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STANDARDIZATION OF PROTOCOL FOR *AGROBACTERIUM*-MEDIATED TRANSFORMATION IN POTATO (*Solanum tuberosum* L.)

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Abstract

The experiment was conducted at the Laboratory of Biotechnology, Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur-1701 during July 2007 to June 2008. An efficient and reproducible protocol for the production of transgenic potato plants was developed by inoculating internode explants of potato with *Agrobacterium tumefaciens* strain LBA4404 carrying a binary vector pBI121 having one reporter gene (*gus*) and selectable marker gene (*nptII*) resistant to Kanamycin. The transformation experiment was done by optimizing two important parameters names infection time and co-cultivation period. Most of the explants produced shoots within 21 days on 5 mg/l Zeatin riboside (ZR) and 50 mg/l Kanamycin supplemented MS medium without introducing callus. The infected explants produced 8.27 and 6.42 shoots in Asterix and Diamant varieties, respectively within 21 days. Transgenes were confirmed by molecular analysis. DNA from well established rooted plants confirmed *nptII* positive through PCR analysis. The transformation rates were 28.97 and 24.37% in Asterix and Diamant, respectively. Putative transformed plants of Diamant and Asterix varieties produced roots in ½MS medium supplemented with 50/mg Cefotaxim, 50 mg/l Kanamycin and 0.5 mg/l IBA.

Keywords: Potato, *Agrobacterium* transformation, *nptII* gene, internode explant

Introduction

Potato (*Solanum tuberosum* L.) belongs to the family Solanaceae is used as the most important food for a large number of people 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). Potato is usually propagated asexually by means of tubers. Potato growers produced 320.71 million tones of potato annually from 19.26 million hectares of land (http//:www.potato 2008.org©FAO2007). In Bangladesh, about 6.65 million tons of potato produced from 0.402 million hectares of lands with an average yield of 16.53 t/ha (BBS, 2009). 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, shortage of quality seed and environmental stresses are the

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major constraints for the low yield of potato in Bangladesh. Moreover the crop losses occur 30-40% due to pest and diseases infestation. It may turn even 100% if the crop is attacked during the early stage (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 (Fraley, 1983). The 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. *Agrobacterium*-mediated 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) against the biotic and abiotic stresses. Thus the present work has been undertaken to standardize the transformation protocol for potato in accordance with the optimization of pre-culture and co-cultivation period for *Agrobacterium*-mediated genetic transformation, so that the gene of interest can be inserted efficiently in the economically important potato varieties of Bangladesh.

Materials and Method

Plant materials

The internode explants of two potato varieties viz., Diamant and Asterix were used as plant materials in this experiment.

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain LBA4404 with the binary plasmid pBI121 was used for this study. The binary vector pBI121 has the background of pBIN19. It contains a reporter gene *GUS* (*β-glucurunidase*) driven by a CaMV35S promoter and NOS terminator. In addition, it has a selectable merker gene *nptII* fused between NOS terminator. It encodes for neomycin phosphotransferase that confers kanamycin resistance (Herrera-Estrella *et al*., 1983). Two culture media YMB (Yeast extract Mannitol Broth) and LB (Luria Broth) were used for the *Agrobacterium* strain. One for maintaining *Agrobacterium* stock and the other 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. The Petri dish was sealed with Para film and kept in the incubator at 28ºC for at least 48 hours. This was then kept at 4ºC to check over growth. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The culture was sub-cultured regularly at each week in freshly prepared medium to maintain the stock. For infection, single streak was taken in an inoculation loop and was inoculated in a conical flask containing liquid LB medium with 50 mg/l Kanamycin. The culture was allowed to grow at 28ºC to get optimum population of *Agrobacterium* for infection and co-cultivation of explants.

Media used

For preparation of explants, instant MS (1962) medium including vitamins and minerals (4.4 g/l) (Duchefa, Netherlands) was used to prepare explants of potato for transformation work. Instant MS medium (4.4 g/l) supplemented with growth hormone (5 mg/l ZR, 1 mg/l IAA, 3 mg/l GA_3 and 50 mg/l Acetosyringone), 20 g/l sucrose and 2 g/l gel rite were used for co-cultivation medium. Filter sterilized ZR was added under laminar air flow cabinet. After 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 mg/l Cefotaxime. For post cultivation and shoot differentiation, MS medium supplemented with 5 mg/l ZR, 1 mg/l IAA, 3 mg/l GA_3, 50 mg/l Kanamicin, 200 mg/l Carbenecillin, 20 g/l sucrose and 2 g/l gel rite were used. IAA (1 mg/l) and GA_3 (3 mg/l) were used following Cearley and Bolyard (1997). To eliminate untransformed tissues, the regenerating explants were sub-cultured after 2-3 times on fresh regeneration medium initially with 50 mg/l Kanamycin. The concentration of Kanamycin was increased every fortnight when fresh subculture was made until its level reached 100 mg/l. During each sub-culture 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. For root induction, ½MS medium supplemented with 50 mg/l Cefotaxime, 50 mg/l Kanamycin and 0.5-1.0 mg/l IBA were used.

Infection and incubation

The *Agrobacteria* grown in liquid LB medium was used for infection and incubation maintaining the optical density of OD_{600}=0.6 to get suitable and sufficient infection of explants. 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 was prepared by adding liquid bacterial culture with liquid MS in an appropriate ratio and acetosyringone 50 mg/l was added to enhance bacterial activities.

Data recording and analysis method

The experiment was laid out in factorial CRD having 7 replications. Each Petri dish containing 7 explants was considered as a single replication. Data were recorded - responsive explants (%) , days required for shoot initiation, number of shoots per explant, length of visible shoots at 21 days, number of visible leaves at 21 days, diameter of visible shoot at 21 days and regeneration frequency and transformed plants. Data were analyzed using MSTAT-C program. Differences among the means were compared following DMRT at 5% and 1% levels of

significance. The percentage data were subjected to appropriate transformation according to Gomez and Gomez (1984).

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 (Biobasic, 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 *nptII* gene in potato genomic DNA was analyzed using PCR. For the detection of the *nptII* coding sequence, DNA was subjected to PCR using forward and reverse primer comprising 5´-CATTAGTCCATGCAAGTT T-3´ and 5´-AAGATTATACCGAGGTAT G-3´, respectively (Sigma,USA). All primers were used at a concentration of 100 pmol/µl . Master mix for PCR was prepared using standard protocol. The number of cycles used for *nptII* gene was 29. The amplified DNA was run on 1.0% agarose gel and stained with ethidium bromide (0.05 µg/ml). To confirm the transformation, 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 mg/l) Kanamycin exposed plants. Genomic DNA from non-transformed plants was also isolated using the same kit. The quality of the plant DNA was confirmed by a positive control PCR reaction. Fifty putatively transformed plants were tested for *nptII* gene.

Results and Discussion

Results presented in Table 1 show that all the explants responded equally to all treatments. Days required for shoot appearance, length of shoot, diameter of shoot, and no. of leaves per shoot varied among the treatments but differences were not significant. Number of shoots per explant significantly influenced due to variety, infection time and co-culture period

Internode explants of variety Asterix produced the highest number of shoots (9.50) per explant when treated as 30-minute infection and 3-day co-culture followed by 20- minute infection with 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-culture (Fig. 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 produced 7-9 shoots per explant. 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.

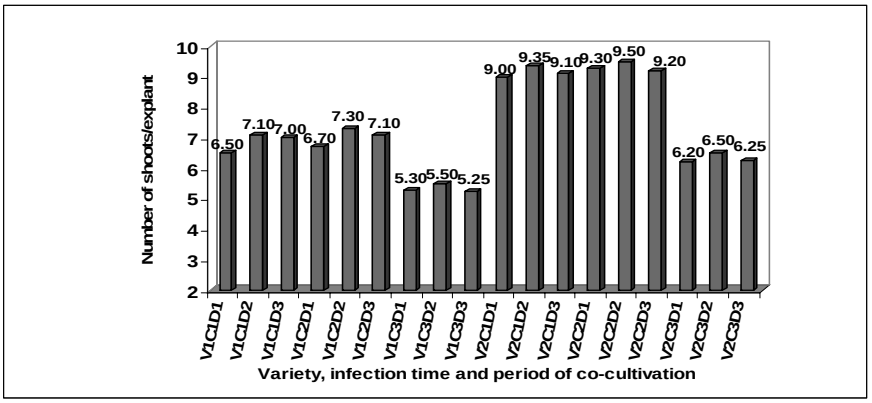

Fig. 1. Effect of variety, infection time and co-culture period on number of shoot production per explant at 28 days.
Where, V_1 = Diamant, V_2 = Asterix, C_1 = Infection time (20 min.), C_2 = Infection time (30 min.), C_3= Infection time (40 min.), D_1 = Co-culture period (2 days), D_2 = Co-culture period (3 days), D_3 = Co-culture period (4 days).

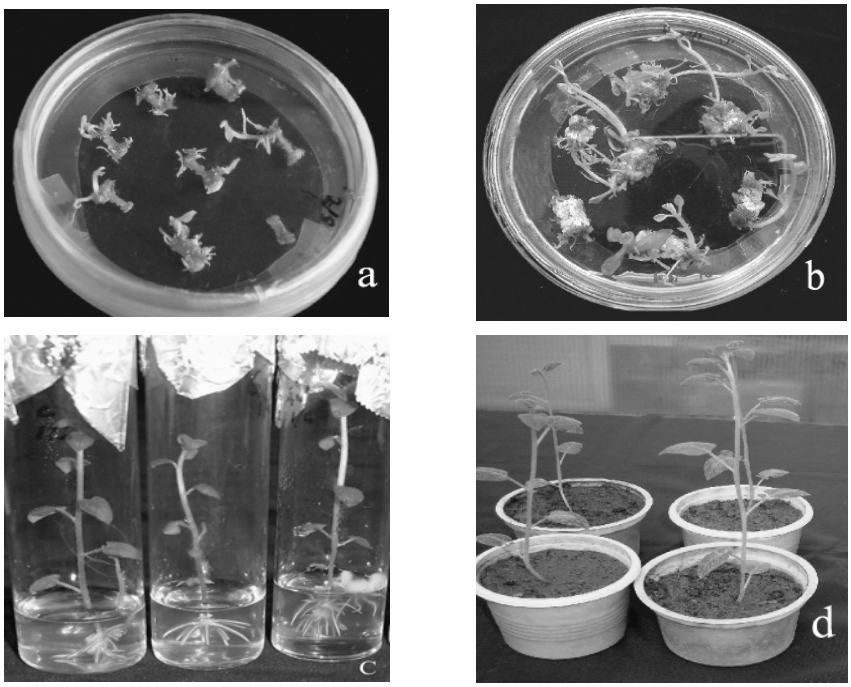

Plate 1: (a) Shoot induction in selection medium at 14 days, (b) Shoot induction in selection medium at 28 days, (c) *In vitro* transformed rooted plantlets at 28 days cv. Asterix, (c) E*x vitro* established plants.

Regeneration frequency percent was significantly influenced due to the effect of variety, infection time, and co-culture period which lay in between 80.43 to 100%. The maximum regeneration frequency percent (100%) was recorded in 30-minute infected explants of variety Asterix with 3-day co-culture (Table 1, Plate 1 a,b). On the other hand, the minimum regeneration frequency percent (80.43%) was recorded in 40-minute infected explants of variety Asterix with 4-day co-culture. The reason for lowest regeneration frequency at 40-minute infection might be due to bacterial over growth in the surface of the explants. Gustafson *et al*. (2006) obtained 67% regeneration frequency in stem explants of potato from MS medium supplemented with 0.1 mg/l IAA + 0.1 mg/l ZR + 5 mg/l Kanamycin + 300 mg/l Cefotaxim. Beaujean *et al.* (1998) stated that internode explants of potato with 30-minute infection and 3-day co-culture performed 90% regeneration frequency.

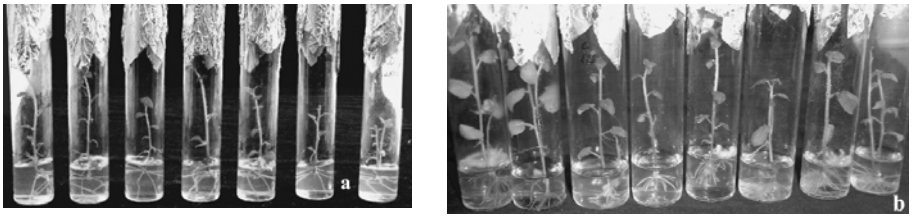

Fig. 5. Kanamycin selected transformed plants at 21 days (a) var. Diamant (b) var. Asterix

PCR analysis revealed that putative transformed plants displayed 700 bp size band (Fig. 6). Primer sets designed to represent the positive control template which also showed 700 bp band (Fig. 6). The genomic DNA from non-transformed control potato plants did not show any band in PCR reaction (Fig. 6). Rooting on selection (high Kanamycin dose) medium was found to be a good indicator of transformation (Fig. 5a, 5b), as 100% of rooted shoots were positive when tested by PCR which was missing in non transformed control plant (Table 2, Fig. 6). The above results indicated that *nptII* gene is conserved in respective region of the plasmid.

Fig. 6. Detection of *nptII* gene by PCR from genomic DNA of transformed potato: M= DNA ladder, Lane 1= positive control, Lane 2-6 = PCR product of transformed plants. Lane 7= negative control.

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

Treatment	Responsive explants (%)	Days required for shoot appearance (day)	Length of shoot (cm)	No. of leaves /shoot	Shoot diameter (mm)	Regeneration frequency (%)
Diamant × 20-min. × 2-day	100	20.40	3.10	2.90	8.30	89.13 g
Diamant × 20-min.× 3-day	100	21.50	2.75	2.70	8.40	97. 39 c
Diamant × 20-min.× 4-day	100	21.80	2.80	2.80	8.20	81.54 l
Diamant × 30-min.× 2-day	100	20.90	2.74	2.85	8.40	94.68 d
Diamant × 30-min.× 3-day	100	21.30	2.75	275	8.80	94.90 d
Diamant × 30-min.× 4-day	100	22.25	2.60	2.65	8.40	90.47 f
Diamant × 40-min.× 2-day	100	21.90	2.85	2.85	8.25	88.49 g
Diamant × 40-min.× 3-day	100	22.40	2.00	2.50	8.30	90.73 ef
Diamant × 40-min.× 4-day	100	22.00	2.20	2.60	8.10	82.33 k
Asterix × 20-min. × 2-day	100	19.70	2.90	2.80	9.20	87.62 h
Asterix × 20-min. × 3-day	100	18.25	3.56	3.35	9.50	98.00 c
Asterix × 20-min. × 4-day	100	19.90	2.90	2.75	9.40	83.42 j
Asterix × 30-min. × 2-day	100	19.85	2.85	2.75	9.40	99.00 b
Asterix × 30-min. × 3-day	100	18.40	3.65	3.30	9.80	100.00 a
Asterix × 30-min. × 4-day	100	19.65	3.35	2.80	9.60	89.23 g
Asterix × 40-min. × 2-day	100	19.25	3.50	2.90	9.10	85.19 i
Asterix × 30-min. × 3-day	100	19.00	3.50	2.90	9.30	91.23 e
Asterix × 30-min. × 4-day	100	20.10	2.50	2.73	9.10	80.43 l
CV(%)		8.9	6.86	8.03	3.93	4.75

Means bearing same letters do not differ significantly at 1% level of probability, - = Absent.

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

Variety	No. of putative transgenic plants		DNA isolation and PCR test for *nptII* gene	*nptII* positive (%)
	T_1 generation	T_2 generation		
Diamant	84	186	56	24.69
Asterix	112	256	56	28.97

PCR amplification confirmed *nptII* gene both in the T_1 and T_2 generation. The *nptII* gene has been stably integrated to the potato genome and shown the appropriate bands after PCR amplification. 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).

Table 3. Effects of different combinations of phytohormone in ½MS medium on root initiation of transgenic shoot of potato.

Treatment	Varieties	No. of shoots showing root initiation	Root initiation (%)
½MS, 50mg/l Cefotaxime, 50 mg/l Kanamycin and 0.0 IBA	Diamant	4 [c]	20.0 [c] (25.61)
	Asterix	6 [b]	30.0 [b] (32.04)
½MS, 50mg/l Cefotaxime, 50 mg/l Kanamycin and 0.5 mg/l IBA	Diamant	20 [a]	100.0 [a] (86.82)
	Asterix	20 [a]	100.0 [a] (86.82)
½MS, 50mg/l Cefotaxime, 50 mg/l Kanamycin and 1 mg/l 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 letters do not differ significantly at 1% level of probability, Data within parentheses represents the arcsine transformed values.

Rooting of transgenic shoots was influenced due to different concentrations (0, 0.5, 1.0 mg/l) of IBA. ½MS medium supplemented with 0.5-1.0 mg/l IBA along with 50 mg/l Cefotaxime and 50 mg/l Kanamycin showed the excellent performance for root induction of transgenic potato shoots (Table 3). Cent percent (100%) transgenic shoots produced root in these media which were statistically identical (Table 3).

Conclusion

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

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