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Improved *Agrobacterium* Mediated Transformation in Cowpea *Vigna unguiculata* L. Walp.

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Abstract: *Agrobacterium tumefaciens* strain LBA4404 carrying binary vector pCAMBIA1305.1, with a hygromycin phosphotransferase gene (hpt) and a β-glucuronidase (GUS) gene interrupted with an intron, was used for transformation of *Vigna unguiculata* cotyledonary node explants. High concentration of acetosyringone (200 μM) in the *Agrobacterium* culture and co-cultivation medium with 1 g L^{-1} L-cysteine, 250 mg L^{-1} Na-thiosulphate and 150 mg L^{-1} dithiothretiol (DTT) proved to be indispensable for successful transformation. Three days old cotyledonary nodes were used for transformation studies. Binary vector pCAMBIA 1305.1 proved to be very efficient for transformation. Stable transformation with 1.61% efficiency was achieved using optimized conditions. Transformed green shoots were rooted on medium containing hygromycin. Transformed shoots tested positive for hpt gene by polymerase chain reaction. GUS activity was detected in cotyledonary nodes and leaves of the putative transformants. Southern analysis of putative transformants showed the integration of hpt into the plant genome.

Key words: Transgenic plants, hpt gene, legume, L-cysteine, Acetosyringone

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) is an important food legume and a good source of protein. It is widely cultivated in the tropics and subtropics and temperate zones of the world. It occupies an area of 12.5 million ha in the world (FAO, 2000) with a total production of more than 3.3 million tones of dry grains. The average yield of cowpea is very low, due to numerous biotic and abiotic stresses. Genetic improvement will be useful for the development of cowpea with increased quality and yield (Popelka *et al*., 2004). However, routine genetic transformation protocols are limited in most of the tropical grain legumes, due to their inability to regenerate under tissue culture conditions (Somers *et al*., 2003). Generally, the family Leguminosae is highly recalcitrant due to high level of phenolics leading to oxidation of explants (Anthony *et al*., 1999).

Development of transformation procedure for any plant, especially for recalcitrant species involves a gene delivery method for targeting foreign DNA to regenerable cells. The most widely used method for the introduction of new genes into plants is based on the natural DNA transfer capability of *Agrobacterium tumefaciens*. In spite of the broad natural host range of *A. tumefaciens*, till today only a few grain legumes have been stably transformed using disarmed vectors (Jaiwal and Singh, 2003; Somers *et al*., 2003; Popelka *et al*., 2004).

The current trend in genetic transformation of recalcitrant grain legumes is the choice of meristem as a source of totipotent cells (Somers *et al*., 2003). Cotyledonary nodes are good explants for plant transformation because regeneration *via* direct shoot formation minimizes the risks of somaclonal variation. The optimization of some important aspects of transformation system components that affect the overall transformation efficiency is therefore, essential to enhance the virulence so as to increase the transformation frequency. In this paper, we describe a meristem targeted rapid and efficient transformation system in cowpea.

MATERIALS AND METHODS

Plant material, bacterial strain and vector: The mature seeds of *V. unguiculata* cultivars VBN-1, VBN-2, Co-6 and Co(cp)-7 were obtained in the year of 2006 from the Tamil Nadu Agricultural University, Coimbatore, India. Of these, the commercially grown cultivar Co(cp)-7 was used for detailed studies. The disarmed *A. tumefaciens* strain LBA4404, harboring a binary vector pCAMBIA1305.1, which contained a β-glucuronidase (GUS) gene and a hygromycin phosphotransferase gene (hpt) both driven

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by the *Cauliflower mosaic virus* (CaMV)35S promoter, was used for transformation (Fig. 1). The GUS gene contained an intron in its coding region to ensure that the observed GUS activity occured in the plant cell and was not due to the presence of residual *Agrobacterium* cells.

Preparation of explants and plant regeneration: The preparation of explants and regeneration of plants were done following the method of Raveendar *et al.* (2009). Healthy uniform seeds were rinsed in 70% alcohol for 1 min and then surface sterilized with 0.1% aqueous solution of $HgCl_2$ (w/v) for 5 min. Various media used in the study are listed in Table 1. The seeds were subsequently washed several times in sterile distilled water and cultured on MSB_5-G medium containing 13.3 μM BAP for 3 days at 25±2°C under dark conditions. The cotyledonary node explants (5 mm) were excised from 3-d-old *in vitro* raised seedlings by removing both the cotyledons and cutting both the epicotyls and hypocotyls approximately 2 mm above and below the nodal region. The explants were cultured in a vertical upright position with the hypocotyls end slightly embedded in MSB_5 medium containing 6.6 μM BAP (MSB_5-MB) and solidified with 0.7% agar (Hi-media, Mumbai). The cultures were maintained at 25±2°C under 16 h cool white fluorescent light of 50 μmol m^{-2} sec^{-1} intensity. After 2-3 weeks of multiple bud induction, the shoot buds were transferred to shoot elongation medium containing 0.5 μM BAP (MSB_5-SE). After 2-3 weeks, the individual shoots were separated from the explants and transferred to MSB_5 basal medium (MSB_5-R) for rooting. The rooted shoots were transferred to pots containing vermiculite: soil at 1:1 ratio. Each pot was covered with a polythene bag to maintain high humidity initially for the first few days. Subsequently, the humidity was reduced by making holes in the polythene bags to harden the plants.

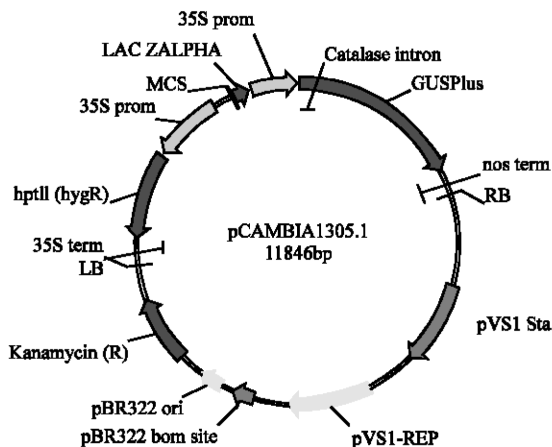

Fig. 1: Binary vector pCAMBIA1305.1 used for transformation

Transformation and plant regeneration: *A. tumefaciens* strain LBA4404 (pCAMBIA1305.1) was grown on AB solid medium containing 50 mg L^{-1} kanamycin and 10 mg L^{-1} rifampicin at 28°C. A single bacterial colony was inoculated into 3 mL of liquid AB medium containing the same antibiotics and grown overnight on a rotary shaker at 180 rpm at 28°C. Three milliliter aliquot of bacterial suspension was added to 30 mL of AB liquid medium containing 50 mg L^{-1} kanamycin and 10 mg L^{-1} rifampicin and grown overnight. Bacteria were pelleted at 6000 rpm for 10 min and resuspended in liquid MSB_5 medium (MSB_5-AS) containing 6.6 μM BAP and 200 μM acetosyringone (AS) at a density of 1OD at 600nm. The cotyledonary node explants excised from 3-d-old seedlings were immersed in bacterial suspension for 25-30 min with occasional shaking. The *Agrobacterium* treated explants were then blotted on sterile filter paper and transferred to coculture medium (MSB_5-CCM) containing 0.8% agar and supplemented with 1 m L^{-1} L-cysteine, 250 mg L^{-1} Na-thiosulphate and 150 mg L^{-1} dithiothretiol (DTT) for 3 days under 16 h photoperiod at 25±2°C. After cocultivation, the explants were washed three to four times with sterile distilled water and blotted dry on sterile filter paper. The explants were cultured on MSB_5 selection medium (MSB_5-MBH) containing 6.6 μM BAP, 30 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime for shoot regeneration. The explants were transferred onto shoot elongation medium

Table 1: Media used in the study

Medium	Components
MSB_5	MS salts, B_5 Vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar, pH 5.7
MSB_5-G	MSB_5 + 13.3 μM BAP
MSB_5-MB	MSB_5 + 6.6 μM BAP
MSB_5-SE	MSB_5 + 0.5 μM BAP
MSB_5-R	MSB_5 basal medium for rooting
MSB_5-AS	MS salts, B_5 Vitamins, 2% (w/v) sucrose, 6.6 μM BAP, 200 μM acetosyringone (AS), pH 5.5
MSB_5-CCM	MSB_5 + 6.6 μM BAP, 200 μM acetosyringone, 1 g L^{-1} L-cysteine, 250 mg L^{-1} Na-thiosulphate, 150 mg L^{-1} dithiothretiol (DTT), pH 5.5
MSB_5-MBH	MSB_5-MB + (30 mg L^{-1}) hygromycin and (500 mg L^{-1}) cefotaxime
MSB_5-SEH	MSB_5-SE + (30 mg L^{-1}) hygromycin and (500 mg L^{-1}) cefotaxime
MSB_5-RH	MSB_5-R + (5 mg L^{-1}) hygromycin and (500 mg L^{-1}) cefotaxime

(MSB_5-SEH) containing 0.5 μM BAP and the same levels of antibiotics after every 2 weeks for a total of 4-6 weeks, until the shoots attained a height of 2-3 cm. The shoots were rooted on MSB_5 basal medium (MSB_5-RH) containing 5 mg L^{-1} hygromycin. The putative transformed plants were established in soil and transferred to greenhouse.

Histochemical assay for the GUS gene: The histochemical assay of GUS gene expression (β-D-Glucuronidase) was performed in cotyledonary node and leaf explants with 5-Bromo-4-Chloro-3-Indolyl Glucuronide (X-Gluc) as a substrate using the established method (Jefferson, 1987). The putative transformed explants were incubated in sodium phosphate buffer (50 mM $NaPO_4$, pH 6.8) that contained 1% Triton X-100 at 37°C for 1 h and were later incubated overnight in a solution containing 1.0 mM X-Gluc, 10 mM ETDA, 100 Mm NaH_2PO_4, 0.1x TritonX-100 and 50% methanol (pH 5.8). The tissue was washed twice in 99% methanol for 2 h to remove chlorophyll pigment. The number of tissue that stained blue was counted as GUS positive.

PCR analysis of putative transgenic plants: Total genomic DNA was extracted from fresh leaves of putative transformed (T_0) and non-transformed (control) plants by the CTAB (cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1987). PCR analysis was carried out using the following primers (5' primer, 5' AAAGCCTGAACTCACCGC 3' 3'primer, 5' GCTTTCCACTATCGGCGA 3'). These primers amplified a 1.1kp fragment from the hpt gene. PCR analysis was carried out in a reaction volume of 25 μL containing the template genomic DNA (100 ng), 2.5 μL 10X PCR amplification buffer, 0.5 μL 10 mM $dNTP_S$, 1.2 μL 50 mM $MgCl_2$, 3 μM (2.5 μL) of each primer, 13.6 μL sterile distilled water, 1 unit (0.20 μL of Taq DNA polymerase (Genei). The samples were heated to 94°C for 5 min and then subjected to 30 cycles of 30 sec melting at 94°C, 30 sec annealing at 60°C and 1 min synthesis at 72°C and followed by another 10 min final extension at 72°C. The amplified products were assayed by electrophoresis on 0.8% agarose gels, stained with ethidium bromide (EtBr; 0.5 μg mL^{-1}), visualized and photographed under ultraviolet light.

Southern blot analysis: The T_0 tranformants were subjected to southern blot hybridization using the coding sequence of hpt gene. Southern blot analysis was done by using PCR samples of putative transgenics. The PCR samples were resolved on 0.8% agarose gels. DNA was transferred to positively charged nylon membrane (as per the manufacturer's instructions) for southern hybridization (Southern, 1975). The coding sequences of hpt genes were labelled with biotin -11-dUTP using biotin decalabel DNA labeling kits (Fermentas Life sciences) and used as a probe. The blot was subjected to detection by overnight colour development using biotin chromogenic detection kit (Fermentas Life sciences).

RESULTS

Sensitivity of explants to hygromycin and cefotaxime: Prior to transformation, an effective concentration of antibiotic for the selection of transformants was determined by culturing cotyledonary node explants on MSB_5-MB and MSB_5-SE media, respectively, containing various concentrations of hygromycin (0, 10, 20, 30, 40 and 50 mg L^{-1}). Hygromycin at a concentration of 30 mg L^{-1} caused almost total inhibition of shoot initiation on cotyledonary node explants (Table 2); hence this concentration was used for selection of transformed shoots. All the cotyledonary node explants produced shoots on hygromycin-free MSB_5-MB medium. Influence of cefotaxime on shoot initiation and on subsequent growth was also checked by culturing explants on MSB_5-MB and MSB_5-SE medium containing 500 mg L^{-1} cefotaxime. This concentration had no effect on shoot initiation and subsequent growth, but effectively controlled the *Agrobacterium* growth.

Transformation: Inoculation of explants in the *Agrobacterium* culture for 25-30 min was done as longer periods of incubation posed problems in the elimination of bacteria and contamination in subsequent cultures of cowpea explants *in vitro*. After inoculation of explants for 25-30 min the explants were transferred to MSB_5-CCM medium and incubated for cocultivation for 3 days. At the end of the cocultivation period of 3 days, the explants were transferred to selection medium (MSB_5-MBH) and cultured for 2 weeks. After 2 weeks of selection, the fresh

Table 2: Effect of different concentrations of hygromycin on shoot regeneration from 3-day-old cotyledonary node explants of cowpea cv. Co(cp)-7

Hygromycin concentration (mg L^{-1})	Survival of explants (%)	Shoot regeneration per explants (%)	Average number of shoots/explants*
0	100	36/36(100)	12.0± 0.7[d]
10	80	29/36(80)	7.4±0.5[c]
20	8.3	3/36(8.3)	3.0±0.8[b]
30	0	0/36(0)	0.0±0.0[a]
40	0	0/36(0)	0.0±0.0[a]
50	0	0/36(0)	0.0±0.0[a]

*Data represent Mean±SD of six replicates. Means followed by the same letters within a column do not differ significantly according to Duncan's multiple range test at a 5% probability level. Data were scored after 6 weeks of culture

survived explants were transferred to MSB_5-SEH medium for second round of selection that selected only those cells receiving and expressing the gene transfer casette. A few explants (25 out of 305 explants treated with *Agrobacterium* strain with pCAMBIA1305.1) survived and formed shoots (Fig. 2a, b) on MSB_5-SEH medium containing (30 mg L^{-1}) hygromycin after 4-5 weeks of culture. The shoots did not grow and elongate further after 6 weeks of culture on MSB_5-SEH medium supplemented with 30 mg L^{-1} hygromycin. The untreated explants cultured on MSB_5-SEH medium containing 30 mg L^{-1} hygromycin did not survive. Following cocultivation with *Agrobacterium*, the regenerating explants were placed for selection medium (MSB_5-SH) containing 30 mg hygromycin for 2 week followed by 2-3 weeks in MSB_5-SEH medium. A total of 305 explants, in three different experiments, produced 25 shoots on hygromycin selection medium (Table 3). After 6 weeks, the elongated shoots were transferred to rooting medium containing lower concentration (5 mg L^{-1}) of hygromycin, where only 16 shoots (64%) produced roots.

GUS analysis: All the cotyledonary node explants showed GUS activity after cocultivation with *A. tumefaciens* LBA4404/pCAMBIA1305.1 harboring GUS gene, predominantly in the regenerable sites (Fig. 2). While endogenous gues expression was not detected in the tissues of control plants, a strong and stable gus

Table 3: Summary of the transformation of 3-day-old cotyledonary node explants of *Vigna unguiculata* cv. Co(cp)-7 co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pCAMBIA1305.1

Exp. No.	No. of explants inoculated in *Agrobacterium* suspension[a]	No. of shoots recovered on selection medium[b]	No. of shoots rooted in the presence of selective agent[c]	Selection efficiency (%)[d]	No. of plants positive for gus and hpt genes by PCR	Transformation efficiency (%)[e]
1	116	8	6	6.89	2	1.72
2	122	12	7	9.83	2	1.63
3	67	5	3	7.46	1	1.49
Total	305	25	16	24.18[f]	5	1.61[f]

[a]Explants cocultured with *Agrobacterium* for 3 days. [b]Explants produced shoots on selection medium: MSB_5-SE + hygromycin (30 mg L^{-1}) and cefotaxime (500 mg L^{-1}) for 4-6 weeks of culture. [c]Shoots rooted on MSB_5-R + hygromycin (5 mg L^{-1}) and (500 mg L^{-1})cefotaxime. [d]Selection efficiency: percentage of hygromycin-resistant plants. [e]Transformation efficiency: percentage of initial explants that developed to plants positive for *gus* and hpt genes by PCR. [f]Average

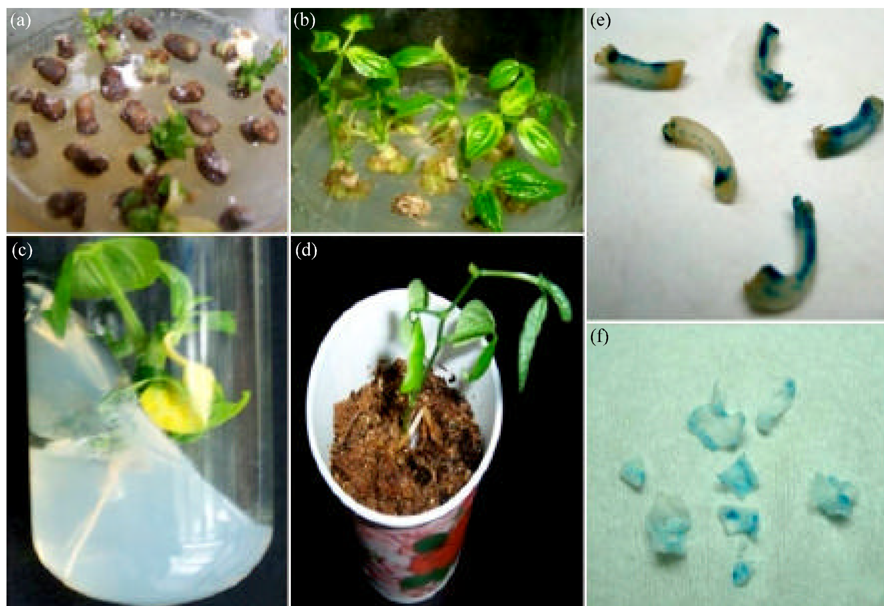

Fig. 2: *Agrobacterium*-mediated transformation of *Vigna unguiculata* cv. Co(cp)-7. (a) Explants inoculated with *Agrobacterium tumefaciens* strain LBA4404 (pCAMBIA1305.1) and cultured on MSB_5-MBH medium containing 30 mg L^{-1} hygromycin showing regeneration of green shoots. (b) Direct shoot regeneration from cotyledonary node explants on MSB_5-SEH medium supplemented with 0.5 μM BAP after 4-5 weeks of culture. (c) Induction of roots from *in vitro* regenerated shoots. (d) A fertile transgenic plant growing in pot. (e) Cotyledonary node explant showing transient GUS activity after 3 days of cocultivation with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1305.1). (f) Stable GUS activity in leaf of putative tyransformants

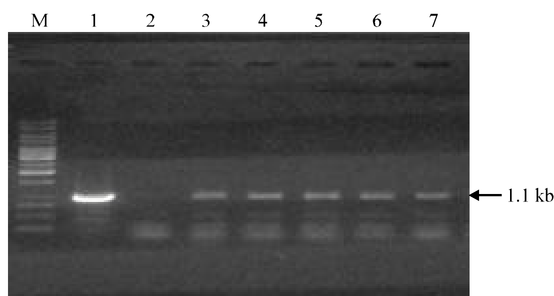

Fig. 3: PCR analysis of primary transformants using the hpt primers. Lane M, Marker DNA; lane 1: +ve, plasmid DNA; lane 2: DNA from untransformed control; lanes 3-7: T_0 transformed plants

Fig. 4: Southern blot analysis of putative transformants

expression was observed in cotyledonary node and leaf of all the hygromycin resistant putative transformants. We determined the stable transformation efficiency based on the plants positive for Gus and hpt genes by PCR. A total of five independently derived transgenic plants were obtained from a total of 305 explants, giving an average transformation frequency of 1.61%.

Molecular analysis of putative transgenics: PCR amplification was performed using hpt gene primers; five plants out of 16 plants showed PCR amplification (Fig. 3). Further these PCR samples were tested for confirmation in Southern analysis; five samples, which showed clear amplification in PCR, were Southern positive (Fig. 4). Southern hybridization was carried out with genomic DNA from GUS expressing, hygromycin resistant shoots. The hpt gene sequence was detected as a fragment of expected size (1.1 kb) in transformed plants. Since hpt probe hybridized to genomic DNA from transgenic plants but not to DNA from non-transformed control plants, the result indicated that hpt DNA was incorporated into cowpea genome. PCR amplified plant DNA sequence was subsequently probed with hpt coding region to identify fragments between the T-DNA border. An internal 1.1 kb fragment corresponding to hpt gene was detected in transgenic plants by hpt probe (Fig. 4). The bands from genomic DNA, identified with hpt probe, contained sequences derived from plant DNA. The size of the bands (1.1 kb) confirmed the integration of the hpt gene into plant nuclear genome.

DISCUSSION

Reports on stable *Agrobacterium*-mediated transformation confirmed by southern analysis of primary transformants are available (Muthukumar *et al*., 1996; Kononowicz *et al*., 1997; Monti *et al*., 1997). Muthukumar *et al*. (1996) used cotyledons excised from 3 day old seedlings. The cotyledons were cocultivated with *A. tumefaciens* and transformed tissues selected on 25 mg L^{-1} hygromycin. Our preliminary work on the effect of hygromycin on *in vitro* regeneration of untransformed cowpea has established significant inhibition levels at 30 mg L^{-1} hygromycin (Table 2) which is similar to the earlier report of Obembe *et al*. (2000). Muthukumar *et al*. (1996) reported that 15-19% of explants produced shoots on hygromycin selection medium.

Our protocol for *Agrobacterium*-mediated transformation of cowpea is summarized in Fig. 5. The 3 days old cotyledonary node explants were inoculated with an *Agrobacterium* culture. After 3 days of cocultivation, the explants were transferred to selection and regeneration media. After 4-6 weeks, regenerated shoots were transferred to rooting medium. The resulting rooted shoots were subjected to screening for transformants by PCR. The positive plants were then potted into soil. A major advantage of our cowpea transformation method is that regenerated transformants can be obtained within 7 weeks after *Agrobacterium* inoculation.

The difference in transformation efficiency in different studies may be attributed to the genotype of the crop species, explant type, cocultivation procedure followed and the *Agrobacterium* strains used in the genetic transformation experiments. Solleti *et al*. (2008) reported transgenic cowpea using cotyledonary node as explants and *A. tumefaciens* LBA4404 harboring plasmid pSB1, while Chaudhury *et al*. (2007) used Agrobacterium strain EHA105. Similarly variation in transformation frequency was observed in cowpea using different strains of *Agrobacterium* and genotypes (Kononowicz *et al*., 1997; Monti *et al*., 1997). In the present study, transformation efficiency was measured as number of confirmed transgenic plants out of total number explants used for transformation experiment. In the present study cowpea variety Co(cp)-7 was transformed with *Agrobacterium* strain LBA4404 carrying the binary vector pCAMBIA1305.1 and the percentage of confirmed transgenic plants appeared to be similar when compared to the previous reports of cowpea (1.64%) reported by Solleti *et al*. (2008).

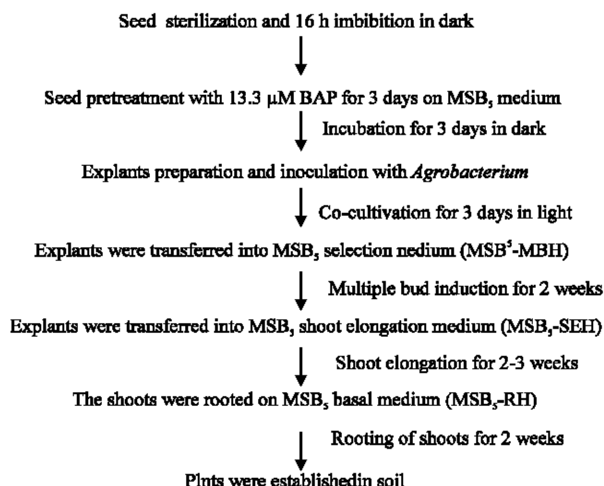

Fig. 5: Steps of transformation protocols for cowpea

The T-DNA transfer is mediated by products encoded by the vir (virulence) region of the Ti-plasmid, which are activated by signal molecules, mainly small phenolics, certain class of monosaccharides and acidic pH acting synergistically with phenolic compounds (De la Riva *et al*., 1998). Addition of 200 µM acetosyringone to the bacterial resuspension medium as well as cocultivation medium resulted in significant increase in transformation frequency from 7.6% in cultures without acetosyringone to 82.6% with acetosyringone with large GUS positive sector(s). Acetosyringone enhanced vir functions during transformation (Stachel *et al*., 1986) and has been found to increase transformation potential of *Agrobacterium* strain with moderately virulent vir region in several plant species (Atkinson and Gardner, 1991; Janssen and Gardner, 1993; Kaneyoshi *et al*., 1994).

Cocultivation of explants in the presence of mixtures of thiol compounds, DTT and L-cysteine increased the frequency of *Agrobacterium*-mediated transformation and recovery of transgenic plants at a high frequency. Cocultivation in presence of thiol compounds has been described to be beneficial in recovering transgenic plants in soybean (Olhoft *et al*., 2001). Moreover, cocultivation of explants in the presence of mixtures of the thiol compounds combined with geneticin selection resulted in enhanced recovery of transgenic plants to an average of 1.67%. Transformation efficiencies for thiol compounds treated cotyledonary node explants using PPT selection in cowpea were 0.001-0.003% (Popelka *et al*., 2006). Employing extra copies of vir genes and geneticin-based selection and recovery of transgenics in cowpea were also reported. (Chaudhury *et al*., 2007). In the present study, high concentrations of acetosyringone (200 µM) in the

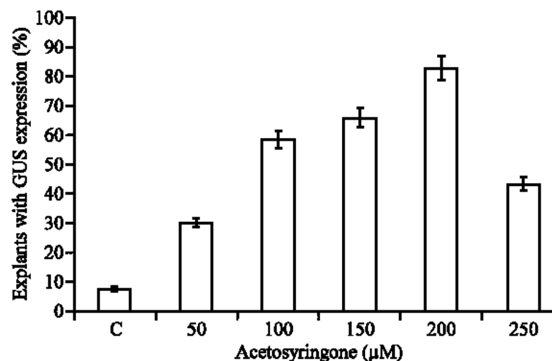

Fig. 6: Effects of Acetosyrigone on transformation of 3-day-old cotyledonary node explants of *Vigna unguiculata* cv. Co(cp)-7 co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pCAMBIA1305.1

Agrobacterium culture and cocultivation medium with 1 mg L^{-1} L-cysteine, 250 mg L^{-1} Na-thiosulphate and 150 mg L^{-1} dithiothretiol (DTT) proved to be indispensable for successful transformation (Fig. 6, 7). Recovery of fertile transgenic plants in our case took approximately 2 months which was significantly less than previous reports in cowpea (Sahoo *et al*., 2000; Popelka *et al*., 2006; Chaudhury *et al*., 2007).

Confirmation of the transgenic nature of the plants was done using PCR amplification and Southern hybridization. When probed with the 1.1 kb PCR amplified hpt gene fragment as a probe, the expected fragment of 1.1 kb was detected in the Southern analysis (Fig. 4). In the T_0 generation plants, the plant genomic DNA fragments after PCR amplification that hybridized to the non-radioactive hpt probe were longer than 1.1 kb proving that the T-DNA got stably integrated into the cowpea

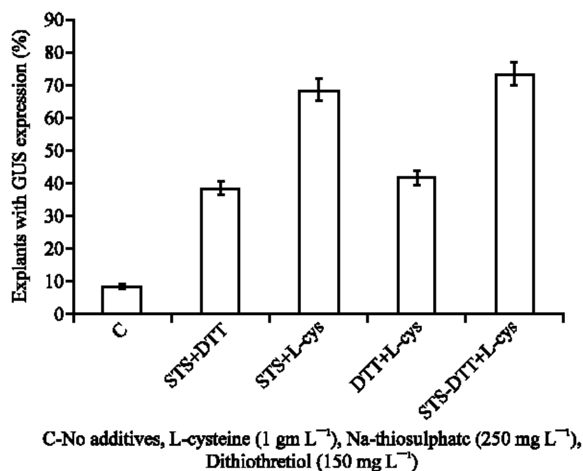

Fig. 7: Synergistic effects of thiol compounds on transformation of 3-day-old cotyledonary node explants of *Vigna unguiculata* cv. Co(cp)-7 co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pCAMBIA1305.1

plant genome. Our method is efficient for the recovery of stable transgenics and introduction of desirable agronomic traits into cowpea.

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