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Research article

AGROBACTERIUM MEDIATED TRANSFORMATION OF PIGEONPEA (CAJANUS CAJAN L MILLLSP) VAR LRG-41 FROM AXILLARY BUD

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ABSTRACT: A reliable method of plant regeneration has been achieved from Axillary buds. Shoots appeared from explants when cultured on Murashige and skoog (MS) medium supplemented with BAP (Benzyl amino purine), Napthalene acetic acid (NAA) and Kinetin at various combinations. Elongated shoots were rooted with 70.6% rooting frequency in MS medium with indole buteric acid (IBA) at 1.0mg/l. The rooted plantlets were established well in soilrite mixture medium with 91% success and days taken for acclimatization were 12.8 days. This protocol was used in Agrobacterium mediated transformation. The transformation was carried out using the Agrobacterium strain LBA4404 containing the binary vector pCAMBIA2301 harboring npt II as selectable marker and GUS as reporter gene.

Key words: Pigeonpea, Regeneration, Agrobacterium, Transformation, Reporter gene.

INTRODUCTION

Pigeonpea (*Cajanus cajan* L. Millsp.) or redgram is one of the most popular legume grains in the world, especially in the Indian subcontinent. Due to its multiple uses, pigeonpea is widely used in intercropping systems in semi-arid regions and ranks fifth in area after soybean, common bean, peanut and chickpea. It is used in diverse ways as a source of food, feed, and fertilizer. It provides the main source of protein for the poorest populations and plays an important role in reducing malnutrition for millions of people around the world. Improvement of pigeonpea cultivars possessing resistance to pest and diseases, tolerance to abiotic stresses and low allergenic proteins in seeds is therefore desirable. Plant regeneraton in pigeonpea has been reported through cotyledons (George and Eapen 1994; Geetha *et al*. 1998), cotyledonary nodes (Mehta and Mohan Ram 1980; Kumar *et al* 1983,1984; Shiva Prakash *et al* 1994; Naidu *et al* 1995; Geetha *et al* 1998) and Embryonal axes (Franklin *et al*. 2000). In these reported regeneraton systems, time required for the formation of shoot buds and the recovery of fully differentiated plants was long thus making such systems inefficient for genetic transformation work. Hence in the present study, the major emphasis was on the establishment of a regeneration protocol that would provide transgenic plants in large numbers for routine work on the genetic enhancement of pigeonpea.

MATERIALS AND METHODS

Plant material

Seeds of pigeonpea (*Cajanus cajan* L. Millsp) variety LRG-41 used in the experiment were obtained from plant breeding (pulses) section, Regional Agricultural Research Station, Acharya NG Ranga Agricultural University, Tirupati-517502, A.P. India

Culture media and conditions

MS basal medium containing 3% sucrose was used for all in vitro cultures. The pH of the medium was adjusted to 5.8 prior to adding 0.8% agar; media were autoclaved at 121^{0}C for 15 min. Cultures were maintained at 26 ±1^{0}C under continuous light provided by white cool fluorescent tubes of 60 μE m^{-2} s^{-1} light intensity. The growth regulators BAP, kinetin, NAA and IBA were filter-sterilized prior to addition to culture media. The explants were cultured on sterile Petri dishes containing SIM (Shoot induction medium), explants bearing adventitious shoot buds were subsequently transferred to culture tubes for shoot elongation and rooting of shoots.

Data on the frequency of shoot bud regeneration from each explant was recorded. All experiments were repeated three times and the data were analyzed by calculating mean and standard error. The data were analysed using analysis of variance for a completely randomized design and the treatment means were compared.

Explant preparation and hormonal combinations

The seeds of pigeonpea var .LRG-41 were washed with 0.1% mercuric chloride for 10 minutes. Axillary buds were used as explants. MS basal medium with different hormonal concentrations of BAP, Kinetin and NAA were evaluated for their effect on days taken for shoot bud initiation, Number of explants producing shoot buds, shooting frequency, mean number of shoots / explant and length of the shoots. The regenerated shoots were transferred to different combinations of IBA alone and in combination with BAP and Kinetin with full strength MS medium for root induction. The data on days taken for root initiation, rooting frequency (%), mean number of roots/shoot and mean length of roots (cm) were recorded. Acclimation: The *in vitro* rooted plants were transferred to different soil mineral mixtures viz soil rite, sand, soil, and soil:sand:soilrite. The data on number of days taken for acclimatization and survival percentage were recorded (Table 3)

Agrobacterium strains and gene constructs

The Agrobacterium tumefaciens strain LBA4404 containing the binary vector pCAMBIA2301 harboring npt II as selectable marker and β-glucoronidase (GUS) as reporter gene. The disarmed Agrobacterium tumefaciens strain LBA harbouring a binary plasmid pCAMBIA was used as a vector for transformation. The most common and versatile reporter gene is the β-glucoronidase (GUS) gene from E.*coli* .The enzyme cleaves a wide range of β-glucoronidase substrates and the activity of expression can be conveniently measured using fluorometric assay (Miyoshi *et al.,* 1995), spectrophotometer or histochemical assays (Jefferson, 1987).

Determination of lethal dose of kanamycin for pigeonpea explants

The LD_{50} of kanamycin used as a selective pressure was determined by culturing Axillary bud explants (3 petri dishes per treatment) per treatment in MS medium and supplemented with various concentrations of kanamycin (0,25,50,75 and 100 mg/l). The cultures were scored after an incubation of 4-5 weeks at 25 ± 2^0c under irradiance of 38 µmol m^{-2} s^{-1} (16 hour photoperiod) provided by cool white fluorescent tubes.

Co-cultivation and transformation

Several conditions were standardized to develop an efficient transformation protocol for the variety LRG-41.An experiment was conducted to optimize several factors such as duration of inoculums with *Agrobacterium* culture, co-cultivation period, concentration of cefotaxime and culture period of co-cultivated explants on cefotaxime medium to prevent bacterial growth (Table-4).

Regeneration of shoots

The shoots regenerated on MS medium containing 75 mg/l Kanamycin and 500 mg/l cefotaxime after 4 weeks of incubation were transferred to a freshly prepared MS medium containing 500 mg/l of cefotaxime and 75 mg/l kanamycin in test tubes and were incubated for another 4 weeks and then green shoots were then transferred to MS medium supplemented with 75 mg/l kanamycin and incubated for another 4 weeks. The shoots surviving on MS medium supplemented with 75 mg/l kanamycin for 8 weeks were scored for GUS activity and or green fluorescence.

Rooting and hardening

The putative transformants surviving on MS medium after co-cultivation were transferred to rooting medium and incubated for 3 weeks. The rooted plantlets were hardened and maintained in the controlled environment at $25\pm2\ ^0$c under 16-hour photoperiod with irradiance of 38 µmol m^{-2} s^{-1} for 2 weeks.

Tissue staining for GUS activity

Histochemical analysis was carried out to determine the β-glucoronidase activity in leaf bits. The leaf was cut in to small leaf bits (0.5 cm) and immersed in X-Gluc solution [50mM Na_2PO_4 buffer (Ph 7.0), 10 mM EDTA (Ph 7.0), 0.5 mM potassium ferricyanide (Ph 7.0), 1 mM X- Glucuronide,0.1% TritonX-100 in microtiter multiwall plates and incubated over night at 37°C. The tissues were bleached in 100% ethanol before observation.

Fluorescence microscopy

Visualization of GFP fluorescence in plant tissues was achieved using a Leica wild MPS32 stereo microscope fitted with G filter. Photographs were taken using Leica MPS 32 photoautomat camera set to take automatic exposure (under dark field) on Fujichrome 400 ASA film.

Isolation of genomic DNA by CTAB method (N-Cetyl-N, N-Trimethyl Ammonium Bromide

Genomic DNA was extracted from putatively transformed plants and untransformed plants. 0.5 g of leaf tissue was weighed from each putatively transformed plant and a control. .The leaves were cut in to pieces and ground well in liquid nitrogen using pestle and mortar to powder.10 ml of hot 2 x CTAB buffer (65^0C) was added.

Contents were transferred to centrifuge tubes. 10 ml of $CHCl_3$+Isoamyl alcohol (24:1) mixture was added to remove chlorophyll and cell debris. This was mixed thoroughly and centrifuged for 10 min at 1000 rpm and supernatant were collected in fresh tubes. To this 1/10 volume of CTAB was added and mixed and centrifuged for 1000rpm for 5 min. The aqueous layer was collected and precipitation buffer was added and centrifuged at 10000rpm.To dissolve the pellet high salt TE buffer was added and to 2 volumes of ice cold absolute ethanol was added to precipitate DNA followed by centrifugation for 15 min at 10000 rpm to obtain DNA pellet. (Two putatively transformed plants obtained (0.5 g tissue) 16 and 18 weeks after co-cultivation with Agrobacterium strain LBA4404 containing binary vector pCAMBIA-2301 were used.)

DNA estimation

DNA isolated by above methods was estimated at 260mm and its purity was determined by measuring O.D ratio at 260/280 nm, A_{260}/A_{280} is 1.8-2.0.Ratio less than 1.8 indicate that the preparation is contaminated either with phenols or proteins.

Confirmation of the presence of GUS gene in the putative transformants by PCR

This was done using PCR and primers specific to GUS. Polymerase chain reaction is a very simple method for *in vitro* amplification of specific nucleic acids using hot start *Taq* DNA polymerase and short stretch of oligonucleotides which are specific to the DNA to be amplified.

Electrophoresis and visualization of amplified products

Electrophoresis is usually carried out in solid matrix like agarose or polyacrylamide gels. In a gel, the shape and size of the DNA fragment to be separated and the concentration of the agarose used influence the migration rate. Agarose powder was dissolved in TBE buffer by melting at 100°C. The solution was cooled to 50°C and 4μl of ethidium bromide (10 mg/ml stock) was added to 100 ml of the solution, poured into the gel frame and allowed to set. After setting the gel, the comb was removed and the gel was transferred to the gel tank such that the wells were towards the negative electrode. The gel tank was filled with 1X TBE buffer just enough to cover the surface of the gel. Amplified DNA samples of 25 μl were mixed with 4μl of loading buffer (6X) and loaded in the wells of the submerged gel using a micro-pipette. 10 μl of 1 kb DNA ladder (Fermentas, USA) was also mixed with loading buffer and loaded on to one of the wells. The gel was then visualized on UV - transilluminator and the picture was taken from (Alpha innotech) Gel documentation system.

RESULTS AND DISCUSSION

Axillary buds obtained from the germinated seedlings were used as explants. Among different combinations, MS medium + BAP 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} recorded significantly lesser number of days taken for shoot bud initiation (17 days),and also recorded highest number of shoots/explants (4.6) and highest shoot length (4.8 cm) shown in Table 1 . Sharma *et al.* (2006) reported that adventitious shoot buds from axillary meristem explants of pigeonpea cultured on shoot induction medium for 10 to 12days and then implanted on MS medium supplemented with BA at a concentration of 22.0 μM. For root induction different combinations of IBA, NAA alone and BAP and Kinetin in combination with IBA were used with full strength MS medium. Mean number of days taken for root initiation was significantly lower when MS medium was supplemented with IBA 1.0 mg l^{-1} (17.9 days) compared to all other combinations shown in Table 2 . Sharma *et al.* (2006) reported that elongated shoots of pigeonpea rooted on MS medium containing 25 μM IAA. However Yadav and Padmaja (2003) reported that low concentrations of growth regulators at 1.0 mg l^{-1} of IAA/NAA + 0.1 mg l^{-1} of Kinetin triggered root induction.

Determination of lethal dose and selection of shoots

Axillary bud explants formed shoots on MS medium without kanamycin after 4 weeks of incubation. The shoot formation was also observed after 4 weeks of culture on MS medium supplemented with 25 and 50 mg/l kanamycin while no shoot formation was observed on MS medium with 75 and 100 mg/l kanamycin. Therefore 50mg/l kanamycin was found to be effective in inhibiting the regeneration totally in non-transformed plants is shown in Table 5.

Table 1.Effect of MS medium with different hormonal treatments on shoot induction and elongation in axillary bud explants of Redgram var. LRG-41

S. No	Concentrations (mg l^{-1})	Days taken for shoot bud initiation	No. of explants producing shoot buds	Shooting frequency (%)	Mean no of shoots/ explant	Length of shoots (cm)
1	MS+BAP 0.5	NR	0.6	8.91(17.36)	0	1.8
2	MS+BAP 1.0	NR	1.0	17.80(24.65)	0	2.2
3	MS+BAP 2.0	21.2	1.7	27.85(31.82)	1.0	2.6
4	MS+BAP 3.0	22.4	1.3	24.28(29.47)	1.3	2.4
5	MS+BAP 4.0	26.2	2.0	28.56(32.27)	1.6	1.6
6	MS+BAP 5.0	21.2	2.3	35.71(36.69)	2.0	2.0
7	MS+Kinetin 0.5	NR	0	0	0	0
8	MS+Kinetin 1.0	NR	0	0	0	0
9	MS+Kinetin 2.0	26.4	3.0	42.83(40.86)	0	3.2
10	MS+Kinetin 3.0	28.4	2.0	28.56(32.27)	2.0	1.6
11	MS+Kinetin 4.0	29.2	1.3	24.28(29.47)	1.6	3.4
12	MS+Kinetin 5.0	26.4	1.0	17.80(24.95)	2.3	3.6
13	MS+BAP0.2+NAA0.1	21.2	1.7	27.85(31.82)	1.3	3.8
14	MS+BAP0.4+NAA0.1	19.8	2.7	38.85(38.53)	1.0	2.0
15	MS+BAP0.6+NAA0.1	26.0	2.3	35.71(36.69)	2.6	2.6
16	MS+BAP0.8+NAA0.1	24.2	3.0	42.83(40.86)	2.3	2.8
17	MS+BAP1.0+NAA0.1	29.2	3.3	47.61(43.62)	2.0	2.9
18	MS+BAP1.2+NAA0.1	23.4	3.6	52.37(46.32)	2.3	2.5
19	MS+BAP1.4+NAA0.1	20.2	2.7	38.85(38.53)	3.0	2.1
20	MS+BAP1.6+NAA0.1	19.8	3.0	42.83(40.86)	1.6	1.7
21	MS+BAP1.8+NAA0.1	18.2	2.3	35.71(36.69)	1.3	2.3
22	MS+BAP2.0+NAA0.1	17.2	4.7	65.84(54.21)	4.6	4.8
23	MS+BAP2.2+NAA0.1	16.2	2.3	35.71(36.69)	3.3	3.3
24	MS+BAP2.4+NAA0.1	18.2	1.7	27.85(31.82)	2.3	2.7
25	MS+BAP3.0+NAA0.1	16.4	2.0	28.56(32.27)	1.0	3.2
26	MS+NAA0.5+Kinetin0.5	28.2	3.3	47.61(43.62)	1.3	3.6
27	MS+NAA1.0+Kinetin1.0	24.4	3.0	42.83(40.86)	2.6	3.7
28	MS+NAA1.5+Kinetin1.5	22.2	3.6	52.37(46.32)	2.0	2.6
29	MS+NAA2.0+Kinetin2.0	29.0	1.0	17.80(24.95)	2.6	2.8
30	MS+NAA2.5+Kinetin2.5	26.4	1.7	27.85(31.82)	2.3	2.2
31	MS+NAA3.0+Kinetin3.0	21.8	1.3	24.28(29.47)	3.0	1.9
	(±)S.Em	0.573	0.45	1.630	0.39	1.23
	C.D at 5%	1.62	0.79	4.618	0.82	0.64

Note: Figures in parantheses represent arc sine transformed values No multiple shoots observed **NR:** No Response

The Axillary bud explants after treatment with *Agrobacterium* suspension were cultured on MS medium containing 500 mg/l cefotaxime and a selection pressure of 50mg/l kanamycin was applied after one week instead of immediate application of selection pressure as reported by Geetha *et al*. (1999) and Lawrence and Koundal (2001). Kanamycin selection was beneficial in producing transgenic calli and shoots as the selection pressure enriched the growth of transformed tissues and suppressed the growth of un-transformed tissues similar to observation of an earlier report on soyabean (Hinchee *et al* 1998).By contrast, no selection pressure was applied at all in a report on cowpea by penza *et al*. (1991). The putative transformants were identified by the virtue of their survival on MS medium containing 75 mg/l kanamycin in all the experiments in which kanamycin selection was applied after 1 week following *Agrobacterium* co-culture and is similar to an earlier report in chickpea (Fontana *et al*.1993) where the selection pressure was applied 3 weeks after co-cultivation and is in contrast to an earlier report on soybean(Hinchee *et al* 1988), where kanamycin selection was applied immediately following *Agrobacterium* treatment.

Table 2: Effect of different MS media fortified with different hormonal treatments on Rooting from shootlets in var. LRG-41

S. No	Concentration (mg l^{-1})	Days taken for root initiation	No. of shoots producing roots	Frequency of rooting (%)	Mean no of roots /shoot	Mean length of roots (cm)
1	MS+IBA 0.2	NR	0	0	0	0
2	MS+IBA 0.4	22.2	0.6	13.3(21.38)	3.3	2.4
3	MS+IBA 0.6	20.8	1.3	26.6(31.04)	3.8	2.56
4	MS+IBA 0.8	18.6	1.0	20.0(26.56)	2.6	2.63
5	MS+IBA 1.0	17.9	3.53	70.6(57.11)	8.6	5.3
6	MS+BAP0.2+IBA0.2	28.4	1.6	33.3(35.24)	4.3	2.8
7	MS+BAP0.4+IBA0.4	19.8	2.52	50.2(45.11)	6.9	3.2
8	MS+BAP0.6+IBA0.6	21.2	2.64	53.3(46.89)	7.4	3.4
9	MS+BAP0.8+IBA0.8	18.2	3.08	61.6(51.17)	6.3	4.0
10	MS+Kinetin0.5+IBA0.2	23.6	3.16	63.3(52.71)	7.9	3.0
11	MS+Kinetin1.0+IBA0.4	18.9	2.52	50.2(45.11)	6.9	3.8
12	MS+Kinetin1.5+IBA0.6	18.0	3.25	65.0(53.72)	7.0	2.8
13	MS+Kinetin2.0+IBA0.8	0.594	2.64	53.3(46.89)	7.3	4.3
	(±)S.Em	1.728	0.463	0.539	0.54	0.574
	C.D at 5%	0.594	1.341	1.559	1.563	1.664

Note : Figures in parentheses represent arc sine transformed values Observations were taken from five shootlets
NR: No Response

Table 3: Effect of different soil mineral mixtures on acclimatization and survival of regenerated plantlets in Redgram var. LRG-41.

S. No	Soil mineral mixture	No. of days taken for acclimatization	Survival percentage
1	Soilrite	12.8	91(71.57)
2	Sand	16.6	45(42.13)
3	Soil	15.3	62(51.94)
4	Soil:sand:soilrite	14.3	76(60.67)
	± S.Em	0.38	0.43
	C.D at 5%	1.3	1.51

Note: Figures in parentheses represent arc sine transformed values

Table 4: Effect of inoculation time (min) and period of Co-cultivation (days) on growth of *Agrobacterium* (±) and health of explants (%) recorded in redgram Axillary buds

S.No	Time (min)	Co- cultivation period (days)			Mean
		1 Day Health of explants (%)	2 Days Health of explants (%)	3 Days Health of explants (%)	
1.	5	100 -	100-	75-	92
2.	10	100-	100-	100-	100
3.	15	100-	100+	75++	92
4.	20	100-	90+	25+++	72

Indications of *Agrobacterium* over growth on plants +++ High ++ Low + Very Low - No

Table 5 : Effect of Kanamycin on regeneration response of non transformed Axillary bud explants of redgram var. LRG-41

S. No	Concentration of Kanamycin (mg l-1)	Regeneration response
1.	0	+
2.	25	+
3.	50	-
4.	75	-
5.	100	-

+ Normal shoot let Regeneration - No regeneration

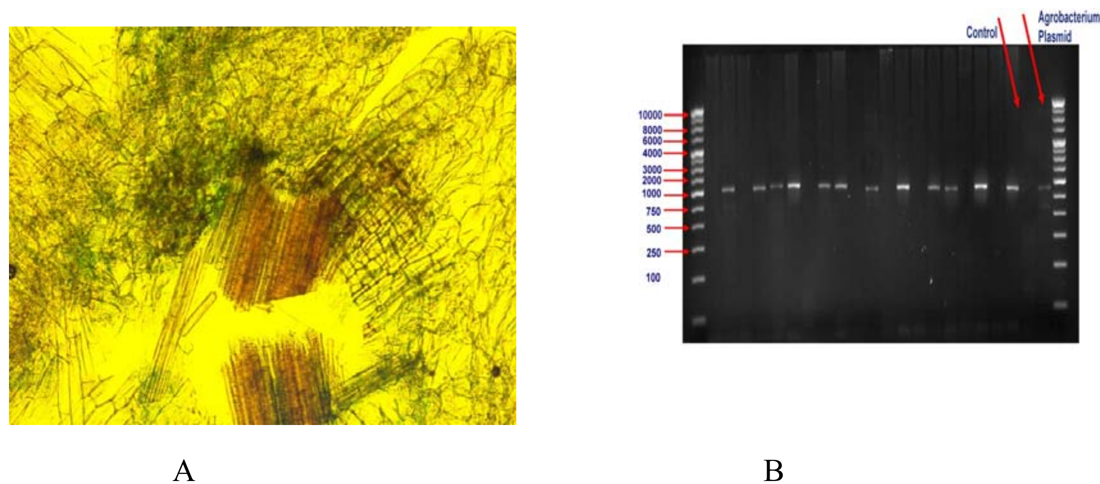
Table 6 : Effect of cefotaxime concentration (mg l^{-1}) **and culture period (days) on suppression of *Agrobacterium* growth in co-cultivated explants of redgram var. LRG-41**

S. No.	Concentration of cefotaxime (mg l-1)	Percent explants found free from Agrobacterium growth during Culture period(days)				
		Day 1	Day 2	Day 3	Day 4	Day 5
1.	400	45	60	65	70	90
2.	500	55	70	85	100	100
3	600	55	70	85	100	100

A B

C D

Fig 1. Regeneration of multiple shoots from explants (A) Axillary bud explants inoculated on MS medium supplemented with BAP and NAA (B) shoot buds originating from explants(C) Multiple shoot production (D) Root induction



A B

Fig-2. A. Expression of GUS gene in plant tissue of transformed shoots B. Gel showing PCR amplification of GUS gene in the plasmid DNA and DNA of transformed and control plants of variety LRG-41

The selection of putative transformants is the prime step in the process of developing transformed plants. After 4 days of culturing on MS medium + BAP 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + 500 mg l^{-1} cefotaxime the explants were transferred to the same medium containing 75 mg l^{-1} kanamycin for selection as it is proved that non transformed shoot let regeneration was minimum (Table 6). The shoots which regenerated on this medium were considered as putative transformants and on an average the number of putative transformants obtained in the present study were 22 out of 90 explants and the per cent putative transformants was 24.4 % presented in Table 7.

Table 7: Transformation frequency of Axillary bud explants of redgram var.LRG-41 for GUS gene assay

S.No	Genotype	LRG-41
1.	No. of co-cultivated explants	90
2.	No. of putative transformants	22
3.	Putative transformants (%)	24.4
4.	No. of GUS positive shoots	13
5.	No. of PCR positive shoots	13
6.	Transformation efficiency (%)	59.09
7.	Transformation frequency (%)	14.4
8.	Transient GUS gene expression (%)	59.09

In order to confirm the presence of gene at an early stage itself, histochemical assay was carried out for GUS expression from the leaf bits of putatively transformed *in vitro* shoots. The leaf bits from putatively transformed shoots of var. LRG-41 were picked up and stained with GUS staining solution for one day, followed by treatment with absolute alcohol. The distinct blue colour was viewed microscopically and 59.09 % of transient GUS gene expression level was observed (Table 7). Histochemical staining for GUS gene transient expression has been reported by Gassama-Dia *et al.* (2004), Tripathi *et al.* (2005), Patnaik *et al.* (2006) and Cevik *et al.* (2006). PCR amplification of GUS gene was done to confirm the stable integration of transgene in the putative transformants. Plasmid DNA isolated from *Agrobacterium*, genomic DNA from both control and putatively transformed plants were subjected to PCR amplification of GUS gene using the specific primers. Out of 22 putative transformants subjected to PCR analysis, 13 showed amplification of GUS gene in var. LRG-41. An 1100 bp band was found amplified in both plasmid DNA and two of putative transformants tested whereas the same band was found absent in control plants. The putative transformants were checked for the presence of the transgene by GUS assay and PCR. The transformation efficiency of 59.09 % and transformation frequency of 14.4 % was observed (Table 7). The histochemical staining also gave the similar results as that of PCR analysis.

Transformation efficiencies in redgram have ranged from50 % (Dayal *et al*. 2003); 51-67 % (Satyavathi *et al.* 2003); 45 % (Kumar *et al*. 2004); 60-65 % (Prasad *et al.* 2004); 60 % (Sharma *et al.*2006) and 40-60 % (Surekha *et al.* 2007) in various studies. From the discussion above, it can be conclude that in the present study established the regeneration protocol with high shoot and rooting frequencies in redgram. *Agrobacterium* mediated transfer of GUS gene into LRG-41 cultivar genetic background has been proved to be simple and efficient, which can be further exploited for the development of transgenic plants with desired traits *viz.,* biotic and abiotic stress resistance in redgram.

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