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Plant regeneration from decapitated mature embryo axis and *Agrobacterium* mediated genetic transformation of pigeonpea

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Abstract

A reliable method of plant regeneration has been achieved from decapitated mature embryo axes (DCMEA) explants. Shoots appear directly from explants of genotype T-15-15 when cultured on Maheswaran and Williams (EC_6) basal medium supplemented with N^6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) at various combinations. The shoots elongated on half strength Murashige and Skoog (MS) medium fortified with 3 μM gibberellic acid. Elongated shoots were rooted with 80 - 85 % efficiency on half strength MS medium with 0.5 μM indole-3-butyric acid. Survival of plants in the pots was 75 - 80 %. This protocol was used in *Agrobacterium* mediated transformation. The DCMEA explants were treated independently with two *A. tumefaciens* (LBA 4404) strains harbouring a binary vector carrying the green fluorescent protein (GFP) and β-glucuronidase (GUS) reporter genes, respectively. Both the strains contained neomycin phosphotransferase selectable marker gene. After co-cultivation, the explants were cultured on EC_6 basal medium supplemented with 5 μM BAP and 1 μM IAA. The selection of putative transformants was on a medium containing 50 mg dm^{-3} kanamycin. Expression of GUS and GFP gene was confirmed by histochemical assay and fluorescence microscopy, respectively. The elongated shoots expressing GFP reporter gene were rooted and transferred to pots for hardening. The integration of GFP gene into the genome of putative transformants was confirmed by Southern blotting.

Additional key words: *Cajanus cajan*, EC_6 medium, β-glucuronidase gene, green fluorescent protein.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a major food legume of the semi-arid tropics and is rich in protein. Pigeonpea suffers from heavy losses due to fungal diseases (mainly *Fusarium* wilt) and insect pests (mainly pod borer - *Helicoverpa armigera*). Cross incompatibility of cultivated and wild species has hindered the improvement of crop by conventional plant breeding. The introduction of specific genes into pigeonpea to improve pest and disease resistance and also to improve nutritional quality could be achieved by genetic engineering. An efficient plant regeneration and transformation system is a prerequisite for successful introduction of desirable traits.

Plant regeneration of pigeonpea *via* callus cultures (Kumar *et al.* 1983) and direct differentiation from leaf (Eapen and George 1993, Eapen *et al.* 1998) have been reported. George and Eapen (1994) have reported organogenesis and embryogenesis from diverse explants of pigeonpea. Somatic embryogenesis has also been reported in pigeonpea by Patel *et al.* (1994), Mallikarjuna *et al.* (1996), Srinivasu *et al.* (1998), Anbazhagan and Ganapathi (1999), and Mohan and Krishnamurhy (2002). Multiple shoot production was achieved from cotyledonary node explants (Mehta and Mohan Ram 1980, Kumar *et al.* 1984, Shiva Prakash *et al.* 1994, Naidu *et al.*

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Abbreviations: BAP - N^6-benzyl amino purine; DCMEA - decapitated mature embryo axis; EC_6 - Maheswaran and Williams (1986) medium; GA_3 - gibberellic acid; GFP - green fluorescent protein; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog's (1962) medium.

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1995, Sudarsana Rao *et al.* 2001) and from epicotyl explants (Kumar *et al.* 1984, Naidu *et al.* 1995). According to Shama Rao and Narayanaswamy (1975) hypocotyl segments, obtained from γ-irradiated seeds, produced abundant calli and shoot buds in 50 % of the cultures. George and Eapen (1994) observed shoot regeneration from the distal end of cotyledons when whole cotyledons were cultured. Geetha *et al.* (1998) obtained multiple shooting in different seedling explants such as leaf, hypocotyl, epicotyl, cotyledon and cotyledonary node explants. Only a few reports describing genetic transformation of pigeonpea are available (Geetha *et al.* 1999, Lawrence and Koundal 2001) probably because of lack of an efficient, high frequency direct regeneration system with reproducibility.

The aim of present investigation was to find an efficient method of direct organogenesis from mature embryo derived explant DCMEA and its suitability for genetic transformation studies of pigeonpea using GUS and GFP as reporter genes and *nptII* gene as selectable marker.

Materials and methods

Plants and regeneration media: Seeds of pigeonpea [*Cajanus cajan* (L.) Millsp.] genotype T-15-15 used in the experiment were obtained from Pulses Scheme, Model Farm, Gujarat Agricultural University, Vadodara, India. Seeds were surface sterilized as described in Mohan and Krishnamurthy (1998). These seeds were soaked in sterile distilled water and incubated on a gyratory shaker at 200 rpm at 28 °C for 18 h in dark. The pre-soaked seeds were washed twice with sterile distilled water and cotyledons were split open. The embryo was extracted and the shoot apex and the root pole were removed (Fig. 1*A*). The explant referred to as decapitated mature embryo axis (DCMEA) (Fig. 1*B*) was cultured on EC_6 basal medium with 3 % sucrose, gelled with 0.8 % agar-agar (*Qualigens*, Mumbai, India) for shoot regeneration for a period of 4 weeks. The basal medium was supplemented with BAP (5 μM) in combination with IAA (0.5, 1.0, 1.5, 2.0, 3.0 μM) for standardization of regeneration medium. The standardized EC_6 basal medium supplemented with 5 μM BAP and 1.0 μM IAA (M1 medium) was used in transformation experiments. There were 20 explants per treatment and the experiments were repeated thrice. The shoots, with the explants attached, were then transferred to half strength MS medium with 3 μM GA_3 for elongation (M2 medium). The cultures were incubated at 25 ± 2 °C under cool white fluorescent light (irradiance of 38 μmol m^{-2} s^{-1}) under 16-h photoperiod. The regenerated shoots were transferred to half strength MS medium containing 0.5 μM IBA for rooting (M3 medium). The rooted plantlets were hardened in pots with soil:vermiculite (1:1) mixture at 25 ± 2 °C under irradiance of 4.6 μmol m^{-2} s^{-1} (16-h photoperiod).

DCMEA explants were also cultured on EC_6 basal medium supplemented with 3 % sucrose, 0.8 % agar-agar, 0.5 μM BAP and 5 μM 2,4-D for callus formation and maintenance (M4 medium). The data were analyzed by Analysis of Variance for a Completely Randomized Design and the treatment means were compared (Panse and Sukhatme 1967).

***Agrobacterium* strains and gene constructs:** The *Agrobacterium tumefaciens* strain LBA4404 containing the pGV2260-35S-GUSINT plasmid is pBIN19 derivative, which carries a chimeric NPTII gene and a GUS gene construct with a ST-LS 1 gene derived intron (Vancanneyt *et al.* 1990) (Fig. 1*C*) was kindly provided by Dr. Deepak Pental (University of Delhi, South Campus, New Delhi, India).

The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.* 1983) harbouring a binary plasmid pBIN 35S-mgfp5-ER (Fig. 1*D*) was used as vector for transformation. The construct was kindly provided by Dr. Jim P. Haseloff from MRC Laboratory of Molecular Biology, Cambridge, England. pBIN 35S-mgfp5-ER is a plant binary vector derived from pBI121 (Jefferson *et al.* 1987) in which the BamHI-SstI fragment containing the GUS reporter gene has been replaced with a BamHI-SstI fragment containing mgfp5-ER gene (Haseloff *et al.* 1997). GFP5 has dual excitation peaks (395 nm and 473 nm) of approximately equal amplitude which can be visualized well with either long wavelength UV (*e.g.* hand-held lamp) or blue light (*e.g.* argon laser) (Siemering *et al.* 1996).

Determination of lethal dose of kanamycin for pigeonpea explants: The LD_{50} of kanamycin used as selective pressure was determined by culturing 30 DCMEA explants (6 explants per Petri dish and 5 Petri dishes per treatment) per treatment in M1 medium and supplemented with various concentrations of kanamycin (25, 50, 75, 100, 200, 300 and 400 mg dm^{-3}). The cultures were scored after an incubation of 4 - 5 weeks at 25 ± 2 °C under irradiance of 38 μmol m^{-2} s^{-1} (16-h photoperiod) provided by cool white fluorescent tubes.

Cocultivation and transformation: A single bacterial colony of *Agrobacterium* was incubated in 5 cm^3 of liquid YEB medium (1 g dm^{-3} of *Bacto*-yeast extract; 5 g dm^{-3} beef extract; 5 g dm^{-3} *Bacto*-peptone; 5 g dm^{-3} sucrose; 0.5 g dm^{-3} $MgSO_4$ (Shaw 1988) containing 100 mg dm^{-3}

kanamycin, 50 mg dm^{-3} streptomycin, 100 mg dm^{-3} rifampicin for GUS gene construct and 50 mg dm^{-3} kanamycin, 250 mg dm^{-3} rifampicin for GFP gene construct and incubated at 28 °C on gyratory shaker at 200 rpm for 48 h. 0.05 cm^3 of this culture was suspended in 5 cm^3 of YEB medium with antibiotics and incubated at 28 °C on a shaker at 200 rpm for 18 h. Later it was centrifuged at 2 000 *g* at 4 °C for 10 min and the pellet was resuspended in 2.5 cm^3 of medium containing 50 mM MES (pH 5.6), 0.5 % glucose, l× AB salts, 2 mM $NaPO_4$ and 100 µM acetosyringone. Treatment of explants was carried out by dipping DCMEA explants in a batch of 20 explants in the *Agrobacterium suspension* for 20 min as longer periods of incubation lead to problems in the elimination of bacteria and contamination in subsequent cultures of DCMEA explants *in vitro*. All the explants treated by dipping were blotted dry on a sterile filter paper and co-cultivated on M1 medium for 72 h. These were then washed with sterile distilled water, blotted dry on sterile filter paper and incubated on M1 medium containing 500 mg dm^{-3} cefotaxime (*Claforan-Russel*, Mumbai, India) for one week. After one week of culture the DCMEA explants were transferred to M1 medium containing 250 mg dm^{-3} cefotaxime and 50 mg dm^{-3} kanamycin for 4 weeks. Around 180 - 240 explants were used per experiment for co-cultivation. Forty explants inoculated on the regenerating media without treatment with *Agrobacterium*, served as control. The experiments were repeated six times.

Regeneration of shoots: The shoots regenerated on M1 medium containing 50 mg dm^{-3} kanamycin and 250 mg dm^{-3} cefotaxime after 4 weeks of incubation were transferred to a freshly prepared M1 medium containing 125 mg dm^{-3} of cefotaxime and 50 mg dm^{-3} kanamycin in test tubes and were incubated for another 4 weeks and the green shoots were then transferred to M2 medium supplemented with 50 mg dm^{-3} kanamycin and incubated for another 4 weeks. The shoots surviving on M1 medium supplemented with 50 mg dm^{-3} kanamycin for 8 weeks were scored for GUS activity and/or green fluorescence.

Rooting and hardening: The putative transformants surviving on M2 medium after 13 weeks of co-cultivation were transferred to M3 medium for rooting and incubated for 3 weeks. The rooted plantlets were hardened and maintained in the controlled environment at 25 ± 2 °C under 16-h photoperiod with irradiance of 38 µmol m^{-2} s^{-1} for 2 weeks.

Callus development from DCMEA explants: In a separate experiment, 20 DCMEA explants treated with *Agrobacterium* suspension by dipping were blotted dry on a sterile filter paper and co-cultivated in M4 medium for 72 h. These were then washed with sterile distilled water, blotted dry on sterile filter paper and incubated on M4 medium containing 500 mg dm^{-3} cefotaxime for one week and then transferred to M4 medium containing 250 mg dm^{-3} cefotaxime and 50 mg dm^{-3} kanamycin for 4 weeks. Calli developed were maintained on M4 medium supplemented with kanamycin (50 mg dm^{-3}). The calli obtained on M4 medium from DCMEA explants not treated with *Agrobacterium* served as controls. Calli were tested for GUS activity and green fluorescence after 8 weeks of their culture in M4 medium.

Tissue staining for GUS activity: Histochemical analysis was carried out to determine the β-glucuronidase activity in callus and leaves of the shoots. The tissue was cut into small bits (0.5 cm) and immersed in X-Gluc solution [0.1 M Na_2PO_4 buffer (pH 7.0), 10 mM EDTA (pH 7.0), 0.5 mM K-ferricyanide (pH 7.0), 0.5 mM K-ferrocyanide (pH 7.0), 1.0 mM X-glucuronide, 0.1 % Triton X-100] in microtiter multiwell plates (*Sigma Chemical Co.*, St. Louis, USA) and incubated overnight at 37 °C (Stomp 1992). 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 (*Leitz*, Wetzlar, Germany) 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.

DNA extraction and Southern hybridization: Genomic DNA was extracted from putatively transformed plants, calli and untransformed pigeonpea plants and callus using Doyle and Doyle (1987) method with modifications. Two putatively transformed plants obtained (0.5 g tissue) 16 and 18 weeks after co-cultivation with *Agrobacterium* strain pBIN 35S-mgfp5-ER were used. The callus (1 g) developed and maintained for 12 weeks on M4 medium supplemented with 50 mg dm^{-3} kanamycin after treating DCMEA explants with *Agrobacterium* strain pBIN 35S-mgfp5-ER was used as transformed callus. 15 µg of DNA was digested with restriction endonuclease EcoRI, separated through a 1 % agarose gel prepared in l× TAE and transferred (Sambrook *et al.* 1989) and blotted on to *Hybond* N^+ nylon membrane (*Amersham Biosciences*, Piscataway, USA) by vacuum transfer. The 880 bp fragment of mgfp5-ER was labelled with ^{32}[α-dATP] and used for hybridization performed according to Sambrook *et al.* (1989) in 6× SSPE, 5× Denhardt's solution, 0.4 % SDS, 0.1 mg cm^{-3} denatured salmon sperm for 24 h at 55 °C before washing membranes with 2× SSPE, 0.5 % SDS solution at 55 °C. Hybridizing bands were detected by 5 d exposure to *Kodak X-OMAT AR* autoradiography film at -70 °C.

Results and discussion

Plant regeneration from DCMEA explants: The DCMEA explants turned green and produced shoots at shoot apex region (Fig. 1*E*) within 3 weeks of culture and many shoot buds at cotyledonary node region. The response of explants to form shoots varied from 5 to 30 % and the number of shoots per explant varied from 3.0 to 5.2. The highest formation of shoots (30 %) and the number of shoots per explant (5.2) were observed in EC_6 basal medium with 5 μM BAP and 1 μM IAA (Table 1). Similar results were obtained by Geetha *et al.* (1998) in a combination of BAP and IAA.

Table 1. Effect of 5 μM BAP and different concentrations of IAA on shoot regeneration on DCMEA explants of pigeonpea. Only shoots arising from shoot apex region were considered and the shoots arising from cotyledonary node region were not counted. Means ± SE, $n = 20$, means with different letters differ significantly at $P = 0.05$.

IAA [μM]	Induction [%]	Number of shoots [explant^{-1}]
0	6 ± 1a	4.5 ± 0.6cd
0.5	5 ± 1a	3.0 ± 0.6ab
1.0	30 ± 13d	5.2 ± 0.8d
1.5	13 ± 3b	3.0 ± 0.9ab
2.0	22 ± 6c	3.9 ± 0.2bc
3.0	13 ± 3b	2.5 ± 1.0a

Regenerating explants with small shoots were transferred to half strength MS medium with 3 μM GA_3. GA_3 was reported to be useful for elongation of shoot buds into shoots previously (Naidu *et al.* 1995, Geetha *et al.* 1998, Mohan and Krishnamurthy 1998). Within 3 weeks of subculture, elongation of the shoots (Fig. 1*F*) was observed in 25 % of the explants (1.3 ± 0.3 per explant), when they were separated and transferred to glass tubes for rooting. Rooting (Fig. 1*G*) of elongated shoots was done on half strength MS medium containing 0.5 μM IBA. The frequency of rooting was 80 - 85 % and rooting took place in 15 - 20 d. Similar observations on rooting were observed by Naidu *et al.* (1995) and Shiva Prakash *et al.* (1994). Geetha *et al.* (1998) found IBA as the best auxin for rooting. Other auxins used for rooting were NAA (Mehta and Mohan Ram 1980, Eapen and George 1993, George and Eapen 1994, Eapen *et al.* 1998, Geetha *et al.* 1998) and IAA (Kumar *et al.* 1983, Geetha *et al.* 1998). Overall, in 45 - 50 d, rooted plantlets were ready to be transferred to pots (Fig. 1*H*) filled with soil:vermiculite (1:1) mixture for hardening. 70 to 80 % of the plants survived after hardening.

Determination of lethal dose and selection of shoots: DCMEA explants formed shoots on M1 medium without kanamycin (control) after 4 weeks of incubation. The shoot formation was also observed after 4 weeks of culture on M1 medium supplemented with 25 and 50 mg dm^{-3} kanamycin while no shoot formation was observed on M1 medium with 75, 100, 200, 300 and 400 mg dm^{-3} of kanamycin. Further transfer of regenerated shoots on to M1 medium with 25 and 50 mg dm^{-3} kanamycin, respectively, for another 4 weeks resulted in the survival of a few shoots in the former while bleaching of shoots in the later. Therefore 50 mg dm^{-3} kanamycin was used as the optimal selective pressure for transformation experiments. In previous reports of pigeonpea transformation (Geetha *et al.* 1999, Lawrence and Koundal 2001), 50 mg dm^{-3} kanamycin was used as the selection pressure.

The DCMEA explants after treatment with *A. tumefaciens* suspension were cultured on M1 medium containing 500 mg dm^{-3} cefotaxime and a selection pressure of 50 mg dm^{-3} 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 soybean (Hinchee *et al.* 1988). 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 M1 medium containing 50 mg dm^{-3} 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 selective 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.

Only 3 out of 616 explants treated with *Agrobacterium* strain with pGV2260-p35SGUSINT survived and formed shoots on M1 medium containing kanamycin (50 mg dm^{-3}) after 4 weeks of culture. However, the shoots did not grow further and elongate after 8 weeks of culture on M1 medium supplemented with 50 mg dm^{-3} kanamycin and the leaves also failed to show GUS expression. The untreated explants cultured on M1 medium containing 50 mg dm^{-3} kanamycin did not survive. Compared to untransformed callus, transformed calli obtained from DCMEA explants showed intense blue colour after 8 weeks of culture (Fig. 2*A*). Similarly absence of GUS activity in shoots and presence of strong GUS activity in calli was made in cotton by Banerjee

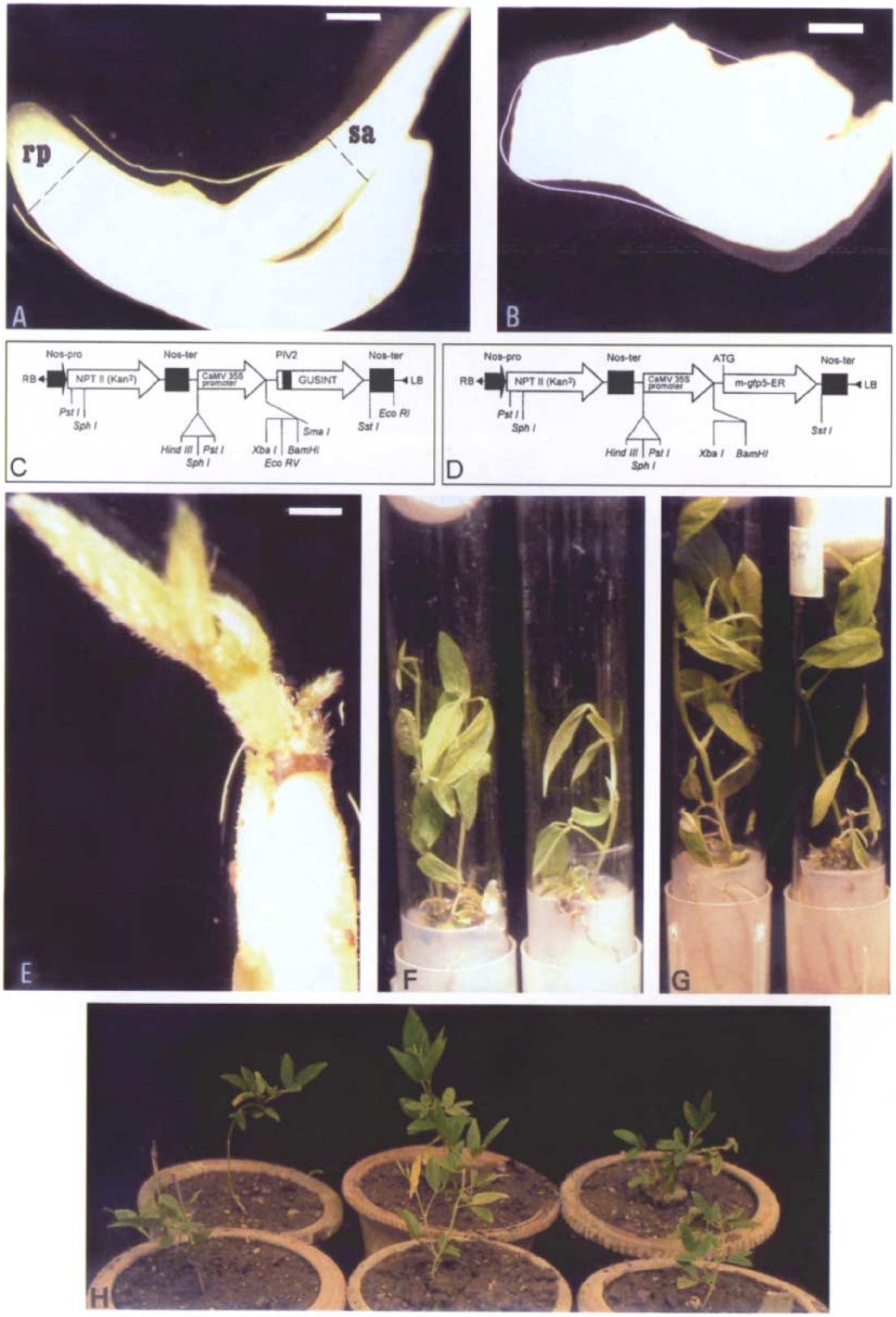

Fig. 1. Plant regeneration from DCMEA explants of pigeonpea and constructs used for genetic transformation experiments: *A* - embryo extracted from mature seed (*bar* = 1000 μm); *B* - DCMEA explant at the time of culture (*bar* = 600 μm); *C* - GUS gene construct used for transformation; *D* - GFP gene construct used for transformation; *E* - small shoots arising from DCMEA explants (*bar* = 750 μm); *F* - elongated shoots; *G* - rooted plantlets; *H* - plants growing in pots.

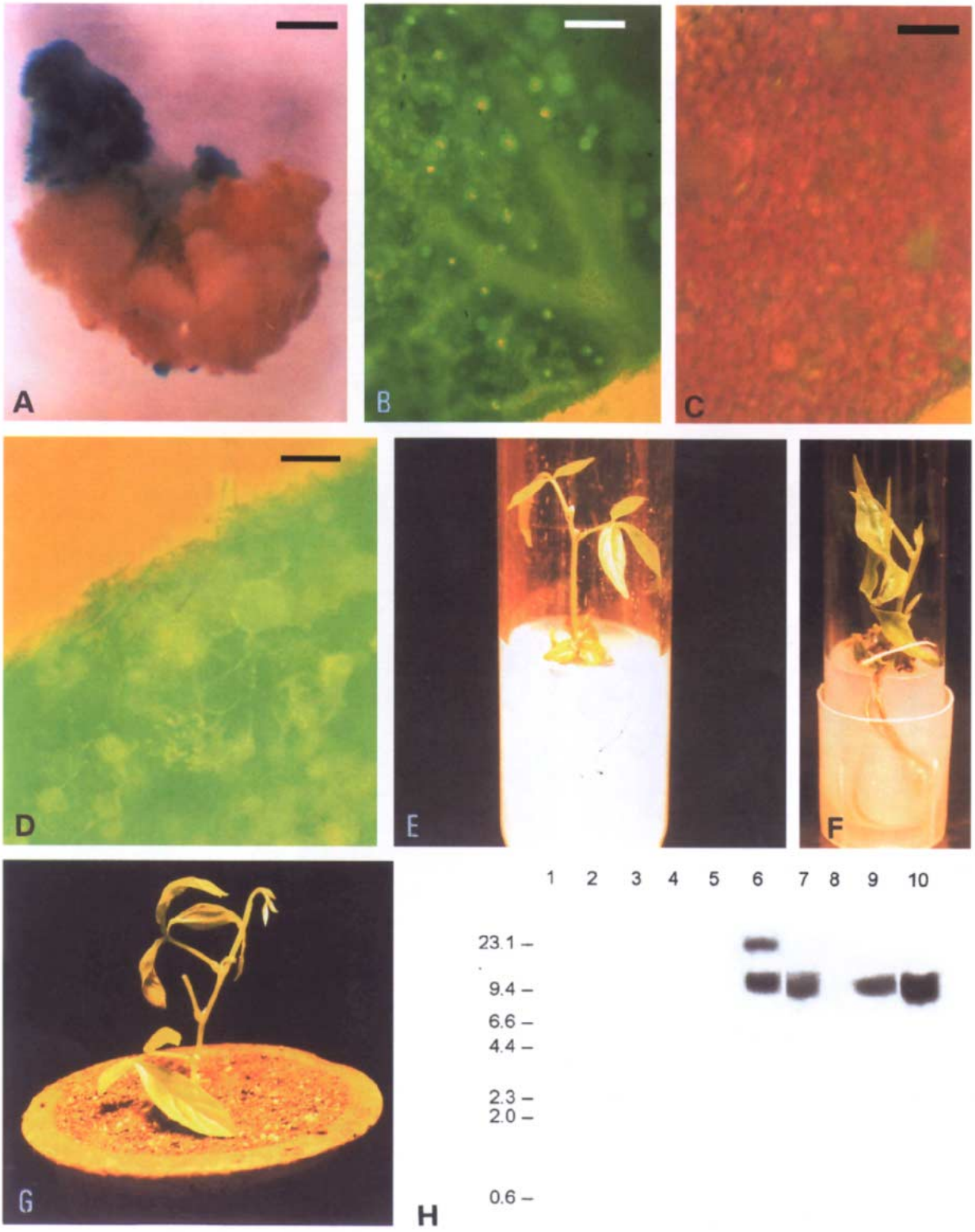

Fig. 2. Genetic transformation of pigeonpea using *Agrobacterium* mediated method: *A* - callus showing intense GUS staining (*bar* = 1000 μm); *B* - section of leaf showing green fluorescence (*bar* = 600 μm); *C* - control leaf showing red autofluorescence (*bar* = 900 μm); *D* - transformed callus showing green fluorescence (*bar* = 600 μm); *E* - putatively transformed shoot elongated on M2 medium; *F* - rooted putatively transformed plant; *G* - putatively transformed plant transferred to pot; *H* - autoradiograph of putatively transformed plants and calli (*lane 1, 2, 5, 8* - blank, *lane 3* - control plant, *lane 4* - control callus, *lane 6, 7* - transformed callus, *lane 9, 10* - putatively transformed plants).

et al. (2002). Absence of GUS activity in shoots may be due to premature termination of the introduced T-DNA during the transfer process (Cousins *et al.* 1991).

Even though varied number of explants survived and developed shoots after treating with *Agrobacterium* strain pBIN 35S-mgfp5-ER on M1 medium supplemented with 50 mg dm^{-3} of kanamycin depending on the batch, their number decreased in subsequent transfers to fresh medium. The initial survival of explants and formation of shoots varied from 8.11 to 26.79 % after 4 weeks of incubation, but only 1.7 to 6.7 % of the explants with shoots survived on kanamycin after 8 weeks of culture, while shoots obtained from untreated explants bleached and died after 8 weeks of culture (Table 2). However, healthy and phenotypically normal kanamycin resistant shoots were obtained when shoots produced on M1 medium after 8 weeks were transferred on M2 medium containing 50 mg dm^{-3} of kanamycin for another 4 weeks. The shoots growing on selection pressure (M1 medium supplemented with 50 mg dm^{-3} kanamycin) for 8 weeks were selected for analysis of green fluorescence. The leaves of control plants for analysis of green fluorescence were taken from the shoots obtained from DCMEA explants, not treated with *Agrobacterium*, growing for 8 weeks on M1 medium without kanamycin.

Table 2. Shoots regenerated in various experiments after treatment with *Agrobacterium* strain pBIN 35S-mgfp5-ER and selection on 50 mg dm^{-3} kanamycin. Survival was determined after 8 weeks of selection and were used for analysis of green fluorescence. C1 - control without selection pressure, C2 - control with selection pressure of kanamycin, 1 - 6 - different batches of co-cultivation experiments.

	Shoot formation [%]	Survival [%]	Green fluorescence
C1	52.0	52.0	-
C2	28.0	0.0	-
1	20.4	5.9	4
2	8.1	3.2	0
3	12.8	3.0	0
4	26.8	6.7	5
5	13.2	3.7	1
6	9.0	1.7	0

The leaf bits analyzed for GFP presence by fluorescence microscopy showed green fluorescence (Fig. 2*B*) as compared to red auto-fluorescence (Fig. 2*C*) by the leaves of control plants. The calli growing on kanamycin containing medium were also analyzed for green fluorescence. The transformed callus also showed green fluorescence (Fig. 2*D*). Out of all the experiments 10 shoots showed green fluorescence. Most of the shoots showing green fluorescence eventually died under selection pressure in 1 - 2 transfers on M2 medium supplemented with 50 mg dm^{-3} kanamycin at 4 weeks interval and only 2 shoots from all the experiments could be recovered (Fig. 2*E*).

The putatively transformed shoots obtained from DCMEA explants treated with *Agrobacterium* strain pBIN 35S-mgfp5-ER, which survived selection pressure on M2 medium supplemented with 50 mg dm^{-3} kanamycin produced roots (Fig. 2*F*) on M3 medium in 3 weeks. One shoot started wilting during rooting stage and the other rooted plant was hardened under controlled conditions. However, hardened plant (Fig. 2*G*) could not survive long and started wilting after 2 weeks. Both these plants were used for Southern analysis.

Southern hybridization of plants confirmed the integration of the GFP gene confirming that pigeonpea is susceptible/amenable to transformation by *A. tumefaciens*. Southern hybridization also confirmed the presence of the gene in transgenic calli. No hybridization could be detected for un-transformed plants and callus (Fig. 2*H*).

Genetic transformation using GFP reporter gene has not been reported so far in pigeonpea and this forms the first report of its nature in the literature. However, Geetha *et al.* (1999) and Lawrence and Koundal (2001) have recently reported *Agrobacterium*-mediated transformation in pigeonpea using GUS (*uidA*) reporter gene and cowpea protease inhibitor gene, respectively.

Conclusions: In the present study, an attempt was made to standardize protocol for *Agrobacterium*-mediated genetic transformation system by co-cultivation method using *Agrobacterium tumefaciens* strains harbouring plasmids pGV2260-p35SGUSINT and pBIN 35S-mgfp5-ER. The callus showing GUS activity was obtained whereas, the putatively transformed shoots obtained on treatment with pGV2260-p35SGUSINT did not show GUS activity. The integration of GFP gene in callus and putatively transformed plants has been confirmed by both fluorescence microscopy and Southern analysis.

GFP is increasingly being used in plant biology from the cellular level to whole plant level. GFP is the first truly *in vivo* reporter system useful in whole plants (Leffel *et al.* 1997). Insertion of the GFP gene into plant viruses has allowed the direct observation of viral movement through host plants during infection (Baulcombe *et al.* 1995, Casper and Holt 1996). Another use of GFP in whole plants is to use it as an *in vivo* marker to monitor transgene spread in the environment. Large scale releases of transgenics may lead to invasiveness and competition of transgenic weeds containing a transgene conferring an increment of fitness. No tracking system is in place to monitor transgene introgression into unintended hosts, however, GFP is the best candidate for this application (Stewart 1996).

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