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Agrobacterium tumefaciens-infection Strategies for Greater Transgenic Recovery in *Nicotiana tabacum* cv. TAPM26

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ABSTRACT

The efficiency of *vir* gene induction and conditions of T-DNA delivery are important parameters for developing an efficient *Agrobacterium*-mediated plant transformation system. Reliable selection regimes coupled with an efficient regeneration method are important for obtaining stably transformed plants. This study reported a modified protocol for greater recovery of transgenic tobacco (*Nicotiana tabacum* cv. TAPM 26) plant using *Agrobacterium tumefaciens*-mediated transformation combined with herbicide Basta as the selection agent. Effective post co-cultivation conditions were developed by investigating the selection regime *in vitro* and *ex vitro*. The effects of carbenicillin on *Agrobacterium* growth and plant regeneration to determine non-phytotoxic concentration of carbenicillin was also evaluated as antibiotics used for *Agrobacterium* growth suppression have known to adversely affect plant regeneration. It was discovered that the efficient conditions that led to greater recovery of transformants includes the exposure to 400 mg L^{-1} of carbenicillin post-co-cultivation for *Agrobacterium* elimination, 1 week of recovery period prior to Basta selection and prolonged selection pressure extended to rooting process which significantly reduces chimeric events. Basta concentration of 1.5 mg L^{-1} at tissue culture level was most effective in maintaining selection pressure without affecting the plants' regeneration competency. Using this improved protocol, an average of 7.00±0.33 Basta resistant shoots were obtained per leaf explant transformed and a total of 371 independently transformed tobacco plants were regenerated in this study. This highly reproducible protocol could be used to produce transgenic tobacco plants expressing useful traits or can be utilized as model system to study the expression of particular genes.

Key words: Tobacco, *Agrobacterium tumefaciens*, optimization, Basta, transformation, regeneration, herbicide

INTRODUCTION

Genetic transformation of plants is the most crucial technique required for any particular crop improvement. *Agrobacterium tumefaciens*-mediated transformation is considered as the most effective gene transfer method for plant species. It has s number of advantages over direct gene transfer technique, namely insertion of lower copy number of transgene which helps to minimize

the risk of gene silencing, production of fertile transformants, precise gene transfer and integration of DNA sequences with defined ends, the ability to transfer larger fragments of T-DNA and technically simple method (Veluthambi *et al*., 2003; Zhu *et al*., 2000; Zupan *et al*., 2000).

Most scientists focus solely on optimizing the pre-infection conditions which depends on transient expression of transferred gene to increase their transformation efficiency, however, the strategy does not always effective to produce stably transformed transgenic plants. This may be due to lack of correlation between T-DNA delivery and the stable transformation events, inefficient selection regimes and regeneration system (Janssen and Gardner, 1990). A reliable selection procedure is highly essential for the recovery and regeneration of minority group of transformed cells from the majority of untransformed cells as described by Song *et al*. (2004). However, to impose an efficient selection system while maintaining the competence for regeneration, type of selection agent used and timing of selection application in culture need to be studied thoroughly. In addition, an optimal concentration of selection agents is essential in recovering transformed cells and regenerating whole transgenic plants. A high concentration of selection agent may results in cell death of weakly expressing transformed plant (Parveez *et al*., 2007). On the other hand, a much lower selection pressure may results in formation of chimeric plants or escapes.

It was reviewed that there are more than fifty selectable marker genes being used in plant genetic transformation studies (Miki and McHugh, 2004). Most commonly used selectable marker genes in plant transformation are neomycin phosphotransferase *(nptII)* (Bevan *et al*., 1983) and hygromycin phosphotransferase (*hptII*) (Gritz and Davies, 1983), which confers resistance towards antibiotics kanamycin and hygromycin, respectively. Phosphinothricin acetyltransferase produced by *bar* gene confers resistance to phosphinothricin, an active ingredient of a commercial herbicide, Basta (Thompson *et al*., 1987). All these selectable marker genes are categorized as negative selection marker, in which the untransformed cells are usually killed by the selection agent supplemented in the medium which allowing only the transformed cells to survive. In contrast, positive selection marker provides metabolic or developmental advantages to the transformed cells and promote faster proliferation and regeneration. The untransformed shoots are therefore easily recognized by their suppressed growth compared to the well developed transformed shoots (Veluthambi *et al*., 2003). One example of positive selectable marker gene is *pmi,* encoding for phosphomannose isomerase (*pmi*) which could utilize mannose as a carbon source by converting mannose-6-phosphate into fructose-6-phosphate (Joersbo and Okkels, 1996). Mannose usually could not be utilized by plant cells, therefore when sucrose is replaced with mannose, the untransformed cells will starve and resulting in growth suppression.

In post co-cultivation process, a range of antibiotics and their concentrations were used to eliminate *Agrobacterium* cells from cultures. Failure to control or suppress *Agrobacterium* growth lead to overgrowth of *Agrobacterium* cells and subsequently necrosis to explant tissues. Common antibiotics to control *Agrobacterium* growth are carbenicillin, cefotaxime, augmentin and timentin. However, carbenicillin and cefotaxime are the most extensively used in transformation studies (Pollock *et al*., 1983: Mathias and Boyd, 1986). In transformation studies, one of the less explored factors is the effect of these antibiotics on plant morphogenesis. Antibiotics have been known to have plant hormone-like effects on cultured plant tissues at particular concentrations.

Therefore, this study aimed at developing effective post co-cultivation conditions for *Agrobacterium tumefaciens* transformation of tobacco by evaluating the effects of carbenicillin on shoot regeneration and also developing the most effective selection mechanism using herbicide Basta as the selection agent, *in vitro* and *ex vitro*.

MATERIALS AND METHODS

Plant material: Seeds of *Nicotiana tabacum* cv. TAPM 26 were obtained from the National Tobacco and Kenaf Board, Kota Bharu, Malaysia in year 2009. Seeds were surface sterilized by immersing in 20 % (v/v) of CLOROX® (commercial bleach with 5.25% of sodium hypochlorite) for 20 min with gentle agitation. The seeds were then thoroughly rinsed several times with sterile double distilled water. Sterilized seeds were placed on MS (Murashige and Skoog, 1962) solid medium containing 0.1 g myo-inositol, 30 g sucrose and 2.7 g phytagel per liter for germination process. pH of the medium was adjusted to 5.7-5.8. Petri dishes (Brandon™) with 10 seeds per plate (90×15 mm) on solid medium were incubated in tissue culture chamber at 25°C under a 16/8 h light/dark photoperiod with light intensity of 12.16 μmol/m^2/sec. After 5 to 6 weeks, young, green and expanded leaves were harvested from *in vitro* grown seedlings and used as explants for the transformation studies.

Preparation of bacterial inoculum: Bacterial stock cultures of *Agrobacterium tumefaciens* strain LBA 4404 harbouring pCambar was kindly provided by Dr. Norwati Adnan from Forest Research Institute of Malaysia (FRIM), Kepong, Malaysia. pCamBar, originally derived from pCambia 1301 (CAMBIA, Canberra, Australia), contains *nptII* gene encoding resistance to kanamycin and *bar* gene encoding resistance to phosphinothricin (PPT), which were used for bacterial selection and plant selection, respectively. The construct also contains *E. coli gus*A reporter gene as a visual marker used in GUS assay system.

Agrobacterium tumefaciens from glycerol stock culture was streaked onto solid YEP medium containing filter sterilized solutions of 100 mg L^{-1} kanamycin (SIGMA) and 100 mg L^{-1} streptomycin (SIGMA) and incubated at 28°C for 2 days. A single colony was isolated and inoculated in 2 mL of YEP medium with appropriate antibiotics and grown overnight at 28°C, with 200 rpm agitation. Overnight culture was then added into 30 mL of liquid YEP medium with antibiotics and 100 μM acetosyringone (AS) (FLUKA) and was grown and incubated at 28°C for 2 days.

Bacterial cells were harvested at A_{600nm} 0.8 (1×10^7 cfu mL^{-1}) by centrifugation (Zentrifugen) for 15 min at 1000 g. Pellet obtained was re-suspended in 10 mL of liquid MS medium. A 1 mL of the re-suspended pellet was used as inoculum for the infection of 10 leaves at a time, in 15 mL of MS liquid medium.

Determination of minimal inhibitory concentration of Basta for wild type *Nicotiana tabacum* cv. TAPM 26: Herbicide Basta (Bayer CropsScience) was used as the selection agent in this study to facilitate the recovery of transgenic tobacco cells. Basta was prepared as 1 mg mL^{-1} stock solution and filter sterilized using 0.2 μM Acrodisc. MS solid medium for shoot induction contained 4 mg L^{-1} BAP and 0.1 mg L^{-1} IBA was used for the assessment of effects of Basta on explants at tissue culture level. Basta was added into the shoot induction medium at concentration of 0.5, 1, 1.5, 2, 2.5 and 3 mg L^{-1}. Six replicates (plates) were used for each concentration, including for the control experiment (without Basta). The petri dishes were incubated at 26°C in tissue culture chambers. The percentage of surviving explants for each treatment were calculated based on number of either shoot forming/ callus forming/ green explants over total number of explants used.

Basta leaf painting assays was conducted using *in vitro* grown *Nicotiana tabacum* which has been acclimatized to *ex vitro* conditions. Basta at concentrations of 0.5, 1, 1.5, 2.0, 2.5 and 3% (v/v)

were applied to determine the minimal concentration required to induce damage to the leaves. Plants were painted on the upper surface of leaves with concentrations of Basta as specified and scored for herbicide sensitivity 3 to 5 days later.

Determination of effective concentration of carbenicillin for the elimination of *Agrobacterium tumefaciens*: In order to mimic the actual transformation conditions, leaf explants harvested from seedlings were co-incubated with *Agrobacterium tumefaciens* suspension. The bacterial inoculums for the infection process was prepared as described earlier. Leaf explants which were previously wounded and pre-cultured for 48 h were co-incubated with *Agrobacterium* inoculum for 30 min. After two days of co-cultivation, explants were washed vigorously with sterile distilled water and placed on MS medium (containing 4 mg L^{-1} BAP and 0.1 mg L^{-1} IBA for shoot initiation) which was supplemented with various concentrations of carbenicillin (SIGMA) (100, 200, 300, 400, 500 and 600 mg L^{-1}). Bactericidal effects of carbenicillin were evaluated by observing the explant culture plates for *Agrobacterium tumefaciens* growth.

Determination of non phytotoxic concentrations of carbenicillin: In order to evaluate the effect of carbenicillin on shoot regeneration, leaf explants obtained from *Nicotiana tabacum* seedlings (5-6 weeks old) were cultured on MS medium with 4 mg L^{-1} BAP, 0.1 mg L^{-1} IBA and different concentrations of carbenicillin (0, 100, 200, 300, 400, 500 and 600 mg L^{-1}). Explants were incubated in tissue culture chambers.

Plant transformation: Transformation of tobacco was performed according to Horsch *et al.* (1985), with minor modifications. *Nicotiana tabacum* leaves from 5-6 weeks old seedlings were harvested with the petiole attached. Tiny pricks were made on explant surface using a sterile scalpel. An approximately 1 mm of the margins of the leaves was cut, leaving the petiole intact. Explants were immersed in pre-culture medium (liquid MS medium) and were incubated at room temperature for two days (Sunilkumar *et al.*, 1999). Bacterial inoculum was prepared as described earlier.

After pre-culture, explants were retrieved from the pre-culture medium and were immersed in infection medium containing 15 mL of liquid MS basal medium (Murashige and Skoog, 1962) and 1 mL of bacterial inoculum. The co-incubation of explants with bacterial suspension was carried out for 30 min at room temperature. After the infection process, leaf explants were blotted dry on sterile filter paper, to remove excess bacterial suspension. The infected leaf explants were then placed on solidified co-cultivation MS medium supplied with 200 μM acetosyringone (AS) (Fluka), 4 mg L^{-1} BAP and 0.1 mg L^{-1} IBA and were incubated in the dark for 2 days at co-cultivation temperature of 22°C. After co-cultivation, explants were washed several times using sterile distilled water. Subsequently, the explants were blotted dry on sterile filter paper before being placed on either Resting Medium (RM) or Selection Medium (SM). Both medium consisted of MS basal salts, vitamins, 4 mg L^{-1} BAP and 0.1 mg L^{-1} IBA, 400 mg L^{-1} carbenicillin, whereas, SM had 1.5 mg L^{-1} Basta (Bayer CropsScience) added as a selection agent. Explants cultured on RM were sub-cultured onto SM after resting period of one week. Explants were sub-cultured every two weeks and usage of carbenicillin was discarded after the first two weeks of culture. All cultures were incubated under 16/8 h of light/dark photoperiod at 25°C.

Shoots regenerated from leaf explants which had reached 1-2 cm in length were eventually sub-cultured on Elongation Medium (EM) (hormone free basal MS medium). Browning shoots or

tissue segments showing signs of necrosis were constantly excised or trimmed before each subculture was carried out. Eventually, after the shoots had reached 3-4 cm in length, they were transferred to solid Root Induction Medium (RIM) to induce roots. RIM MS medium was supplemented with 0.5 mg L^{-1} IBA. Basta at 1.5 mg L^{-1} was continually supplemented in all cultures to maintain selection pressure and prevent escapes. Once the shoots had well developed roots, they were acclimatized to *ex vitro* conditions according to Burrow *et al.* (1990).

β-Glucoronidase (GUS) histochemical assay: Histochemical assay for the visual detection of GUS gene expression was carried out according to Jefferson (1987). Histochemical analysis was performed on segments of Basta resistant shoots regenerated on selection medium, at two stages. The first GUS assay was conducted on the primary shoot segments which were induced on SIM medium and the subsequent GUS assay was carried out on shoot segments obtained from RIM medium. The analysis was carried out by incubating the shoot segments in GUS substrate buffer at 37°C for 18-24 h. Segments that displayed blue colouration, which indicates stable integration of the GUS gene, were recorded as GUS positive. The GUS substrate buffer consisted of X-Gluc (1 mM), potassium ferrocyanide (5 mM), potassium ferricyanide (5 mM), sodium phosphate buffer (100 mM), pH 7.0, TritonX-100 (0.1%) and methanol (10%). After GUS staining, the explants were immersed in ethanol (70% (v/v)) for 3 to 4 h to remove chlorophyll for ease of observation.

Polymerase Chain Reaction (PCR) analysis: Molecular analysis was performed on the putatively transformed plants of T_0 generation using Polymerase Chain Reaction (PCR). Genomic DNA was extracted from putative transgenic plants using QuickExtract™ plant DNA extraction (EPICENTRE®, Biotechnologies, USA) solution. Genomic DNA extractions were carried out according to manufacturer's instructions. Genomic DNA extracted from untransformed plants were used as negative control while plasmid DNA extracted from *Agrobacterium tumefaciens* LBA 4404 was used as positive control. Plasmid DNA was extracted using QIAprep® Spin Miniprep kit (QIAGEN). The primer for amplification of *bar* (0.5 kb) and *gus* (1.1 kb) gene fragments were: 5'- TCA AAT CTC GGT GAC GGG CA -3' and 5'- GGT CTG CAC CAT CGT CAA CC -3' for *bar* gene; 5- AACTGGACAAGGCACTAGCG -3' and 5'- CACCGAAGTTCATCGCAGTC -3' for *gus* gene. The amplification reaction was carried out using a Bio-Rad MyCycler™ under the following conditions: 35 cycles of denaturation at 95° for 30 sec, annealing at 62° for 40 sec and extension at 72° for 1 min.

Herbicide leaf painting assay: Leaf painting assay using herbicide Basta was carried out to test putative transgenic plants' tolerance against the toxicity of herbicide Basta at 0.5% (v/v). Basta resistance was tested on putative transgenic plants that have been transferred onto soil and untransformed plants were used as control. Basta solution at 0.5% (v/v) was painted upon leaves of transformed and untransformed plants and the results were scored after one week. Transformed plants which remained undamaged were recorded to determine transformation efficiency.

Data analysis: All data were subjected to Analysis of Variance (ANOVA) statistical test using SPSS software version 15.0 (SPSS Inc, USA). The means were compared for significant differences at $p<0.05$ level. All experiments were repeated at least thrice.

RESULTS

Determination of minimal inhibitory concentration of Basta for wild type *Nicotiana tabacum* cv. TAPM26: The minimal inhibitory concentration of Basta for *in vitro* selection of transgenic plant cells after co-cultivation process was evaluated using leaf explants from wild type *Nicotiana tabacum*. The leaves were incubated on medium containing various concentrations of Basta. Results obtained are presented in Table 1, within two weeks; all explants culture at 1.5 mg L^{-1} and above turned necrotic and died. Tobacco leaves were found to be highly sensitive towards Basta concentration as low as 2 mg L^{-1}, to which the cultured explants responded by turning into dark brown in colour within 4 to 6 days. These reactions show that the explants had acute and massive cell death. Therefore, these concentrations were considered too high for transgenic cell recovery. On the other hand, 0.5 mg L^{-1} of Basta was proven non-lethal to explants as callus and shoot formation was detected, though they appeared to be growing at slower pace, whereas at 1 mg L^{-1} of Basta concentration, only callus growth was observed and eventually cell death occurred after 5 weeks. Basta concentration at 1.5 mg L^{-1} effectively suppressed the development of shoots and within two weeks all explants were completely necrotic, representative explants cultured at different concentration of Basta are shown in Fig. 1a-d.

As for leaf painting assay, Basta concentration at 1% (v/v) and above was toxic to the tobacco plants and the affected plants wilted and died within a week. At 0.5% (v/v), the plants were less affected compared to 1% (v/v) and above. Basta concentration higher than 2% (v/v) completely turned the plant tissue necrotic and became brown within 3 days. Therefore, 0.5% (v/v) could be used for the initial leaf painting experiment, to test the transgenic shoots resistant in further experiment. In this way, level of *bar* gene expression can be determined without killing it by applying very high concentrations of Basta. Representative leaves from Basta treated plants are shown in Fig. 2a-d.

Determination of the carbenicillin concentration for the elimination of *Agrobacterium tumefaciens*: Results in Table 2 showed that the carbenicillin concentration at 400 mg L^{-1} was the most effective in suppressing *Agrobacterium*'s growth. Lower concentrations of carbenicillin (100-300 mg L^{-1}) were proven not effective as explants cultured at these concentrations were excessively infected with *Agrobacterium* cells and subsequently became necrotic and died.

Current results suggested that carbenicillin concentration at 500 and 600 mg L^{-1} had a negative impact on shoot regeneration capability of the explants tested as shown by the significant decline in shoot regeneration compared to that of the control explants. Carbenicillin concentrations up to 400 mg L^{-1} showed no significant differences in shoot regeneration as compared to the control medium. Based on statistical analysis, carbenicillin concentrations below 500 mg L^{-1} had

Table 1: The effect of different concentrations of Basta on *Nicotiana tabacum* explant after 2 weeks in culture

Basta concentrations (mg L^{-1})	Surviving explant (%)
0	100^{a}
0.5	100^{a}
1	62.2^{c}
1.5	0^{b}
2	0^{b}
2.5	0^{b}
3	0^{b}

Data within same column followed by the same letter(s) indicate no significance at 5% level

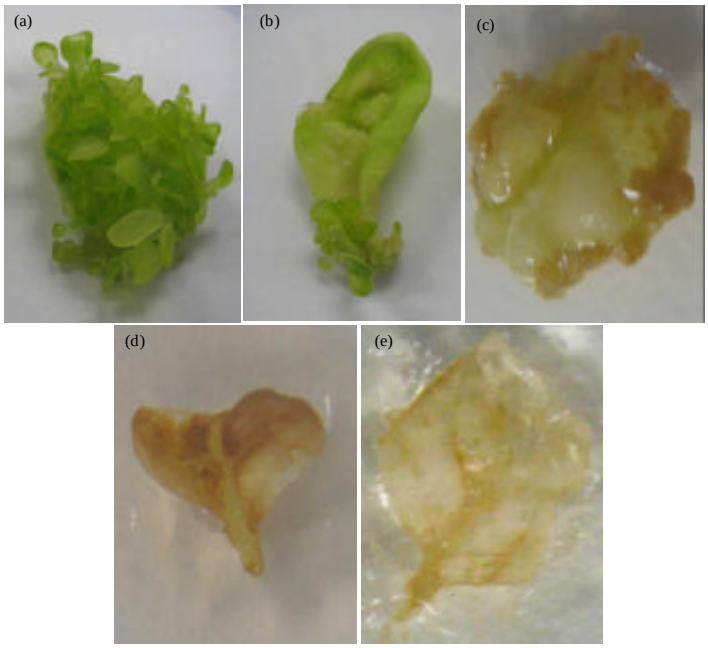

Fig. 1: Tobacco explants exposed at different concentrations of Basta. (a) Control (0 mg L^{-1}), (b) at 0.5 mg L^{-1}, (c) at 1 mg L^{-1}, (d) at 1.5 mg L^{-1} and (e) at 2.0 mg L^{-1}

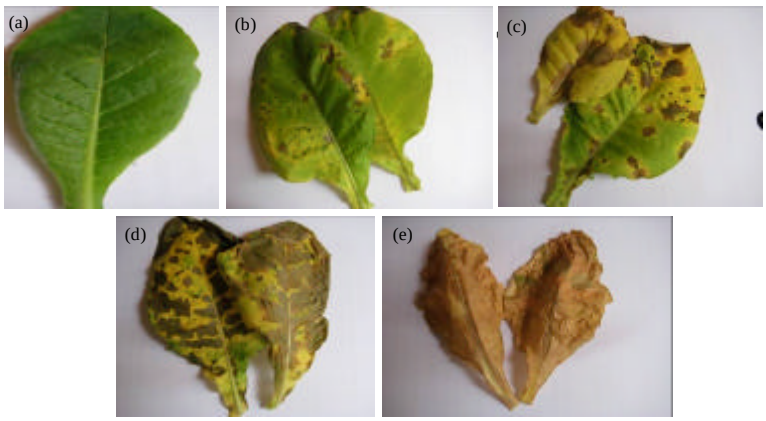

Fig. 2: Leaf panting assay, showing tobacco leaf exposed to various concentrations of Basta, after 5 days. (a) Control (0 mg L^{-1}), (b) at 0.5 mg L^{-1}, (c) at 1 mg L^{-1}, (d) at 1.5 mg L^{-1} and (e) at 2.0 mg L^{-1}

Table 2: The effect of carbenicillin on *Agrobacterium tumefaciens* LBA 4404's growth and shoot regeneration of leaf explants

Carbenicillin concentrations (mg L^{-1})	Percentage of infected explants (%)	No. of shoots per explant (Mean±SE)
0	100[a]	12.07±0.32[a]
100	100[a]	12.20±0.35[a]
200	96.7[a]	12.07±0.45[a]
300	53.3[a]	11.90±0.38[a]
400	0[b]	12.17±0.48[a]
500	0[b]	9.87±0.22[b]
600	0[b]	3.93±0.40[c]

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

Table 3: The efficiency of the transformation of *Nicotiana tabacum* using optimized conditions

No. of inoculated explants	60
No. of Basta resistant explants[a]	60
No. of shoots per explant[b]	7.00±0.33
PCR positive explants	
bar$^+$	60
gus$^+$	56
GUS positive shoots	
Primary shoots	18.3 (81.7*)
Secondary shoots	85 (15*)
No. of plantlets regenerated on selection medium with Basta	386
No. of *bar* positive regenerated plants	371

[a]Explants which were able to form shoots were considered as Basta resistant. [b]Explants with at least one PCR positive shoot. *Chimeric shoots with partial GUS expression

no significant effect on *Nicotiana tabacum* shoot regeneration. Therefore, carbenicillin concentration at 400 mg L^{-1} was used in selection medium for the elimination of *Agrobacterium* cells.

Plant transformation: A total of 386 transgenic plantlets demonstrating resistance towards the toxicity of the herbicide Basta were regenerated using the optimized conditions. From the 386 plantlets analyzed using PCR analysis, only 371 were tested positive for the presence of *bar* gene (Table 3).

Our initial attempts to regenerate Basta resistant shoots resulted in extremely low number of shoots which appeared stunted and eventually turned brown and died. Considering that the direct culture of infected explants on selection medium immediately after co-cultivation may have attributed to the failure of attaining resistant shoots, another attempt was made with slight modifications. The regeneration protocol was modified to include one week of recover and proliferation period, during which the co-cultivated explants were cultured on SIM medium containing carbenicillin without Basta.

Using the modified approach, the co-cultivated explants were transferred to the selection medium with 1.5 mg L^{-1} Basta one week post transformation. Six weeks after exposure to Basta, shoot buds begin to appear from the co-cultivated explants (Fig. 3b). Adventitious shoots were induced especially at the wounded sites on the explants (Fig. 3c). Shoot regeneration was completely inhibited in non-infected control explants and these explants turned necrotic and died within two weeks of culture on selection medium (Fig. 3a). After several rounds of subcultures, the primary shoots were succeeded by the formation of secondary shoots which grew at faster phase than the primary shoots (Fig. 3d). Individual shoots or shoot clumps (1-2 cm in length) were continuously sub-cultured on selection medium containing 1.5 mg L^{-1} Basta. Continuous culture of the regenerated shoots on selection medium eventually led to reduce number of regenerating shoots. Some of the shoots or shoot segments turned brown and died, indicating that these were chimeric shoots, resulting from mixture of transformed and untransformed cells. These shoots were removed before the following subculture process.

On average, it was demonstrated that 7.00±0.33 (Mean±SE) shoots per explant were obtained with shoot induction frequency of 100%. Elongated shoots (3-4 cm in length) were transferred to RIM medium to induce roots. A total of 386 plantlets were regenerated on Basta containing RIM

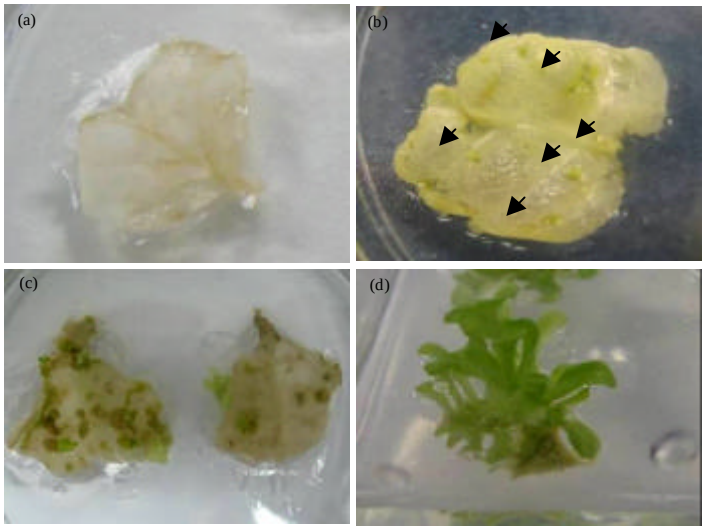

Fig. 3: Regeneration of Basta resistant shoots from *Nicotiana tabacum* leaf explants. (a) Untransformed control explant showing necrosis. (b) Shoot buds (indicated by arrows) induced on co-cultivated explant at 6th week. (c) Primary shoots formed on explants at 8th week of culture. (d) Induction of secondary shoots following the individual transfer of primary shoots to fresh medium

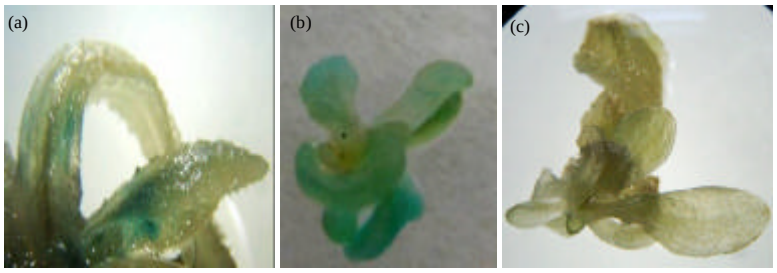

Fig. 4: Histochemical GUS assay conducted on Basta resistant shoots. (a) Primary shoots expressing partial GUS expression. (b) Secondary shoots expressing uniform GUS expression. (c) Untransformed control shoots without blue coloration

medium. Root induction and development took 5 weeks upon culture on RIM, which shows delayed root induction as compared to the control shoots in RIM without Basta, which produced roots within 2 weeks. Nevertheless, plantlets obtained were successfully acclimatized to *ex vitro* conditions.

GUS assay performed on 60 randomly chosen primary shoots and secondary shoot segments revealed different pattern of GUS expressions. Primary shoots exhibited partial or scattered blue sectors, therefore, these were considered as plant cells with chimeric nature (Fig. 4a). Secondary shoots obtained at a later stage of the regeneration process expressed uniform blue coloration throughout the shoots, indicating that the shoots consisted of fully transformed cells (Fig. 4b). On the other hand, no blue spots or blue sectors were observed on non-infected control explants (Fig. 4c). Overall results for GUS assay shows that although many primary shoots were of chimeric nature, continuous culture in selection medium with Basta progressively reduced the number of chimaeras or escapes.

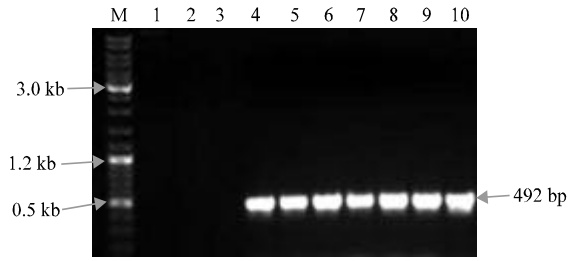

Fig. 5: PCR analysis of genomic DNA extracted from selected Basta resistant shoots shows the amplification of the 492 bp fragment of the *bar* gene. Lane M: 1 kb molecular weight marker; Lane 1-3: DNA from untransformed plants (negative controls); Lane 4-9: DNA from individual putatively transformed plants; Lane 10: pCamBar plasmid DNA (positive control)

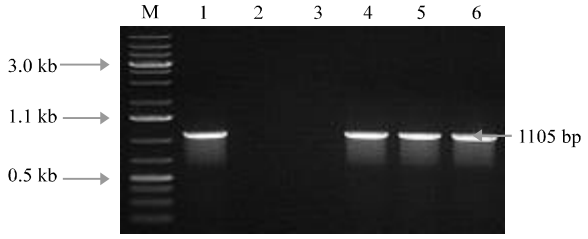

Fig. 6: PCR analysis of genomic DNA extracted from selected Basta resistant shoots shows the amplification of the 1105 bp fragment of the *gus* gene (Lane 4-6). Lane M: 1 kb molecular weight marker; Lane 1: pCamBar plasmid DNA (positive control). Lane 2-3: DNA from untransformed plants (negative controls)

Bar and *gus* genes integration into plant genome were analyzed at molecular level via Polymerase Chain Reaction (PCR) technique (Fig. 5 and 6). PCR analysis of putative transgenic shoots induced on selection medium, revealed 79% of total shoots analyzed were positive for *bar* and *gus*, whereas 17% of shoots tested positive for *bar* gene only. Overall, 96% of *bar* positive transgenic plants were obtained. Integration and expression of *bar* gene in plants were further evaluated using Basta leaf painting assay on transformed plants which has been acclimatized on soil for 1 month. *In vitro* grown untransformed plants were used as control. Out of 96 plants tested for tolerance against Basta, 93% remained undamaged or only showed negligible level of damages. Most of these plants showed slight transient discoloration and appeared to be inhibited of growth for a short period of time. One week later, these plants resumed growth and appeared morphologically normal, similar to wild types. Interestingly, several plants which were initially tested positive for *bar* gene in PCR analysis, could not survive the leaf painting assay and died. Similarly, control plants displayed dark brown lesions on surface and wilted within a week of leaf painting. Survival of majority of the transformed plants after Basta application, verified the functional expression of *bar* gene in transgenic plants.

DISCUSSION

Plant transformation involves delivering, integrating and expressing defined foreign genes into plant cells, which eventually can be regenerated into a whole transgenic plant. Efficiency of stable gene transfer is considered low even in most successful transfer system because only a few cells

would have been transformed and had stably integrated with the foreign gene in its genome. Antibiotic or herbicide resistance marker genes allow transformed cells to be selected out of the non transformed cells' population. For example Basta, which inhibits glutamine synthesis in plants is added into the selection medium (Thompson *et al.*, 1987). Plant cells which are transformed will be able to neutralize the toxic effects of selection agent and continued to survive whereas the untransformed cells will be affected by the toxic herbicide and eventually die.

Sensitivity to selection agents depend on many factors such as explant type, developmental stage, tissue culture conditions and the genotype. As in the case of the current study, a low concentration of Basta (1.5 mg L^{-1}) was sufficient to induce toxicity to the tobacco explants tested. High concentration of Basta (4 mg L^{-1}) was used for selection of transgenic tobacco by Lutz *et al.* (2001). In contrast, current study found that a concentration of Basta above 2 mg L^{-1} was proven to be too high as acute cell death was observed in the explants tested. Therefore, 1.5 mg L^{-1} of Basta was considered as the effective concentration for selection of transformed plant cells. Low level of PPT (an active ingredient in Basta) concentration was reported in maize (Frame *et al.*, 2002), soybean (Dang and Wei, 2007), Kenaf (Srivatanakul *et al.*, 2001), Acacia (Vengadesan *et al.*, 2006) and cucumber (Vengadesan *et al.*, 2004). Other studies reported higher concentrations of Basta (ranging from 5-20 mg L^{-1}) were effective in selecting a number of transgenic plants such as oil palm (Parveez *et al.*, 2007), sugarcane (Manickavasagam *et al.*, 2004), soybean (Paz *et al.*, 2004), *Arabidopsis* (Hadi *et al.*, 2002) and wheat (Melchiorre *et al.*, 2002).

In the events, when a high level of selection pressure is required, then the selection could be applied at a later stage, such as one or two weeks after the infection and co-cultivation period. In this way, single transgenic cells will have time to multiply and become more resistant to the selection agent. However, a study conducted on sugarcane has shown that delayed selection resulted in untransformed cells to develop into shoots as well (Bower and Birch, 1992). It was suggested that transformed cells located near or surrounding the untransformed cells provided cross-protection to the untransformed cells and made it possible to survive against the selection agent's toxicity. Nevertheless, prolonged period of incubation of explants on selection medium has shown to overcome problem of escape events (Manickavasagam *et al.*, 2004).

The effects of carbenicillin at different concentrations were evaluated in terms of phytotoxicity and effective elimination of *Agrobacterium tumefaciens* during post co-cultivation period. Results from this study revealed that the effective concentration of carbenicillin to suppress the growth of *Agrobacterium tumefaciens* strain LBA 4404 was achieved at 400 mg L^{-1}. Carbenicillin, along with cefotaxime were the two most commonly used antibiotics to control growth of *Agrobacterium* after co-cultivation period. Both antibiotics belong to **β**-lactam group, that inhibit cell wall synthesis in bacterial cells leading to subsequent death of the bacteria (Bycroft and Shute, 1987). However, in plant transformation, carbenicillin at higher concentrations has been shown to cause phytotoxicity in terms of regeneration. Schmitt *et al.* (1997) found that tobacco callus appeared to be completely inhibited at shoot regeneration and this was attributed in response to higher concentration of carbenicillin (above 500 mg L^{-1}). Similar observations were demonstrated in the current study, where carbenicillin at or above 500 mg L^{-1}, significantly affected the regeneration of shoots from tobacco leaf explants. In regeneration of transgenic rose, application of carbenicillin was found to completely inhibit shoot formation at all concentration (Li *et al.*, 2002). (Silva and Fukai, 2001) claimed that carbenicillin effects on plant regeneration was indeed found to be dependent on cultivar. According to Yepes and Aldwinekle (1994) carbenicillin at 250 mg L^{-1} enhanced regeneration and shoot development in apple, however at higher concentration of 500 mg L^{-1}

massive callus formation was stimulated and regeneration of shoot was completely inhibited. Lin *et al.* (1995) proposed that the phytotoxicity of carbenicillin might be partially linked to its auxin-like functional structures.

In current study, a selection process preceded by a resting period of one week, at which explants were cultured on medium without Basta. Initial attempts to regenerate transgenic shoots without using a resting period failed to obtain transgenic events. A resting period, during which no selection pressure is applied, allows the small number of cells which have been transformed to undergo several cycles of cell divisions and when the transformed cell mass increases, the expression level of transgene can be increased as well. As a result, the transformed cells would have higher chances to survive under selection pressure.

Christou and Ford (1995), reported that besides the type of selection agent used, the timing of selection application and culture practices highly influenced the recovery of transgenic plants. Similar findings have been reported by Sreeramanan *et al.* (2006) and Lee *et al.* (2006), where several days of unselected period prior to selection greatly enhanced transgenic plant recovery.

Chimeric events could be minimized or eliminated altogether by employing a prolonged selection period, which has been proven effective in the current study. GUS analysis of primary shoots revealed 81.7% of shoots were of chimeric nature, however GUS analysis of secondary shoots obtained at much later stage gave only 15% chimeric events. Thus, it is evident that continuous culture under selection pressure progressively reduced the growth of untransformed cells and only allowed the growth of transformed shoots.

Leaf painting assay using the herbicide Basta showed that the majority of transgenic plants obtained from this study were resistant against Basta. Even though these plants continued to grow healthily, a slight discolouration and inhibition of growth was observed for a short period of time after being painted with Basta solution. Inhibition of photosynthesis was one of the herbicidal role of Basta, thus the resumed growth suggested that successful expression of *bar* gene which encodes for PAT (phosphinothricin acetyltransferase) that neutralizes the effect of Basta. A study by Cao *et al.* (1992) revealed that *bar* gene driven by CaMV (Cauliflower Mosaic Virus) 35S promoter only allowed low level of expression in transformed rice, which was reflected by accumulation of ammonia level in transgenic rice plant after spraying with Basta. However, by day 5, the ammonia level was reduced to basal level which shows that sufficient amount of (PAT) has been produced.

Some transgenic plants which tested positive for the presence of *bar* via PCR, failed to survive the toxic effects of Basta. This could have been caused by lower expression of the *bar* in these plants. Transgene expression levels could be highly variable among different independently generated transgenic plants. Such variability in transgene expression could be explained by differences in multiple copies of transgene or insertion site of the transgene in plant genome (Finnegan and McElroy, 1996). Multiple copies of a transgene in a cell can result in post transcriptional gene silencing, which is also known as co-suppression. Following co-suppression, even though the transgene is transcribed, resulting mRNA will be unstable and therefore transgene expression will be severely reduced. The mechanism of co-suppression appears to involve gene methylation, which is natural defense mechanism to protect plants foreign objects' invasion (Meins, 2000; Meyer, 2000). Several methods such as co-transformation, site specific homologous recombination and transposon mediated single copy gene delivery have been identified and proposed to overcome transgene silencing and inactivation in transgenic plants (Koprek *et al.*, 2001). Transgenic tobacco generated using current method was able to demonstrate resistance to Basta. This indicates stable integration and expression of *bar* gene within the plant genome.

In conclusion, a simple and efficient plant transformation of *Nicotiana tabacum* using optimized *Agrobacterium tumefaciens* mediated transformation protocol was successfully demonstrated. In addition, current protocol also gave high efficiency of transformation and high number of transgenic plants recovery.

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