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A preliminary attempt for efficient genetic transformation and regeneration of legume *Mucuna pruriens* L. mediated by *Agrobacterium tumefaciens*

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Abstract: *Mucuna pruriens* belongs to the family Papillonaceae. The seeds and leaves of these plants contain a large amount of L-DOPA. L-DOPA (L-3,4-dihydroxyphenylalanine) is one of the most widely used drugs in the treatment of Parkinson disease. Development of an efficient gene transfer method is an absolute requirement for genetically improving *Mucuna pruriens* and creating plants with more desirable traits. A simple protocol was developed for the *Agrobacterium*-mediated stable genetic transformation of *M. pruriens*. *Agrobacterium tumefaciens* strain EHA 101, containing the vector pCAMBIA1305 and the *hptII* and GUS plus genes, was used for the gene transfer experiments. Putative transgenic shoots were obtained on medium supplemented with kanamycin (50 mg L^{-1}) and cefotaxime (400 mg L^{-1}). GUS histochemical analysis of the putative transgenic tissues further confirmed the transformation event. Genomic polymerase chain reaction (PCR) analysis was performed to verify the presence of transgenes and their stable integration. Transformation mediated by *Agrobacterium* in *M. pruriens* is reported for the first time.

Key words: *M. pruriens*, genetic transformation, *Agrobacterium*, GUS, PCR

Introduction

Mucuna pruriens, also called velvet bean, is a tropical legume belonging to the family Papillonaceae. *M. pruriens* is a twiner with trifoliate leaves, purple flowers, and pods covered with hairs. It is indigenous to tropical regions, especially Africa, India, and the West Indies. It is widespread over most of the subcontinent and is found in bushes, hedges, and dry deciduous, low forests throughout the plains of India (1). All parts of *M. pruriens* possess valuable medicinal properties (2), and there is a heavy demand for *Mucuna* in Indian and international drug markets (3).

Plant genetic transformation is a core research tool in modern plant biology and agricultural biotechnology. *Agrobacterium tumefaciens* remains the preferred method to genetically transform plants (4). Legumes are not readily amenable to stable genetic transformation and, hence, protocols for high throughput generation of transgenic legume plants are not available. In general, the difficulty for achieving efficient genetic transformation of legumes is related to their low responsiveness to in vitro regeneration. The best approach for obtaining stable transgenic legumes has been through indirect in vitro

regeneration, defined as the induction of regenerative somatic embryos or shoots from morphogenetic calli (5). To date, no report of genetic transformation in *M. pruriens* is available. In the present study, we report the development of a simple and reliable method for the genetic transformation of *M. pruriens* mediated via *A. tumefaciens*.

Materials and methods

Plant material

Seeds were procured from the University of Agriculture, Bangalore, India. The seeds were surface sterilized with 1% mercuric chloride for 5 min and then washed with sterile distilled water 5-6 times to remove traces of surface sterilant. Seeds were germinated on basal Murashige and Skoog (MS) (6) medium. Plant identity was verified by senior taxonomist Prof S.B. Kamalakar, Department of Botany, Sahyadri Science College, Shimoga, India. Plants grown in this way were used as an explant source.

Preparation of explants

Nodal explants were used for regeneration, and leaves (disks) were used for callus induction. Explants were aseptically trimmed (1.5 to 2.0 cm) and inoculated onto MS medium supplemented with sucrose (30 g L^{-1}), high concentrations of 6-benzylaminopurine (BAP) and low concentrations of indole acetic acid (IAA) for regeneration, and 0.2 mg L^{-1} BAP and 2 mg L^{-1} IAA for callus formation. The media were solidified with 0.8% (w/v) agar and adjusted to pH 5.8 prior to autoclaving at 121 °C for 20 min. Cultures were maintained at 26 ± 2 °C under a 16-h light/8-h dark photoperiod with light provided by cool white fluorescent lamps (25 µmol m^{-2} s^{-1}) in a growth chamber.

Determination of antibiotic sensitivity

The effects of different concentrations of kanamycin (10-80 mg L^{-1}) and cefotaxime (100-500 mg L^{-1}) (Sigma Aldrich, St. Louis, MO, USA) on shoot regeneration were investigated to standardize the antibiotic concentrations for transformation and regeneration. All antibiotics were added to the medium after filter sterilization (0.22 µm and 0.45 µm; Millipore Intertech Inc., Milford, MA, USA). Each treatment was replicated at least 3 times.

Agrobacterium tumefaciens strain and vector used for transformation

The *A. tumefaciens* strain EHA 101 (7) (Department of Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India) harboring the binary vector pCAMBIA1305 (CAMBIA, Canberra, Australia; http://www.cambia.org) was used for transformation experiments. The T-DNA region of the plasmid has an *hptII* gene and the histochemical reporter gene *uidA* (β-D-glucuronidase, GUS plus), in which the coding sequence is interrupted by an intron and is controlled by the constitutive CaMV 35S promoter.

Preparation of bacterial culture

Bacterial stock cultures were maintained on yeast extract peptone agar (YEP) medium (8) containing 0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.05% $MgSO_4.7H_20$, 0.5% sucrose, and 1.5% agar (pH 7.0). The medium for the EHA 101 strain always contained kanamycin sulfate at 50 mg L^{-1} (81% free base). Bacterial suspensions were prepared for inoculation in YEP liquid medium and were cultured for 18 h. The bacterial suspensions were centrifuged at 2000 rpm at room temperature to pellet down the cells. Cell density was adjusted to obtain an OD value of 1.5 at 600 nm. The bacterial cells were resuspended in MS liquid medium supplemented with 100 µM acetosyringone (Sigma Aldrich) and used for the infection of explants.

Agrobacterium tumefaciens transformation

The processing of plant tissue material or explants was performed as described above for explant preparation. The in vitro germinated explants were wounded mechanically with the help of a sterile scalpel, then transferred to bacterial suspension and incubated for 1-2 min. The explants were blotted on sterile filter paper to remove excess bacteria and transferred to MS medium containing varying concentrations of BAP and IAA for regeneration and 0.2 mg L^{-1} BAP and 2 mg L^{-1} IAA for callus formation. The medium also contained 50 mg L^{-1} kanamycin and 400 mg L^{-1} cefotaxime, bactericidal antibiotics. The cultures were incubated in the dark for 24 h and later transferred to a culture room maintained at standard culture conditions for regeneration as mentioned above.

Histochemical analysis of β-glucuronidase activity

GUS expression in putative transformed and nontransformed plant tissues was determined histochemically according to the method of Jefferson et al. (9). Tissues of both transformed and nontransformed (control) plantlets and calli were washed with sterile distilled water, blot-dried, and stained in 2 mM X-Gluc (100 mM phosphate buffer, 5 mM K3Fe(CN)$_6$, 5 mM $K_4Fe(CN)_6$, 1 mM EDTA, and 0.01% Triton X-100) overnight at 37 °C. After the incubation, chlorophyll was cleared from the stained tissues by washing with 70% ethanol.

Molecular analysis, genomic DNA isolation, and polymerase chain reaction analysis

Total genomic DNA was isolated from leaves and stems of putative transformed plants and nontransformed control plants using the modified CTAB method (10). The polymerase chain reaction (PCR) amplification was performed in a 20-µL reaction volume consisting of 10X buffer, 50 mM KCl, 1.5 mM $MgCl_2$, 100 mM dNTPs, 0.5 U Taq DNA polymerase, 250 nM primers, and 20 ng template DNA. To amplify the GUS gene, the following primer pair was used:

5'-ATCAGGAAGTGATGGAGCATCAGG-3' and 5'-TGCCACGCAAGTCCGCATCT-3'.

The PCR amplification profile consisted of an initial denaturation at 94 °C for 4 min, followed by 30 cycles of strand separation (at 94 °C for 1 min), annealing at 45 °C (GUS) and 55 °C (*hptII*) for 90 s, and extension at 72 °C for 2 min; the reaction was concluded by a final cycle at 94 °C for 7 min. Plasmid DNA was used as a positive control, and nontransgenic plant DNA was included as a negative control. The amplicons were separated on 1.2% agarose gels containing ethidium bromide, visualized, and photographed under UV light.

Data analysis

Experiments were performed with 3 replicates per treatment, both in explant regeneration and genetic transformation, and in each treatment, 30 explants were used. The significant difference among treatments was determined using Duncan's multiple range test and data were represented as mean ± SE.

Results

Plant regeneration

Plant regeneration from nodal explants was achieved on MS medium supplemented with 3 mg L^{-1} BAP and 0.2 mg L^{-1} IAA, and callus was induced from leaf disks on medium supplemented with 0.2 mg L^{-1} BAP and 2 mg L^{-1} IAA (Table 1). Successful transformation was established for shoots regenerated in vitro (Figure 1) on MS medium supplemented with BAP (3 mg L^{-1})

Table 1. Effect of cytokinin-auxin combinations on shoot regeneration in nodal explants of *Mucuna pruriens* L. after 40 days of incubation.

BAP (mg L^{-1})	IAA (mg L^{-1})	Average no. of shoots per culture	Average shoot length (cm)	Survival percentage
0	0.0	–	–	–
0.5	0.2	1.00 ± 0.33	0.82 ± 0.23	15.47 ± 0.67
1	0.2	1.83 ± 0.41	1.33 ± 0.19	25.53 ± 1.97
1.5	0.2	1.92 ± 0.29	1.83 ± 0.21	46.85 ± 2.53
2	0.2	1.27 ± 0.51	2.25 ± 0.28	57.00 ± 1.53
2.5	0.2	1.17 ± 0.97	3.28 ± 0.36	62.17 ± 1.56
3	0.2	3.42 ± 1.69	7.42 ± 0.61	97.00 ± 1.09
3.5	0.2	14.25 ± 2.46	5.83 ± 0.53	89.09 ± 1.80
4	0.2	1.33 ± 0.66	3.58 ± 0.45	64.18 ± 0.32
4.5	0.2	1.42 ± 0.01	2.00 ± 0.35	52.17 ± 1.17
5	0.2	1.32 ± 0.34	1.83 ± 0.21	41.15 ± 1.23

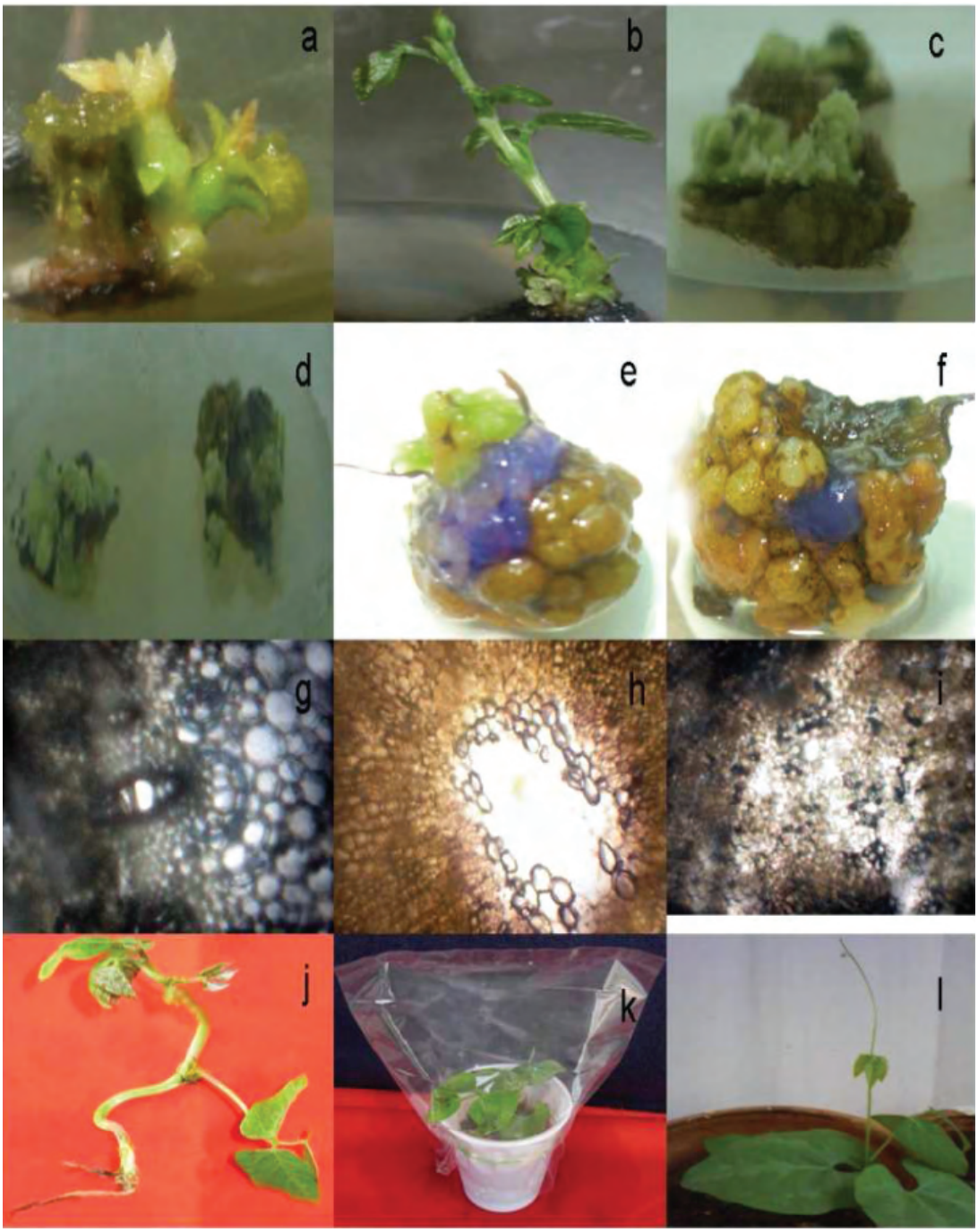

Figure 1. *A. tumefaciens*-mediated genetic transformation and histochemical localization of GUS in *Mucuna pruriens*. a-b: Regeneration in *Mucuna pruriens*; c-d: callus formation from leaf explants; e: regenerated plant stained with 2 mM X-Gluc in phosphate buffer (pH 7.0) overnight; f: callus stained with 2 mM X-Gluc in phosphate buffer (pH 7.0) overnight; g: transverse section of callus stained with X-Gluc; h: transverse section of stem stained with X-Gluc; i: transverse section of leaf stained with X-Gluc; j: rooting in shoots of *Mucuna pruriens*; k-l: acclimatization of transformed *Mucuna pruriens* plantlets.

and IAA (0.2 mg L^{-1}) in the presence of kanamycin and cefotaxime after standardizing the antibiotic concentration; these antibiotics are detrimental to tissue proliferation and growth at higher concentrations and fail to suppress bacterial growth at low concentrations. Successful root establishment was achieved in individual shoots on MS medium (half-strength) supplemented with NAA (0.5 mg L^{-1}) alone in the presence of 0.1% activated charcoal after 30 days of incubation. The regenerated plantlets were acclimatized successfully to natural environmental conditions with a success rate of 98% (Figures 1j, 1k, and 1l).

Determination of antibiotic sensitivity

The effects of different concentrations of kanamycin (10-80 mg L^{-1}) and cefotaxime (100-500 mg L^{-1}) on the morphogenesis of *M. pruriens* were studied. The results revealed that 50 mg L^{-1} kanamycin was optimal for sustaining the regenerability of explants (Table 2). Shoots were induced in the explants, and the survival percentage of regenerants was found to be optimal (98%) at this concentration of antibiotic. Cefotaxime was selected as the bactericidal antibiotic, and cefotaxime at 400 mg L^{-1} was the most suitable and effective in suppressing bacterial overgrowth with only a moderate effect on regeneration (data not shown). Based on these results, 400 mg L^{-1} cefotaxime was used to suppress bacterial growth in transformation, and 50 mg L^{-1} kanamycin was used to select transformants.

Histochemical analysis

Histochemical analysis of GUS activity was carried out to confirm transformation. GUS expression was examined in the leaves, shoots, and callus of *M. pruriens*. GUS positive blue coloration was detected in all transgenic tissues stained with X-Gluc reagent, whereas no blue color was observed in the nontransformed control tissues. The staining patterns as well as intensity of blue color were not uniform and differed between transgenic lines (Figures 1e and 1f). Similar findings were also reported by Padmanabhan and Sahi (11) for genetic transformation and regeneration of *Sesbania drummondii*.

PCR analysis

Successful genetic transformation was further confirmed by PCR analysis. PCR analysis conducted with genomic DNA showed the amplification of the predicted 610-bp product corresponding to the *gus A* gene in transformed plants as well as in the positive controls (Figure 2, lanes 2-6). No specific amplification products were detected in the nontransgenic control plant (Figure 2, lane 1).

Table 2. Effect of kanamycin on morphogenesis of *Mucuna pruriens* L.

Kanamycin concentration (mg L^{-1})	Average no. of shoots per culture	Average shoot length (cm)	Survival percentage
10	2.47 ± 0.32	1.82 ± 0.23	38.75 ± 1.33
20	3.10 ± 0.32	1.73 ± 0.21	42.75 ± 1.32
30	3.21 ± 0.36	1.63 ± 0.24	46.75 ± 2.35
40	12.65 ± 1.32	2.24 ± 0.26	52.00 ± 1.62
50	84.40 ± 1.63	7.24 ± 0.51	98.00 ± 1.07
60	1.53 ± 0.10	0.42 ± 0.21	12.52 ± 0.82
70	1.32 ± 0.31	0.72 ± 0.21	11.32 ± 0.47
80	–	–	–
90	–	–	–
100	–	–	–

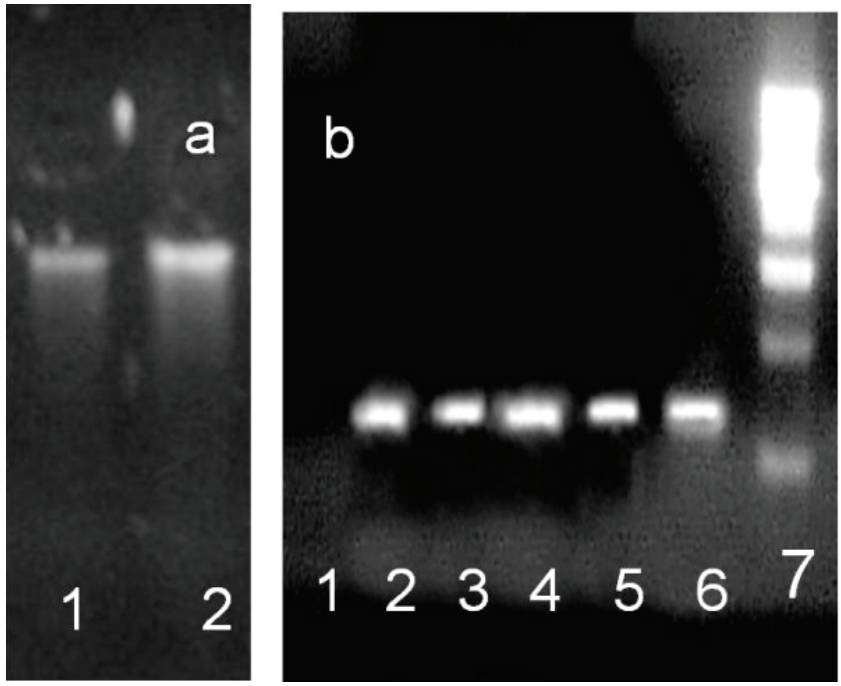

Figure 2. PCR analysis. a: Plant genomic DNA was isolated from transformed and untransformed plant tissue; line 1: *M. pruriens* nontransformed plant (control), line 2: *M. pruriens* transformed DNA. b: PCR assay of putative transformed plants; genomic DNA was amplified with primers corresponding to *uidA* (GUS) gene sequence; line 1: nontransformed plant, lines 2-6: transformed *M. pruriens*, line 7: molecular weight ladder (containing 10 DNA fragments of sizes 500 bp, 1000 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 3500 bp, 4000 bp, 4500 bp, and 5000 bp).

Discussion

Development of an efficient gene transfer method is an absolute requirement for the genetic improvement of plants. Legumes in general have been resistant to in vitro regeneration, and this is a bottleneck for genetic transformation (12). In the present study, an attempt was made to transform *M. pruriens* L. genetically. Plant regeneration is a prerequisite for transformation. Plant regeneration in *M. pruriens* was reported earlier (13-15) using high cytokinin/low auxin supplemented media. In the present study, the plantlets regenerated on MS medium supplemented with 3 mg L^{-1} BAP and 0.2 mg L^{-1} IAA.

Various forms of antibiotics and their different concentrations have either positive or negative effects on cell growth and/or plant regeneration. Therefore, evaluating the effects of antibiotics on shoot regeneration is a prerequisite for any plant transformation effort (16). During the present study, 50 mg L^{-1} kanamycin was found to be optimal for plant regeneration. In a transformation study of rose, Li et al. (17) also reported that 50 mg L^{-1} kanamycin was ideal for plant sustenance. The effective elimination of bacterial contamination following cocultivation is a crucial step for the regeneration of transformed tissues. Cefotaxime has been used for the elimination of *Agrobacterium* from the transformed plant tissues of many plants (18-20). It has been reported that the use of high concentrations of bactericidal antibiotics such as cefotaxime, carbenicillin, and augmentin had inhibitory effects on the regeneration of many legumes (21). However, the positive or beneficial effects of antibiotics on regeneration have also been reported in genetic transformation studies of other plants (22). In the present study, cefotaxime at 400 mg L^{-1} was found to be optimal for the elimination of *Agrobacterium* from transformed *M. pruriens* explants.

In conclusion, a simple and reliable gene transfer protocol was developed for the genetic transformation of *M. pruriens*. This is the first report on the genetic transformation of *M. pruriens* L. These data might serve as a basis for future genetic engineering experiments in *Mucuna* species. This was a preliminary attempt to transform this plant, and more refinements in technique are required to further improve the transformation frequency. This, in turn, will aid in the metabolic engineering of this medicinally important plant.

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References

1. Rastogi RP, Mehrotra BN. Compendium of Indian Medicinal Plants. CDRI. Lucknow; 1994.
2. Caius JF. The Medicinal and Poisonous Legumes of India. Scientific Publishers. Jodhpur; 1989.
3. Manyam BV, Dhanasekaran M, Hare TA. Effect of antiparkinson drug HP-200 (*Mucuna pruriens*) on the central monoaminergic neurotransmitters. Phytother Res 18: 97-101, 2004.
4. Gheysen G, Angenon G, Van Montagu M. *Agrobacterium*-mediated plant transformation: a scientifically intriguing story with significant applications. In: Lindsey K. ed. Transgenic Plant Research. Harwood; 1988: pp. 1-33.
5. Arellano J, Fuentes SI, Castillo-Espana P et al. Regeneration of different cultivars of common bean (*Phaseolus vulgaris* L.) via indirect organogenesis. Plant Cell Tiss Org Cul 96: 11-18, 2009.
6. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15: 473-497, 1962.
7. Hood EE, Helmer GL, Fraley RT et al. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bact 168: 1291-1301, 1986.
8. Wang HM, Qi M, Cutler AJ. A simple method of preparing plant samples for PCR. Nucleic Acids Res 21: 4153-4154, 1993.
9. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907, 1987.
10. Doyle JJ, Doyle JL. A rapid DNA isolation procedure from small quantities of fresh leaf tissue. Phytochem Bull 19: 11-15, 1987.
11. Priya P, Shivendra SV. Genetic transformation and regeneration of *Sesbania drummondii* using cotyledonary nodes. Plant Cell Rep 28: 31-40, 2009.
12. Popelka JC, Terryn N, Higgins THV. Gene technology for grain legumes: can it contribute to the food challenge in developing countries? Plant Sci 167: 195-206, 2004.
13. Sharmila C, Datta SK, Mahato SB. Rapid micropropagation for *Mucuna pruriens* L. Plant Cell Rep 15: 271-273, 1995.
14. Faisal M, Siddique I, Anis M. An efficient plant regeneration system for *Mucuna Pruriens* L. (DC.) using cotyledonary node explants. In Vitro Cell Dev Biol Plant 42: 59-64, 2006.
15. Sathyanarayana N, Bharath kumar TN, Vikas PB et al. *In vitro* clonal propagation of *Mucuna pruriens* var. *utilis* and its evaluation of genetic stability through RAPD markers. Afr J Biotechnol 7: 973-980, 2008.
16. Lin JJ, Assad-Garcia N, Kuo J. Plant hormone effect of antibiotics on the transformation efficiency of plant tissues by *Agrobacterium tumefaciens* cells. Plant Sci 109: 171-177, 1995.
17. Li X, Krasnyanski SF, Korban, SS. Optimization of the *uid A* gene transfer into somatic embryos of rose via *Agrobacterium tumefaciens*. Plant Physiol Bioch 40: 453-459, 2002.
18. Derks FHM, van Dijk AJ, Hänisch ten Cate CH et al. Prolongation of vase life of cut roses via introduction of genes coding for antibacterial activity, somatic embryogenesis and *Agrobacterium*-mediated transformation. Acta Horti 405: 205-209, 1995.

19. Yan Y, Wang Z. Genetic transformation of the medicinal plant *Salvia miltiorrhiza* by *Agrobacterium tumefaciens*-mediated method. Plant Cell Tiss Org Cult 88: 175-184, 2007.

20. Yong WTL, Abdullah JO, Mahmood M. *Agrobacterium*-mediated transformation of *Melastoma malabathricum* and *Tibouchina semidecandra* with sense and antisense dihydroflavonol-4-reductase (DFR) genes. Plant Cell Tiss Org Cult 96: 59-67, 2009.

21. Barik DP, Mohapatra U, Chand PK. Transgenic grass pea (*Lathyrus sativus* L.): factors influencing *Agrobacterium*-mediated transformation and regeneration. Plant Cell Rep 24: 523-531, 2005.

22. Suzuki S, Nakano M. *Agrobacterium*-mediated production of transgenic plants *Muscari armeniacum* Leichtl. ex Bark. Plant Cell Rep 20: 835-841, 2002.