusA* gene expression in single buds with different *Agrobacterium* strains. (A) EHA 101 (pIG-121Hm); (B) LBA 4404 (pCambia 1304). All pictures were taken under 40X magnification using a stereomicroscope

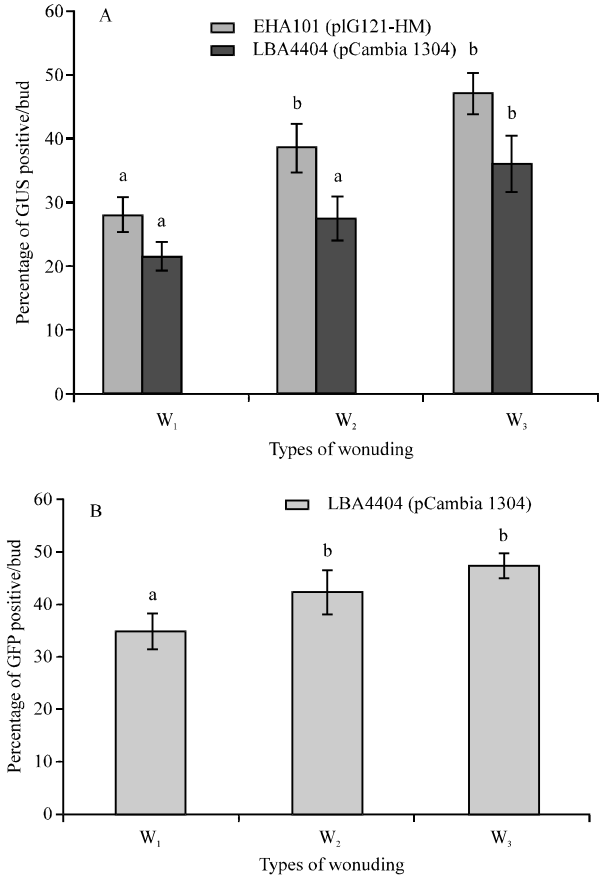


Fig. 8: Effect types of wounding on transient expression based on GUS (A) and GFP (B). For each parameter, four replicates were used containing ten single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

Transient GUS expression levels were higher using the *Agrobacterium* strain, EHA 101 (pIG 121-Hm) compared to LBA 4404 (pCambia 1304). The advantage of naked gold particles via particle bombardment that wounds cells underlying the act surfaces of the targeted buds. This treatment significantly enhanced the level of GUS transient expression level on single buds using both *Agrobacterium tumefaciens* strains EHA 101 (pIG 121-Hm) and LBA 4404 (pCambia 1304) after inoculation when compared to the method W 1.

Generally, wounding is necessary in order to provide *Agrobacterium* accessibility to transformation competent cells by allows the entry of the bacteria into plant cells and provides phenolic compounds. It has been proposed that inactivation of active cell division upon wounding improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites and production of *vir*-inducing compounds by the metabolically active cells improved the transformation efficiency (Park *et al*., 1996; Bean *et al*., 1997). Subjecting plant tissues to brief periods of ultrasound by sonication, which allows *Agrobacterium* entry throughout the tissues, is another method of increasing transformation efficiency (Teixeira and Fukai, 2003). Accordingly, microprojectile bombardment and sonication-based wounding treatments have also been shown to enhance *Agrobacterium*-mediated transformation of different plant tissues, including somatic and zygotic embryos, immature cotyledons, leaves, stems, apical meristems, roots and whole seedlings (Bidney *et al*., 1992; Trick and Finer, 1997; Santarem *et al*., 1998; Tang, 2003). This phenomenon could be explained by the attraction of *Agrobacterium* to wounded sites and increased access of *Agrobacterium* to plant cells. Wounding of sunflower embryogenic axes by bombardment with naked particles had also been shown to increase the transformation efficiency by subsequent co -culture with *Agrobacterium* (Bidney *et al*., 1992).

Few of the transient transformation events were observed in the unwounded parts of single buds. Since there were no significant differences between the second and third wounding levels, using the second wounding level is preferable as the microprojectile method is more costly and time consuming.

**Size of single buds:** The size of the single buds, which was used as an indication of the developmental stage of the tissue, was considered in order to select the right size of target that is most suitable for *Agrobacterium*-mediated transformation. Four week old single buds, measuring from 3, 5 and 10 mm were subjected to transformation. The result of the effect of different size of single buds on transient GUS and GFP expressions is presented in Fig. 9. Variation in transient GUS expression was observed in all sizes of the single buds using EHA 101 (pIG 121-Hm) and LBA 4404 (pCambia 1304).

The percentage of buds shown GFP expression is higher than GUS obtained from *Agrobacterium tumefaciens*, LBA 4404 strain. The highest GUS and GFP expressions were exhibited by the 3 mm size range. This could be due to the small and delicate explants, perhaps received more T-DNA strands. Size ranging from 10 mm was found to have significantly lower levels of GUS and GFP expression (Fig. 9). This could be due to larger surface area and bigger sizes of single buds which displayed lower transient expression and potential chimaeric explants regeneration in later stage during the

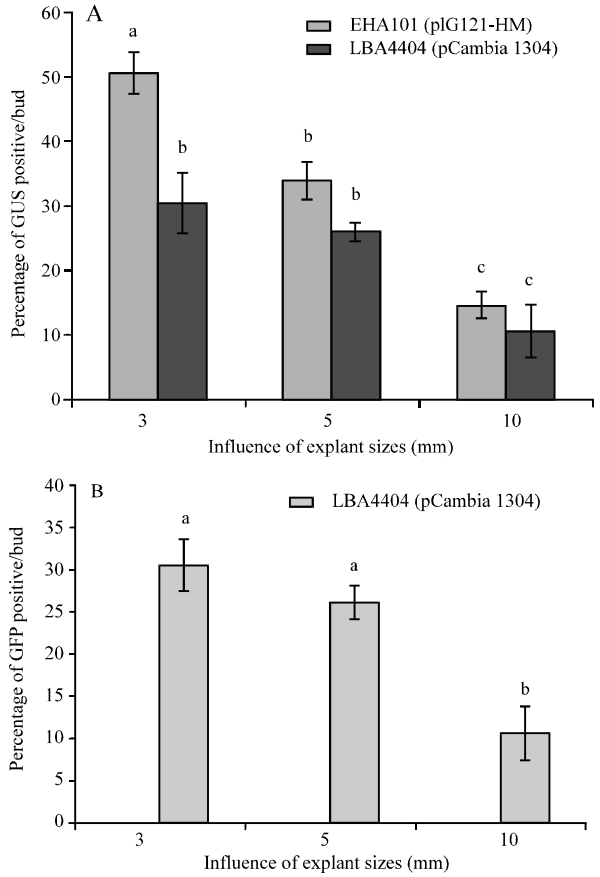


Fig. 9: Effect of different single bud sizes on transient GUS (A) and GFP (B) expression. For each parameter, four replicates were used containing ten single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

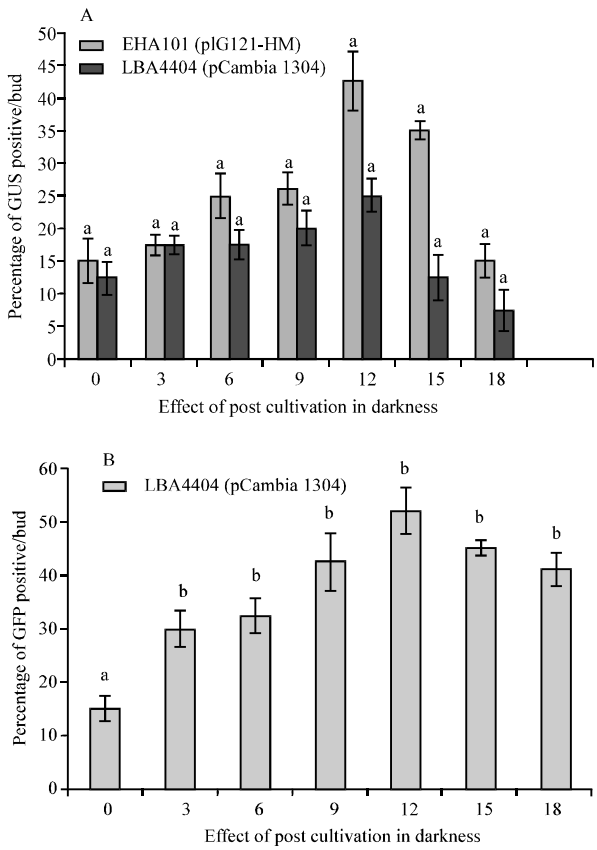


Fig. 10: Effect of post cultivation on transient GUS (A) and GFP (B) expression. For each parameter, four replicates were used containing ten single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

antibiotic selections. Therefore, 3 mm size of single bud was chosen as a suitable target size for transformation studies.

**Post-cultivation periods:** Optimizing the number of days for GUS and GFP expression are also essential as this will allow the maximum expression of the gene in a foreign tissue background. The results showed that post-cultivation for 12 days gave the highest transient GUS expression in *Agrobacterium tumefaciens*, EHA 101 (pIG 121-Hm) (Fig. 10). Similar results were obtained for GUS and GFP expression using *Agrobacterium tumefaciens*, LBA 4404 (pCambia 1304).

The sharp increase in GUS and GFP expression at day 12 reflects the division of cells with stably incorporated T-DNA segments. It could be that T-DNA strands required certain time frame as transgenes to express in a new environment. Based from the results, the single buds should subject to antibiotic selection pressure after 12 days during the transformation events.

It was suggested that such an allowance of unselected period would enable the transformed cells to undergo several wounds of cell division to reach a threshold of cell mass that had higher chances of surviving under the selection pressure (Ozias-Atkins *et al*., 1993).

## CONCLUSIONS

Systematic evaluation of the various parameters enabled the improvement of the transformation techniques by obtaining high levels of transient GUS and GFP expression in banana cultivar, Rastali (AAB)

single buds. The experimental protocol includes the following conditions: (1) pre-culture period 2 days prior to infection; (2) co-cultivation period of 3 days; (3) 100 μM acetosyringone concentration during co-cultivation in *Agrobacterium* suspensions; (4) single buds injured with scalpel; (5) an *Agrobacterium* strain, EHA 101; (6) 3 mm of single bud size and (7) using antibiotic selection pressure after 12 days based on result obtained from post-cultivation period. The key factors in transformation of Pisang Rastali (AAB) that were discussed in this paper will very likely prove also to be key factors in the transformation of other important banana cultivars. It seems probable that *Agrobacterium*-mediated techniques of gene transfer will be extended to other recalcitrant banana cultivars as soon as the methodological parameters are optimized.

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