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Optimization and Transformation of Garden Balsam, *Impatiens balsamina*, Mediated by Microprojectile Bombardment

[1]A.M. Taha, [1]A. Wagiran, [2]H. Ghazali, [1]F. Huyop and [3]G.K.A. Parveez
[1]Department of Biotechnology Industry, Faculty of Biosciences and Bioengineering,
Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[2]Biotechnology Research Center, Malaysian Agriculture Research and Development Institute,
P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
[3]Advanced Biotechnology and Breeding Center, Biological Research Division, Malaysian Palm Oil Board,
P.O. Box 10620, 50720 Kuala Lumpur, Malaysia

Abstract: In this study, a transformation system was developed by initially optimizing the microprojectile bombardment parameters for cotyledonous explants. The parameters optimized were combination of distance from stopping screen to target tissue and helium pressure, number of bombardment, preculture duration prior to bombardment, DNA amount, osmoticum treatment and post-bombardment incubation time. Determination of minimal inhibitory concentration for efficient selection of transformants was also carried out for hygromycin. Different concentrations of hygromycin (25, 50, 75 and 100 mg L^{-1}) were tested against different ages of explants (0, 1, 3 and 5 weeks) for efficient selection of transformants. Using the optimized parameters, transformation of cotyledons was carried out followed by selection on 75 mg L^{-1} hygromycin at different weeks of post-bombardment. Transgenic plants were successfully regenerated from bombarded explants and confirmed via histochemical GUS assay and PCR analysis.

Key words: Plant transformation, microprojectile bombardment, *Impatiens balsamina*

INTRODUCTION

Impatiens balsamina is locally known as garden balsam and cultivated as an ornamental plant in many parts of Asia. This annual soft stem's plant produces four different colors of flower and offers potential for producing novel colors through genetic engineering approach. The plant parts have been claimed to have medicinal properties such antifungal activity against *Candida albicans* (Lee *et al*., 1999). The use of biotechnology techniques to develop the transformation system of other species of *Impatiens* sp. has been reported. One of the targets of genetic engineering of *Impatiens* is to develop virus resistant plants (Altpeter *et al*., 2005). *Impatiens balsamina* is one of the host plants for *Impatiens* Necrotic Spot Virus (INSV), the virus that could infect the crops in many species. Efforts to develop a transformation system using biolistics for producing INSV resistant plants have been initiated (Daughtrey *et al*., 1997).

Although the regeneration systems for *Impatiens* have been established, there has been no report on the stable transformation of *Impatiens balsamina* species. This study focused on developing a reliable transformation system for *Impatiens balsamina* using biolistics which is widely employed method for the transformation of many monocotyledonous plants. However, it has been reported that optimization of physical and biological parameters is required for a particular plant or a target tissue as the conversion rate of transient expression over stable transformation is only 0.1-2% (Sanford *et al*., 1993). Jain *et al*. (1996) suggested that there could be several reasons to account for the low efficiency including the variation in the optimal bombardment conditions, type and quality of the target cells and tissues and differences in the media requirements for bombardments. The biolistic system is also highly variable due to the nature of the machine and therefore requires optimization (Taylor and Vasil, 1991; Kikkert *et al*., 2004).

Optimization of DNA delivery conditions based on transient *gusA* gene expression is important for developing an efficient and stable transformation system for a particular species or target tissue. Using the

Corresponding Author: Dr. G.K. Ahmad Parveez, Advanced Biotechnology and Breeding Center, Biological Research Division, Malaysian Palm Oil Board, P.O. Box 10620, 50720 Kuala Lumpur, Malaysia
Tel: +603 87694584 Fax: +603 89261337

optimized conditions, successful production of transgenic plants has been reported for plants such as oil palm and chickpea (Parveez, 2000; Indurker *et al*., 2007). Besides optimization of transformation parameters, the ability to isolate and regenerate cells containing a stably integrated foreign gene from majority of untransformed cells is critical in the production of transgenic plants. This can be achieved using selective agent, either antibiotic or herbicide, at a minimum concentration at which all the non-transformed cells will be killed and allow for the transformed cells to survive and finally regenerate.

The combination of the optimized physical and biological parameters and selective agent would be the best condition for efficiently transforming, selecting and eventually regenerating transgenic plants for a particular species. As, there is currently no report on stable transformation of *Impatiens balsamina*, efforts to develop such a system is required before any genetic improvement of this species could be carried out. Therefore, in this study, the optimization of biolistics parameters, determination of minimal inhibitory concentration of hygromycin and regeneration of *Impatiens balsamina* from cotyledon will be elaborated.

MATERIALS AND METHODS

Plant materials and seed sterilization: All seeds were washed under running tap water for one hour. This followed by surface sterilization by soaking the seeds in 70% (v/v) ethanol for 1 min and 10% (v/v) commercial sodium hypochlorite solution for 10 min. Seeds were rinsed with sterile distilled water three times and blotted dry on sterile filter paper.

Germination of seeds: Seeds were germinated on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, IB vitamins (2 mg L^{-1} glycine; 100 mg L^{-1} myo-inositol; 0.5 mg L^{-1} nicotinic acid; 0.5 mg L^{-1} pyridoxine HCl and 0.1 mg L^{-1} thiamine HCl) and solidified with 0.8% (w/v) Bacto Difco agar. The pH of media was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCl prior to sterilization.

***In vitro* regeneration:** Proximal section of cotyledons were cultured onto optimal shooting MS macro- and micronutrient supplemented with 1 mg L^{-1} BAP for three weeks. The shoots were transferred onto optimal rooting media in half strength MS media supplemented with 0.1 mg L^{-1} IAA. After two weeks on rooting media, plantlets were sub-cultured on different media for development observation. Half of the plantlets were sub-cultured onto half strength MS media supplemented with 0.1 mg L^{-1} IAA and the other half was transferred onto hormone-free MS media. Development of plantlets, measured as height, was observed up to three weeks. The plantlets were cultured in an incubator at 25±2°C with 16 h photoperiod, 17 µmol m^{-2} sec^{-1} supplied by cool light Daylight fluorescent tubes (LICOR, USA).

Plasmid DNA isolation: In this study, plasmid pRQ6, carrying the ß-glucuronidase (*uid*A) gene and hygromycin phosphotransferase (*hph)* gene conferring resistance to antibiotic hygromycin was used for transformation. Both genes were driven by CaMV 35S promoter. One milliliter of fresh overnight culture was inoculated into 500 mL of LB medium (5 g NaCl, 5 g tryptone and 2.5 g yeast extract) containing 75 µg mL^{-1} of ampicillin. The overnight culture was transferred into large centrifuge bottles and the bacteria were pelleted by centrifugation at 20,000 g at 4°C for 10 min. DNA isolation was later carried out using the QIAGEN Plasmid Maxi kit. The plasmid DNA obtained was dissolved in 1 mL TE buffer and confirmed by restriction enzyme analysis.

DNA-microcarrier preparation and optimization of bombardment condition: DNA precipitation onto 1.0 µm gold microcarriers was carried out according to manufacturer's instructions for the Biolistic PDS/He 1000 (Bio-Rad) device. Twenty microliter of DNA solution (1 µg $µL^{-1}$), 100 µL of $CaCl_2$ (2.5 M) and 40 µL spermidine (0.1 M, free base form) were added sequentially to the 100 µL particle suspension. The mixture was vortexed for 3 min, spun for 10 sec at 20,000 g and the supernatant discarded. The pellet was washed with 250 µL of absolute ethanol and resuspended in 60 µL of absolute ethanol. Twelve microlitres of the solution were loaded onto the centre of the macrocarrier and air dried.

Bombardments of cotyledon explants were carried out on MS media supplemented with IB vitamins, sucrose 3% (w/v), 1 mg L^{-1} BAP and 0.2 M mannitol/0.2 M sorbitol (pH 5.7) and solidified with Bacto agar (0.8 % (w/v). Cotyledons (20 pieces) were placed at the center of 9 cm Petri dish containing bombardment media prior to bombardment. Vacuum pressure was maintained at 28 Hg. Each experiment was performed in three replicates and repeated twice. Unbombarded cotyledon explant was used as control. Optimization of the physical factors was carried out under the following conditions, combination of helium pressure (650, 900 or 1100 psi) and distance from stopping screen to the target tissue (6, 9 or 12 cm) and the number of bombardments. The biological parameters optimized included the pre-culture time prior to bombardment (4, 16 or 32 h), DNA amount (0.2, 0.5 and 1.0 µg), post-bombardment incubation time (4, 24 or

48 h) and osmotic treatments (bombardment media) using mannitol, sorbitol and combination of both appropriately.

Histochemical GUS (ß-glucuronidase) assay for transient gene expression (for optimization) was performed 24 h post-bombardment. Explants were incubated at 37°C for 24 h in GUS buffer (1 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-Gluc), 0.2 M sodium phosphate (pH 7.0) and 0.1% (v/v) Triton X-100) (Jefferson *et al*., 1987). After incubation, explants were soaked in 70% (v/v) ethanol for 15 min to remove the chlorophyll. The presence of GUS spots on explants was examined under a dissecting microscope. The GUS gene expression was recorded as percentage of GUS positive explants and average number of GUS spots per explants. All data were subjected to one way ANOVA using SPSS 12.0 (SPSS Inc., Chicago, USA) with significant level at 0.05.

Determination of minimal inhibitory concentration of hygromycin on explants: The minimum concentration of hygromycin required for the selection of transformants was determined by placing the unbombarded tissues on MS media supplemented with 25, 50, 75 or 100 mg L^{-1} hygromycin. The growth and development of the explants in culture were monitored and scored for a period of 5 weeks. The minimum inhibitory concentration was determined, based on minimum concentration of hygromycin required for giving complete mortality.

The effect of different explants ages on hygromycin media: The bombarded explants were cultured on MS media supplemented with 75 mg L^{-1} hygromycin at various explant ages of 0, 1, 3 and 5 week post-bombardment. The best explant age should give the highest percentage of plants after five weeks in the selection media. Furthermore, transgenic status of these plants needs to be proven.

Selection and regeneration of transgenic plants: Bombarded cotyledons from proximal section were cultured on optimal shooting MS media supplemented with IB vitamins, 3% (w/v) sucrose, 1 mg L^{-1} BAP and 0.2 M mannitol/0.2 M sorbitol and 75 mg L^{-1} hygromycin (pH 5.7) for a few weeks until resistant shoots were obtained. The shoots were then transferred onto optimal rooting media in half strength MS media supplemented with IB vitamins, sucrose 3% (w/v), 0.2 M mannitol / 0.2 M sorbitol, 0.1 mg L^{-1} IAA and 75 mg L^{-1} hygromycin. After two weeks on rooting media, half of the plantlets was sub-cultured onto half strength MS media supplemented with 0.1 mg L^{-1} IAA, IB vitamins, 3% (w/v) sucrose, 0.2 M mannitol/0.2 M sorbitol and 75 mg L^{-1} hygromycin. The other half was transferred onto hormone-free MS media containing 75 mg L^{-1} hygromycin. Development of plantlets, measured as height, was observed up to three weeks. The plantlets were cultured in an incubator at 25±2°C with 16 h photoperiod, 17 µmol m^{-2} sec^{-1} supplied by coolight daylight fluorescent tubes.

Molecular analysis of transformants

Genomic DNA preparation of *Impatiens balsamina*: A 0.5 g of leaf sample was homogenized in liquid nitrogen using mortar and pestle. The homogenized tissues were transferred into 15 mL corex tube and 1 mL of 65°C CTAB extraction buffer containing 2% cetyl methyl ammonium bromide (CTAB) (w/v), 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% (w/v) polyvinyl-pyrrolidone (PVP) and 3 M sodium acetate (pH 5.2) were added (Doyle and Doyle, 1987). The tube was incubated at 65°C for 5 min. Equal volume of chloroform: isoamylalcohol (24:1) was added and the mixture was centrifuged at 10,000 g for 10 min to separate the phases. The supernatant was carefully decanted and transferred to a new tube and 0.2 v of 5% (w/v) CTAB solution was added. The above steps were repeated twice. The supernatant was precipitated with 95% (w/v) ethanol and 0.1 v sodium acetate (pH 5.2) was added. The DNA pellet was obtained by centrifuging the supernatant at 10,000 g for 10 min. The DNA pellet were washed using 100% ethanol, dried and resuspended in 100 µL TE buffer.

PCR analysis: Plants regenerated following bombardment and hygromycin selection were analyzed by PCR. The DNA derived from selected transformed plants was tested for the presence of *hph* gene using specific primers which produce a 0.8 kb fragment. PCR reaction was performed with Gene Amp PCR System 2400 (Perkin Elmer). The primers used for amplification of *hph* gene were 5'-GGGGGGTCGGTTTCCACTA-3' and 5'-ATCGTTATGTTTATCGGCACTTTG-3'. PCR amplification was carried out using initial denaturation of 2 min at 94°C, followed by 29 cycles of 45 sec at 94°C, 30 sec at 55°C annealing temperature, 30 sec at 72°C and a final elongation cycle for 5 min at 72°C. PCR products were analyzed in 1.2% (w/v) agarose gel electrophoresis.

RESULTS

Transformation system for *Impatiens balsamina* was developed by initially optimizing the physical and biological parameters affecting DNA delivery and integration into the genome. It has been reported that optimization of physical and biological parameters is

required for a particular plant or a target tissue as the conversion rate of transient expression over stable transformation is only 0.1-2% (Sanford *et al.*, 1993).

The effect of distance of stopping screen to target tissue and helium pressure on transient *gusA* gene expression: This experiment was carried out to determine the best correlation between the distance of stopping screen to target tissue and helium pressure on DNA delivery into *Impatiens balsamina* explants during transformation procedure. In the present study, while keeping the DNA concentration (1.0 µg) and osmoticum treatment (MS media supplemented with 0.2 M mannitol and 0.2 M sorbitol and 16 h prior bombardment incubation period) constant, it was observed that the distance of 9 cm combined with 1100 psi helium pressure showed the highest average number of blue spots as compared to other parameter combinations (Fig. 1). The average number of blue spots was 137.4 for explants bombarded at this condition. Helium pressure lower than 1100 psi resulted in lower number of blue spots. Increasing the distance from 9 to 12 cm resulted in severe reduction in the number of blue spots as compared to reducing the distance from 9 to 6 cm.

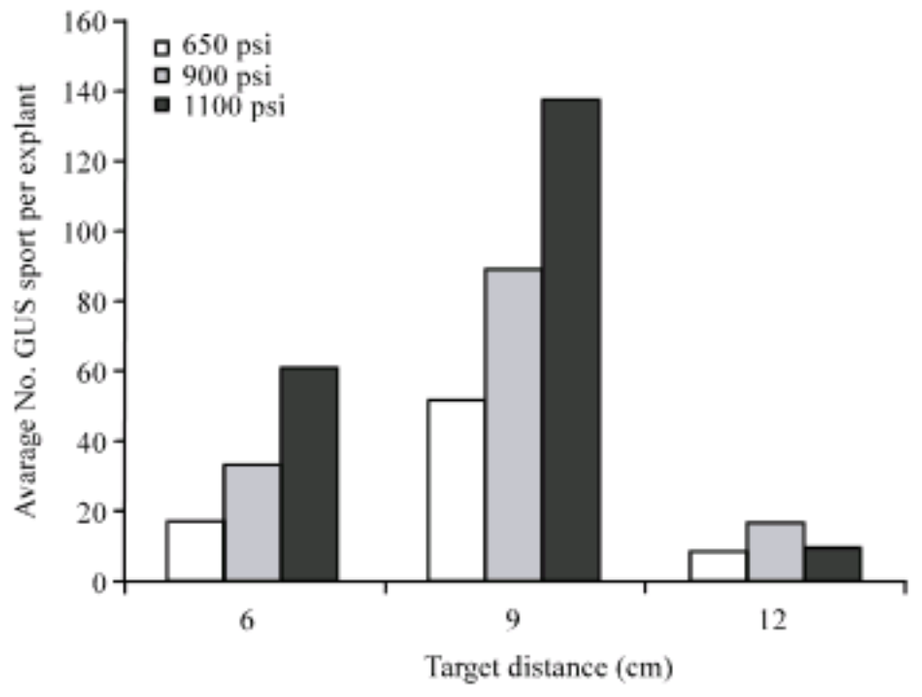

Fig. 1: The effect of distance from stopping screen to target tissue and helium pressure on transient *gusA* gene expression 24 h post bombardment

The effect of bombardments number on transient *gusA* gene expression: It was observed that there was no significant difference in the number of blue spots in both numbers of bombardments when bombarded at 9 cm target distance and 1100 psi helium pressure. One time bombardment was chosen to enhance transformants recovery and reduce the cell damage (Fig. 2).

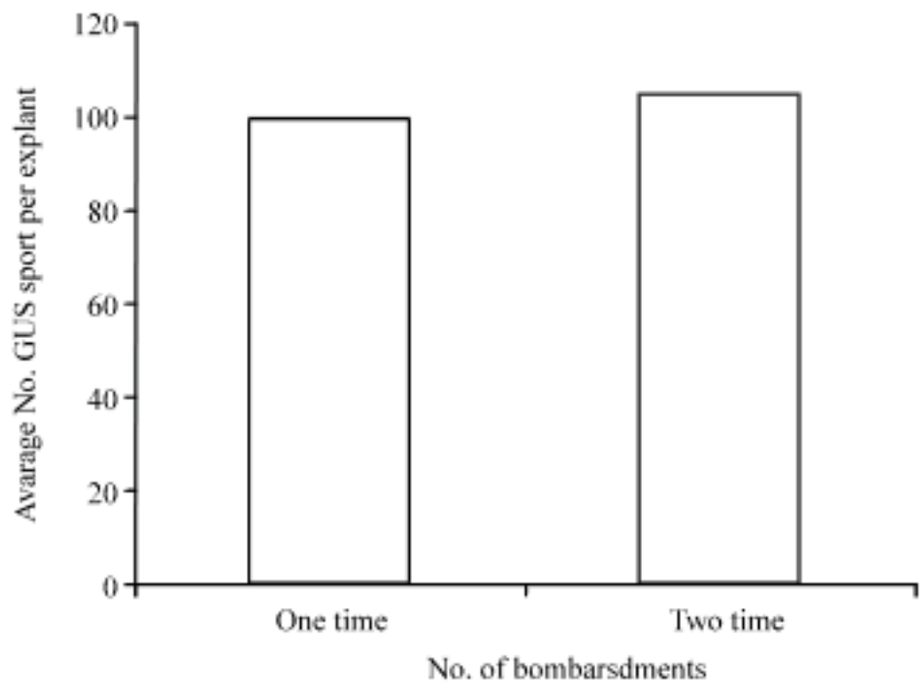

Fig. 2: The effect of bombardment number on transient GUS spots per explant 24 h post-bombardment

The pre-culture duration prior to bombardment on transient *gusA* gene expression: One of the biological factors examined was the pre-culture duration prior to bombardment. The result obtained demonstrated that higher number of blue spots was obtained for 16 h pre-culture duration. The reduction of pre-culture time to 4 h resulted in the lowest number of blue spots. Four hours pre-culture duration possibly insufficient for cell to acclimatize in the osmotic media. A longer pre-culture time (32 h), however, reduced the number of blue spots (Fig. 3). This long pre-culture period may cause the explants to dehydrate and inhibit shoot induction.

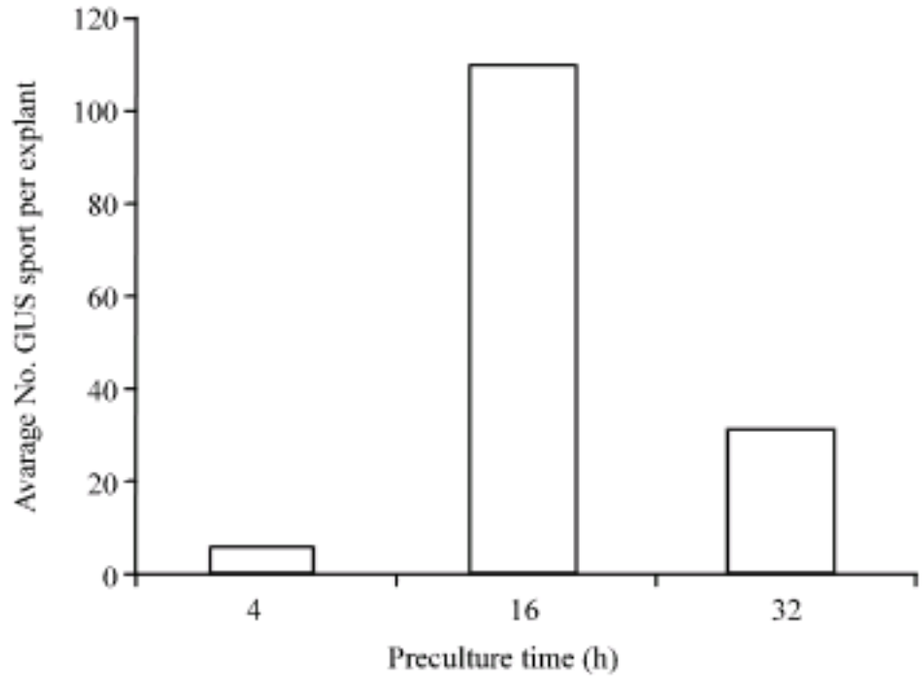

Fig. 3: The effect of pre-culture duration of explants on osmotic media on transient *gusA* gene expression after bombardment

The effect of DNA amount on transient *gusA* gene expression: The effects of DNA amount on transient *gusA* gene expression in *Impatiens balsamina* were evaluated in the range between 0.5 to 1.5 µg. The result

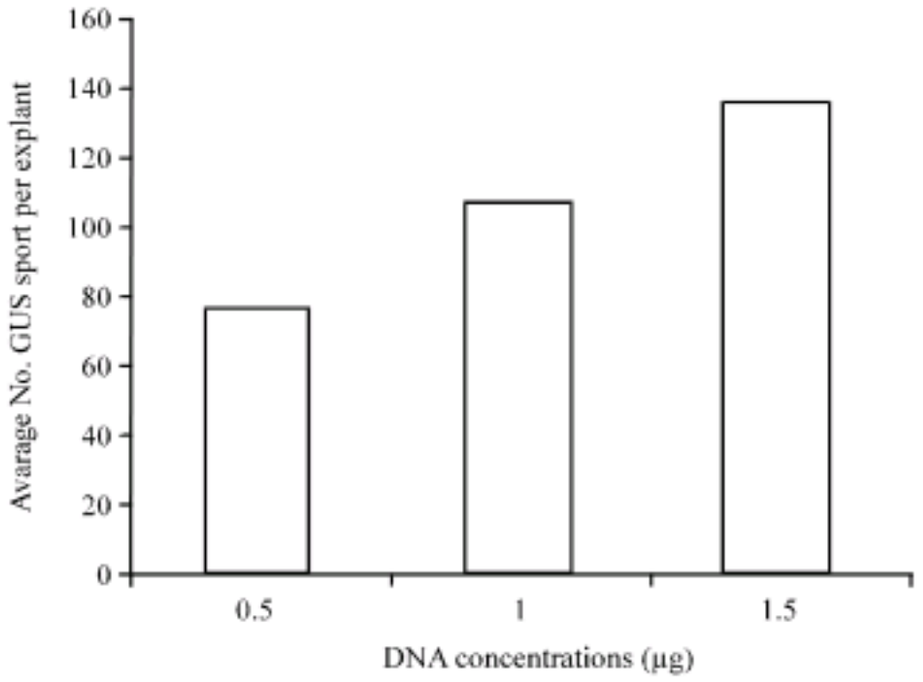

Fig. 4: The effect of DNA concentrations (µg) on transient GUS spots per explant 24 h post-bombardment

showed that the highest transient *gusA* gene expression was obtained using 1.5 µg DNA (Fig. 4). The higher the amount of DNA resulted in highest number of transient expression. Aggregation of DNA coated gold particle was observed under the light microscope when higher amount of DNA used. Particle aggregation may reduce transformation efficiency due to higher cell damages. Subsequently, 1.0 µg plasmid DNA was used throughout the experiment.

The effect of pre-culture osmoticum treatment on transient *gusA* gene expression: The optimization of osmoticum type and concentration was carried out to determine the best osmoticum combination that could reduce cell injury during bombardment procedure. In the present study, the osmoticum treatments for 16 h consisted of mixture of sorbitol and mannitol showed up to 7.2-fold higher number of blue spots as compared to the bombardment without osmoticum treatment. The same pre-culture treatment of less or more than 16 h showed lower number of blue spots. Therefore, 16 h treatment was used throughout the experiment.

The average number of GUS spots with both manitol and sorbitol treatment (0.4 M each) was 110.2 as compared to without treatment with 15.3 GUS spots. Overall, osmoticum treatment using mannitol alone showed a higher number of blue spots as compared to sorbitol treatments alone (Table 1). However, treatment using combination of both osmoticum gave better results and was used for future transformation experiments for stable transformation.

The effect of post-bombardment incubation time on transient *gusA* gene expression: The bombarded explants were subjected to transient *gusA* gene assay

Table 1: The osmotic treatments on transient *gusA* gene expression 24 h post-bombardment

Osmotic treatments	Concentrations (M)	No. of bombarded explants	Percentage of GUS$^+$ explants (%)	No. of GUS average spots per explants±SE
Mannitol	0.2	80	64	65.8±1.44
	0.4	80	82	85.7±1.96
	0.6	80	71	48.7±1.83
Sorbitol	0.2	80	43	45.2±0.93
	0.4	80	76	81.4±2.15
	0.6	80	62	52.1±1.71
Mannitol	0.2	80	70	86.1±2.64
and l Sorbitol	0.4	80	93	110.2±3.56
	0.6	80	62	71.1±2.18
Control	0.0	80	64	15.3±0.01

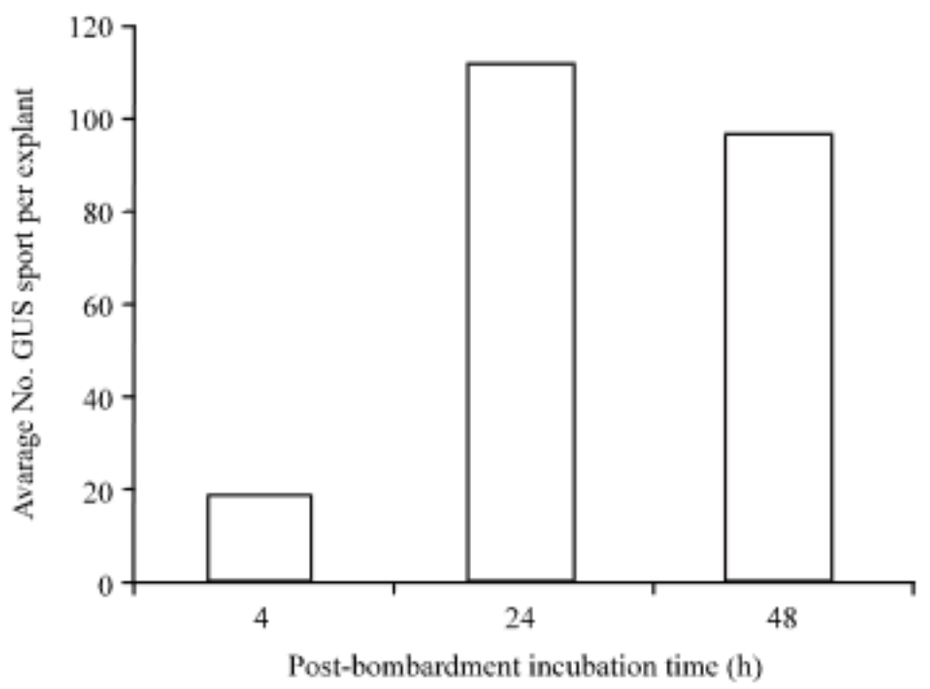

Fig. 5: The effect of post-bombardment incubation time on transient *gusA* gene expression after bombardments

after a number of incubation times. This was to determine the time needed for the highest expression of the transgenes in the cells. The post-bombardment incubation times tested were 4, 24 and 48 h. The results showed that the highest transient expression was obtained at 24 h of post-bombardment. The 4 h post-bombardment incubation showed the least number of transient expressions as compared to 24 h, whereas 48 h showed no significant difference to 24 h treatment (Fig. 5). This may be due to the fact that 4 h is too short for all the cells to express the *gusA* gene transformed.

The effect of optimized conditions on stable *gusA* gene expression and regeneration. The optimized conditions were collectively used to transform and regenerate transgenic plantlets. In the stable transformation experiment, 480 plates of explants were bombarded and 140 of them were used for transient expression evaluation. It was observed that the plasmid pRQ6 gave a

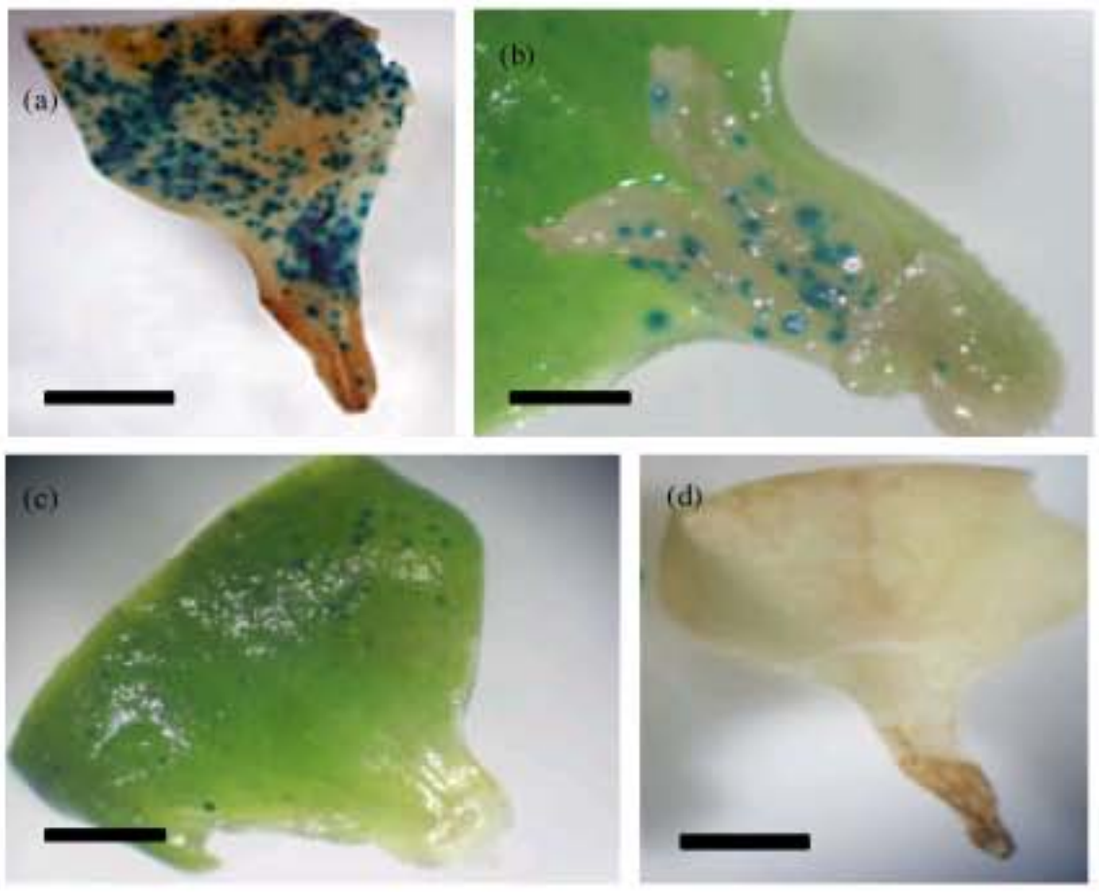

Fig. 6: The effect of bombardments on transient *gusA* gene expression of explants. (a) *gusA* gene expression on explants after 24 h bombardment using the optimal conditions of bombardment. Bar = 0.3 cm, (b) *gusA* gene expression on meristematic region of explants after 48 h bombardment. Bar = 0.1 cm, (c) Lower *gusA* gene expression on explants after 4 h bombardment. Bar = 0.2 cm and (d) Control with no *gusA* gene expression after 24 h bombardment. Bar = 0.3 cm

Table 2: The effect optimal bombardment conditions on transient *gusA* gene expression of explants 24 h post-bombardment

Plasmid	No. of bombarded explants	No. of explants for GUS assay	Average No. GUS spot	Percentage GUS^+ explants (%)±SE
pRQ6	480	140	149.3	95± 1.73

mean blue spots of 149.3 (95%) per bombarded explant (Table 2, Fig. 6). The other bombarded explants, which were not used for transient expression, were subjected to selection using hygromycin.

Production of transgenic plants

Determination of minimal inhibitory concentration for hygromycin: Minimal inhibitory concentration was determined by exposing unbombarded explants at various ages (0-3 weeks) to different concentrations of hygromycin (25-100 mg L^{-1}). It was determined that 75 mg L^{-1} hygromycin give 100% mortality of explants after five weeks in culture (Table 3, Fig. 7). The explants exposed to 25 and 50 mg L^{-1} hygromycin survived even when the cultured period was extended for another five weeks. However, explants surviving 100 mg L^{-1} hygromycin showed inhibition of shoot development. In the subsequent stable transformation experiments, 75 mg L^{-1} hygromycin was used.

The effect of explant ages on hygromycin media: This experiment was carried out to determine the most suitable time to expose the bombarded explants to the selection agent after bombardment. The bombarded explants, at 0, 1, 3 and 5 weeks post-bombardment, were cultured on media supplemented with either 75 or 100 mg L^{-1} hygromycin. Zero and one week post-bombarded explants showed 100% mortality when exposed to both concentrations (Table 4). The effect of optimal concentration of hygromycin (75 mg L^{-1}) on the

Table 3: The effect of different concentrations of hygromycin on various ages of untransformed explants after 5 weeks in culture

Hygromycin (mg L^{-1})	Age of explants (weeks)	Percentage of mortality explants (%)
25	0	0
	1	0
	3	0
	5	0
50	0	25
	1	10
	3	0
	5	0
75	0	100
	1	100
	3	100
	5	100
100	0	100
	1	100
	3	100
	5	100
0	0	0
	1	0
	3	0
	5	0

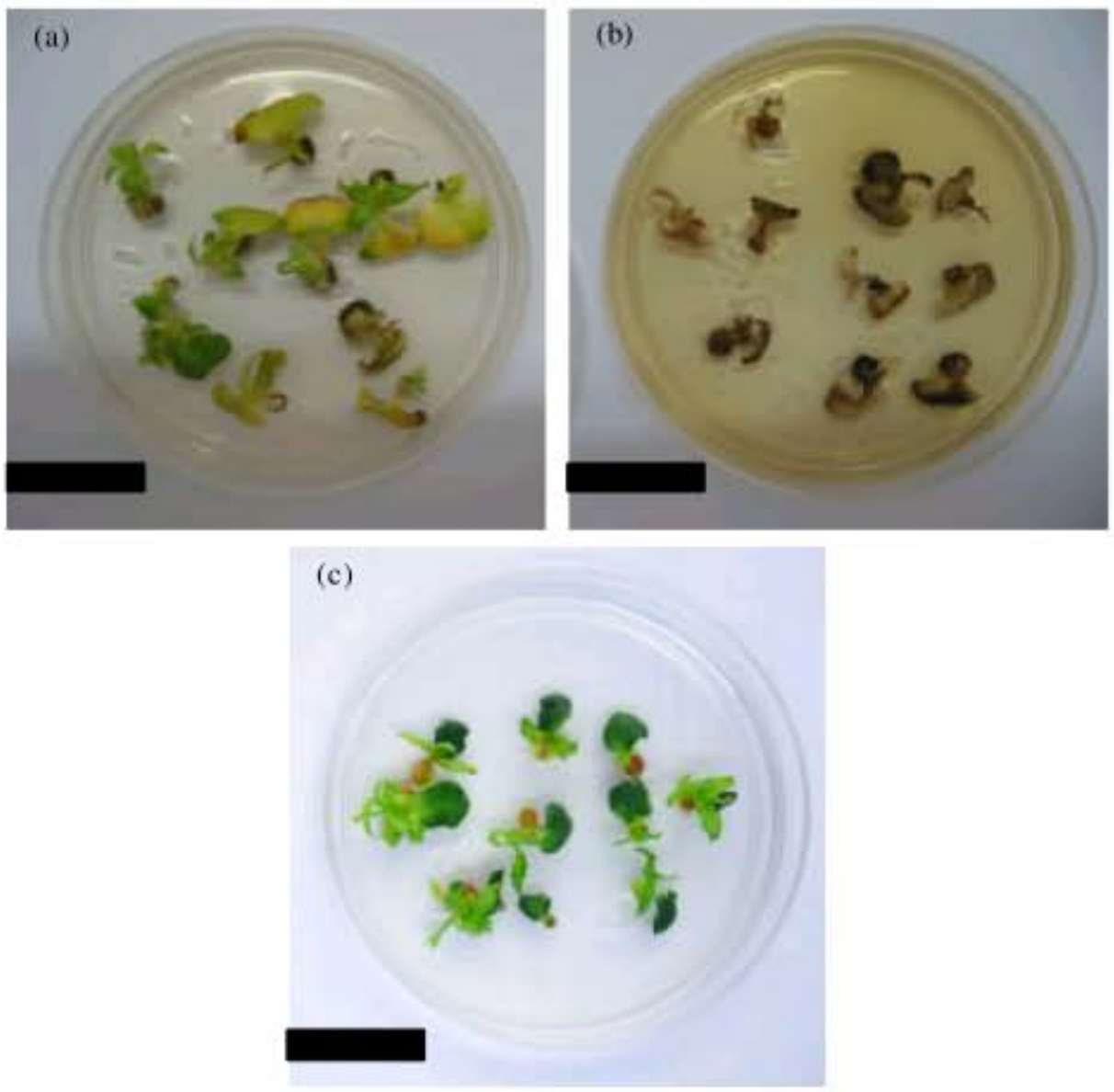

Fig. 7: The effect of different concentrations of hygromycin on explants after five weeks in culture.(a) 50 mg L^{-1}, Bar = 3 cm, (b) 75 mg L^{-1}, Bar = 3 cm and (c) 0 mg L^{-1} , Bar = 3 cm

Table 4: The effect of different ages post-bombardment on regenerating plantlets after 5 weeks in culture

Hygromycin (mg L^{-1})	Age of explants (weeks)	No. of explants bombarded	No. of regenerating explants	Percentage of regenerating plants (%)
100	0	80	0	0
	1	80	0	0
	3	80	5	7
	5	80	18	23
75	0	80	0	0
	1	80	0	0
	3	80	16	20
	5	80	43	54
0				
	0	80	80	100
	1	80	80	100
	3	80	80	100
	5	80	80	100

regeneration was observed. Selection on 5 weeks old explant exhibited 54% of explants survival (Table 4, Fig. 8). When the hygromycin was increased to 100 mg L^{-1}, the explant survival started to decrease to 23%. However, when selection was carried out on 3 weeks old explants, the explants survival reduced drastically to 20 and 7% for 75 and 100 mg L^{-1}, respectively. Based on above observation, in the following experiment, regeneration of transgenic plantlets was carried out on 75 mg L^{-1} hygromycin at 3 or 5 weeks post-bombardment.

Regeneration of transgenic *Impatiens balsamina*: A total of 84 putative transgenic plants were regenerated from 160 explants bombarded with pRQ6 plasmid (Table 5). When 40 of the regenerated plants parts were subjected to GUS assay, 14 of them showed positive GUS results. The GUS positive plants were later subjected to PCR analysis to confirm the presence of *hph* gene in the resistant plants. Results showed that all 14 GUS positive plants were positive for PCR. Figure 9 shows the expected 0.8 kb DNA fragment of *hph* gene amplified from these plants.

Transformation frequency was determined based on the number of GUS and PCR positive transformed plants per number of explants bombarded. Table 4 shows that transformation frequency of 18.3% was obtained in this study. This result demonstrated the successful transformation using biolistic and hygromycin as the selection agent. This procedure could be used as a basic system for future transformation using other useful genes into *Impatiens balsamina*.

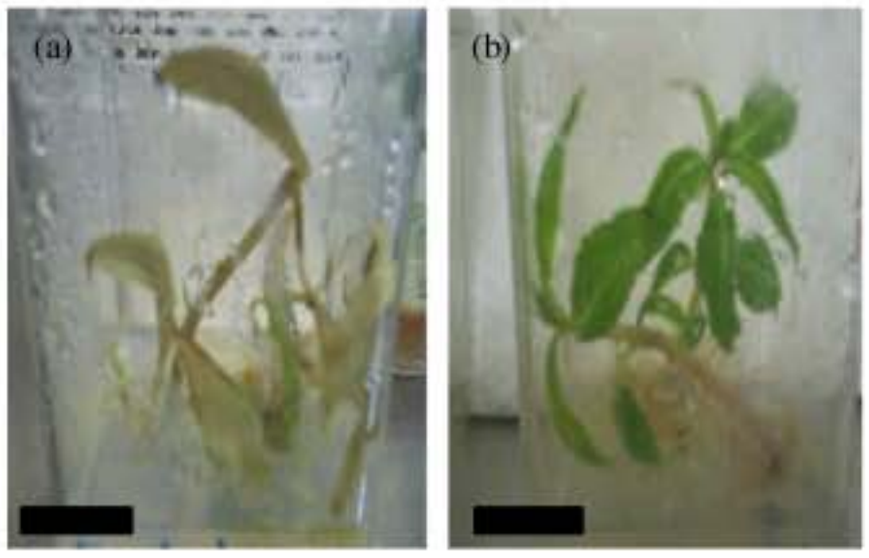

Fig. 8: The effect of hygromycin on plantlet regeneration (a) Untransformed plants in MS media supplemented with 75 mg L^{-1} hygromycin. Bar = 4 cm and (b) Transformed plants in MS media supplemented with 75 mg L^{-1} hygromycin. Bar = 4 cm

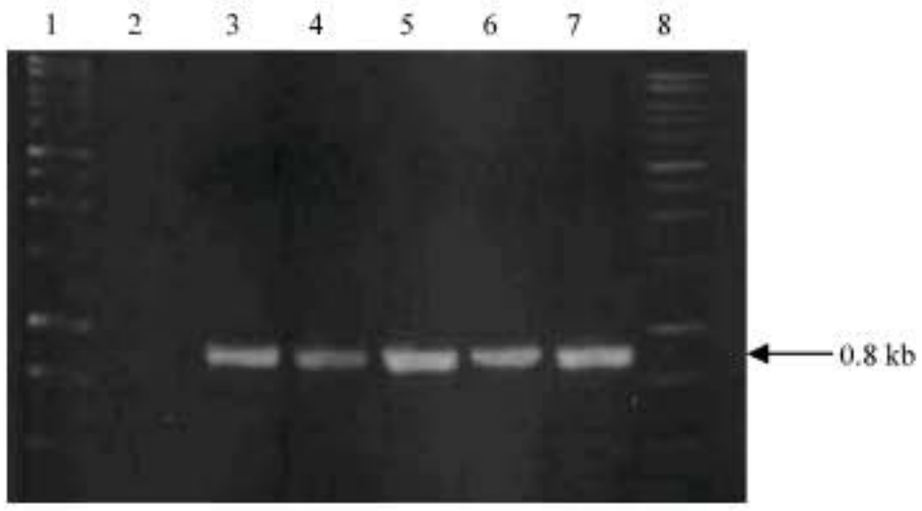

Fig. 9: Agarose gel electrophoresis of PCR product of transformed *Impatiens balsamina*. A 0.8 kb DNA was amplified using specific primers for *hph* gene Lane 1 and 8: 1 kb DNA marker (Promega) Lane 2: non-transformed plant cell Lane 3-6: transformed plants showing 0.8 kb *hph* gene Lane 7: PCR of pRQ6 (*hph*) plasmid

Table 5: The transformation frequency of bombarded explants with 5 weeks post-bombardment incubation period in 75 mg L^{-1} hygromycin

Treatment	No. of explants bombarded	No. of regenerated plants	No. of GUS^+ plants/ Total plants analyzed	Transformation frequency (%)
75 mg L^{-1} hygromycin	160	84	14/40	18.3

DISCUSSION

The present study on biolistic transformation of *Impatiens balsamina* was carried out using plasmid pRQ6 carrying *hph* and *gusA* genes. DNA is commonly bound to tungsten or gold particles by calcium chloride and spermidine co-precipitation. In the present study, the gold particles (1.0 µm) was chosen because tungsten has been reported to acidify and degrade DNA bonds (Sanford *et al*., 1993). It also has a greater tendency to aggregate during precipitation (Christou *et al*., 1990). It was reported in chickpea, transformation using gold particles produced a higher transformation frequency (18%) as compared to tungsten (15%) (Indurker *et al*., 2007). In other reports, gold particles were the preferred microcarriers for biolistic transformation because of their uniformity, spherical shape and inert nature (Jain *et al*., 1996; Russell *et al*., 1992).

Successful transformation using the biolistics depends on the physical and biological parameters. Therefore the optimization of both parameters is important (Kikkert *et al*., 2004). The success of both parameters depends on the velocity using the target distance and helium pressure and these were also subjected to different tissue types and the cell wall thickness to allow penetration of several layers (Birch and Bower, 1994). In present study, the *gusA* gene expression was examined in all different target distances between stopping screen to target tissue and helium pressure tested. The target distance and helium pressure study showed that the highest *gusA* gene expression was obtained when using 9 cm target distance and 1,100 psi helium pressure. These results were achieved as the organized tissues with thicker cell walls required higher particle velocities for penetration than thin walled cells from suspension cultures (Birch and Bower, 1994). In castor, it was reported that a target distance of 6.0 cm with helium pressure of 1,100 psi gave the optimum transformation efficiency (Sailaja *et al*., 2008). Increasing the helium pressure to 1,350 psi will increase transient expression; however, it resulted in drastic reduction in the frequency of surviving shoots and shoots failed to survive after third selection. Helium pressure showed positive correlation to transient expression but negatively to shoot recovery. In sugarcane, a target distance of 7 cm and 1,100 psi helium pressure was found to be optimum (Jain *et al*., 2007). However, in hop (*Humulus lupulus* L.), a target distance of 12 cm and 1,350 psi helium pressure was found to be optimum (Batista *et al*., 2008). At shorter distances, 6 and 9 cm, cellular damage due to particle impact showed negative effect on regeneration by decreasing callus formation and increase in tissue browning. It was also suggested that for most plant applications, 1,100 psi is optimal or nearly optimal (Sanford *et al*., 1993).

Multiple bombardments were normally carried out with the objective of getting better coverage of the target area and also to compensate for misfires from faulty and poorly set rupture discs (King and Kasha, 1994). However, multiple bombardments can also cause higher tissue damage (Taylor and Vasil, 1991). The balance between increased gene transfer and increased cell injury

determines the benefit from multiple bombardments and this is best determined empirically for a specific target tissue and bombardment conditions (Wong, 1994; Birch and Bower, 1994). The double bombardments in the present study apparently caused cell injury as the explant's shoot regeneration was inhibited although the *gusA* gene expression was higher than using one time bombardment. Similarly it was reported for castor that single bombarded explants resulted in significantly higher number of shoots surviving selection as compared to double (two times bombardment) and reverse bombardment (both sides of embryo axis) (Sailaja *et al*., 2008). This probably could be due to the possible extensive explant injury during double bombardments. This shows that higher number of transient *gusA* gene expression is not the main criteria for optimization; it must be followed by the highest number of surviving transformants.

The use of the appropriate amount of DNA is important in order to produce efficient DNA-microcarrier binding. The precipitation of DNA onto gold particles (1.0 μm) at different amount was tested to determine the optimum amount for DNA delivery. The use of high amount of DNA (1.5 μg) in the present study gave the highest *gusA* gene expression. However, increasing the amount of DNA precipitated increases transient expression numbers until particle aggregation occurs, resulting in poor dispersal and increased cell damage (Birch and Bower, 1994). In peanut and bean, increasing the amount of DNA above the optimum quantity (1.25 and 2.5 μg, respectively per bombardment) has also resulted in particle aggregation and reduced *gusA* gene expression (Clemente *et al*., 1992; Aragao *et al*., 1993). Therefore, the use of 1.0 μg of DNA also resulted in the optimum *gusA* gene expression in this study.

Osmoticum has also been shown to have major effects on transformation efficiency in and both chloroplast and nucleus in plants (Birch, 1997). Delivery of DNA into cells requires the penetration of microcarriers by high velocity bombardment. This penetration can disturb the intracellular lipid membrane structure causing cell destruction and ethylene accumulation (Imaseki, 1986). The use of an osmoticum can facilitate stabilization of cell membranes for faster healing of the lesion and reduce turgor pressure of cells to reduce leakage and cell rupture (Perl *et al*., 1992; Ye *et al*., 1994). Moreover, it has been proposed that osmotic treatment could reduce the volume of the vacuoles which could increase the possibility of reaching the nucleus. Consequently, resulting in a larger number of cells could successfully express the introduced gene (Santos *et al*., 2002). In this study highest *gusA* gene expression was obtained using 0.2 M mannitol and 0.2 M sorbitol. In agreement, it was reported that 0.2 M sorbitol and 0.2 M mannitol would resulted in highest transformation efficiency in castor (Sailaja *et al*., 2008). In pearl millet (*Pennisetum glaucum* L) highest transformation efficiency was obtained using 0.25 M sorbitol and 0.25 M mannitol (Latha *et al*., 2006).

In this present study, 4 h pre-culture treatment using osmotic treatment, prior to bombardment, showed that the damages of cell membrane and loss of cytoplasm were due to the ineffective osmotic treatment. The pre-culture treatment using the osmoticum for 16 h prior bombardment gave the highest results for the *gusA* gene expression compared to 32 h prior bombardment. In pearl millet exposure of explants to osmoticum medium for 4 h prior to bombardment and 16 h post-bombardment resulted in the highest transformation efficiency (Latha *et al*., 2006). However, in castor, exposure of explants to osmoticum medium for 2 h prior to bombardment and 2 h post-bombardment resulted in the highest transformation efficiency (Sailaja *et al*., 2008). It was suggested that osmoticum treatment has a significant role to play in successful production of transgenic castor plants.

In order to obtain the minimal inhibitory concentration of hygromycin, the explants were subjected to different concentration of selection agents. The results showed that with 75 mg L^{-1} of hygromycin, all explants were necrotic and 100% of mortality was observed. In Kentucky blue grass, selection of transformants using 100 mg L^{-1} hygromycin was optimum and resulted in a high transformation efficiency (22%) (Gao *et al*., 2006). In contrast, 50 mg L^{-1} hygromycin was the optimal concentrations that inhibited the growth of calli in rice (Lee *et al*., 2003; Li *et al*., 1993), oil palm (Parveez *et al*., 1996) and cotyledons of pepper (Li *et al*., 2003). The timing of selection also influences the transformation efficiency and there were reports that showed delayed exposure to selection agent resulted in higher number of transformants obtained (Ghosh *et al*., 2002). In the present study, the later selection (5 and 3 weeks post bombardment) gave a higher number of resistant plantlets as compared to earlier selection (1 week post bombardment) which resulting in 100% of explants mortality. The resistant plants could be obtained from three weeks bombarded explants on 75 mg L^{-1} hygromycin, however, the percentage was lower in terms of *gusA* gene expression and resistant plants. Similarly, in oil palm, selection using hygromycin 3 weeks post-bombardment resulted in higher number of transformants as compared to selection after 1 week (Parveez and Christou, 1998). However, the differences were not significant. This finding concurs with (Li *et al*., 2003) who reported that the delay selection treatments led to an

increased in differentiation efficiency. In contrast, Men *et al*. (2003) reported that later selection on 30 mg L^{-1} hygromycin resulted in a relatively lower transformation efficiency of orchids. Selection at the third week after bombardment was preferred as it allows transformed cells to divide several times. The amplification process will result in a critical mass of transformed cells which is important for maintaining the survival of cells under selection pressure.

In the present study, the bombarded 5 weeks old explants were cultured on 75 mg L^{-1} hygromycin and produced 55% resistant plantlets. Only 14 out of the 84 plants tested were GUS positive and followed by positive PCR for the presence of the *hph* gene in the plants genome. Initially, the expression of the *gusA* reporter gene was localized to few cells on the cotyledons explants, however, during the development of leaves, the expression increase from few cells to nearly on the whole leaf. This finding concurs with Gobert *et al*. (2006) who reported that in *Arabidopsis*, initially the expression occurs in small clusters of cells that tend to concentrate near the leaf vasculature and cotyledon periphery, creating the spotty pattern. However, during the development of leaf, the expression becomes more ubiquitous around vascular bundles.

It was observed that only 14 out of the 84 regenerated plants (18%) were positive for *gusA* gene expression and PCR analysis. This low rate of positive transformants may be due to escapes or chimeric nature of the transformants. In this study, we take advantage of the meristematic target tissue as it can be excised and regenerated to plants with minimal time in tissue culture. However, the high proportion of transformed regenerants is likely to be chimeric as observed with soybean and cotton (Birch and Bower, 1994). Genotypic segregation, in the progenies, can confirm the chimeric nature of the transformants in question and can allow conclusions to be drawn about the ontogeny of *in vitro* adventitious shoot formation. Currently, we have successfully demonstrated the stable transformation of *Impatiens balsamina* using microprojectile bombardment and hygromycin as the selection agent with the transformation frequency of 18.3% based on 14 GUS and PCR positive regenerated plants from a total of 64 regenerated plants.

CONCLUSIONS

In this study, the optimization of physical and biological parameters affecting transformation and regeneration of transgenic *Impatiens balsamina* using microprojectile bombardment were described. Minimal inhibitory concentration of antibiotic hygromycin was also determined for selecting transformants. Using the optimum parameters and concentration of selection agent, transgenic *Impatiens balsamina* plants were successfully regenerated from cotyledon explants. There is evidence of escapes or chimeric plants production based on GUS and PCR analysis. It is proposed that the hygromycin selection scheme (concentration and time of selection initiation) be further optimized for future production of escapes or chimeric free transgenic *Impatiens balsamina*.

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