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FACTORS AFFECTING *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION IN GROUNDNUT (*ARACHIS HYPOGAEA* L.)

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Key words: *Agrobacterium tumefaciens, Arachis hypogaea, uidA, nptII, Polymerase chain reaction.*

Abstract – The development of *Agrobacterium*-mediated transformation protocol for a recalcitrant species like groundnut requires the identification and optimisation of the factors affecting T-DNA delivery and plant transformation. During the present investigation, we have used de-embryonated cotyledon (DC), leaflet (LL) and cotyledonary node (CN) explants of groundnut cv ICG 7827 and the *Agrobacterium* strain LBA 4404 harbouring the binary vector pBAL2 containing *nptII* and *uidA* (Gus) genes. Factors that affect significant differences in T-DNA delivery and transformation included explant type, duration of pre-culture of explant, incubation period in bacterial suspension, co-cultivation, antibiotics used for selection and the presence of Acetosyringone (AS) in the medium. Three days precultured explants (DC, LL and CN) were incubated in *Agrobacterium* suspension for 20 min. Optimized the cocultivation period in the shoot induction medium with 100 mg/L AS at 25 °C in the dark for 4 days. A concentration of 75 mg/L (LL) and 100 mg/L (DC, CN) Kan was added to shoot induction medium (SIM)) to select transformed shoots. The Kan^R shoots were excised and cultured on modified MS medium (MMS) +50 mg/L Kan+0.5 mg/L BAP for shoot elongation. These elongated micro shoots were rooted on root induction medium (RIM) supplemented with 1 mg/L NAA+50 mg/L Kan. The *in vitro* rooted plantlets were acclimatized and maintained in the green house. The expression of GUS gene (*uidA* gene) in the regenerated plants (T_0) was verified by histochemical GUS assay. Stable integration and expression of the transgenes (*uidA* and *nptII*) in T_0 plants were confirmed by using PCR analysis. Showing the transformation efficiency as, 16.29% for DC, 13.15% for LL and 10.59% for CN explants of groundnut cv ICG 7827. Thus, the optimized factors of *Agrobacterium* mediated genetic transformation showed the enhanced transformation frequencies to transfer gene of interest into groundnut.

INTRODUCTION

Legumes are third largest family of higher plants, wherein Peanut/Groundnut (*Arachis hypogaea* L.) is one of the most important species. It is a premier oilseed crop providing food, oil, fodder and fuel to households. India is the world's second largest producer of groundnut (USDA-FSA, 2014). Its production and productivity is constrained by several biotic and abiotic factors cause serious economic losses in peanut yield and production (Jaiwal and Singh, 2003).

Genetic improvement in this peanut crop by conventional breeding is not as rapid as needed to meet the demands of increasing population. Groundnut has a very narrow genetic base (Kochert, 1996). Hence, genetic transformation is the best choice for improvement of many important traits which are not being improved by conventional methods. Establishment of a suitable gene delivery system and a protocol for subsequent recovery of plants is a basic requirement for recalcitrant peanut varieties. Although, genetic transformation of groundnut has been reported previously but recovery of viable reproductive plants is very limited because of very low transformation efficiencies (Cheng *et al.,* 1996; Qin *et al.,* 2013). The success of *Agrobactenum* mediated transformation appears to be heavily influenced by the peanut cultivar, the strain of *A. tumefaciens* used, the plasmid that the bacterium carries, as well as the co-cultivation conditions and the efficiency of the regeneration system.

Abbreviations: KAN Kanamycin, AS Acetosyringone, CN Cotyledonary Node, LL Leaf Let, DC Deemryonated Cotyledon, nptII Neomycin Phospho Transferase, NAA α-Naphthalene Acetic Acid, BAP 6-Benzylaminopurin.

There is need to improve several commercially grown varieties in India and elsewhere. Tools of genetic engineering can be exploited as an additional method for introduction of agronomically useful traits into established cultivars. For successful genetic modification for the production of transgenic plants, effective regeneration and transformation system is imperative. Hence, recently we have developed the reproducible regeneration protocol in groundnut cv ICG 7827 (Rajinikanth *et al.,* 2013; 2014, 2015).

The various factors that affect significant differences in T-DNA delivery and transformation included explant type, duration of pre-culture of explant, incubation period in bacterial suspension, the concentration of *A. tumefaciens* during infection, the use and concentration of inducers such as acetosyringone during infection, antibiotics used for selection and the extent of the co-culture period were optimized to establish a transformation protocol of common bean (*Phaseolus vulgaris*) (Zhang *et al.,* 1997) and in chickpea (Akbulut *et al.,* 2008). The aim of this work was to develop a method of *A. tumefaciens* mediated genetic transformation and to evaluate the factors that affect the genetic transformation efficiency by using marker genes (*nptII*; *uidA*) in groundnut cv ICG 7827.

MATERIAL AND METHODS

Plant material, culture initiation and maintenance

Mature seeds of groundnut cv ICG 7827 were obtained from the germplasm bank of ICRISAT, Patancheru, Hyderabad, Telangana, India.

Dried mature groundnut seeds were washed under running tap water for 10-15 min followed by treating with Tween-20 (5%-v/v) for 5 min and it was repeated twice followed by rinsing in sterile distilled water thoroughly. Later the seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 8 min followed by rinsing in sterilized distilled water for 3-4 times under aseptic conditions and soaked for 24 h in sterile distilled water.

The regeneration experiments were conducted on modified MS (Murashige and Skoog, 1962) medium (MMS medium) containing MS basal salts and B_5 vitamins (Gamborg *et al.,* 1968), 100 mg/L myo-inositol and 30 g/L sucrose. The pH of the medium was adjusted to 5.8±0.02 with either 0.1N NaOH or 0.1N HCl before addition of agar. The medium was solidified with 0.8% Difco bacto-agar and autoclaved at 121°C under 15 psi for 15-20 min. All the cultures were incubated at 25 ± 2 °C under 16/8 h (light/dark) photoperiod with cool white fluorescent light (1600 lux).

Explants preparation

De-embryonated cotyledon (DC), leaf let (LL) and cotyledonary node (CN) explants were used for optimization of transformation factors. Mature dry seeds were imbibed in sterile distilled water for 24 h after surface sterilization and kept for germination on autoclaved filter paper bridge/boat in ½ strength liquid MMS medium in culture tubes.

In the case of CN explants, they were excised from 7 days old *in vitro* grown seedlings and removed the cotyledons, redical, shoot tip parts and cultured on shoot induction medium (SIM) containing MMS+15 mg/L BAP (Rajinikanth *et al.,* 2013). For LL explants, two weeks old *in vitro* grown seedlings were selected and cut into 0.8-1.0 cm^2 size and cultured on MMS+0.5 mg/L NAA+20 mg/L BAP (SIM) (Rajinikanth *et al.,* 2014), in the case of DC explants, 24 hrs soaked seeds were taken, two cotyledons were separated from embryo, then each cotyledon cut in to two pieces lengthwise and these were inoculated on MMS+2 mg/L $AgNO_3$+10 mg/L BAP+1.0 mg/L NAA (SIM) (Rajinikanth *et al.,* 2015).

Agrobacterium strain and plasmid vector

The *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary plasmid pBAL2 (18.8 Kb) was used for optimization of transformation protocol in groundnut cv ICG 7827. The binary vector pBAL2 consists of β-glucuronidase (*gus*/*uidA*) gene driven by the CaMV 35S promoter and terminator sequences served as reporter gene. The neomycin phophotranferase II (*npt* II) gene driven by the nopaline synthase (NOS) promoter and terminator sequences was used as the selectable marker gene (Fig. 1).

Transformation and regeneration of transformants

The *A. tumefaciens* strain LBA 4404 harbouring pBAL2 was grown on YEM agar medium containing 50 mg/L of Kan and 10 mg/L Rif. A single bacterial colony was inoculated in 50 mL liquid YEM medium containing the same antibiotics and grown overnight at 28°C in an incubator shaker at 120 rpm.

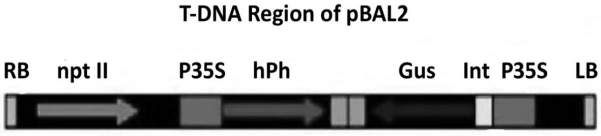


Fig. 1. Linear diagram of T-DNA portion of pBAL2

5 mL of this overnight culture was reinoculated in 50 mL fresh YEM liquid medium containing 50 mg/L Kan and grown overnight. The bacterial cell density was determined at OD600 of 0.5 to 1.5. Later the bacterial suspension was pelleted at 5,000 rpm for 10 min and resuspended in 25 mL hormone-free liquid half-strength MS medium.

The explants, DC, LL and CN were pre-cultured for 1-11 days on shoot induction medium (SIM) prior to infection with bacteria. The trimmed explants were immersed in *Agrobacterium* suspension containing 25-200 mg/L AS for 10-30 minutes and subjected to manual shaking. Subsequently, the explants were blotted dry on sterile tissue paper and co-cultivated for 1-7 days on SIM along with 100 mg/L AS and incubated in the dark at 28±2^{0}C. Following co-cultivation, the explants were washed with 250 mg/L cefotaxime for 5 min, rinsed with sterile distilled water three times for 5 min each with constant stirring, and blotted dry on sterile tissue paper. All these explants were cultured on selection medium containing SIM+75 mg/L Kan for LL and 100 mg/L Kan for DC and CN explants and 250 mg/L cefotaxime for 2-3 weeks. The explants with Kan^R shoots were sub cultured on SIM with 50 mg/L Kan for further proliferation of shoots. Later each micro-shoot was excised and cultured on MMS+50 mg/L Kan+0.5 mg/L BAP for shoot elongation. The elongated micro-shoots were transferred on to root induction medium (RIM) containing 1 mg/L NAA+50 mg/L Kan. All the cultures were incubated at 25±2^{0}C under 16/8 hr photoperiod. These Kan^R plantlets were shifted to plastic cups containing the soil mix, sand: soil: vermiculite (1:1:1) and acclimatized in the growth chamber. Established plantlets were transferred to green house and allowed to grow in an earthenware pots for maturity.

Histochemical GUS analysis

Histochemical GUS assay was carried out in different tissues of T_0 plants as described by Jefferson *et al.,* (1987).

PCR Analysis of putative transformants

The leaf genomic DNA from T_0 plants of cv ICG 7827 was isolated by Cetyl Trimethyl Ammonium Bromide (CTAB) method and used for molecular characterization of putative transgenics by PCR using *nptII* and *uidA* gene specific primers. The *npt II* primer sequences were, (F) 5′-GCT TGG GTG GAG AGG GCT ATT-3′ (R) 5′-AGA ACT CGT CAA GAA GGC GA -3′ and *uidA* gene specific primer sequences were, (F) 52- TTT AAC TAT GCC GGG ATC CAT CGC - 32 (R) 52- CCA GTC GAG CAT CTC TTC AGC GT - 32. The PCR for *nptII* gene was carried out by initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1.30 min and final extension at 72°C for 10 min. A PCR programme for *uidA* gene was carried out by initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 64°C for 1.30 min and 72°C for 2 min and final extension at 72°C for 10 min. The amplified PCR products were separated by gel electrophoresis on a 0.8% agarose gel, using TE buffer were photographed by using gel documentation system (Biorad).

Data Analysis

The culture response was expressed in terms of percentage; number of multiple shoots per explant and transformation efficiency was calculated.

RESULTS AND DISCUSSION

To evaluate the optimization of factors affecting *Agrobacterium* mediated genetic transformation in an individual explant DC, LL, CN, the effect of inoculum concentration (0.5, 1.0 and 2.0 OD600 of *A. tumefaciens*), preculture period (1-11 days), cocultivation period (1-7 days), explant type (DC, CN, LL), acetosyringone concentration (25-200 mg/L) and antibiotic (Kan) concentration (25-150 mg/L) on the efficiency of genetic transformation of groundnut cv ICG 7827 have been studied.

Factors affecting genetic transformation

Effect of concentration of bacterial suspension

The inoculum concentration 1.0 at OD_{600}, resulted the highest percentage of transformation efficiency in DC (78.45%), LL (70.23%), CN (64.12%) explants (Fig. 3a) while at 0.5 and 2.0 ($O.D_{600}$) resulted in decreased effect on transformation efficiency. Higher bacterial density in the medium may lead to severe necrosis of tissue and thus very effective for transformation. Similar results were also reported in *Capsicum Chinense* (Arcos-Ortega *et al.,* 2010).

Effect of pre-culture period (days)

Pre-culture is one of the important parameter for genetic transformation of plants. Three different types of explants DC, LL and CN were pre cultured for 1-11 days. The explants cultured for 3 days

found the best response. The transient transformation efficiency (TTE) was high 69.11% in DC followed by 66.22% in LL, 64.92 in CN explants. Whereas it was found decreased after 3 days of pre culture period (Fig. 3b). Similar observations were also reported in peanut (Vasudavan *et al.,* 2002). Because pre culture period, reduces the wound stress and enhances the attachment of bacterial cells at the wound site by increasing the entry of T-DNA part into the host cells.

Effect of co-cultivation period

The transformation frequency of different explants was highly influenced by co-cultivation period. The transformation frequency was increased the co-cultivation period extending up to 4 days and subsequent decreased the transformation frequency increase in co-cultivation time resulting in bacterial over growth (Fig. 3c). Our results showed that, the TTE was high 62.22% in DC, 60.14% in LL and 57.23 in CN explants after 4 days while it was decreased after 5-7 days of cocultivation. Co-cultivation for 4 days was shown to be acceptable in groundnut for better transformation while the longer co-cultivation period showed over growth of the bacterium leading to death of the explant. Similar results were also observed in pea (Lulsdorf *et al.,* 1991) and in *Phaseolus vulgaris* (Zhang *et al.,* 1997).

Effect of Acetosyringone (AS)

Different levels of AS (0-200 mg/L) was tested and at 100 mg/L AS showed the best transformation efficiency i.e. 76.54% in DC, 71.78% in LL, 68.21% in CN explants, while at 125-200 mg/L decreased the transformation (Fig. 3d). Transformation studies in other plant species have indicated that AS at an appropriate concentration enhances efficiency of transformation as in peanut (Ashutosh *et al.,* 2012) and also recorded in the present investigation.

Effect of antibiotics sensitivity (Kan)

To find out optimum concentration of Kan for the selection of transformed cells, the explants (DC, CN and LL) were cultured on SIM containing various concentrations of Kan (0-150 mg/L) (Fig. 3e). As the concentration of Kan increased, the regeneration efficiency was reduced. 90% of inhibition of shoot induction at 100 mg/L Kan in DC, CN and more than 85% at 75 mg/L Kan in LL explants was recorded (Fig. 2a-i). Beyond that level of Kan, showed the bleaching and necrosis of the explants. Similar findings were also reported in cotton (Bao-Hong *et al.,* 2001).

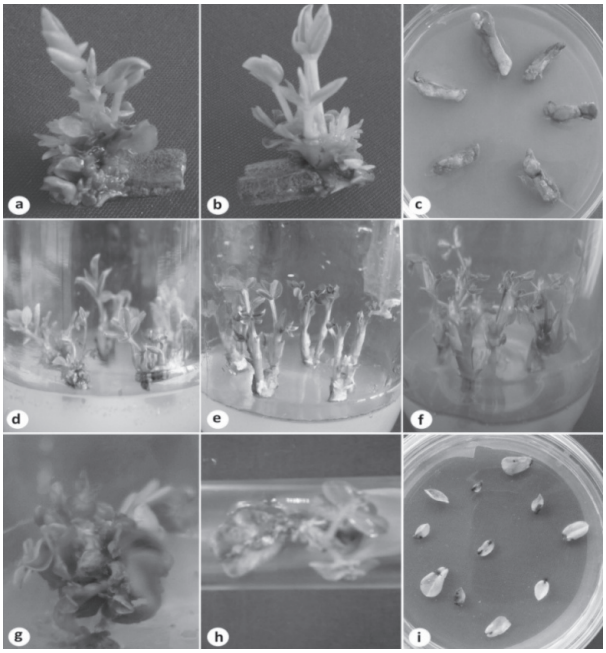
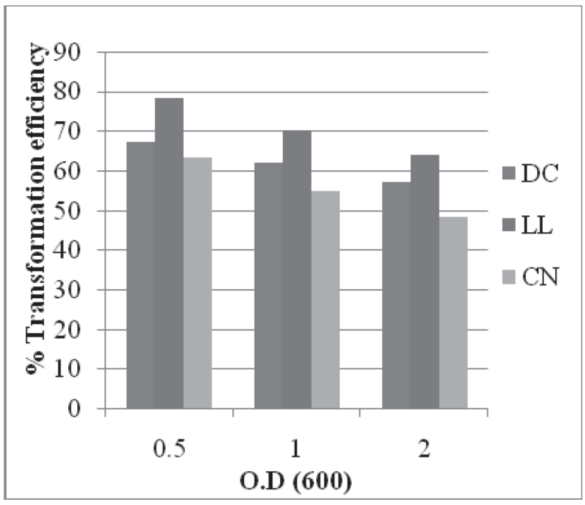


Fig. 2. Determination of antibiotics sensitivity (Kan) in three different types of explants (DC, CN, LL) of groundnut cv ICG 7827.

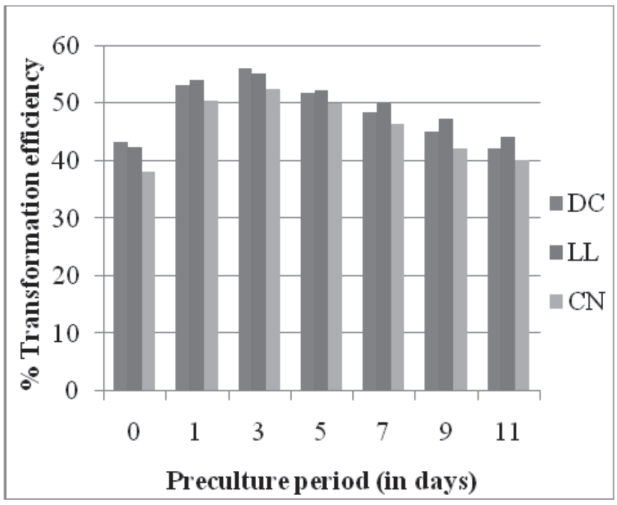
a-c) Shoot buds proliferation on SIM without antibiotics (control), at 100 mg/L Kan and inhibition of shoot buds development at 125 mg/L Kan (Note the necrosis of explants) in DC explants respectively. d-f) Shoot buds proliferation on SIM without antibiotics (control), at 100 mg/L Kan and inhibition of shoot buds development at 125 mg/L Kan (Note the necrosis of explants) in CN explants respectively, g-i) Shoot buds proliferation on SIM without antibiotics (control), at 75 mg/L Kan and inhibition of shoot buds development at 100 mg/L Kan (Note the necrosis of explants) in LL explants respectively.

Effect of explant type

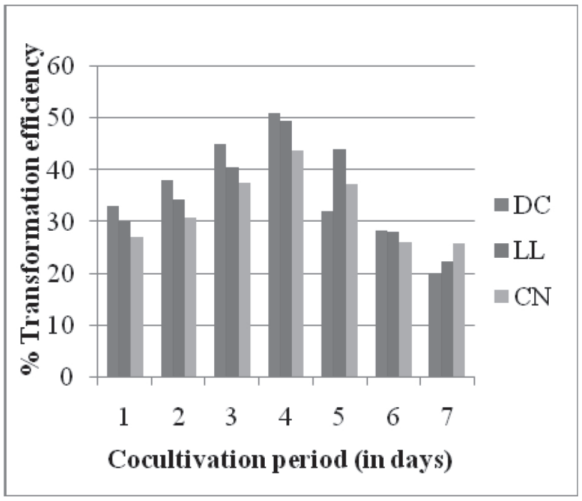
To know the efficiency of explant type, different explants DC, LL and CN of groundnut cv ICG 7827 were cocultivated for four days. After cocultivation, these explants were shifted on to the selection medium. Later from these transformed explants (DC, LL & CN) established the plantlets. Highest number of transgenic plants was developed from the DC explants compared to LL and CN explants (Fig. 3f). The highest transformation frequency was observed in DC (16.29%) followed by LL (13.15%) and CN (10.59%) explants. Transformation of different explants leading to the recovery of transgenic plants has been reported earlier in DC-



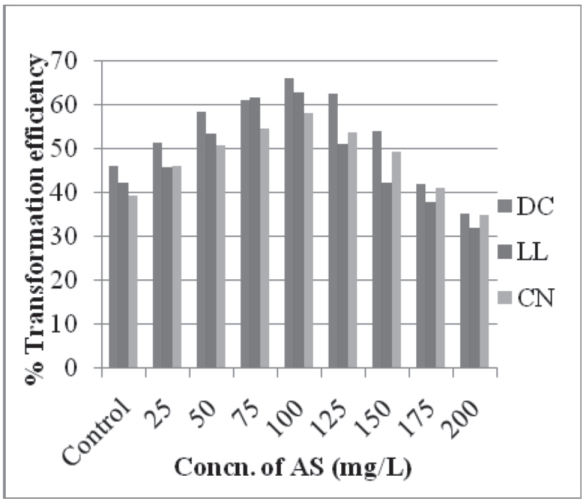
a. Effect of different values of O.D at 600 nm



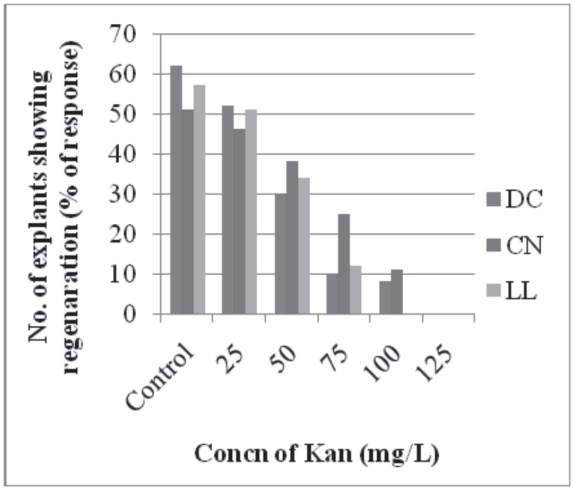
b. Effect of pre-culture period



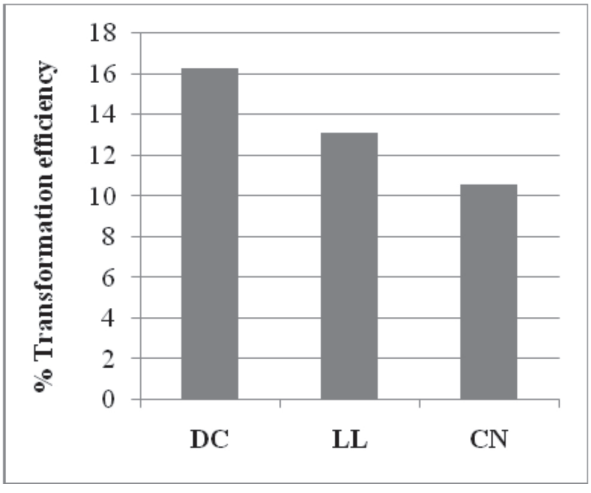
c. Effect of cocultivation period



d. Effect of Acetosyringone concentration



e. Effect of antibiotics sensitivity



f. Effect of explant type

Fig. 3 (a-f) Transformation efficiency with different parameters of groundnut cv ICG 7827.

10.2% **(**Banjara *et al.*, 2012), CN-7.4% (Beena *et al.*, 2008) and LL- 2.2% (Mehta *et al.*, 2013) explants of groundnut.

Confirmation of transformants

Gus assay

Preliminarily GUS staining tests were conducted to know the transformed plants developed from different explants of groundnut cv ICG 7827 (Fig. 4). The putatively transformed plants (*uidA* positive) showed the typical indigo blue colouration of X-gluc treatment (Fig. 4f), while the untransformed plants (*uidA* negative) did not show blue colouration (Fig. 4e). Thus, the GUS +ve plants were separated for further studies.

Molecular conformation

In addition, transformation was also evaluated by molecular analysis by using PCR technique in T_0 plants. The amplification of the presence of *nptII* and *uidA* (gus) genes in Kan^R putatively transformed plants (T_0) was confirmed (Fig. 5). These putatively transformed plants were obtained after three cycles of culture on selection medium after cocultivation with *A. tumefaciens* LBA 4404 strain harbouring the binary vector pBAL2. The gene specific primers for *nptII* and *uid 'A'* genes were used for PCR amplification and the amplified DNA fragments were found 750 bp and 1.9 Kb respectively (Figs. 6, 7). Thus, the transformed (T_0) plants of groundnut cv ICG 7827 were confirmed for the presence of *nptII* and *uidA* genes (Figs. 6, 7, Lanes 1-7). However, PCR products of *uidA* and *nptII* genes were not seen in untransformed (control - WT) plants (Figs. 6, 7, Lane C). As a positive control, the *nptII* and *uidA* genes were also amplified from the pBAL2 plasmid (Figs. 6, 7, Lane P).

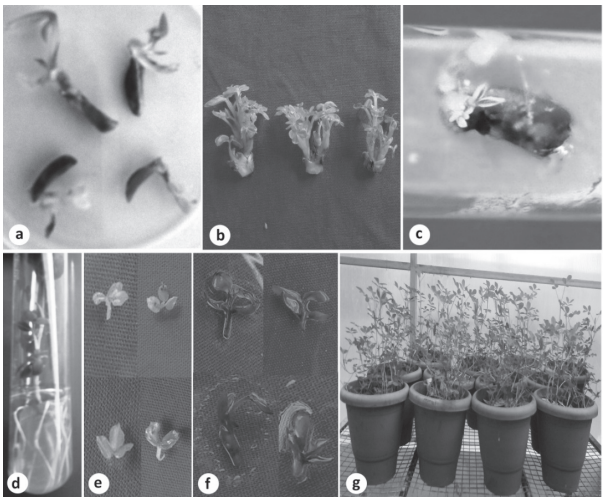


Fig. 4. *Agrobacterium* mediated transformation and GUS Assay of transgenic shoots (Kan^R) developed from DC, CN and LL explants of cv ICG 7827 by using the binary vector pBAL2.
a-c) Induction of Kan^R shoots on selection medium after 4 weeks of incubation from DC, CN and LL respectively, **d)** *In vitro* rooting of shoots on RIM supplemented with 1.0 mg/L NAA+ 50 mg/L Kan (Note profuse rhizogenesis), **e)** Non transformed shoots (control-WT), **f)** Transformed shoots (+ve for GUS), **g)** Transformed plants are shifted to plastic pots containing soil mix and maintained in the green house.

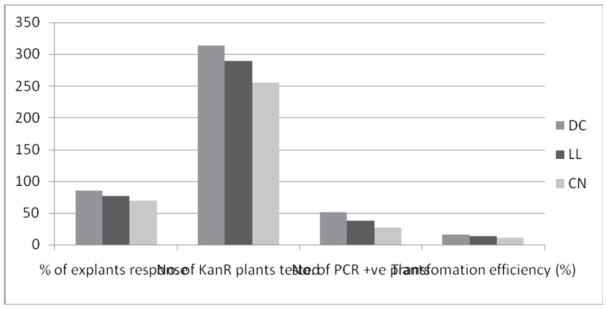


Fig. 5. Transformation efficiency in different explants of groundnut cv ICG 7827.

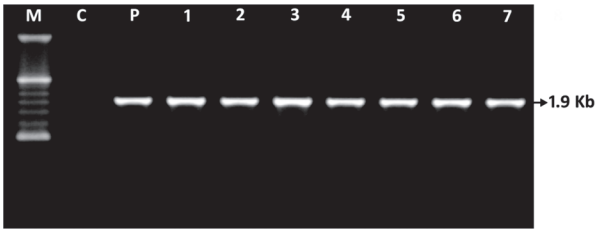


Fig. 6. PCR screening of T_0 plants for the presence of the *uidA* gene using gene-specific primers in cv ICG 7827 of groundnut.

Lane M=DNA marker, Lane C=Control plant DNA (-ve control-WT), Lane P= Plasmid pBAL2 (+ve control), Lanes 1-3= T_0 plants developed from DC, Lanes 4-5= T_0 plants developed from LL, Lanes 6-7= T_0 plants developed from CN explants.

CONCLUSION

On the basis of our results, we found that the most effective conditions for *A. tumefaciens* mediated genetic transformation of groundnut cv ICG 7827, are: Concentration of *A. tumefaciens* at OD_{600}: 1.0, Preculture period: 3 days, Co-cultivation period: 4 days, Kan concentration: 100 mg/L for DC&CN, 75 mg/L for LL explants, Concentration of AS: 100 mg/L. The same parameters can be used to transfer

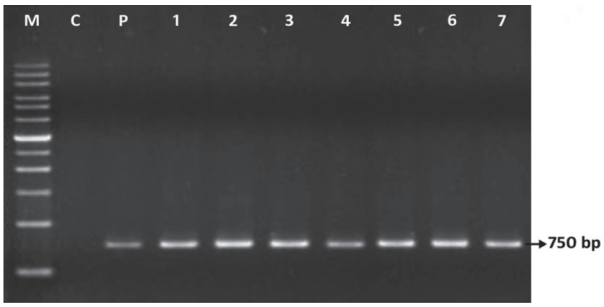


Fig. 7. PCR screening of T_0 plants for the presence of the *nptII* gene using gene-specific primers in cv ICG 7827 of groundnut.
Lane M=DNA Marker, Lane C=Control plant DNA (-ve control-WT), Lane P= Plasmid pBAL2 (+ve control), Lanes 1-3= T_0 transgenic plants developed from DC, Lanes 4-5= T_0 transgenic plants developed from LL, Lanes 6-7= T_0 transgenic plants developed from CN explants.

novel gene (s) into groundnut cv ICG 7827 by using *A. tumefaciens* mediated genetic transformation.

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