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Agrobacterium-mediated Genetic Transformation for Local Cultivars of Potato (*Solanum tuberosum* L.) Using Marker Genes

Rita Sarah Borna, M. I. Hoque and R. H. Sarker*

Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka 1000, Bangladesh

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Abstract

Genetic transformation using nodal and internodal segments from three economically important potato (*Solanum tuberosum* L.) varieties namely, Diamant, Cardinal and Granola was conducted using an *Agrobacterium tumefaciens* strain LBA4404 harbouring binary plasmid pBI12 containing the GUS and *nptII* genes. Node and internodal segments were used for direct regeneration as well as regeneration with the intervention of callus. Best responses were obtained for direct regeneration of shoots when the explants were cultured on MS supplemented with 4.0 mg/l BAP +1.0 mg/l IAA, 1.5 mg/l BAP + 0.5 mg/l IAA and 5.0 mg/l BAP +1.0 mg/l IAA in Diamant, Cardinal and Granola, respectively. In Diamant spontaneous *in vitro* microtuberization was obtained from these proliferated shoots. Further culturing of these *in vitro* grown green microtubers regenerated a large number of shoots on MS containing 4.0 mg/l BAP +1.0 mg/l IAA. By combining the best treatments, this protocol yielded an average transformation rate of 87% of treared explants. Stable expression of GUS gene was visualized in the various parts of transformed shoots through histochemical assay. Genomic DNA was isolated from transformed shoots and stable integration of the GUS and *nptII* genes was confirmed by PCR analysis.

Introduction

Potato (*Solanum tuberosum* L.) is a tuberous crop plant of the Solanaceae family. It is an agronomically important food crop and widely cultivated all over the world. As a major non-cereal crop, potato is exceeded only by wheat, rice and maize for human consumption. In Bangladesh potato is the third important crop and rapid development of modern technology has made potatoes the second largest crop (by weight) in Bangladesh after rice. In 2007 - 08 per hectare yield of potato in Bangladesh was 16.578 MT which is much greater than that of rice (1.639 MT)

*Author for correspondence. <rhsarker2000@yahoo.co.uk>.

(BBS 2009). At present 0.87% of the cultivable lands are used in potato cultivation in Bangladesh. The annual yield is about 6648 MT which is about 7.3% of the total cultivated food crops. However, the production of potato in Bangladesh is still low (16.57 MT/ha) compared to the average yield in Germany and Netherlands (35 and 43 MT/ha, respectively). In South Korea the yield of potato has been increased from 20.0 to 35.0 MT/ha (Joung et al. 1993). This kind of dramatic increase of average yield is possible due to the cultivation of improved varieties of potatoes.

Production of potato becomes increasingly important for Bangladesh agriculture. However, the improvement in potato production faces several constraints. The yield is reduced greatly due to the shortage of quality seed potato. Moreover, the production and quality of this crop are not equivalent to global standard due to its cultivation under biotic and abiotic stresses such as diseases, pests and climatic factors. The major constraints of potato production in several countries including Bangladesh are due to incidence of different types of fungal diseases (late blight, early blight, etc.). Late blight caused by *Phytophthora infestans* is the most damaging disease of potatoes worldwide. This disease affects potato development from emergence to harvest. Genetic resistant to late blight is hard to obtain. As a consequence a large amount of fungicides have been frequently applied leading to pollution of the natural environment.

With the advent of new strains of late blight, pressure to sustain food production and preserve the environment in an alarming proportion. Therefore it is desirable to develop fungus resistant plants through the introduction of foreign fungal resistant genes into them. Conventiontional breeding techniques for the improvement of potato is not applicable in our country, because in this climatic condition it has not been possible to induce flowering. Under these circumstances, genetic transformation may be one of the method of choice to introduce a new character in the gene pool of potato.

Considerable progress has been made on the transformation of potato and a number of transgenes have been designed to improve agronomic and quality traits (Voyda and Belknap 1992, Trujillo et al. 2001). In Bangladesh only a limited number of reports are available on genetic transformation of local cultivars of potato. All preliminary investigations have been conducted through *Agrobacterium*-mediated genetic transformation (Sarker and Mustafa 2002, Sultana 2005, Sarker et al. 2009). For the present set of experiment *Agrobacterium*-mediated genetic transformation was carried out using two different explants from three local varieties of potato. Apart from these studies *in vitro* regeneration methods facilitating transformation of these varieties, namely, Diamant, Cardinal and Granola have been also reported here. Following *Agrobacterium*-mediated genetic transformation expression of GUS reporter gene was detected by

histochemical assay. Integration of GUS and *nptII* gene was confirmed by PCR analysis.

Materials and Methods

Tubers of three varieties of potato (*Solanum tuberosum* L.), namely Diamant, Cardinal and Granola collected from Bangladesh Agricultural Research Institute (BARI), Joydevpur, Gazipur were used in the present investigation. Freshly developed sprouts were used as the primary explants for the establishment of *in vitro* cultures. From the *in vitro* grown shoots, node and internodal segments were used for direct regeneration as well as regeneration with intervention of callus.

The sprouts were first washed three times in distilled water and surface sterilized with 0.1% $HgCl_2$ for 7 - 8 min inside a laminar flow cabinet. The surface sterilized sprouts were cultured on MS supplemented with 4.0 mg/1 BAP and 1.0 mg/1 IAA to obtain shoots. The desired explants were collected from these shoots. MS with various combinations of IAA, NAA, BAP, Kn, GA_3 were used for *in vitro* regeneration. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were maintained in growth room with a photoperiod of 16 hr at 25 ± 1°C. For induction of roots, regenerated shoots (3 - 4 cm long) were excised and transferred to half strength of MS supplemented with different concentrations of IBA.

For genetic transformation, *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pBI121 was used. This plasmid contains a scoreable reporter gene GUS (β-glucuronidase) driven by CaMV35S promoter and NOS terminator and a selectable marker gene *npt II* fused between NOS promoter and NOS terminator encoding for the enzyme neomycin phosphotransferase conferring kanamycin resistance (Herrera-Estrella et al. 1983).

The preparation of *Agrobacterium* suspension and transformation experiments were conducted following the procedures described by Sarker et al. (2003). The explants of node and internodal segments collected from 3 - 4 week old *in vitro* grown shoots were used for transformation. Only the apical three node and internodes were used for this purpose. The explants were prepared with a scalpel while submerged in the *Agrobacterium* suspension. The prepared explants were kept incubated in the *Agrobacterium* suspension in a small Petri dish for an additional 30 min, then blotted dry and cocultured for three days in the dark on MS with 4.0 mg/l BAP and 1.0 mg/l IAA. After coculture the explants were washed in Ticarcillin (500 mg/l) to stop the excess growth of *Agrobacterium*. They were then cultured on the regeneration medium for shoot development and maintained in the growth room.

Since *nptII* gene was present in the plasmid putatively transformed shoots were cultured on different concentrations of kanamycin containing MS for selection of transformants. To eliminate the untransformed developing shoots from the cultures the explants were subcultured on fresh regeneration medium initially with 50 mg/l kanamycin. The selection pressure of kanamycin was gradually increased from 50 mg/l up to 200 and 150 mg/l kanamycin was optimum in killing the non-transformed shoots. In the optimum selection medium containing 150 mg/l kanamycin both albino and green shoots were observed. The survival of green shoots on the optimum selection medium indicated the production of transformed shoots. Transforming ability of the explants as well as stable expression of the GUS gene was monitored by GUS histochemical assay (Jeffereson et al. 1987) by submerging them in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl α-D glucuronide) and incubating them at 37ºC for 24 - 48 hr and subsequently bleached with 70% ethanol before scoring for GUS expression.

Genomic DNA was isolated from the transformed potato shoots and stable integration of GUS and *nptII* genes were confirmed by PCR analysis.

CTAB method (Doyle and Doyle 1990) was used for DNA isolation. For the detection of the *nptII* coding sequence, DNA was subjected to PCR using the following primers: forward-5'-TAG CTT CTT GGG TAT CTT TAA ATA-3′ and reverse-3'-CCA GTT ACC TTC GGA AAA AGA GTT-5'. For the GUS gene the primers were : forward 5′-CCT GTA GAA ACC CCA ACC CG-3′ and reverse 5′-TGG CTG TGA CGC ACA GTT CA-3′. All primers were used at a concentration of 100 pmol/µl. The DNA isolated from transgenic tobacco was used as the positive control. Master mixture was prepared by mixing all of the PCR components e.g. 10x buffer, dNTPs, $MgCl_2$ Primer- F and R, etc. except the component against which the optimization strategy was intended. In each reaction, the volume of PCR buffer was used one tenth of the total reaction volume which was 25 µl. For PCR amplification of the GUS gene, DNA was denatured at 94ºC for 5 min and then amplified in 30 cycles using 94ºC for 1 min, 65ºC for 1 min (annealing) and 72ºC for 1 min followed by 5 min at 72ºC. For *nptII* gene the cycling conditions were 5 min at 94ºC denaturtion and 30 amplification cycles using 94ºC for 1 min, 55ºC for 1 min (annealing) and 72ºC for 1 min followed by 5 min at 72ºC. The amplified DNA was run on 0.80% agarose gel and stained with ethidium bromide (0.05 µl/ml).

Results and Discussion

The explants of nodal and internodal segments used in this study were obtained from the shoots developed through the *in vitro* culture of potato sprouts. Therefore, potato sprouts from all the varieties were used as primary explants.

The surface sterilized sprouts were cultured on MS supplemented with 4.0 mg/1 BAP and 1.0 mg/1 IAA. In all the three varieties, optimum responses towards development of shoots from these sprouts was achieved when the sprouts were surface sterilized for 7 - 8 min with 0.1% $HgCl_2$. The developing shoots from such sprouts is presented Fig. 1.

Agar solidified MS supplemented with various concentrations of BAP, Kn, IAA and GA_3 were used for the induction of multiple shoots from nodal and internodal segments of all the three potato varieties. Among the two cytokinins (BAP and Kn) used in the present study BAP was found to induce the best response towards the development of multiple shoots per explants. Best response towards shoot induction was obtained when both the explants from Diamant were cultured on MS supplemented with 2.0 mg/1 BAP and 1.0 mg/1 Kn. In case of Cardinal, MS supplemented with 2.5 mg/1 BAP and 1.0 mg/1 Kn can induce considerable number of shoots. The explants from Granola also responded best when cultured on MS supplemented with 2.5 mg/1 BAP and 1.0 mg/1 Kn.

A number of combinations of BAP and IAA were also used for direct regeneration of multiple shoots from node and internodal explants. These combinations of BAP and IAA responded better than the combination of BAP and Kn in inducing shoots. In Diamant about 10 - 12 shoots regenerated when the explants were cultured on MS supplemented with 4.0 mg/1 BAP and 1.0 mg/1 IAA (Fig. 2).

In case of Cardinal maximum shoots were obtained on MS supplemented with 1.5 mg/1 BAP and 0.5 mg/1 IAA. But for multiple shoot induction in Granola comparatively high concentration of BAP was required. Granola responded best on MS supplemented with 5.0 mg/1 BAP and 1.0 mg/1 IAA. The results are presented in the Table 1. These variable responses towards *in vitro* shoot development were also reported by some previous workers (Hussey and Stacey 1981, Miller et al. 1985, Islam 1990, Mila 1991, Hossain 1994, Bajaj 1981, Khan and Rabbani 1999, Sarker and Mustafa 2002 and Sultana 2005).

In the present investigation, node and internodal segments were also used to regenerate plants through callus culture. Here, BAP and NAA supplemented medium has been found to be effective for callus formation. High callus formation frequency was achieved in Granola (80.0%) following the use of MS containing 4.0 mg/1 BAP + 1.0 mg/1 NAA. Similar frequency of callus induction was obtained in Diamant in the same media. In case of Cardinal, best callus was obtained in MS containing 3.0 mg/l BAP + 1.0 mg/1 NAA. Different concentrations of BAP and IAA were employed on MS for shoot development from callus tissue. Shoot buds were found to initiate from those green friable callus within one month (Fig. 3).

Table 1. Effects of various combinations of BAP and IAA on regeneration and proliferation of multiple shoots from nodal and internodal segments of potato (var. Diamant, Cardinal and Granola).

Explants	BAP + IAA (mg/l)	Number of explants inoculated			Mean No. of shoot buds/explant after 30 days of inoculation			Mean number of shoot buds/explant after 60 days of inoculation		
		D	C	G	D	C	G	D	C	G
Node	1.0 + 0.5	25	25	30	6.1	6.1	4.1	10.5	15.8	10.1
	1.5 + 0.5	25	25	30	5.3	7.5	4.4	12.7	18.5	10.8
	2.0 + 1.0	25	25	30	7.4	7.1	5.0	12.4	12.0	13.1
	3.0 + 1.0	25	25	30	8.0	7.0	5.8	15.1	12.0	14.8
	4.0 + 0.5	25	25	30	10.5	6.8	6.1	20.0	10.6	16.2
	4.0 + 1.0	25	25	30	12.0	6.6	6.6	30.5	11.1	18.5
Internode	1.0 + 0.5	25	25	30	4.3	4.5	4.0	5.2	12.2	10.2
	1.5 + 0.5	25	25	30	4.6	6.8	4.2	6.0	15.7	11.1
	2.0 + 1.0	25	25	30	5.5	6.2	4.9	6.7	17.1	13.2
	3.0 + 1.0	25	25	30	6	5.5	5.6	8.8	13.1	14.1
	4.0 + 0.5	25	25	30	6.1	5.4	5.9	11.1	10.3	17.1
	4.0 + 1.0	25	25	30	10.5	5.1	6.3	12.0	10.2	17.2

D = Diamant, C = Cardinal, G = Granola.

Apart from these regeneration experiments spontaneous microtuberization (Fig. 4) was obtained from these proliferated shoots when they were subcultured on MS containing 4.0 mg/1 BAP + 1.0 mg/1 IAA. These spontaneous microtuberization was found to initiate within six - seven week of culture. Further culturing of these green *in vitro* grown microtubers was found to regenerate a large number of shoots which were almost identical in size and shape (Fig. 5). These microtuber formation and the proliferation of shoots from these microtubers was carried in case of Diament and Cardinal.

Half strength of MS without hormonal supplement was sufficient in initiating and developing healthy roots. Among IAA, IBA and NAA used for root induction, MS containing 0.2 mg/1 IBA showed best response in developing roots (Fig. 6). However, in some indigenous potato varieties best response in root induction was obtained in MS supplemented with 0.1 mg/1 IAA (Sarker and Mustafa 2002).

Transformation experiments for the three potato varieties (Diamant, Cardinal and Granola) were conducted by using nodal and internodal segments as explants. Transformation ability of various explants was detected using GUS histochemical assay (Fig. 7). In all the varieties nodes were found to show the best transformation ability, followed by internodal segments. Results of these observations are presented in Table 2. In the present investigation transformation efficiency of explants in all the varieties was found to be maximum with bacterial

suspension having on optical density of about 1.0 at 600 nm. An incubation period of 60 minutes followed by 72 hr of cocultivation was maintained towards effective transformation.

Figs 1-9: Plant regeneration and genetic transformation in potato. 1. Development of shoots from sprouts on MS supplemented with 4.0 mg/1 BAP and 1.0 mg/1 IAA in Diamant. 2. Multiple shoots regenerated from nodal segment in Diamant on MS supplemented with 4.0 mg/1 BAP + 1 mg/l IAA. 3. Shoot buds developing from the callus in Diamant on MS with 1.5 mg/l BAP and 1.0 mg/l IAA. 4. Spontaneous microtuberization in nine week culture of Diamant in 4.0 mg/l BAP and 1.0 mg/l IAA. 5. *In vitro* shoots regenerated from microtubers of Diamant. 6. Rooting on MS with 0.2 mg/l IBA. 7. Stereomicroscopic view of histochemical localization of GUS activity (blue coloured zones) at the entire cut surface internodal segments (× 27). 8. Selection of transformed shoots of Diamant in presence of 150 mg/l kanamycin (note that the non transformed shoots became albino). 9. Magnified view of GUS positive cells within the internal tissues of the shoot primodia developed from the internodal explant (× 10).

In a separate set of experiments Sarker et al. (2009) reported that an incubation period of 40 min was optimum in case of variety Cardinal and Atlas. Lecardonnel et al. (1999) also used 40 min for infection and coculture was maintained for three days in potato. Influence of regulatory factors on the transformation efficiency of various explants was also observed by Islam (1998) in peanut and by Mansur et al. (1993) in peanut.

Selection of the transformants was carried out using various concentrations of kanamycin. However, the presence of kanamycin greatly hampers growth of the explants and as a result many putative transformants may have been lost. For this reason immediately after cocultivation kanamycin was not applied for selection, rather regeneration was encouraged from the cocultivated explants. Well developed shoots were obtained from infected nodal and internodal segment after 15 - 18 days of culture and well developed calluses were obtained from infected explants following three days of culture.

Table 2. Responses of various explants of potato towards genetic transformation with *Agrobacterium* strain LBA4404 containing the binary plasmid pBI121 analyzed by transient GUS histochemical assay.

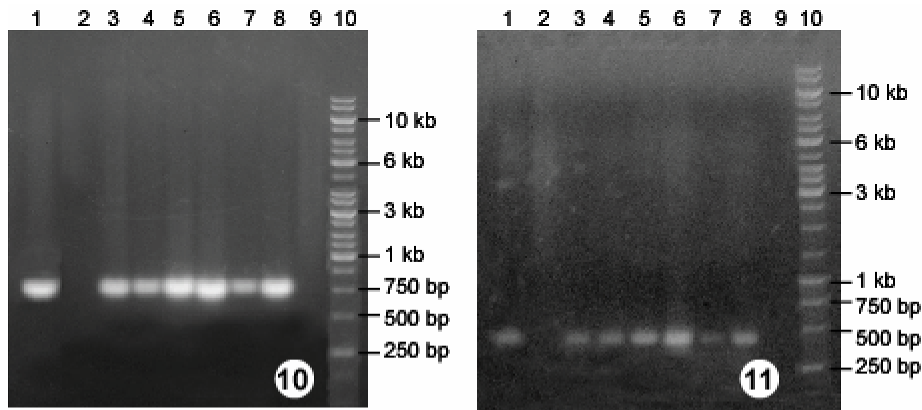
Variety	Explant	No. of explants infected	No. of explants assayed for GUS expression	No. of explants assayed +ve for GUS	% of GUS positive explant
Diamant	Node	500	50	46	92
	Internode	500	50	42	84
Cardinal	Node	500	50	43	86
	Internode	500	50	41	81
Granola	Node	500	50	44	88
	Internode	500	50	45	90

In the present investigation higher concentrations of kanamycin were applied to screen the transformed shoots effectively. For this purpose kanamycin concentration was gradually increased from 50 to 200 mg/l. It was found that all the control shoots failed to survive at 150 mg/l kanamycin within 15 days. Therefore, the shoots that survived in the medium containing 150 mg/l kanamycin were considered as transformed (Fig. 8). All the shoots obtained though selection were subjected to rooting in a medium with 50 mg/1 kanamycin where the roots were found to develop properly. Here, it may be pointed out that the selection procedure developed during this study has been found to be effective in recovering transformed plantlets. Stable expression of GUS gene was visualized through histochemical staining in the regenerating shoots from nodes developed in presence of selection pressure of 150 mg/l kanamycin (Fig. 9). These tests were carried out regularly for more than three months and the results of these experiments are presented in Table 3. The regenerated roots from the transformed shoots also showed GUS expression .

Table 3. Stable GUS expressions in the putative transformed plantlets following three months of selection in presence of higher concentration of kanamycin (150 - 200 mg/1).

Plant parts tested	Total No. of parts tested	No. of GUS positive parts	Percentage of GUS positive parts
Leaf	20	8	40
shoot	20	5	25

The transgenic nature of the shoots was confirmed by PCR amplification of the GUS and *nptII* gene present within the genomic DNA (Figs 10 and 11) of six randomly selected transformants. Specific primers were used for this purpose as detailed in the Materials and Methods section. Amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that the single band formed in each of the six transformed plantlets were identical to the amplified DNA of transformed tobacco marker. This result indicated that both the GUS and *nptII* genes were inserted in the genomic DNA of six transformed plantlets.



Figs 10-11: 10. PCR amplification of GUS gene from transformed plants. Lane-1. Genomic DNA of transformed tobacco plant. Lane-2. Genomic DNA of non-transformed potato plant. Lane-3-8. Amplified DNA of transformed potato plants. Lane-9. Water control. Lane-10. Ladder. 11. Same as 10 but with *nptII* gene.

The protocol of *Agrobacterium*-mediated genetic transformation developed through the present investigation can be used for the production of transgenic potato plants for specific purpose. In this case the protocol was primarily developed using screenable marker gene like GUS and selectable marker gene like *nptII*. Using this protocol, in future agronomically important gene/s can be transferred to the potato varieties grown in Bangladesh. Particularly for the development of fungal disease resistant potato variety this technique of transformation can be exploited.

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References

Bajaj YPS (1981) Regeneration of plants from potato meristems freeze preserved for 24 months. Euphytica **30**(1): 141-145.

BBS (2009) Bangladesh Bureau of Statistics, Agriculture Wing. Ministry of Planning, People's republic of Bangladesh.

Doyle JJ and **Doyle JL** (1990) Isolation of Plant DNA from fresh tissue Focus **12**: 13-15.

Herrera-Estrella L, Depicker A, Van Montague M and **Schell J** (1983) Expression of chimeric genes transferred into plant cells using a Ti-plasmid derived vector. Nature **303**: 209.

Hossain MJ (1994) *In vitro* propagation of potato (*Solanum tuberosum* L.). Plant Tissue Cult. **4**(1): 33-37.

Hussey G and **Stacey NJ** (1981). *In vitro* propagation of potato (*Solanum tuberosum* L.) Ann. Bot. **48**: 787-796.

Islam A (1990) Studies of *in vitro* microtuber formation in potato (*Solanum tuberosum* L.). M. Sc. thesis, Plant Breeding and Tissue Culture Lab., Department of Botany, University of Dhaka.

Islam A (1998) *In vitro* regeneration and genetic transformation of peanut (*Arachis hypogaea* L.). M. Sc. thesis, Plant Breeding and Tissue Culture Lab.,' Department of Botany, University of Dhaka.

Jefferson RA, Kavanagh TA and **Bevan MW** (1987) GUS Fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6** : 3901-3907.

Joung H, Jeon J H, Park S W and **Kim S** (1993) Potential impact of mass produced potato microtubers on conventional potato agriculture. Paper presented in Int. Plant Tissue Culture Conference, held from 19-21 Dec. 1993 at Botany Department of Dhaka University.

Khan SAKU and **Rabbani MG** (1999). Effect of preincubation of tubers on *in vitro* culture of three potato varieties. Plant Tissue Cult. **9**(1): 1-9.

Lecardonnel A, Prevost G, Beaujean A, Sangwang RS and **Norrell BSS** (1999) Genetic transformation of potato with *nptII*-GUS marker gene enhances foliage consumption by Colorado potato beetle larvae. Molecular Breed. **5**: 441-451.

Mansur EA, Lacorte C, Defriitus VG, Deoliviera DF, Timmerman B and **Cordeiro AR** (1993) Regulation of transformation efficiency of peanut (*Arachis hypogaea* L.) explant by *Agrobacterium tumefaciens.* Plant Sci. **89**: 93-99.

Mila NB (1991) Optimization of *in vitro* microtuber formation in potato (*Solanum tuberosum* L.). M. Sc. thesis, Plant Breeding and Tissue Culture Lab., Department of Botany, University of Dhaka.

Miller PR, Amirouche L, Stuchbury T and **Mathews S** (1985) The use of plant growth regulators in micropropagation of slow-growing potato cultivars. Potato Research. **28**: 479-486.

Sarker RH and **Mustafa BM** (2002) Regeneration and *Agrobacterium*-mediaied genetic transformation of two indigenous potato varieties of Bangladesh. Plant Tissue Cult. **12**(1): 69-77.

Sarker RH, Mustafa BM, Biswas A, Mahbub S, Nahar M, Hashem R and **Hoque MI** (2003) *Agrobacterium*-mediate transformation of lentil (*Lens culinaris* Medik.) Plant Tissue Cult. **13**(1): 1-12.

Sarker SR, Hossain M and **Shirin F** (2009) Precise Incubation Period for the *Agrobacterium*-mediate transformation efficiency in potato (*Solanim tuberosum*) cvs. Cardinal and Atlas. Plant Tissue Cult. & Biotech. **19(2)**: 227-235.

Sultana P (2005) *In vitro* regeneration and *Agrobacterium*- mediate genetic transformation of potato (*Solanum tuberosum* L.), M. S. thesis, Plant breeding and Biotechnology lab., Department of Botany, University of Dhaka.

Trujillo C, Rodriguez-Arango, Jaramillo S, Hoyos R, Orduz S and **Arango R** (2001) One-step transformation of two Andean potato cultivers (*Solanum tuberosum* L.) subsp. andigend). Plant Cell Reports **20**: 637-641.

Voyda ME and **Belknap WR** (1992) The emergence of transgenic potato as commercial products and tools for basic research. Transgenic Res. **1:** 149-163.