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Full Length Research Paper

Optimization of parameters for *Agrobacterium* mediated transformation of black gram (*Vigna mungo* L. Hepper) using cotyledon explants

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Agrobacterium tumefaciens **strain LBA4404 harbouring binary vector pCAMBIA 2301, which contains a neomycin phosphotransferase gene (*nptII*) and a β-glucuronidase (*GUS*) gene (*uid A*) was used for transformation of *Vigna mungo* cotyledon derived calli. Wounding of explants before infection, osmotic effects of infection and cocultivation media had an effect on the competence of the tissue as well as transforming ability of *Agrobacterium* cells. Transient GUS expression studies revealed that a cell density of 10^8 cells/ml, 100 µM acetosyringone and 330 µM cysteine were effective in increasing the transformation frequency and obtaining stable transformants with a 3.8% transformation efficiency. IBA pulse treatment was effective in root induction of kanamycin selected putative transformants. Molecular analysis using polymerase chain reaction (PCR) of *nptII* gene confirmed the transgenic nature of T_0 transformants.**

Key words: Black gram, genetic transformation, *Agrobacterium tumefaciens*, cotyledon.

INTRODUCTION

Black gram (*Vigna mungo* L. Hepper), is an important grain legume cultivated mainly for its protein rich edible dry seeds. It provides an inexpensive source of nutritionally rich vegetable protein that complements the cereals to provide a balanced diet (Bhomkar et al., 2008). This crop is subjected to various biotic and abiotic stresses which limit its productivity (Eapen, 2008). The high susceptibility of the crop to yellow mosaic virus (VMYMV), fungal pathogens (powdery mildew, cercospora leaf spot), insects (bruchids) (Sahoo et al., 2002) and drought result in significant yield losses. Until recently, the genetic improvement of black gram has been carried out through traditional plant breeding methods, but the limited genetic variability in black gram germplasm has considerably slowed the pace of breeding varieties for agronomically important traits. Though genes conferring resistance to biotic and abiotic stresses have been reported in many wild and related species, these are sexually incompatible with the cultivated ones. Some of such genes have been isolated, characterized and cloned from other plant species and microorganisms (Grover et al., 2003). Genetic engineering allows these genes to be transferred to cultivated varieties in order to enhance the tolerance to various stresses and hence stabilize yield (Dita et al., 2006; Kaur and Murphy, 2012).

Genetic transformation of large seeded grain legumes, including *V. mungo* has been difficult and challenging as these are notoriously recalcitrant to *in vitro* regeneration (Saini and Jaiwal, 2007). *In vitro* plant regeneration has been reported in black gram via direct organogenesis (Igancimuthu et al., 1997; Sen and Guha-Mukherjee,

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Abbreviations: VMYMV, *Vigna mungo* yellow mosaic virus; **IBA,** indole-3-butyric acid; **CTAB,** cetyl trimethylammonium bromide; **PCR,** polymerase chain reaction.

1998) and indirect organogenesis (Geetha and Rao, 1997; Das et al., 1998). However, success with recovering transgenics has been limited.

Since regeneration and transformation are genotype and explant dependent, optimization of various factors that influence *Agrobacterium* mediated genetic transformation need to be optimized to develop transgenic plants with more precision and reproducibility (Somers et al., 2003; Sathyanarayana et al., 2012). *Agrobacterium tumefaciens* is most commonly used when compared with microprojectile bombardment due to the development of highly virulent strains and binary vectors that are useful for legume transformation. Moreover, *A. tumefaciens* only delivers the T-DNA and the resulting transgene loci are less complex. Studies have also shown that strain by genotype interactions, addition of acetosyringone and various thiol compounds have increased transformation efficiencies and the production of transgenic plants. However, there is no overall trend evident from reviewing the data, suggesting strong interactions between the selection system, culture type, and genotype within a species that require substantial experimentation to optimize.

Therefore the present report was aimed at optimization of several *A. tumefaciens* and plant tissue specific factors known to influence the transfer of T-DNA and its integration into the plant genome. Transient β-glucuronidase (GUS) assay was used to monitor the expression. Also the present study differs from the previous reports in many aspects such as bacterial strain, explant type and cocultivation conditions for the selection and regeneration of transformed cells. This is the first report of optimizing transformation parameters using cotyledon explants.

MATERIALS AND METHODS

Black gram seeds of variety T-9 were washed with 70% ethanol for 1 min followed by 0.1% mercuric chloride for 5 min. The seeds were then rinsed in sterile distilled water 3 to 4 times and soaked in sterile water for 18 h in dark. The imbibed seeds were decoated and the cotyledons were carefully separated. The cotyledon along with the embryonal axis was placed on MSB5 medium (Murashige and Skoog salts, B5 vitamins, 3% sucrose supplemented with 4 mg/l BAP). Ten days old embryogenic calli were used as explants for transformation experiments.

A. tumefaciens strain LBA4404 harboring binary vector pCAMBIA 2301, containing the β-glucuronidase (*uidA*) gene as reporter gene and neomycin phosphotransferase gene (*nptII*) as selectable marker both driven by the CaMV35S promoter was used for transformation studies.

The *uidA* gene contains an intron in the coding region to ensure that the observed GUS activity occurred in the plant cell and not due to residual *Agrobacterium* cells.

The transformation events were quantified by directly observing the GUS blue staining in the explants. The bacterial culture was grown in liquid YEM (Yeast extract 0.4 g/l, mannitol 10 g/l, NaCl 0.1 g/l, $MgSO_4$ $7H_2O$ 2.0 g/l, KH_2PO_4 0.5 g/l, pH 7.0) medium containing 50 mg/l kanamycin and 10 mg/l rifampicilin overnight at 28°C. The density of bacterial suspension was checked at 600 nm and dilutions were made for obtaining different concentrations of bacterial cells.

Freshly subcultured 10 d old calli were immersed in infection media (MSB5 + 200mM sucrose and 200mM glucose pH 5.2) for 12 min with different concentrations of bacterial cells (10^6 to 10^9 cells/ml), transferred to cocultivation media (MSB5 + 4 mg/L BAP + 200 mM sucrose and 200 mM glucose, pH 5.7) and incubated for 48 h. In an effort to increase transformation efficiency, different concentrations of both acetosyringone (0, 50, 100 and 200 μM) and cysteine (0, 330 and 660 μM) were tested. Acetosyringone was used in both the inoculation and cocultivation media while cysteine was added in cocultivation and selection media.

Cocultivated explants were then washed in sterile distilled water containing cefotaxime (250 μg/ml) 3 to 4 times till no turbidity was seen. The explants were blot dried and analyzed for GUS histochemical assay (Jefferson et al., 1987). For GUS assay the putative transformed and nontransformed explants were immersed in a solution containing 50 mM sodium phosphate pH 7.0, 0.1% Triton X-100, 10 mM EDTA and 1mM 5-bromo-4-chloro-3indolyl-β-D-glucuronidase and incubated at 37°C for 24 h. For each treatment, 30 explants were used and each experiment was repeated thrice. The frequency of transient GUS expression was calculated for each experiment. The optimized parameters were then used to generate the transgenics. The cocultivated explants were selected on MSB5 medium containing 100 μg/ml kanamycin for selecting the transformed cells and 250 μg/ml cefotaxime to eliminate the bacteria. The transformed shoots were transferred to ½ strength MSB5 medium for elongation. Since rooting in transgenics was difficult, the putatively transformed shoots were subjected to indole-3-butyric acid (IBA) (1 mg/ml) treatment for 15 min and transferred to rooting media (½MSB5). The putative transformed plants were established in soil and grown to maturity to collect T_0 seeds. Explants cultured without cocultivation were used to regenerate untransformed control plants.

Genomic DNA was isolated from putative transgenics and untransformed control following cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Polymerase chain reaction (PCR) analysis was carried out on putative T_0 and T_1 transgenics using *npt II* gene specific primers. The sequences of the primers used were forward primer: 5' AATATCACGGGTAGCCAACG 3' and reverse primer 5' GCTTGGGTGGAGAGGCTATT 3'. The PCR conditions used were initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 60°C for 30 s, 72°C for 30 s followed by a final extension at 72°C for 5 min. PCR amplification was carried out using Gene AmpR PCR system 9700 (Applied Biosystems, Foster city, CA, USA). The genomic DNA from untransformed control plants and plasmid pCAMBIA 2301 were used as negative and positive controls, respectively. Segregation analysis was carried out based on PCR analysis using *nptII* primers on the progeny of two T_0 transformants (L1, L2). The stable GUS activity was monitored in the germinating T_1 seedlings and flowers. The standard error and chi square test were done according to standard statistical procedures described in Gomez and Gomez (1984).

RESULTS

Agrobacterium mediated transformation of black gram using cotyledon derived calli was optimized based on GUS histochemical assay. Subculturing of the explants prior to infection caused mechanical injury facilitating bacterial penetration into the tissue as well as production of *vir* gene inducers and enhanced plant cell competence for transformation. When previously subcultured explants

Table 1. Effect of different transformation parameters on transient GUS expression of cotyledon derived callus explants of black gram cv. T-9 inoculated with *Agrobacterium tumefaciens* strain LBA4404 harbouring vector pCAMBIA 2301.

Factor	Frequency of transient GUS expression (%)
Bacterial culture (cells/ml)	
10^6	65.26 ± 1.38
10^7	93.00 ± 1.53
10^8	100.00 ± 0.0
10^9	77.67 ± 1.45
Acetosyringone (μM)	
0	64.33 ± 1.20
50	80.60 ± 0.60
100	100.00 ± 0.0
200	67.97 ± 1.02
Cysteine (μM)	
0	89.96 ± 0.61
330	100.00 ± 0.0
660	67.33 ± 0.67

were infected, frequency of GUS expression was low. Inclusion of higher concentrations of sucrose and glucose in infection and cocultivation media resulted in osmotic effects and improved T-DNA delivery when compared to the media without osmoticum. The transformation frequency increased with the increase in concentration of *Agrobacterium* cells upto10^8 cells/ml (Table 1). Higher culture concentration resulted in severe necrosis of explants and also limited the survival of the explants on selection medium. However, at lower culture concentrations, the number of bacterial cells was insufficient to infect and transfer T-DNA into plant cell and therefore the transformation efficiency was low. In the presence of acetosyringone, the transformation frequency increased with increase in concentration upto 100 μM concentration and decreased thereafter (Table 1). At the concentration of 200 μM acetosyringone, the GUS frequency decreased to 68%. This could be due to the fact that high concentration of acetosyringone is toxic to explants as a result of harmful effect of supra-optimal concentration of acetosyringone and alcohol solvent used for preparation. Also, necrotic zones were formed on the explants at higher concentrations, finally affecting transformation efficiency. Addition of cysteine in the cocultivation medium increased the transient GUS activity when used at 330 μM. The increased transient GUS expression was through thiol inhibition of peroxidase and polyphenol oxidase in explants. These optimized transformation parameters that is, culture density of 10^8 cells/ml, 100 μM acetosyringone and 330 μM cysteine were used for the stable genetic transformation of *Vigna mungo* (Table 1).

Calli on selection medium showed shoot initiation within 15 days (Figure 1a) and were transferred to ½ strength MS basal medium for elongation and maintained for 24 days (Figure 1b). IBA pulse treatment was effective in initiation of roots (Figure 1c). Plants survived, grew to maturity and produced normal flowers and pods with viable seeds (Figure 1d). These seeds were collected and sown to obtain T_1 transgenic plants. Thousand cotyledon derived calli cocultured with *Agrobacterium* produced a total of 451 shoots on kanamycin selection medium. Thirty eight plants survived and grew to maturity with a transformation efficiency of 3.8% (Table 2).

Transient and stable GUS expression was observed in the cotyledon explants, germinating T_0 seeds and flowers (Figure 2a, b, c) upon histochemical assay. PCR analysis of putative T_0 transformants and the progeny of two transgenics (L1, L2) using *nptII* gene specific primers amplified the 645 bp band corresponding to neomycin phosphotransferase gene. No such amplification was observed in the untransformed control (Figure 3a).

Segregation analysis carried out on the progeny of two T_0 transformants (L1, L2) revealed that out of the 42 progeny tested for L1, 32 plants were positive and out of 28 tested for L2, 17 were positive (Figure 3b, c). T_0 transformant L1 segregated in the 3:1 ratio while the transformant L2 segregated in the ratio of 2.43:1.47 (Table 3).

DISCUSSION

Agrobacterium-mediated transformation involves interact-tion between two biological systems and is affected by various physiological conditions. Therefore optimization of some of the aspects that enhance the virulence of *Agrobacterium* for T-DNA transfer and factors that improve survival and regeneration of transformed cells is crucial for a recalcitrant crop like black gram. Transient GUS expression provides an easy and clear indication of the expression of transferred genes and can be used to assess the frequency of transformation. In the present study, subculturing of explants prior to agroinfection facilitated entry of *Agrobacterium* cells and production of *vir* gene inducers (Stachel et al., 1985; Saini et al., 2003; Khawar et al., 2004). Inclusion of higher concentrations of sucrose and glucose in infection and cocultivation media resulted in osmotic effects and improved T-DNA delivery as manifested in rice (Uze et al., 1997). These results are in agreement with earlier reports in *Nicotiana tabacum* and *Arabidopsis thaliana* (Lin et al., 1994) and in most grain legumes (Bean et al., 1997**,** Saini and Jaiwal, 2007).

At culture concentrations higher than 10^8 cells/ml, it was very difficult to overcome the *Agrobacterium* growth. The selection of culture density was therefore, a

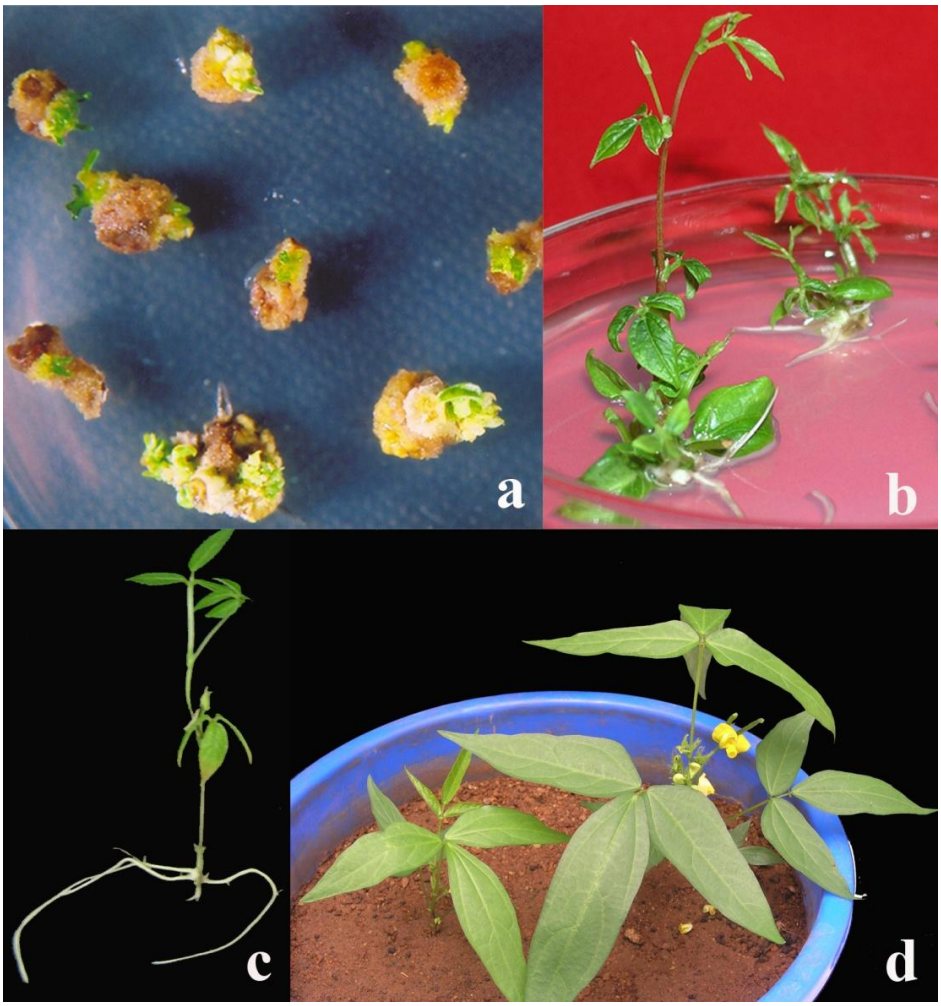

Figure 1. Regeneration of transgenic plants from cotyledon explants. (a) Transformed calli growing on selection media (b) Transformed shoots growing in regeneration media (c) Rooting (d) Putative transgenics growing in pots.

Table 2. Frequency of regeneration and transformation of cotyledon derived calli of black gram infected using plasmid pCAMBIA 2301 vector.

Total No. of calli infected	No. of explants regenerating on kanamycin (100 mg/l)	PCR positive regenerants	Transformation efficiency[a] (%)
1000	451	38	3.8

[a]Transformation efficiency = No. of explants regenerating and showing PCR amplification / Total number of explants infected X 100.

compromise between the efficiency of infection and survival rate after transfer. Inclusion of synthetic phenolic compound acetosyringone in cocultivation medium enhanced transient expression of *GUS* when used at a final concentration of 100 µM. Acetsyringone enhances *vir* functions during transformation (Stachel et al., 1986) and has been shown 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). Also the acidic pH (5.2) used in the infection medium acts synergistically with acetosyringone for increasing the transformation efficiency (De la Riva et al., 1998).

In the present study, addition of cysteine (330µM) in the selection medium improved the appearance and survival of the explants. Olhoft and Somers (2001) reported that the addition of cysteine at 3.3 to 8.3 mM in the cocultivation medium increased T-DNA transfer in cultivar Bert cotyledonary nodes based on transient GUS expression. The antioxidant properties of cysteine reduce

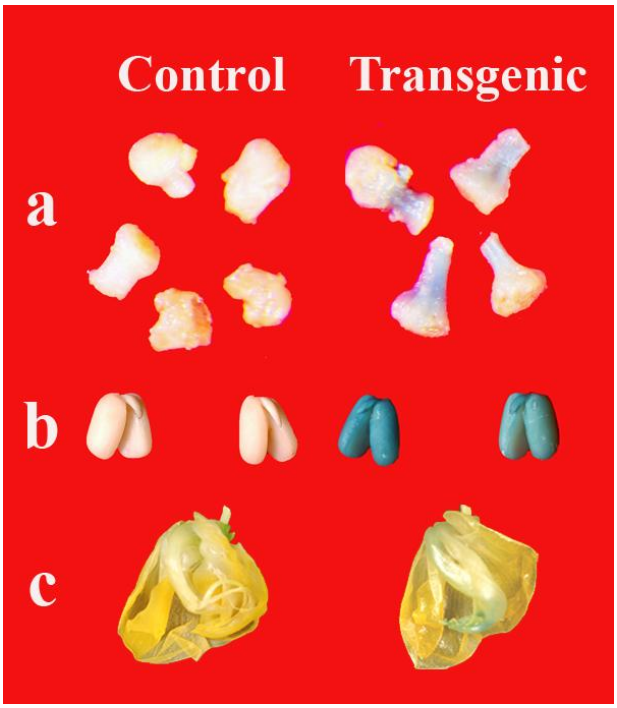

Figure 2. (a) Transient GUS expression in 10 days old calli (b) Stable GUS activity in T_0 germinating seeds (c) Flowers

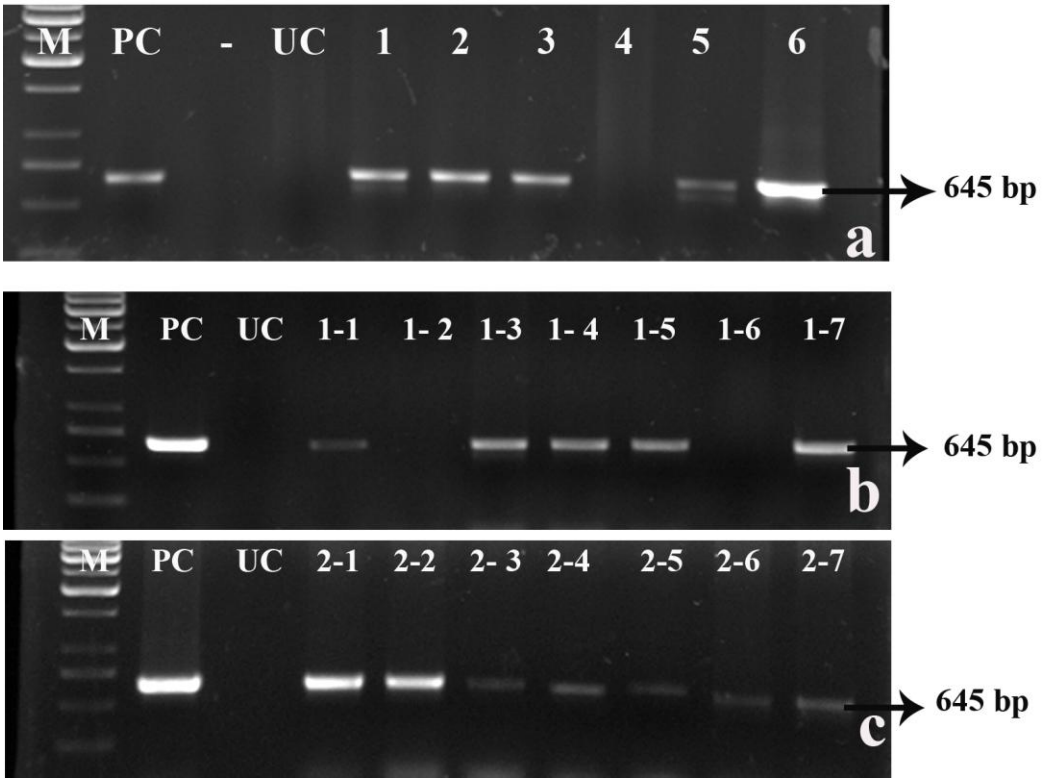

Figure 3. PCR confirmation of putative transgenics using *nptII* gene specific primers. Lane M – 1kb ladder, lane PC – positive control (plasmid pCAMBIA 2301), UC – untransformed control, (a) T_0 transgenics. Lanes 1- 6 T_0 transgenics, (b, c) Progeny of two T_0 transgenic lines (L1, L2). Lanes 1-1 to 1-7 progeny of transgenic line L1, lanes 2-1 to 2-7 progeny of transgenic line L2.

Table 3. Segregation analysis of the progeny of two T_0 transgenic lines based on inheritance of *npt II* gene analyzed by PCR.

T_0 transgenic line	No. of T_1 plants tested	No. of plants positive to PCR	No. of plants negative to PCR	Segregation ratio	χ^2 value
L1	42	32	10	3:1	0.030
L2	28	17	11	2.43:1.57	3.046

Tabular $\chi 2$ values for 1 d.f. = 3.841 at 5% level of significance.

browning of wounded explants and resulted in better recovery of infected explants. Also, the less oxidized tissue would improve the interaction between *Agrobacterium* and plant cells as suggested by Olhoft et al. (2001). Of the different parameters analyzed, subculturing of explants prior to agroinfection, addition of acetosyringone were useful in improving the *Agrobacterium* infectivity of the explants. Addition of cysteine prevented the necrosis of the transformed tissues and improved the efficiency of regeneration.

The blue coloration in the infected cotyledon explants and germinating T_o seeds upon histochemical assay suggested the introduction and expression of *GUS* gene. Based on molecular characterization using PCR, the integration of *nptII* gene was confirmed in transgenics. The untransformed control did not show any amplification with these primers. Two T_o transgenic lines (L1, L2) and their progeny were found to be stably expressing the *nptII* gene. Segregation analysis based on PCR analysis of *nptII* gene revealed a ratio of 3:1 for L1 confirming the Mendelian segregation for single gene insert. However, line L2, did not follow Mendelian segregation ratio.

In summary, the results presented in this communication clearly demonstrate the optimization of parameters for *Agrobacterium* mediated transformation of black gram using cotyledon explants. Also the transgenic plants carrying *nptII* and *uidA* genes have been produced. Transgene inherited and expressed in the progenies of transformants. The transformation protocol is repeatable and can be used to mobilize genes conferring tolerance to various biotic and abiotic stresses from diverse sources into *V. mungo* with more precision. Expanding the range of genotypes within a species that undergo transformation will allow faster varietal improvement for enhancing crop yield.

REFERENCES

Atkinson RG, Gardner R (1991). *Agrobacterium* – mediated transformation of *Pepino* and regeneration of transgenic plants. Plant Cell Rep. 10:208-212.

Bean SJ, Gooding PS, Mullineaux PM, Davies DR (1997). A simple system for pea transformation. Plant Cell Rep. 16:513-519.

Bhomkar P, Upadhyay CP, Saxena M, Muthusamy A, Shiva Prakash N, Pooggin M, Hohn T, Sarin NB (2008). Salt stress alleviation in transgenic *Vigna mungo* L. Hepper (blackgram) by overexpression of the *glyoxalase I gene* using a novel Cestrum yellow leaf curling virus (CmYLCV) promoter. Mol. Breed. 22:169-181.

Das DK, Shiva-Prakash N, Bhalla-Sarin N (1998). An efficient regeneration system of blackgram (*Vigna mungo* L. Hepper) through organogenesis. Plant Sci. 134:199-206.

De la Riva GA, Cabrera JG, Padron RV, Pardo CA (1998). *Agrobacterium tumefaciens*: a natural tool for plant transformation. Eur. J. Biotechnol. 1:1-18.

Dita MA, Rispail N, Prats E, Rubiales D, Singh KB (2006). Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. Euphytica 147:1-24.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Eapen S (2008). Advances in development of transgenic pulse crops. Biotechnol. Adv. 26:162-168.

Geetha NVP, Rao GR (1997). Plant regeneration and propagation of blackgram (*Vigna mungo* L. Hepper) through tissue culture. Trop. Agric. 74:73-76.

Gomez KA, Gomez AA (1984). Statistical procedures in agricultural research. Wiley publishers, New York.

Grover A, Agarwal PK, Kapoor A, Katiyar Agarwal S, Agarwal M, Chandramouli A (2003). Addressing abiotic stresses in agriculture through transgene technology. Curr. Sci. 84:355-367.

Ignacimuthu S, Franklin G, Melchias G (1997). Multiple shoot formation and *in vitro* fruiting from cotyledonary nodes of *Vigna mungo* L. Hepper. Curr. Sci. 73:733-735.

Janssen BJ, Gardner RC (1993). The use of transient GUS expression to develop an *Agrobacterium*-mediated gene transfer system for kiwi fruit. Plant Cell Rep. 13:28-31.

Jefferson RA, Kavanagh TA, Bevan MW (1987). GUS fusions β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Kaneyoshi J, Kobayashi S, Nakamura Y, Shigemoto N, Doi Y (1994). A simple and efficient gene transfer system of trifoliate orange. Plant Cell Rep. 13:541-545.

Kaur N, Murphy JB (2012). Enhanced isoflavone biosynthesis in transgenic cowpea (*Vigna unguiculata* l.) Callus. Plant Mol. Biol. Biotechnol. 3(1):1- 8

Khawar KM, Gulbitti-Onarici S, Cocu S, Erisen S, Sancak C, Ozcan S (2004). *In vitro* crown galls induced by *Agrobacterium tumefaciens* strain A281 (pTiBo542) in *Trigonella foenum-graecum*. Biol. Plant. 48(3):441-444.

Lin JJ, Garcia-Assad N, Kuo J (1994). Effect of *Agrobacterium* cell concentration on the transformation efficiency of tobacco and *Arabidopsis thaliana*. Focus 16:72-77.

Olhoft PM, Somers DA (2001). L-cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep. 20:706-711.

Sahoo L, Sugla T, Jaiwal PK (2002). *In vitro* regeneration and genetic transformation of Vigna species. In: Jaiwal PK , Singh RP (Eds). Biotechnology for improvement of legumes. Kluwer Academic Publishers, Dordrecht. pp. 1-48.

Saini R, Jaiwal PK (2003). Stable genetic transformation of *Vigna mungo* L. Hepper via *Agrobacterium tumefaciens*. Plant Cell Rep. 21:851-859.

Saini R, Jaiwal PK (2007). *Agrobacterium tumefaciens*–mediated transformation of blackgram: an assessment of factors influencing the efficiency of *uidA* gene transfer. Biol. Plant. 51:69-74.

Sathyanarayana R, Kumar V, Ramesh CK, Parmesha M, Khan MHM (2012). A preliminary attempt for efficient genetic transformation and regeneration of legume *Mucuna pruriens* L. mediated by *Agrobacterium tumefaciens*. Turk. J. Biol. 36:285-292.

Sen J, Guha-Mukherjee S (1998). *In vitro* induction of multiple shoots and plant regeneration in *Vigna*. *In Vitro* Cell. Dev. Biol. (Plant). 34:

276-280.
Somers DA, Samac DA, Olhoft PM (2003). Recent advances in legume transformation. Plant Physiol. 131:892-899.
Stachel SE, Messens E, Van Montagu M, Zambraki P (1985). Identification of signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens.* Nature 318:624-629.
Stachel SE, Nester EW, Zambryski P (1986). A plant cell factor induces *Agrobacterium tumefaciens* via gene expression. Proc. Nat. Acad. Sci. 83:379-383.
Uze M, Wunn J, Pounti-Kaelas J, Potrykus I, Sauter C (1997). Plasmolysis of precultured immature embryos improves *Agrobacterium* mediated gene transfer to rice (*Oryza sativa* L). Plant Sci. 130:87-95.