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AGROBACTERIUM-MEDIATED GENE TRANSFER IN POTATO FOR ABIOTIC STRESS TOLERANCE

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ABSTRACT

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The experiment was carried out to standardize the callus induction potentiality and transformation ability of leaf and callus explants of potato cv. Granola during the period of January to June 2009 in the Department of Biotechnology, Bangladesh Agricultural University, Mymensingh. Callus induction ability of leaf explants was observed in response to different concentrations and combinations of growth regulators supplemented to MS medium. The highest number of calli per vial (5.67), percentage of callus induction (94.44%) and callus weight (2.66 g) were observed in MS medium containing 3.0 mgL-1 NAA and 2.5 mgL-1 BAP. No callus was observed in medium without growth regulators. Leaf discs and callus explants of potato cv. Granola were inoculated with *Agrobaterium tumefaciens* strain LB4404 carrying CIPK (abiotic stress tolerant) sense gene and a binary vector pBI121, which contains GUS (reporter gene) and nptII (kanamycin resistance) gene. The transformation work was performed with different infection times and co-cultivation periods. The leaf discs and callus of potato cv. Granola were found GUS positive after inoculation and selection. The highest GUS positive response (83.33%) was recorded from the leaf disc when inoculated for 7 minutes and co-cultured for 5 days. The highest GUS positive response (88.89 %) was observed for 3 minutes inoculation time and 5 days co-cultivation periods in case of callus of potato cv. Granola. These results of GUS histochemical assay indicated that, abiotic stress tolerant CIPK sense gene was transferred in potato cv. Granola.

Key words: *Agrobaterium, gene, transfer, stress, tolerance*

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important tuber crop mainly used as vegetables belonging to the family Solanaceae. It is not only a cash crop but also a substitute of food crop next to rice and wheat. It grows well both in temperate and tropical countries. It is also important crop in terms of dry matter production (2.2 t ha^{-1}), energy (216 MJ ha^{-1} day^{-1}) and nutrition (Beukema and Vander Zaag 1990). Potato ranks 7th in respect of land occupancy and 5th in its production among global food crops (Horton 1987; FAO 2003), while it ranks 1st both in area and production among the vegetable crops grown in Bangladesh (BBS 2008).

Bangladesh has a high agro-ecological potential of growing potato but the average yield is 14.83 t ha^{-1} (BBS 2005), which is very low in compare to many other countries like Netherlands (45 t ha^{-1}), Germany (46 t ha^{-1}), Scandinavian countries (48-52 t ha^{-1}) (Rashid *et al*. 1993). Lack of quality seed, several abiotic and biotic stresses, and poor management practices etc. are the important factors for this low yield. Abiotic stresses include drought, salinity, extreme temperature, chemical toxicity and oxidative stress, which are serious threats to agriculture and the natural states of environment (Wang *et al.* 2003).

Conventional breeding methods have the potential to develop various crop species with environmental stress tolerance but they are time consuming and laborious (Cullins 1991). To improve upon the characters of agronomic importance in potato, conventional breeding methods were tried. Genetic modification of plants using recombinant DNA techniques holds the promise of increased crop productivity, product quality and reduced dependence on chemical inputs for pest control (Asano *et al.* 1991). Modern plant genetic engineering involves the transfer of desired genes into the plant genome and then regeneration of a whole plant from the transformed tissue.

High frequency regeneration of plant from *in vitro* cultured tissues and cells is a pre-requisite for successful application of tissue culture and genetic engineering technologies for crop improvement. Several protocols for genetic transformation using leaf discs have been successfully employed to generate transgenic plant resistant to biotic stresses but the achievements is not satisfactory in potato (Bencheckroun *et al.* 1995). Both the regeneration and transformation processes depend on optimum growth conditions, suitable explants and variety. Keeping the above facts in mind, the present research work has been undertaken to standardize the callus induction potentiality and to develop abiotic stress tolerant potato through *Agrobacterium*-mediated genetic transformation.

MATERIALS AND METHODS

The experiment was conducted during period of January to June, 2009 in the Biotechnology Laboratory of Bangladesh Agricultural University, Mymensingh to observe the regeneration potentiality and to transform the genetic make up of potato cv. Granola through *Agrobacterium tumefaciens* starin LBA4404 containing pBI121. This vector includes *CIPK* sense gene encoding calcineurin B-like protein conferring abiotic stress tolerance.

Experimental design and treatments

The experiments were laid in Completely Randomized Design (CRD) with four replications. For callus induction, two different factors were included:

i) Different concentrations of NAA (0.0, 2.5, 3.0 and 3.5 mg/L)
ii) Different concentrations of BAP (0.0, 2.0, 2.5 and 3.0 mg/L)

Agrobacterium- mediated genetic transformation of potato cv. Granola consisted of four factors:

i) Different concentrations of cefotaxime (100, 200 and 500 mg/L)
ii) Different concentrations of kanamycin (20, 40 and 50 mg/L)
iii) Inoculation time (3, 5, 7 and 9 minutes)
iv) Co-cultivation period (3, 5 and 7 days)

Culture media

For pre-culture and the induction of callus, MS (Murashige and Skoog, 1962) medium supplemented with different concentration of BAP and NAA were used. Two different types of culture media, namely YMB (Yeast Extract Mannitol Broth) medium and LB (Luria Broth) medium were used with kanamycin as antibiotic to grow the strain of genetically engineered Agrobacterium tumefaciens. MS medium without growth hormones was used for co-cultivation. Cefotaxime (500 mg L^{-1}) was used for washing the explants after co-cultivation. MS medium supplemented with 3.0 mg L^{-1} NAA and 2.5 mg L^{-1} BAP and 200 mg L^{-1} cefotaxime was used for post-cultivation and regeneration purpose. For low selection medium, MS medium supplemented with 3.0 mg L^{-1} NAA, 2.5 mg L^{-1} BAP, 20 mg L^{-1} kanamycin and 100 mg L^{-1} cefotaxime; whereas for high selection medium, MS medium supplemented with 3.0 mg L^{-1} NAA, 2.5 mg L^{-1} BAP, 40 mg L^{-1} kanamycin and 100 mg L^{-1} cefotaxime were used. MS medium supplemented with 3.0 mg L^{-1} NAA, 2.5 mg L^{-1} BAP, 40 mg L^{-1} kanamycine and 100 mg L^{-1} cefotaxime were used for regeneration.

Culture techniques

Six pieces of leaf discs were separately cultured on MS medium supplemented with different combinations of BAP and NAA. The culture plates were placed under dark in a room with controlled temperature (25± 2°C).Leaf discs were arranged horizontally on each petridish and gently pressed into the surface of the sterilized pre-culture medium. The culture plates were then placed under dark in a room with controlled temperature (25±2°C). All the explants (callus) were maintained in pre-culture media for 5 to 7 days depending on the explants.

For maintenance, one single colony from previously maintained *Agrobacterium* stocks was streaked on to freshly prepared petridish containing YMB medium having kanamycin. The petridish was sealed with parafilm and kept at room temperature for at last 48 hours. This was then kept at 4°C to check over growth. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The cultures were subcultured regularly at each week in freshly prepared media 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 mg L^{-1} kanamycin. The culture was allowed to grow at 28°C for three days to get optimum population of *Agrobacterium* for infection and co-cultivation of explants.

Following the determination of density, the pre-culture explants were dipped into bacterial suspension (OD600= 0.60) for 3, 5, 7 or 9 minutes before transferring them to co-cultivation medium. All the explants were maintained in co-cultivation media for 3, 5 and 7 days. Co-cultured vial containing explants were placed under fluorescent illumination (1500 lux) for 16/8 hours light/dark cycle at 25±2°C.After co-cultivation, the infected explants were washed twice with sterile distilled water and once with sterile distilled water containing 500 mg L^{-1} cefotaxime. Then the explants were transferred onto post-cultivation medium containing 200 mg L^{-1} cefotaxime. Following post-cultivation, the explants were transferred onto low selective medium. After low selection the calli were transferred onto high selection medium.

For this experiment, survived calli were immersed in X-gluc (5-bromo-4-chloro-3-indoly-l- glucuronide) solution and were incubated at 37°C overnight. A characteristic blue color would be the expression of GUS *(β-Glucuronidase)* gene in the plant tissue. After ten days the survived callus were transferred to regeneration medium.

Recording data

Callus initiation was started after eight days of incubation of explants. The number of callus initiated over a number of days was recorded. The number of explants producing callus in each vial was recorded. The percentage of callus induction was calculated by the following formula

$$\text{Per cent callus induction} = \frac{\text{No. of explants induced calli}}{\text{No. of explants incubated}} \times 100$$

The weight of callus was measured on the basis of the difference between the weight of vial containing leaf explants before and after callusing. The number of leaf and callus giving positive response to GUS histochemical assay is recorded. The percentage of GUS positive explants is calculated on the basis of the number of calluses assayed for GUS and the total number of calli positive for GUS.

RESULTS AND DISCUSSION

Callus induction

Potato cv. Granola was taken for the callus induction where leaves from sub-cultured micro plants (Fig. A) were used as explants and cultured on MS medium supplemented with different combinations and concentrations of auxin (NAA) and cytokinin (BAP) to observe their callus induction potentiality (Fig. E). Percentage of callus induction, days to callusing and weight of callus varied for different phytohormone concentrations (Table 1). The highest callus per vial (94.44%) was recorded in MS medium containing 3.0 mg/L NAA and 2.5 mg/L BAP followed by 3.0 mg/L NAA × 3.0 mg/L BAP and 2.5 mg/L NAA × 3.0 mg/L BAP. Whereas no callus was observed in MS medium without growth regulators. It was also found that, calli were induced in the medium supplemented with a combination of NAA and BAP which were in support with the results obtained by Ali *et al.* (2005). Effect of different concentrations of NAA and BAP was significant on days to callus initiation. The minimum number of days was required in MS medium containing 3.5 mg/L NAA and 2.5 mg/L BAP (12.67 days). On the other hand, the maximum number of days (22.67 days) was required for without NAA and 2.0 mg/L BAP and also for without for BAP and 2.5 mg/L NAA.

Higher callus weight indicates the higher callus induction potentiality. Results indicated that, the highest weight of callus (2.66 g) was found in MS medium containing 3.0 mg/L NAA and 2.5 mg/L BAP followed by 3.0 mg/L NAA and 3.0 mg/L BAP. No callus was observed in MS medium without growth regulators. Lowest result (1.21) was achieved at 2.5 mg/L NAA and 2.0 mg/L BAP.

Transformation potentiality of potato cv. Granola

After antibiotic treatment of explant, highest per cent of survived calli was observed for 20 mg/L kanamycin (27.78%) in selection media (Fig. F) and the lowest was observed in MS medium supplemented with 40 mg/L kanamycin (22.22%) (Table 2).

Transformation ability was assessed through histochemical assay of GUS reporter gene. GUS assay was done after co-cultivation of explants (Fig. D) with the *Agrobacterium tumefaciens*. Before co-cultivation, infection of explant was done by *Agrobacterium tumefaciens* which was maintained in YMB solid medium (Fig. B & C). Conspicuous GUS positive (blue color) regions were detected at the entire surface of the explants which are in support of the results obtained by Jefferson *et al.* (1987). The detailed results of this investigation are produced in table 2 and 3 and figure G, H, I and J. From the GUS histochemical assay the result indicated that, the potato cv. Granola showed positive responses towards transformation.

Inoculation time and co-cultivation period are the important factors of *Agrobacterium-mediated* gene transformation. Leaves and callus were inoculated for 3, 5, 7 or 9 minutes and co-cultivated for 3, 5 and 7 days and wide range of variation of explants for GUS assay were found. Response of transformed leaf in GUS assay was increased with the time of inoculation and co-cultivation period. Leaf disc showed the highest response (83.33 %) to the assay in case of 7 minutes inoculation time and 5 days co-cultivation period. Same result was also found in case of 5 minutes inoculation time and 7 days co-cultivation period in leaf disc (Table 2). The lowest GUS positive response (0.00%) was recorded in 9 minutes inoculation and 7 days co-cultivation.

In case of callus, 3 minutes inoculation time and 5 days co-cultivation gave the highest result (88.89% GUS positive). The lowest GUS positive response (27.78%) was observed for 9 minutes inoculation time and 7 days co-cultivation periods (Table 3).

Table 1. Effect of different concentrations of NAA and BAP on number of calli, per cent callus induction, days to callus initiation and weight of callus (g) of potato cv. Granola

BAP (mg L^{-1})	NAA (mg L^{-1})	No. of calli per vial	Callus induction (%)	Days to callus initiation	Weight of callus (g)
0.0	0.0	0.000 g	0.000 g	0.000	0.000 j
	2.5	2.000 f	33.333 f	22.667	0.663 i
	3.0	3.333 def	55.557 cde	20.667	0.990 h
	3.5	3.000 def	50.000 def	20.667	1.257 g
2.0	0.0	2.333 ef	38.887 ef	22.667	0.950 h
	2.5	2.667 def	44.443 def	21.667	1.210 g
	3.0	4.667 abc	77.777 abc	15.333	1.890 d
	3.5	4.000 bcd	66.667 bcd	16.667	1.703 e
2.5	0.0	3.000 def	50.000 def	18.000	1.557 c
	2.5	4.000 bcd	66.667 bcd	15.333	2.150 c
	3.0	5.667 a	94.443 a	12.667	2.660 a
	3.5	3.667 bcde	61.113 bcde	16.000	1.863 d
3.0	0.0	3.667 bcde	61.1131 bcde	16.000	1.953 d
	2.5	4.667 abc	77.777 abc	13.000	2.107 c
	3.0	5.000 ab	83.333 ab	15.000	2.350 b
	3.5	3.667 bcde	66.667 bcde	14.667	1.867 d
CV (%)		21.69	22.34	5.60	3.26

Note: Values having common letter are identical and those having different letters are statistically different

Table 2. Effect of kanamycin on number of survived calli per vial and per cent survived calli

Kanamycin (mg/L)	Number of survived calli/vial	Per cent survived calli
20	1.667	27.777
40	1.333	22.223

Table 3. Effect of inoculation time and co-cultivation period on gene transformation of leaf disc of potato cv. Granola

Inoculation time (minute)	Co-cultivation time (days)	No. of leaf explants positive for GUS	% GUS positive explants
3	3	2.000 d	33.330 d
	5	2.000 d	33.330 d
	7	3.000 c	50.000 c
5	3	3.000 c	50.000 c
	5	4.000 b	66.667 b
	7	4.000 b	66.667 b
7	3	4.000 b	66.667 b
	5	5.000 a	83.330 a
	7	4.000 b	66.667 b
9	3	1.000 e	16.667 e
	5	1.000 e	16.667 e
	7	0.000 f	0.000 f
CV (%)		10.05	0.76

Note: Values having common letter are identical and those having different letters are statistically different

Table 4. Effect of inoculation time and co-cultivation period on gene transformation of callus of potato cv. Granola

Inoculation time (minutes)	Co-cultivation time (days)	No. of callus positive for GUS	% GUS positive callus
3	3	4.333 ab	72.223 ab
	5	5.333 a	88.887 a
	7	3.333 abc	55.557 abc
5	3	3.667 ab	61.113 ab
	5	4.333 a	72.223 a
	7	2.333 bcd	38.890 bcd
7	3	3.333 abc	55.557 abc
	5	3.333 abc	55.557 abc
	7	2.000 cd	33.333 cd
9	3	2.000 cd	33.333 cd
	5	4.000 a	66.667 a
	7	1.667 d	27.777 d
CV (%)		23.11	23.10

Note: Values having common letter are identical and those having different letters are statistically different

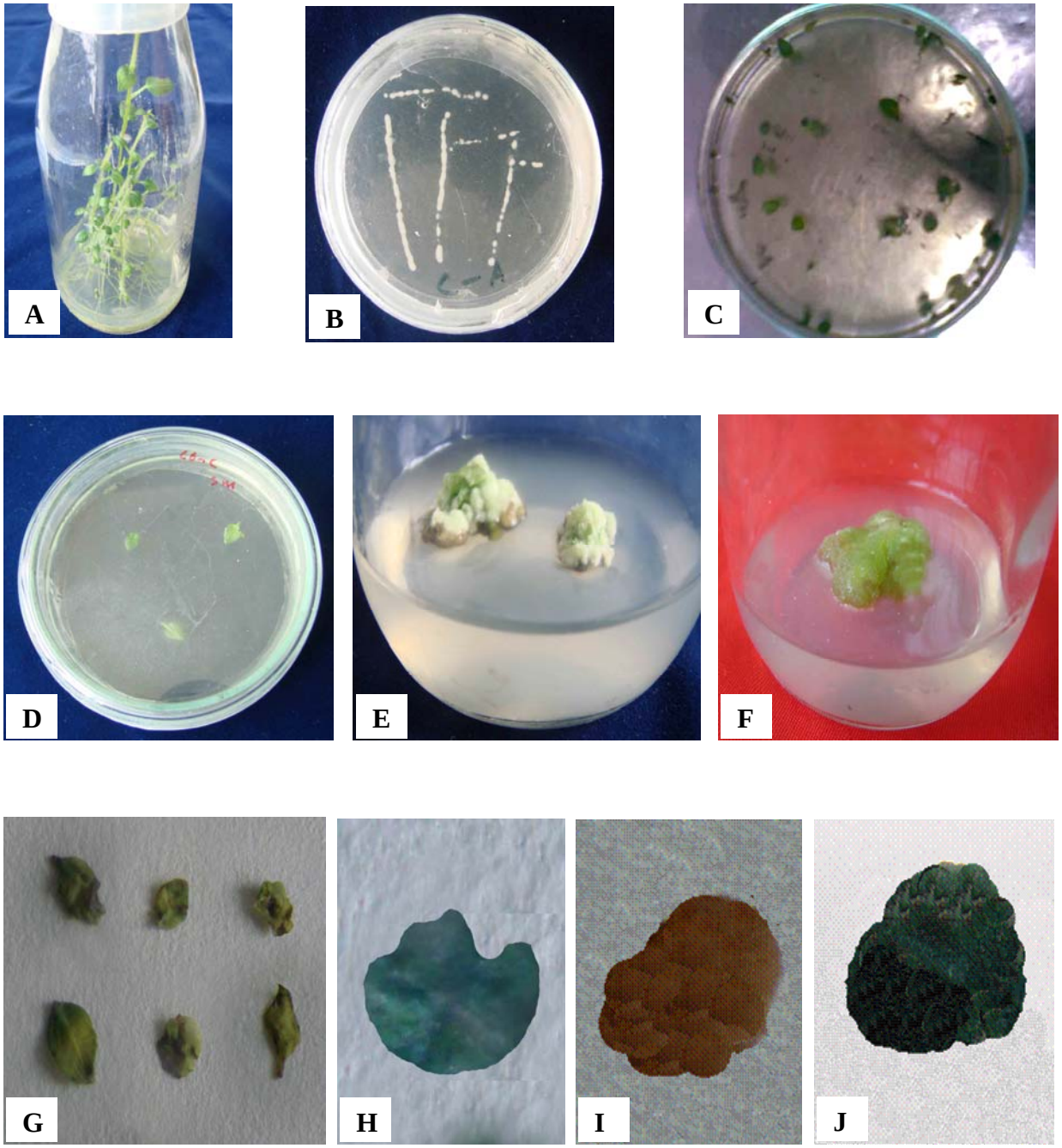

Fig. A) Sub-cultured micro plant of potato cv. Granola, B) Maintenance of *Agrobacterium tumefaciens* on solid media (YMB media) containing kanamycin, C) Infection of *Agrobacterium tumefaciens* into the leaf explants of potato cv. Granola, D) Co-cultivation of *Agrobacterium tumefaciens* and leaf explants of potato in petridishes, E) Callus induction from leaf disc of potato cv. Granola, F) Selection of kanamycin resistant calli from leaf explants of potato cv. Granola, G) Infected leaves of potato cv. Granola, H) Gus Positive leaf tissue of potato cv. Granola, I) Non-transformed callus of cv. Granola, J) Gus Positive callus of potato of potato cv. Granola.

CONCLUSION

Histochemical GUS assay performed soon after co-cultivation of explants with *Agrobacterium tumefaciens* strain LBA4404 containing the gene for GUS expression produced characteristic blue colour with X-gluc. Therefore, the presence of this blue colour gave a preliminary indication of GUS gene transfer from bacterial plasmid into the plant cell. The result of GUS assay depended upon many factors like, variety, inoculation time and co-cultivation periods. From the GUS histochemical assay, the result indicated that the potato cv. Granola showed positive responses towards transformation. The leaf discs and callus of potato cv. Granola were found GUS positive after inoculation and selection.

Agrobacterium tumefaciens strain LBA4404 carrying a binary vector pBI121 herbouring CIPK sense gene was used for infection of leaf explants. CIPK sense gives defense against a veriety of abiotic stresses tolerance including drought, light, cold and wounding. *Agrobacterium tumefaciens* strain LBA4404 has nptII gene within its T-DNA, which is abiotic stress tolerant. The results of GUS histochemical assay indicated that abiotic stress tolerant CIPK sense gene was transferred in potato cv. Granola. In this way, we can develop abiotic stress tolerant transgenic potato. Thus, using this protocol, agronomically and economically important gene/genes can be transferred to the locally grown potato variety in further program.

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