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AGROBACTERIUM MEDIATED TRANSFORMATION OF SOYBEAN (*GLYCINE MAX* L.): SOME CONDITIONS STANDARDIZATION

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Abstract

Present study was aimed to standardize some transformation conditions for soybean cultivar NARC-4 using *Agrobacterium tumefaciens* strain EHA 101 harboring pGUSintNPTII. Soybean half seed cotyledonary node method was opted. It was found that explant preparation in Agro-suspension culture resulted in highest transformation efficiency (48.3%) than in infection medium and water. One hour infection time was found optimum (55.9% transformation efficiency) in culture OD_{600} 1.0. Co-cultivation of soybean half seed explants with *Agrobacterium* for five days showed better results as compared with three and four days. Two hr washing in washing medium containing 1g/L cefotaxime controlled prevalence of *Agrobacterium* in further steps. It was found that varying kanamycin concentration in selection medium resulted in high survival rate of transformed shoots. NARC-4 soybean cultivar showed better transformation efficiency than NARC-7 when genotype dependency was examined using *Agrobacterium* strain EHA101.

Introduction

Plant genetic engineering mainly depends upon *Agrobacterium tumefaciens* mediated transformation that accounts so far production of 80% transgenics (Wang & Fang, 1998). Soybean transformation has shown significant improvement and enabled public and private sector for production of commercial cultivars with transgenic traits.

Hinchee *et al*., (1988) first time reported soybean transformation with *Agrobacterium* strain. They successfully regenerated plants on media containing kanamycin or glyphosate but transformation efficiency was quite low (Hinchee *et al*., 1988) but modifications in regeneration protocol may produce high rate of transformants (Parrott *et al*., 1989; McKenzie & Cress, 1992). Although a number of factors that affect on transformation efficiency has been studied that includes sonication assisted *Agrobacterium* mediated transformation (Trick & Finer, 1997); use of cystine, dithiothretiol and thiol compounds (Olhoft *et al*., 2007); co-cultivation at 22°C and use of Silwet-77 as surfactant (Liu *et al*., 2007); use of antioxidant during co-cultivation (Wang & Xue, 2008); 4 day co-cultivation time period (Ko & Korban, 2004) and selection by direct placement of explant at low concentration of antibiotic (Yan *et al*., 2000). But still soybean genetic transformation is limited to few laboratories due to low transformation and as well as regeneration efficiencies.

Mainly soybean transformation is carried out using immature cotyledons as explant but very few reports are available describing other explants as T-DNA recipient due to poor regeneration capability. Transformation efficiency was observed upto 15.8% using embryonic tips of soybean pre grown on MS medium containing BAP (Liu *et al*., 2004). While Paz *et al*., (2006) used cotyledonary node of half seeds as an explant. They

reported that half seed explants ranged transformation efficiency 1.4 to 8.7% and this system is simple and does not require deliberate wounding of explants. Donaldson & Simmonds (2000) demonstrated that competent cells, in the case of cotyledonary node transformation, are few so has low transformation competency therefore using cotyledonary nodes as explants present low transformation efficiency.

The objective of the present study was to standardize some *Agrobacterium* mediated transformation conditions for soybean using cotyledonary node as explants.

Materials and Methods

Explant preparation and inoculation: *Glycine max* cultivars NARC-4 was selected to standardize some *Agrobacterium* mediated transformation conditions due to its better regeneration capability. For *Agrobacterium* mediated transformation through mature soybean cotyledonary node (half seed) method as described by Paz *et al*., 2006 was followed with some modifications. Different media used are listed in Table 1.

***Agrobacterium* preparation:** *Agrobacterium tumefaciens* strain EHA101 stock was made by streaking on LB agar medium containing 50mg/L kanamycin and incubated at 25°C until colony formation. EHA101 contain p35SGUSintnptII, GUS gene under the control of 35S Cauliflower Mosaic Virus promoter and nptII gene under the control of NOS promote (Fig. 1). Single colony of EHA101 was re-cultured in 50ml LB medium containing 100mg/L Kanamycin for 24 hrs for better selection. A volume of 100µl was suspended in 50ml LB medium and kept overnight at 120rpm and 25°C. One hr before infection, the culture was centrifuged at 4000rpm and at 4°C for 10 min. The pellet was re-suspended in infection medium until the OD_{600} reached 1.0 (Agro-suspension culture).

Explant preparation and conditions: Soybean seeds were sterilized with 0.1% (w/v) Mercuric chloride followed by three rinses with autoclaved distilled water. The seeds remained immerged in autoclaved distilled water under complete darkness for 16 hr. After that the seed coat was removed and the seeds were vertically bisected along the embryo and half of embryo from pointed end was also removed. Cutting of explants was performed in water, in infection medium and in Agro-suspension culture to analyze the effect on transformation. All *in vitro* cultures were kept in growth chamber at 23±1°C, illuminated with white florescent light (10000 lux) and 16/8 photoperiod.

Half bisected soybean seeds were infected with *Agrobacterium* for different time period i.e., 30min, one hr, three hr or five hr. Infection was carried out at 50rpm at 25°C. The infected explants were transferred on co-cultivation medium as abaxial side down word. The explants were co-cultivated with *Agrobacterium* for three, four and five days. After co-cultivation time period the explants were washed in washing medium for 30min., 1hr and 2hr to kill *Agrobacterium* and again thoroughly rinsed with washing medium containing 500mg/L cefotaxime instead of 1.0g/L. The explants were blotted dry on sterile filter paper, a fresh cut was made on the base and transferred to shoot induction medium-I as adaxial side touching the medium. After 14 days, any regenerated shoot was removed and discarded. The explants were transferred on shoot induction medium-II with a fresh cut on the base again for 14 days. The explants were transferred to shoot elongation medium for 56 days. The media was refreshed after each 14 days and fresh cut was made on the base of explant every time. Different concentrations of kanamycin (20, 30, 40, 50 mg/L) were checked during shoot induction stage II and shoot elongation stages.

Table 1. Media used for transformation / regeneration of soybean cultivars.

Culture medium	Composition
Infection medium	B5 salts with iron source (Gamborg *et al*., 1968), 30g/l sucrose, 3.9g/L MES (pH5.4).Filter sterilized B5 vitamins, 1mg/L BAP, 0.25mg/L GA3, 40mg/L acetosyringone were added after autoclaving
Co-cultivation medium	B5 salts with iron source, 30g/L sucrose, 3.9g/l MES, 0.5%agar (pH 5.4). Filter sterilized B5 vitamin, 0.25 mg/L GA3, 1mg/l BAP, 400mg/L Cystine, 154mg/L DTT and 40mg/L aceosyringone were added after autoclaving. (~10ml/ 100 x 15mm Petri plate) Sterile filter paper was overlaid on medium when solidified.
Washing medium	B5 salts with iron source, 30g/L sucrose, 0.59g/L MES (pH5.7). Filter sterilized B5 vitamins, 1mg/L BAP, 1g/L cefotaxime were added after autoclaving. The flask was placed on shaker at 50rpm/min and 25°C.
Shoot induction medium I	B5 salts with iron source, 30g/l sucrose, 0.7% agar and 0.59g MES (pH 5.7). Filter sterilized B5 vitamins, BAP 1mg/L, 500mg/L cefotaxime were added after autoclaving.
Shoot induction medium II	Same as Shoot induction medium I, in addition with Kanamycin
Shoot elongation medium I-IV	B5 salts with iron source, 30g/l sucrose, 0.7% agar and 0.59g MES (pH 5.7). Filter sterilized B5 vitamins, ZTR 1mg/L, 50mg/l Aspragine, 100mg/L L-pyroglutamic acid, 250mg/l cefotaxime and 20mg/L kanamycin was added after autoclaving.
Rooting medium	Half strength MS salts and iron (Murashige and Skoog, 1962.), 0.59g/l MES, 2% sucrose, 1mg/L IBA solidified with 0.8% agar at pH 5.7.

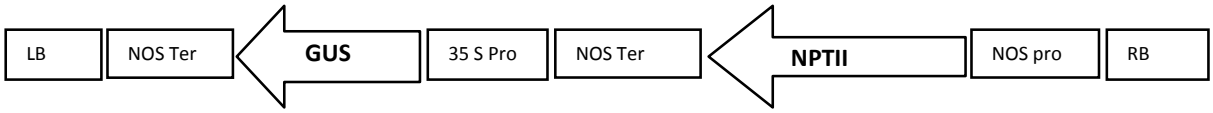

Fig. 1. T-DNA region of transformation vector p35SGUSintnptII. LB Left Border, 35SP 35S promoter, GUS beta-glucuronidase gene, NOST 3-Nopaline synthase terminator, NOSP 3-Nopaline synthase promoter, RB Right border.

The regenerated shoots were selected on kanamycin containing medium when attained height 3-4cm were separated from the base and transferred on ½ MS salt medium without vitamins containing 1.0 mg/L IBA for rooting.

Transient GUS expression: For all parameters studied except kanamycin concentration, after 14 days on SIM-I while for kanamycin concentration as selective agent after 6-8 weeks on SEM-IV complete explant was analyzed for transient GUS expression. Histochemical staining of GUS activity was performed by incubating tissue sections in GUS solution as described by Jefferson *et al*., 1987.

Statistical analysis: For each experiment to optimize transformation conditions, 100 explants were infected. For one condition, other parameters were kept constant (described as a note below each table). Percent response was measured from the explants survived at the end of SIM-I for all parameters studied except kanamycin concentration. For

kanamycin concentration; percentage response was measured by the shoots survived at SEM-IV. Each explant was considered as experimental unit. The data was analyzed by DMRT at probability level 0.05.

Genotype dependency of two soybean cultivars: On basis of all previous results, transformation efficiency of NARC-4 and NARC-7 soybean genotypes was analyzed. The explants were prepared in Agro-suspension culture, infected for 1hr, co-cultivation was done for 5 days and washing was done for 2hr. Regenerated plants were selected at varying concentration of kanamycin in the medium as during SIM-II, Kanamycin 30mg/L; for SEM-I, 20 mg/L; for SEM-II & III, 40 mg/L while for SEM-IV, 20mg/L. If explants were kept on SEM for additional 14 days, kanamycin was not added in SEM-V.

GUS Expression and polymerase chain reaction: The plants survived at SEM-IV were subjected for GUS assay (leaves and stem parts). The plants showing GUS expression were subjected to polymerase chain reaction. Leaf explants were used for extraction of genomic DNA by CTAB method previously described by Doyle & Doyle (1990) and modified by Sharma *et al*., (2003). PCR was performed for detection of npt*II* and GUS genes. The primer sequences used for nptII were 5-AAGATGGATTGCACGCAGGTTC-3 and 5-GAAGAACTCGTCAAGAAGGCGA-3. While for GUS gene, primers used were 5-AACTGGACAAGGCACTAGCGG-3 and 5-TGCGACCTGACCGTACTTGAA-3.

Results

In the present study, some conditions for soybean *Agrobacterium* mediated transformation were standardized using soybean cultivar NARC-4. Later on, genotypic dependency was analyzed using two soybean cultivars NARC-4 and NARC-7 under best optimized conditions.

Effect of explant cutting in different media and infection time: The seed cutting in water, in infection medium or in Agro-suspension culture affected on survival of explants as well as on transformation efficiency. Seed cutting in Agro-suspension culture led maximum survival of explants (60%) as well as T-DNA delivery (48.3% out of survived explants). A total of 29 explants (out of 60 survived) showed blue spots after SIM-I. When seeds were bisected in water and in infection medium, 40.4 and 38.7% GUS expression was observed, respectively. In these mediums survival of explants were 47% and 62%, respectively (Table 2).

After preparation of explants, they were allowed to be incontact with *Agrobacterium* for T-DNA delivery to plant cell. Infection time also affected on survival of explants and percentage of GUS expression. Thirty min infection time was best for survival of explant (74%) but it showed less GUS expression (24.3%) on explants observed after SIM-I (Table 3). While 1 hr infection time proved best. At this infection time, transient GUS expression was maximum (55.9%) with 68% explant survival rate. Increasing infection time upto 3hr or 5 hr showed decline in both cases; explant survival and GUS expression.

Effect of co-cultivation and washing time: It was found that five days co-cultivation time was optimum for maximum GUS expression from explants survived on SIM-I (Table 4). After 5 days co-cultivation time, maximum GUS expression (55.17%) was observed with minimum survival of explants (58%). After 3-days co-cultivation time, 40 explants out of 77 showed GUS expressions (51.9% response) while 31 explants out of 64 (48.4%), survived on SIM I, showed blue coloration in the case of 4-days co-cultivation.

Table 2. Effect of different explant cutting medium * on genetic transformation of soybean cv NARC-4.

Media	No. of explants	Explants survived at SIMI (%)	Explant showing GUS expression at SIMI	% GUS expression♣
Water	100	47 (47%) [b]	19 [c]	40.4 [b]
Infection	100	62 (62%) [a]	24 [b]	38.7 [b]
Agro-suspension	100	60 (60%) [a]	29 [a]	48.3 [a]

*Co-cultivation for 3 days, infection time 1 hr and washing time 1 hr

♣GUS expression = [Explant showing GUS expression / Explants survived SIM-1] x 100

Table 3. Effect of different infection time* on genetic transformation of soybean cv. NARC-4.

Media	No. of explants	Explants survived at SIMI (%)	Explant showing GUS expression at SIMI	% GUS expression♣
30 min	100	74 (74%) [a]	18 [c]	24.3 [c]
1 hr	100	68 (68%) [b]	38 [a]	55.9 [a]
3hr	100	65 (65%) [b]	29 [b]	44.6 [b]
5hr	100	59 (59%) [c]	13 [d]	22.0 [d]

*Explant cutting in infection medium, co-cultivation for 3 days and washing time 1 hr

♣GUS expression = [Explant showing GUS expression / Explants survived SIM-1] x 100

Table 4. Effect of different co-cultivation time* on genetic transformation of soybean cv. NARC-4.

Days	No. of explants	Explants survived at SIMI (%)	Explant showing GUS expression at SIMI	% GUS expression♣
3 days	100	77 (77%) [a]	40 [a]	51.9 [b]
4 days	100	64 (64%) [b]	31 [b]	48.4 [c]
5 days	100	58 (58%) [c]	32 [b]	55.17 [a]

*Explant cutting in infection medium, infection time 1hr and washing time 1 hr

♣GUS expression = [Explant showing GUS expression / Explants survived SIM-1] x 100

Table 5. Effect of different washing time* on genetic transformation of soybean cv. NARC-4.

Time	No. of explants	Explants survived at SIMI (%)	Explant showing GUS expression at SIMI	% GUS expression[5]
30min	100	28 (28%) [b]	11 [b]	39.2 [c]
1hr	100	57(57%) [a]	30 [a]	52.6 [b]
2hr	100	61 (61%) [a]	34 [a]	55.7 [a]

*Explant cutting in infection medium, co-cultivation for 3 days and infection time 1 hr

♣GUS expression = [Explant showing GUS expression / Explants survived SIM-1] x 100

Increasing washing time period scored high number of survival of explants and GUS positive response (Table 5). Thirty four explants out of 61 survived at SIM-I (55.7%) showed GUS expression when washed for 2 hr. While 1 hr washing endured 57 explants, out of those 30 explants (52.6%) showed blue color at explants describing presence and expression of GUS gene in plant cell.

Effect of Kanamycin concentration in selection medium: Kanamycin concentration in selection medium drastically affected on regeneration of shoots emerging from explants. At 20mg/L kanamycin, 54 explants showed shooting response or embryo formation. At SEM-IV average 1.6shoots/explant were counted. Seventeen shoots out of 48 survived on

selection medium (35.4%) showed GUS expression (Table 6). While at selection medium, containing 30mg/L kanamycin, 21 regenerated shoots (63.6%) were positive for GUS activity out of 33 survived on selection medium. Increasing the kanamycin concentration decreased survival of explants as well as regeneration of shoots.

Table 6. Effect of Kanamycin concentration* on genetic transformation of soybean cv. NARC-4.

Conc. (mg/l)	No. of explant	Responding explants at SEM IV	Embryo/shoot per explant at SEM IV	# of shoot survived at SEMIV	GUS positive (stem & leaves)	% GUS response♣
20	100	54 (54%) [a]	1.6 [a]	48 [a]	17 [b]	35.4 [c]
30	100	47 (47%) [b]	1.1 [b]	33 [b]	21 [a]	63.6 [b]
40	100	24 (24%) [c]	0.8 [c]	8 [c]	5 [c]	62.5 [b]
50	100	16 (16%) [d]	0.2 [d]	3 [c]	2 [c]	66.6 [a]

*Explant cutting in infection medium, infection time 1hr, washing time 1hr and co-cultivation for 3 days

♣GUS expression = [Explant showing GUS expression / Explants survived SIM-1] x 100

Table 7. Transformation efficiency of two soybean cultivars by *A. tumefaciens* EHA101 harboring GUSint 35 Snpt II.

Cultivar	No of explants	No of shoots produced	No of GUS +ive shoots	% response	GUS+ plant's rooting response
NARC-4	120	56	34	60.7	21 (17.5%)
NARC-7	70	29	12	41.3	7 (10%)

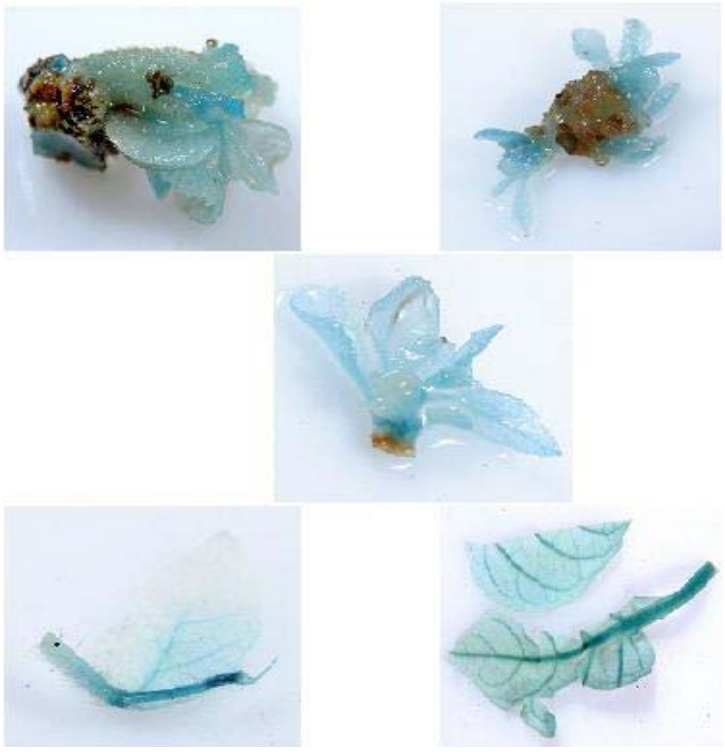

Fig. 2. GUS expression of different Soybean NARC-4 explants at different stages of transformation.

Transformation efficiency of two soybean cultivars NARC-4 & NARC-7: Combining all best conditions with varying concentration of kanamycin in selection medium, response of soybean genotypes NARC-4 and NARC-7 was observed. For NARC-4, 120 explants were infected with *Agrobacterium tumefaciens*. At the end of SEM-IV, 56

shoots were counted, out of that 34 shoots (60.7%) showed blue coloration (Fig. 2). Only 21 shoots out of 34 rooted showing 17.5% transformation efficiency from totals number of explants to start the experiment (Table 7) while 10% transformation efficiency was observed from soybean cultivar NARC-7. At the end 7 out of 12 plants successfully produced roots and showed GUS expression (Fig. 3).

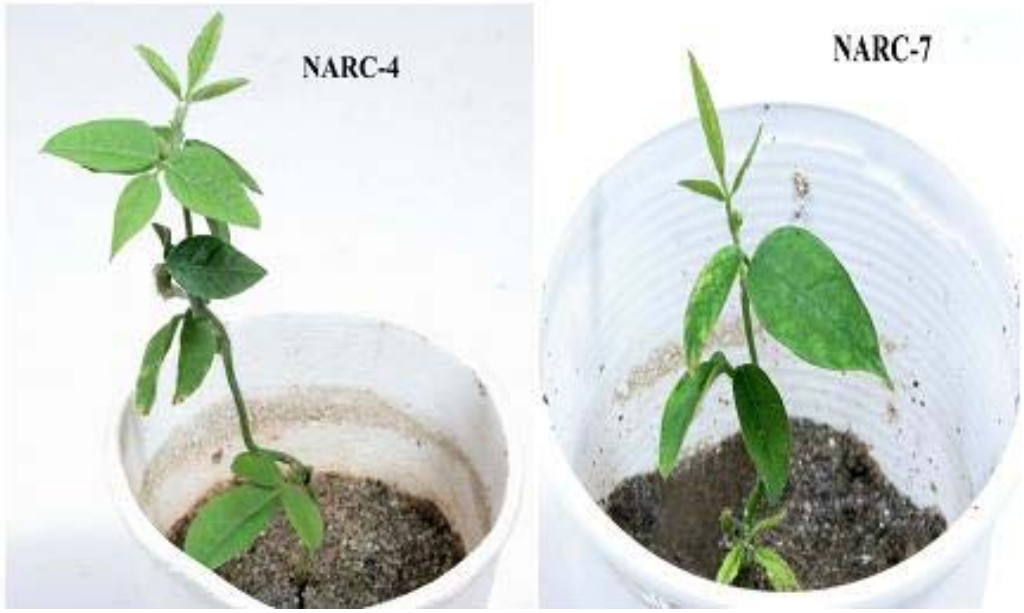

Fig. 3. Acclimatization of Soybean transformed plants.

DNA isolated from leaf of GUS positive and non-transformed plants along with p35SGUSint plasmid isolated from EHA101 (positive control) were subjected for amplification of nptII and GUS genes fragments (Fig. 4). Presence of 1100bp fragment for nptII and 781bp fragment for GUS gene confirmed integration of T-DNA into plant genome. These fragments were not observed in control non-transformed plants.

Discussion

Agrobacterium tumefaciens mediated transformation to a number of plant species is routine work but parameters for improvement in transformation efficiency and regeneration of plant from infected explant are important to be studied. Although, a number of parameters that affect on T-DNA delivery have been studied and found more or less common for many plant species transformation including soybean but to analyze appropriate level related to strain, genotype and explant type is prerequisite.

Effect of explant cutting in different media and infection time: Explant cutting media significantly affected on explants survival rate and transformation efficiency. As a routine matter, explants are prepared and then infected with *Agrobacterium*. In this study, survival rate and percentage of GUS expression was maximum when explants were prepared in Agro-suspension culture leading to infection medium and water. The process of *Agrobacterium*-mediated gene transfer initiates by wounding of plant tissue, which leads to the release of phenolic compounds and monosaccharides and subsequent triggering of the expression of *vir*-genes in *A. tumefaciens* (Sheng & Citovsky, 1996). One vir senses plant phenolic compounds and transduce this signal to induce expression of virA and virG genes (Stachel *et al*., 1986). Because wounding is important for efficient plant transformation, *Agrobacterium* can sense a wounded potential host by perceiving

these phenolic compounds. Infection medium also contained acetosyringone, DTT and acidic pH. These factors have already been proven to facilitate T-DNA transfer and enhance transformation efficiency (Olhoft *et al*., 2003). Present results suggest that when explants were in contact with *Agrobacterium* just after wounded, showed better results. This method of explant preparation not only increased GUS expression but high explant survival rate was also observed.

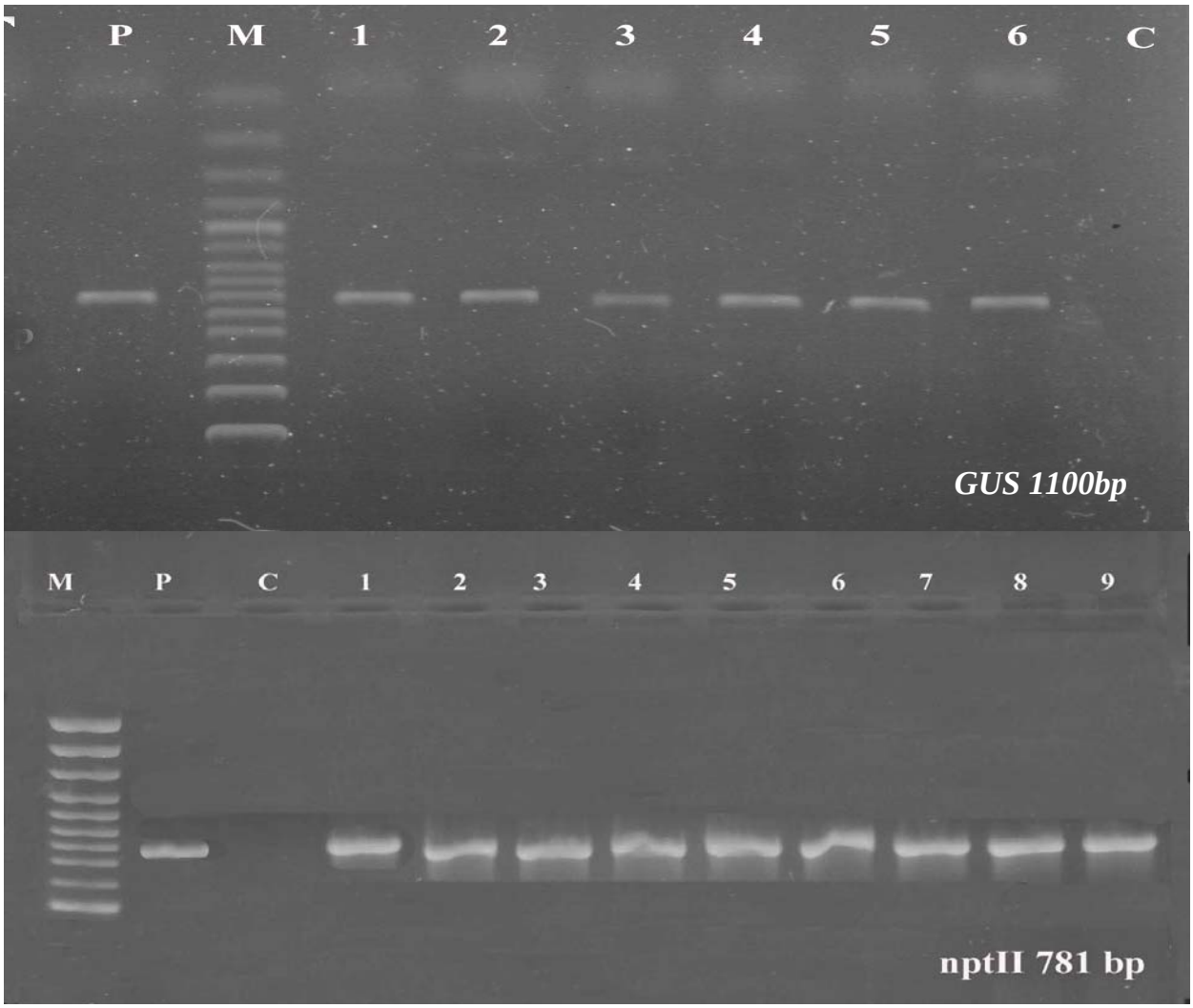

Fig. 4. PCR product of soybean NARC-4 and NARC-7 transformed plants.
M= Marker, C= Control non-transformed, P= Plasmid 1,2,3…… Transformed plants

The T-DNA delivery time depends upon *Agrobacterium* strain, vector and explant used. One hr infection of soybean half seeds with *Agrobacterium* culture (OD 1.0) was found significant to get high number of transforments and also for survival of explants. While less time period (30 min) increased the survival of explants but GUS expression was low. Liu *et al*., (2004) and Ko & Korban (2004) also found same results while standardizing soybean transformation conditions. According to many reports, *Agrobacterium* induces necrosis of explants. The degree of necrotic reaction depends upon several transformation parameters, including explant age, preculture period, bacterial inoculum density and infection duration (Kuta & Tripathi, 2005).

Effect of co-cultivation and washing time: For soybean half seeds transformation methodology, 5 days co-cultivation lead to moderate survival of explants but with higher transformation efficiency. Paz *et al*., (2006) and Xue *et al*., (2006) also placed the half seed explants on co-cultivation medium for 5 days for better transformation efficiency. While Yan *et al*., 2000 found non-significant difference in induction of somatic embryos from immature cotyledons and survival of explants when co-cultivated beyond 3 days. A comparison of co-culture time showed that shorter the co-culture period rated higher the survival rate of explant but transformation percentage was low.

Presence of high concentration of cefotaxime in culture media also effects on induction of somatic embryos and on shoot elongation (Shyamkumar *et al*., 2007) while longer washing time in liquid medium may harm for explant leading to complete necrosis and death. Present findings showed that 30 min washing did not completely kill the *Agrobacterium* cells so less number of survival rate of explant and low GUS expression was observed. Increasing the washing time led to survival of explants and higher transformation efficiency. There is not much difference between 1hr and 2 hr washing time results but it was observed that better control at this stage do not cause contamination problem in next steps.

Effect of Kanamycin concentration in selection medium: Low concentration of kanamycin (20mg/l) in shoot induction and shoot elongation medium produced higher number of somatic embryos per explant but percentage of GUS expression was low. Increasing the kanamycin concentration (40 or 50 mg/l) in selection medium increased transformation efficiency but at these concentrations responding explants and generation of shoots was low. Higher concentration of kanamycin was also found toxic for regeneration system from embryonic tips, cotyledonary node and hypocotyls segments (Liu *et al*., 2004). While Cho *et al*., (2000) also reported that some transformed roots failed to grow in kanamycin and carbenicillin containing medium.

Transformation efficiency of two soybean cultivars NARC-4 & NARC-7: The conditions standardized proved best to generate transformed plants at higher efficiency (17.5% for NARC-4). A total number of 56 shoots were produced at the end; out of that 34 were GUS positive, showing 60% response. While for NARC-7 41% response was observed in regenerated shoots while final transformation efficiency after rooting was 10%. Many hypervirulent strains has been studied for tumorogenic response to soybean as well as for T-DNA delivery by disarmed strain but EHA 105 responded well and commonly used for soybean transformation (Yan *et al*., 2000; Olhoft *et al*., 2004; Yi & Yu, 2006; Liu *et al*., 2007). Meurer *et al*., (1998) reported that KYRT1 was better for soybean transformation efficiency over LBA4404 but it significantly decreased shooting response as compared to EHA105. Ko *et al*., (2003) described that EHA105 was ineffective in producing transgenic somatic embryos while Yi & Yu (2006) concluded that transformation efficiency is associated with *Agrobacterium* strain as well as with soybean cultivar.

Half seed methodology has been used by Xue *et al*., (2006) and Paz *et al*., (2006) successfully; determining final transformation efficiency upto 12%. Present findings conclude that by improving T-DNA delivery system (increasing infection time) and better selection may increase transformation efficiency in soybean.

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