

## Agrobacterium-mediated Transformation in Potato

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### Abstract

*Agrobacterium tumefaciens* strain LBA4404 carrying a binary vector pBI121 having one reporter gene (*gus*) and selectable marker gene (*nptII*) resistant to kanamycin with internode explant was used in this study. Transformation was done by optimizing two important parameters viz. infection time and co-cultivation period. Most of the explants produced shoots within 18-21 days on 5 mg L<sup>-1</sup> zeatin riboside and 50 mg L<sup>-1</sup> kanamycin supplemented MS medium through organogenesis. Explants infection for 30-minute with 3-day co-culture produced maximum 8.27 and 6.42 shoots in Asterix and Diamant varieties, respectively within 18-21 days. Stable integration and expression of the transgenes were confirmed by histochemical and molecular analysis. DNA from well established rooted plants confirmed *nptII* and *gus* positive through PCR analysis and resulted transformation rate 28.97% and 24.37% in Asterix and Diamant varieties, respectively. 100% shoots from putative transformed plants of Diamant and Asterix varieties produced roots on ½MS medium supplemented with 50 mg l<sup>-1</sup> cefotaxim, 50 mg l<sup>-1</sup> kanamycin and 0.5 mg l<sup>-1</sup> IBA.

**Keywords:** transformation, potato, *nptII* gene, *gus* gene, *agrobacterium*

### Introduction

Potato (*Solanum tuberosum* L.) belongs to the family Solanaceae originated in the central Andean mountain of South America. Today, potatoes are grown worldwide from their home to the highlands of Africa and Asia, behind polders and dykes in the Netherlands and in the wetlands of the Ukraine. It is used as the most important food for a large number of peoples in the world. In Bangladesh, potato represents about 53% of the total edible vegetables. It is the most important non-cereal food crop and ranks fourth in terms of total global food production after maize, wheat and rice (Chakraborty et al., 2000; FAO, 2007). Potato is usually propagated asexually by means of tubers. As such potato growers produced about 320.71 million tones of potato annually from 19.26 million hectares of land. In Bangladesh, about 6.65 million tones of potato produced from 0.402 million hectares of lands with an average yield of 16.53 t

ha<sup>-1</sup> (BBS, 2008). However, this yield is much lower than that of many potato growing countries of the world. Lack of high yielding varieties, pest and disease infestation and environmental stresses are the major constraints for the low yield of potato in Bangladesh. The crop loss occurs 30-40% due to pest and diseases infestation. It may turn even 100% if the crop is attack during the early stage of the crop (Hossain, 1997). It is now evident that to overcome the constraints of potato production, improvement of this crop by any means is an essential task.

It is a well known fact that through meristem culture, possible to develop virus free potato planting stocks in a mass scale (Hossain, 1987; Zaman et al., 2001; Sanavy and Moieni, 2003). But it is not yet possible to develop fungal resistant cultivars through *in vitro* culture technique. For this purpose different conventional breeding and biotechnological approaches are being applied in various parts of the world. Although conventional

breeding techniques has been successfully used in improving potato varieties which is time consuming and laborious. Quantitative traits are posing even greater challenge. To overcome such problems in crop improvement, genetic transformation of plants have been evolved, which offers the ability to introduce single new character into a plant cultivar without altering its existing traits (Gardner, 1993). Thus, genetic transformation provides an exciting new technology to supplement traditional crop improvement and accelerate the development of new plant varieties against diseases, pests and environmental stresses. The successful production of transgenic plants of these cultivars will open the way for introducing various agronomical useful traits into any commercially grown cultivars, providing the best opportunity for maximizing the yields with other qualities. *Agrobacterium*-mediated plant transformation has become the most useful method for the introduction of foreign genes into plant cells and the subsequent development of transgenic plants (Gustavo et al., 1998). A number of crop varieties have already been transformed in abroad through this method such as cotton, maize, potato, tobacco, rapeseed, raspberry, soybean, pea, tomato, rice etc. (Sarker et al., 2009). In 2009, biotech crops were grown by 13.3 million farmers in 25 countries covering 125 million hectares of land and 90% of the beneficiary farmers were resource-poor farmers from developing countries, whose increased income from biotech crops contributed to the alleviation of poverty (James, 2009). This progress can be attributed to the better understanding of the underlying process involved in DNA recombination and the development in molecular genetics, plant transformation and regeneration techniques (Akhond and Machray, 2008). *Agrobacterium*-mediated genetic transformation of potatoes is reported in abroad but there are very few reports are available in Bangladesh. The present work was undertaken to optimize the *Agrobacterium*-mediated genetic transformation protocol, so that the gene of interest can be inserted efficiently in the economically important potato varieties of Bangladesh.

## Materials and Methods

### Plant Materials

Diamant and Asterix varieties were collected from Tuber Crops Research Centre, Bangladesh Agricultural Research Institute, Gazipur and sprouts were collected from the tuber and cultured *in vitro*. The internode explants from 21 days old *in vitro* plantlets were used as plant materials in this experiment.

### *Agrobacterium* Strain and Plasmid

*Agrobacterium tumefaciens* strain LBA4404 with the binary plasmid pBI121 was used for transformation for above mentioned two varieties of potato. The binary vector pBI121 has the backbone of pBIN19. It contains a reporter gene *GUS* ( $\beta$ -glucuronidase) NOS promoter and terminator driven by a CaMV35S. In addition it has a selectable marker gene *nptII*. It encodes for neomycin phosphotransferase that confers kanamycin resistance (Herrera-Estrella et al. 1983). Two type of culture media YMB (Yeast extract Mannitol Broth) and LB (Luria Broth) were used for the *Agrobacterium* strain. The first medium was used to maintain *Agrobacterium* stock and the second one was for the infection of explants. For maintenance, on single colony from previously maintained *Agrobacterium* stocks was streaked into freshly prepared Petri dish containing YMB medium having kanamycin ( $50 \text{ mg L}^{-1}$ ). The Petri dish was sealed with Para film and kept in the incubator at  $28^\circ\text{C}$  at least 48 hours. This was then kept at  $4^\circ\text{C}$  to check over growth. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The culture was sub-cultured at each week in fresh prepared medium to maintain the stock. For infection, from this *Agrobacterium* stock single streak was taken in an inoculation loop and was inoculated in a conical flask containing liquid LB medium with  $50 \text{ mg L}^{-1}$  kanamycin. The culture was allowed to grow over night inside the digital incubating shaker (Vision Scientific Co. ltd) at  $28^\circ\text{C}$  @ 250 rpm to get optimum population of *Agrobacterium*.

### Plant Induction Media

For preparation of explants, instant Murashige and Skoog (MS) (1962) medium including vitamins and minerals ( $4.4 \text{ g L}^{-1}$ ) (Duchiffa, Netherlands) was used to prepare explants of potato for transformation work. MS medium supplemented with different concentration of ZR in addition with gibberellic acid ( $\text{GA}_3$ ) ( $0.2 \text{ mg L}^{-1}$ ) and indole-3-acetic acid (IAA) ( $0.01 \text{ mg L}^{-1}$ ) concentration based on Cearly and Bolyard (1997) was used for direct shoot regeneration of potato. Instant MS medium ( $4.4 \text{ g L}^{-1}$ ) supplemented with  $5 \text{ mg L}^{-1}$  zeatin riboside (ZR),  $1 \text{ mg L}^{-1}$  IAA,  $3 \text{ mg L}^{-1}$   $\text{GA}_3$  and  $50 \text{ mg L}^{-1}$  acetosyringone,  $20 \text{ g L}^{-1}$  sucrose and  $2 \text{ g L}^{-1}$  gelrite were used for co-cultivation medium. Filter sterilized ZR was added inside laminar air flow cabinet.

Following co-culture the explants were washed several times with sterile distilled water with gentle shaking until no opaque suspension was seen. The infected explants were finally washed for 3 minutes in MS liquid medium supplemented with  $400 \text{ mg L}^{-1}$  cefotaxime. MS medium supplemented with  $5 \text{ mg L}^{-1}$  ZR,  $1 \text{ mg L}^{-1}$  IAA,  $3 \text{ mg L}^{-1}$   $\text{GA}_3$ ,  $50 \text{ mg L}^{-1}$  kanamycin,  $200 \text{ mg L}^{-1}$  carbenecilline,  $20 \text{ g L}^{-1}$  sucrose and  $2 \text{ g L}^{-1}$  gelrite were used for post cultivation and shoot differentiation.

To eliminate untransformed tissues, the regenerating explants were sub-cultured after 2-3 times on fresh regeneration medium initially with  $50 \text{ mg L}^{-1}$  kanamycin. The concentration of kanamycin was increased at every 2 weeks when fresh subculture was made until its level reached  $100 \text{ mg L}^{-1}$ . During each subculture the dead and deep brown tissues were discarded and green shoots were sub-cultured in a fresh medium containing the next higher concentration of kanamycin.  $\frac{1}{2}$ MS,  $50 \text{ mg L}^{-1}$  cefotaxime,  $50 \text{ mg L}^{-1}$  kanamycin and  $0.5 \text{ mg L}^{-1}$  indole-3-butyric acid (IBA) were used for rooting.

### Infection and Co-cultivation

The *Agrobacterium tumefaciens* LBA4404 harboring binary vector pBI121 grown in liquid LB media were used for infection either co-cultivation maintaining the optical density of  $\text{OD}_{600}=0.6$  to get suitable and sufficient infection of explants. Freshly excised explants were wounded and immersed in a culture of the above bacteria solution for 20, 30 and 40 minutes with gentle shaking and then transferred them to co-cultivation medium. For infection the

explants were dipped in the *Agrobacterium* suspension for 20, 30, 40 minutes and co-cultivation for 2, 3 and 4 days. Infection medium were prepared by adding liquid bacterial culture with liquid MS in 1:1 ratio and acetosyringone (phenolic compound, inducer of *Agrobacterium*)  $50 \text{ mg L}^{-1}$  was added to activate the vir genes.

### Methods of *GUS* ( $\beta$ -glucuronidase)

#### Histochemical Assay

Randomly selected five co-cultured explants from each of the treatments were examined for *GUS* histochemical assay. Co-cultured explants were immersed in fixation solution (formaldehyde ( $750\mu\text{l}$ ,  $0.5\text{M}$  MES ( $2 \text{ mL}$ ), menitol ( $5.47\text{g}$ ) followed by X-gluc ( $5\text{-bromo-4-chloro-3-indolyl glucuronide } 50\text{mM}$  phosphate buffer having pH 7.8) solution and incubated at  $37^\circ\text{C}$  for 24-48h. A characteristic blue color would be the expression of *GUS* ( $\beta$ -glucuronidase) gene in the plant tissue. For control treatment, *GUS* assay was done with the explants from normal plant. Leaves, shoots and roots were assayed from randomly selected plants. Explants and plant parts were dipped into *GUS* solution for 24-48 hours at  $37^\circ\text{C}$ . After 24-48 h treatment, explants were soaked with 70% ethanol to remove chlorophyll. Then the de-greened explants/plant parts were observed under a stereomicroscope (Olympus, Japan).

The experiment was laid out in factorial completely randomized design having seven replications and each petri dish containing 7 (seven) explants were considered as single replication. Data were recorded on responsive explants (%), days required for shoot initiation (days), number of shoots per explant, length of visible shoot at 21 days (cm), number of visible leaves at 21 days, diameter of visible shoot at 21 days (mm) and regeneration frequency, percentage of *GUS* positive and transformed plants. Data were analyzed using MSTAT-C statistical package. Differences among the means were compared following DMRT test at 5% and 1% level of significance. The analysis of variance for different characters was performed and means were compared by the Duncan's Multiple Range Test (DMRT). The percentage data were subjected to appropriate transformation like arcsine and square root wherever applicable according to Gomez and Gomez (1984).

### Control Experiments

As control explants were neither infected nor placed in the selection medium. Rather they were placed in the regeneration medium and were sub-cultured after 21 days interval. For negative control, explants were not infected with *Agrobacterium* but were placed in the selection medium and they were sub-cultured at an interval of 21 days.

### Isolation of Plant Genomic DNA

Genomic DNA was isolated from non-transformed plant and transformants (particularly from leaf tissues) using the methods of EZ 10 spin column DNA isolation kit for plant (Bio Basic, Canada). Approximately 100 mg of leaf sample was collected from transformed plants and was ground with liquid nitrogen. All steps for the DNA extraction were done according to the manufacture's instructions.

### PCR Reactions

The presence of the *GUS* and *nptII* genes in the potato genomic DNA was analyzed by PCR. For the detection of the *nptII* coding sequence, DNA was subjected to PCR using forward and reverse primer comprising 5'-CATTAGTCCATGCAAGT T T-3' and 5'-AAGATTATACCGAGGTAT G-3', respectively. The primers for *GUS* gene 5'-CCTGTAGAAACCCCAACCCG-3' and 5'-TGGCTGTGACGCACAGTTCA-3' as forward and reverse (Sigma, USA) were used at a concentration of 100  $\mu\text{M}$  (stock) and it was diluted 10 times for working concentration. DNA Taq polymerase (3U  $\mu\text{L}^{-1}$ , dNTPs 2 mM and Taq buffer 10 $\times$  @ 0.3 $\mu\text{L}$ , 1.6  $\mu\text{L}$  and 2 $\mu\text{L}$ , respectively all are GeNei, Merck Germany) were used for PCR reaction. DNA concentration was measured by NanoDrop ND 1000 spectrophotometer which was varied from 30-40 ng  $\mu\text{L}^{-1}$  where each sample contained 4  $\mu\text{L}$  DNA template. Master mix of PCR was prepared for 16 samples at a time. After adding the DNA Taq polymerase, dNTPs, Taq buffer and DNA template in PCR master mix, final volume was made using double distilled water. Initial denaturation of PCR at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 1 minute was performed. The annealing temperature being 65°C for 1 minute for *GUS* gene and 55°C

with 30 second for *nptII* gene was adjusted. DNA elongation or extension at 72°C for 1 minute was adjusted. After the last cycle, a final step of 7 minutes at 72°C was added to allow complete extension of all amplified fragments. After completion of cycling program, reaction was held at 4°C. The numbers of cycles used for *GUS* and *nptII* gene was 30. The amplified DNA was run on 0.8% agarose gel and stained with ethidium bromide (0.05  $\mu\text{g mL}^{-1}$ ).

## Results and Discussions

### Days Required for Shoot Initiation

The combination of variety, infection time and co-culture period did not influence the number of days required for shoot initiation which ranged from 18.25 to 22.40 days (Table 1). The highest days required for shoot initiation (22.40 days) was noted in Diamant with 40-minute infection and 3-day co-culture period followed by the same variety with 30-minute infection and 4-day co-culture period (22.25 days). The explant from Asterix variety infection for 20-minute with 3-day co-culture demonstrated the early shoot initiation 18.25 days (Table 1). Rapid shoot bud development helps to ensure more successful and stable genetic transformation (Dandekar and Fisk, 2005). The possible reason for higher days required for shoot appearance due to higher infection period resulted bacterial over grown in the surface of the explants, as a result shoot appearance delayed. Beaujean et al. (1998) reported that internode explant of potato infected with 30-minute and 3 days co-culture needed 28-42 days for shoot initiation.

### Number of Shoots per Explant

The combination of variety, infection time and co-culture period had significant effect on the number of shoots per explant. It was observed that variety Asterix with 30-minute infection and 3-day co-culture produced the highest number of shoots (9.50) per explant followed by Asterix with 20 minutes infection time and same co-culture period (9.35) which was statistically identical. On the other hand, the lowest number of shoots per explant (5.25) was recorded from the variety Diamant with 40-minute infection and 4-day co-

**Table 1** Effect of varieties, infection time and co-cultivation period on shoot regeneration of potato.

Variety × infection time × co-culture period	Responsive explant (%)	Day required for shoot appearance	Length of shoot (cm)	Number of leave /shoot	Shoot diameter (mm)	Regeneration rate (%)
Diamant × 20-min. × 2-day	100	20.40	3.10	2.90	13.30	89.13 g
× 3-day	100	21.50	2.75	2.70	13.40	97.39 c
× 4-day	100	21.80	2.80	2.80	13.20	81.54 l
Diamant × 30-min. × 2-day	100	20.90	2.74	2.85	13.40	94.68 d
× 3-day	100	21.30	2.75	2.75	13.80	94.90 d
× 4 days	100	22.25	2.60	2.65	13.40	90.47 f
Diamant × 40-min. × 2-day	100	21.90	2.85	2.85	13.25	88.49 g
× 3-day	100	22.40	2.00	2.50	13.30	90.73 ef
× 4-day	100	22.00	2.20	2.60	13.10	82.33 k
Asterix × 20-min. × 2-day	100	19.70	2.90	2.80	14.20	87.62 h
× 3-day	100	18.25	3.56	3.35	14.50	98.00 c
× 4-day	100	19.90	2.90	2.75	14.40	83.42 j
Asterix × 30-min. × 2-day	100	19.85	2.85	2.75	14.40	99.00 b
× 3-day	100	18.40	3.65	3.30	14.80	100.00 a
× 4-day	100	19.65	3.35	2.80	14.60	89.23 g
Asterix × 40-min. × 2-day	100	19.25	3.50	2.90	14.10	85.19 i
× 3-day	100	19.00	3.50	2.90	14.30	91.23 e
× 4-day	100	20.10	2.50	2.73	14.10	80.43 l
CV%	-	8.9	6.86	8.03	3.93	4.75

Means bearing same letter/s do not differ significantly at 1% level of probability.

culture period (Figure 1). Beaujean et al. (1998) reported that 30-minute infection and 3-day co-culture of internode explants of potato cultured in MS medium supplemented with ZR 0.8 mg L<sup>-1</sup> gave 7-9 shoots per explant.

Direct regeneration of adventitious shoots from cotyledonary explants and indirect regeneration from callus had been reported by Mukhapadhyay et al. (1992) and Bubic et al. (1998). Mitten et al. (1990) stated that the proper environment for the co-culture of bacterium and plant materials must be provided. A total tissue culture system must be

devised to allow the regeneration of shoots within the context of selection for the marker gene and elimination of the *Agrobacterium*. Winzler et al. (1989) reported that 4-day pre-treatment of tissues and 3-4 day co-culture is common to all *Agrobacterium* base gene transfer system.

#### Length of Shoot

The combined effect of variety, infection time and co-culture period had no significant effect on shoot length. The longest shoot length (3.65 cm) was recorded in 30-minute infected explants of

Asterix variety with 3-day co-culture period, while it was the shortest (2 cm) in 40-minute infected Diamant explants with 3-day co-culture (Table 1, Figures 2b and 3b).

### Number of Leaves per Shoot

The effect of variety, infection time and co-culture period on number of leaves per shoot had not significant difference but it ranged from 2.50 to 3.35 (Table 1). The highest number of leaves per shoot (3.35) was recorded in 20 minutes infected explants of Asterix variety with 3 days co-culture, while it was the lowest (2.50) in 40 minutes infected explants of Diamant variety with 3 days culture period (Table 1).

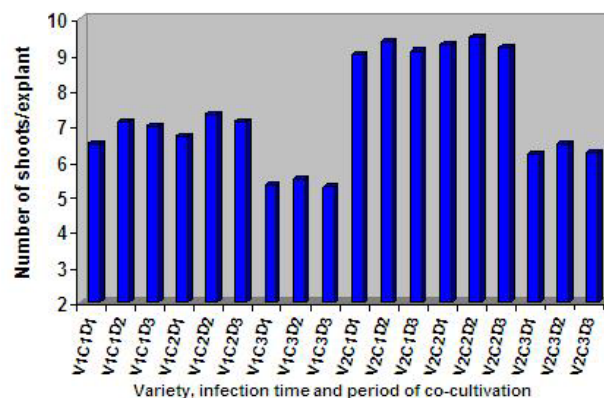
### Diameter of Shoot

The effect of variety, infection time and co-culture period had no significant influence on shoot diameter. The maximum shoot diameter (14.80 mm) was recorded in 30-minute infected explants of Asterix variety with 3-day co-culture followed by same infection time and 4-day co-culture period in the same explant (14.60 mm) (Table 1). The lowest shoot diameter (13.10 mm) was measured in 40 minutes infected explants of Diamant variety co-cultured 4 days. The possible reason for higher shoot diameter might be due to early shoot appearance and higher number of leaves produced more assimilates to source or sink.

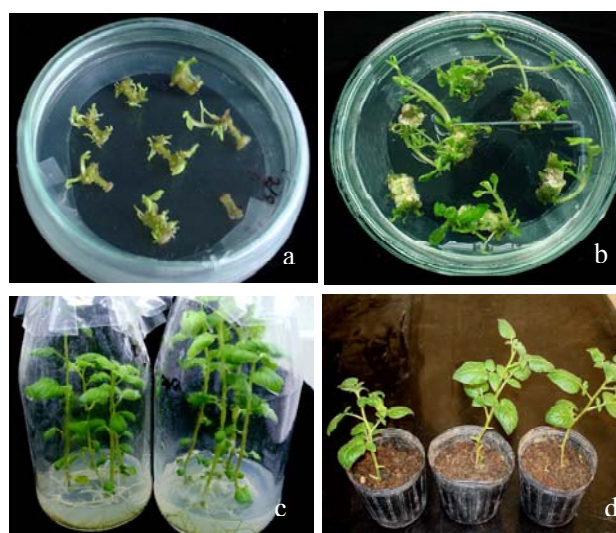
### Regeneration Rate (%)

Regeneration frequency per cent significantly differed due to the combined effect of variety infection time and co-culture period which lay in between 80.43 to 100%. The maximum regeneration frequency per cent (100%) was recorded in 30-minute infected explants of variety Asterix with 3-day co-culture (Table 1). On the other hand, the minimum regeneration frequency per cent (80.43%) was recorded in 40-minute infected explants of variety Asterix with 4-day co-culture. The possible reason for lower regeneration frequency per cent due to higher infection time and co-culture period decreased the shoot induction. Gustafson et al. (2006) obtained 67% regeneration frequency in stem explants of potato from MS medium supplemented with  $0.1 \text{ mg L}^{-1}$  IAA,  $0.1 \text{ mg L}^{-1}$  ZR,  $5 \text{ mg L}^{-1}$  kanamycin and  $300 \text{ mg L}^{-1}$

cefotaxim. Beaujean et al. (1998) stated that internode explants of potato with 30-minute infection and 3-day co-culture performed 90% regeneration frequency. The findings of the present study are also in accordance with the results of Beaujean et al. (1998).



**Figure 1** Effect of variety, infection time and co-culture period on number of shoot production per explant at 28 days. Where, V<sub>1</sub> = Diamant, V<sub>2</sub> = Asterix, C<sub>1</sub> = Infection time (20 min.), C<sub>2</sub> = Infection time (30 min.), C<sub>3</sub> = Infection time (40 min.), D<sub>1</sub> = Co-culture period (2 days), D<sub>2</sub> = Co-culture period (3 days), D<sub>3</sub> = Co-culture period (4 days).



**Figure 2** (a) Shoot induction in selection medium at 14 days, (b) Shoot induction in selection medium at 28 days, (c) *In vitro* transformed plants of 28 days cv. Asterix, (d) *Ex vitro* established plants.

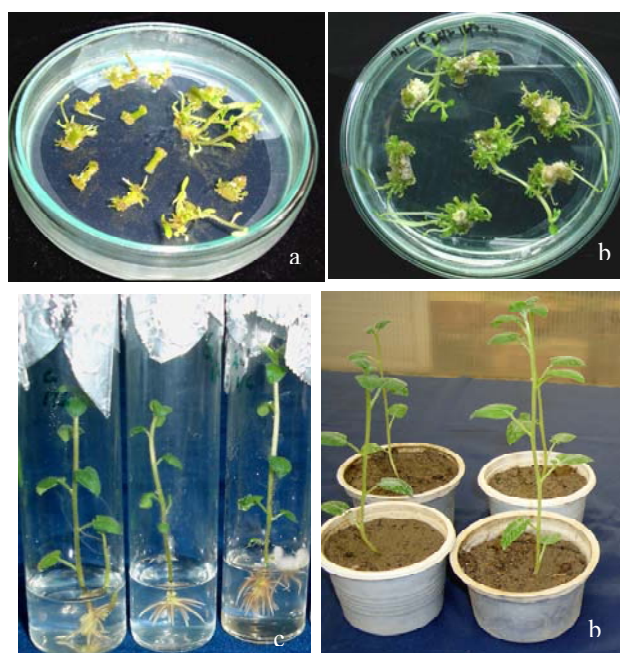
### GUS Activity Staining

*Agrobacterium* strain LBA4404 contained plasmid pBI121 along with *GUS* and *nptII* gene. Presence of *GUS* gene was tested after co-cultivation according to the treatments (results not shown). After *in vitro* establishment of plantlets internode and leaf were tested for *GUS* positive. The results of the study reflected that the highest (78%) *GUS* positive was achieved in internode of Asterix variety from the treatment 30-minute infection  $\times$  3-day co-culture (Figure 5). On the other hand, the second highest *GUS* positive (72.75%) was obtained from the explants of Diamant with same infection time and co-culture period. However, the lowest *GUS* positive (35%) was observed in the explants of Diamant, infected for 20 minutes with 2-day co-culture. In case of leaves, the maximum *GUS* positive (90%) was recorded in Asterix variety from 30-minute infection time with 3-day co-culture period followed by Diamant (85.20%) with same infection time and co-culture period while it was the minimum (50.60%) in the same variety with 20-minute infection and 2-day co-culture. Blue coloration showing in the *GUS* assays confirmed the genetic transformation (Figure 4).

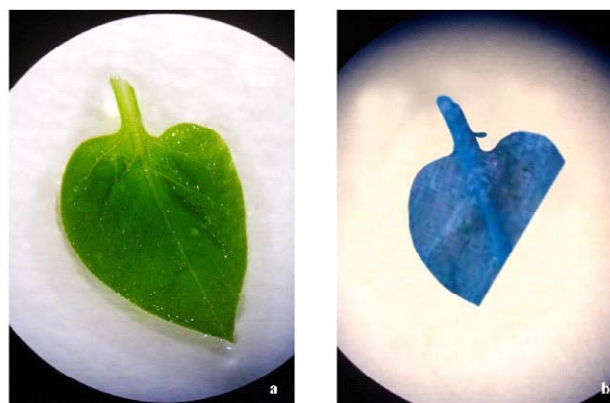
The results of the study to some extent supported by Hossain (2008) who obtained *GUS* positive 80% in internode explants of variety Diamant. This result is also in accordance with Sarker and Mustafa (2002) where histological *GUS* assay showed the expression of *GUS* gene in the leaf tissues of transformed shoots.

### PCR Test for Presences Transgenes

To confirm the stable transformation in the genome, DNA was isolated using DNA isolation EZ-10 spin column genomic DNA isolation kit (Bio Basic, Canada) from putative transformed plants of high dose ( $100 \text{ mg L}^{-1}$ ) kanamycin exposure plants (Figure 6).



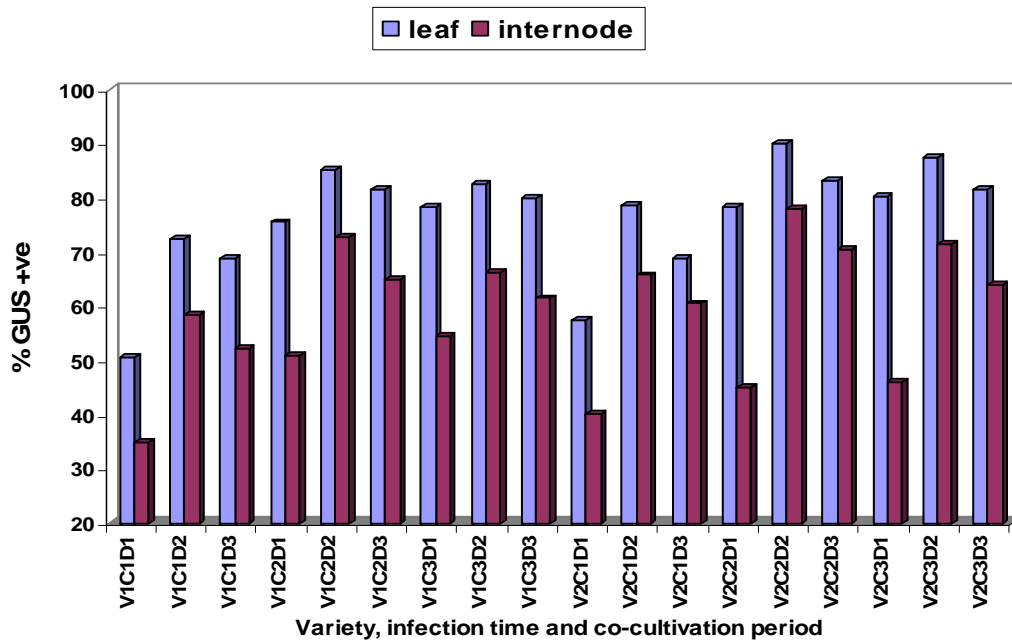
**Figure 3** (a) Shoot induction in selection medium at 18 days (b) Shoot induction in selection medium at 28 days (c) *in vitro* transformed plants of var. Diamant at 28 days (d) *Ex vitro* established plants.



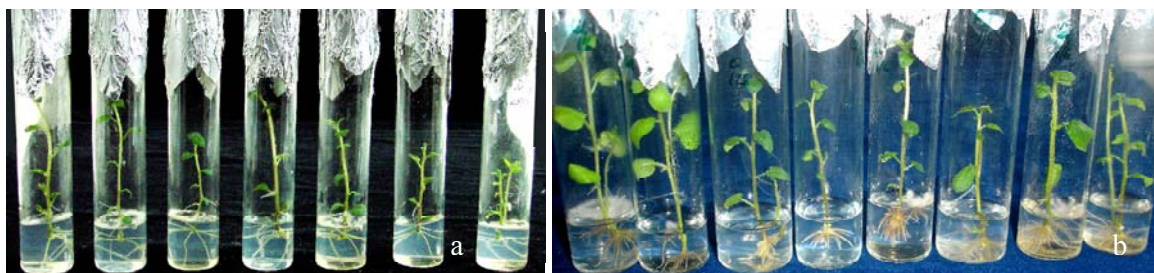
**Figure 4** *GUS* test of potato leaf cv. Asterix from *in vitro* transformed plants: Magnified microscopic view of (a) Fresh leaf and (b) *GUS* positive leaf.

**Table 2** Transformants ( $T_1$  and  $T_2$  generation) identification from the high doses Kanamycin tested plants.

Variety	No. of putative transgenic plants	No. of putative transgenic plants	No. of plants test for <i>GUS</i>	DNA isolation and PCR test for <i>nptII</i> gene	<i>nptII</i> positive (%)
	$T_1$ generation	$T_2$ generation			
Diamant	84	186	50	56	24.69
Asterix	112	256	50	56	28.97



**Figure 5** GUS positive test of variety (Diamant and Asterix), infection time (20, 30, 40 min.) and co-culture period (2, 3, 4 days) at 21 days of established in vitro plants. Where, V<sub>1</sub> = Diamant, V<sub>2</sub> = Asterix, C<sub>1</sub> = Infection time (20 min.), C<sub>2</sub> = Infection time (30 min.), C<sub>3</sub> = Infection time (40 min.), D<sub>1</sub> = Co-culture period (2 days), D<sub>2</sub> = Co-culture period (3 days), D<sub>3</sub> = Co-culture period (4 days).



**Figure 6** (a) Kanamycin selected transformed plants var. Diamant at 21 day. (b). Kanamycin selected transformed plants var. Asterix at 21 days.

Genomic DNA from non-transformed plants was also isolated using the same kit. After transformation with *Agrobacterium* strain LBA4404 contained plasmid pBI121 along with *GUS* and *nptII* gene, isolated DNA was quantified and amplified by PCR using the specific primers. The quality of the plant DNA was confirmed by a positive control PCR reaction. Fifty putatively transformed plants were tested for *nptII* and *GUS* gene. PCR analysis revealed that putative transformed plants displayed expected 700 bp and 600 bp size band (Figures 7a and 7b). Primer sets design to represent the positive control template which shown also the 700 bp and 600 bp band

(Figures 7a and 7b). The genomic DNA from non transformed control potato plants did not show any band in PCR reaction (Figures 7a and 7b).

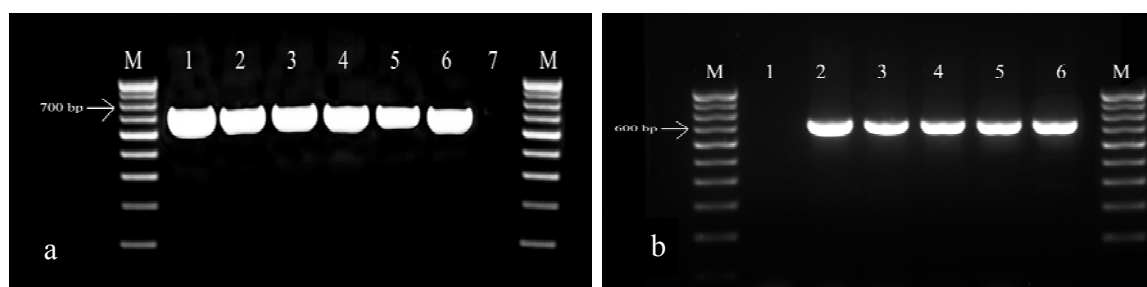
Rooting on selection (high kanamycin dose) medium was found to be a good indicator of transformation (Figures 6a and 6b), as 100% of rooted shoots were positive when tested by PCR which was missing in non transformed control plant (Table 3, Figures 7a and 7b). The above results indicated that *nptII* and *GUS* gene are conserved in respective region of the plasmid.

The *nptII* and *GUS* gene has been stably integrated to the potato genome and has shown the appropriate bands after PCR amplification.

**Table 3** Effects of different combinations of phytohormone in  $\frac{1}{2}$ MS medium on root initiation of transgenic shoot of potato

Treatment	Materials	No. of shoots showing root initiation	% Root initiation
$\frac{1}{2}$ MS, 50 mg L <sup>-1</sup> cefotaxime, 50 mg L <sup>-1</sup> kanamycin and zero IBA	Diamant	4 c	20.0 c (25.61)
	Asterix	6 b	30.0 b (32.04)
$\frac{1}{2}$ MS, 50 mg L <sup>-1</sup> cefotaxime, 50 mg L <sup>-1</sup> kanamycin and 0.5 mg L <sup>-1</sup> IBA	Diamant	20 a	100.0 a (86.82)
	Asterix	20 a	100.0 a (86.82)
$\frac{1}{2}$ MS, 50 mg L <sup>-1</sup> cefotaxime, 50 mg L <sup>-1</sup> kanamycin and 1 mg L <sup>-1</sup> IBA	Diamant	20 a	100.0 a (86.82)
	Asterix	20 a	100.0 a (86.82)
CV %	-	4.57	6.51

Means bearing same letter/s do not differ significantly at 1% level of probability, Data within parentheses represent the arcsine transformed values.



**Figure 7** (a) Detection of *nptII* gene by PCR from genomic DNA of transformed potato: M= DNA ladder, Lane 1= plasmid control, Lane 2-6 = PCR product of transformed plants. Lane 7= negative control. (b). Detection of *GUS* gene by PCR from genomic DNA of transformed potato: M= DNA ladder, Lane 1= negative control, Lane 2= plasmid control, Lane 3-6 = PCR product of transformed plants.

PCR amplification confirmed *GUS* and *nptII* gene both in the T<sub>1</sub> and T<sub>2</sub> generation. Transformation rate was recorded 24.69% and 28.97% against *nptII* gene in the Diamant and Asterix potato variety respectively (Table 2). This result is in agreement with the findings of Beaujean et al. (1998), Sarker et al. (2002) and Gustafson et al. (2006).

### Conclusions

Explants infection for 30-minute with 3-day co-culture produced maximum shoots in Asterix and Diamant variety, respectively within 18-21 days in 5 mg L<sup>-1</sup> zeatin riboside and 50 mg L<sup>-1</sup> kanamycin supplemented MS medium. DNA from well established rooted plants confirmed *nptII* and *GUS* positive through PCR analysis.

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