Transformation of chickpea: effect of genotype, explant, *Agrobacterium*-strain and composition of culture medium

B. BHATTACHARJEE, M. MOHAN[1] and S. NAIR*

International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110067, India

Abstract

Reproducible and high-frequency transgenic plant regeneration from callus and embryo axes of four different genotypes of chickpea (*Cicer arietinum*) was achieved after *Agrobacterium*-mediated transformation. Three different strains of *Agrobacterium* (EHA105, AGL1 and LBA4404) harboring the binary vector pCAMBIA1301 containing β-glucuronidase (*GUS*) and hygromycin phosphotransferase (*hpt*) genes under the control of a CaMV35S promoter were used. The highest number of transgenic plants was obtained from cotyledonary node-derived calli of genotype Pusa-256. A highly efficient rooting was achieved on Murashige and Skoog medium supplemented with indole-3-butyric acid. The stable integration of the gene was confirmed by molecular analyses of the transformed plants. Inheritance of *GUS* and *hpt* gene was followed through two generations and they showed the expected 3:1 inheritance.

Additional key words: *Cicer arietinum*, cotyledon- and cotyledonary node-derived calli, β-glucuronidase, hygromycin phosphotransferase.

Introduction

Agrobacterium-mediated transformation has been successfully attempted in different grain legumes such as pea (Polowick *et al*. 2000), peanut (Rohini and Rao 2000, Sharma and Anjaiah 2000), blackgram (Saini and Jaiwal 2007) and soybean (Olhoft *et al*. 2003, Zhang *et al*. 1999) for over a decade. To date, chickpea regeneration has been possible mainly through somatic embryogenesis and shoot organogenesis with varying degrees of success (Huda *et al*. 2003, Jayanand *et al*. 2003, Singh *et al*. 2002, Somers *et al*. 2003). However, there have been few successful reports of the production of transgenic chickpea plants using *Agrobacterium*-mediated transformation (Fontana *et al*. 1993, Krishnamurthy *et al*. 2000, Polowick *et al*. 2004, Sarmah *et al*. 2004, Senthil *et al*. 2004, Tewari-Singh *et al*. 2004) and a single report of a biolistic transformation (Kar *et al*. 1997). The major difference between ordinary *in vitro* culture procedures and tissue culture for transformation is that the later requires prolonged subculture on antibiotic containing medium as a selection process. Therefore, in most of the transformation events reported earlier variations were observed in *1*) transformation efficiency, *2*) number of transgenic plants recovered, *3*) rooting efficiency and subsequent establishment of the plants in the greenhouse and, *4*) inheritance of transgene in the following generations. For example, Fontana *et al*. (1993) claimed a 4 % success rate to achieve only 3 GUS positive transgenic chickpea plants. Kar *et al*. (1996), Krishnamurthy *et al*. (2000) and Tewari-Singh *et al*. (2004) reported *Agrobacterium*-mediated transformation of chickpea based on multiple shoot formation from embryo axis explants. Only Krishnamurthy *et al*. (2000) and Tewari-Singh *et al*. (2004) reported the inheritance of transgene in T_1 generation of transformed plants of chickpea. The transformation frequency and recovery of transgenic plants was also not very high (0.4 - 4 %). Recently Polowik *et al*. (2004) showed successful *Agrobacterium*-mediated transformation of chickpea, but

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Abbreviations: As - acetosyringone; BAP - 6-benzylaminopurine; Cef - cefotaxime; CIM - callus induction medium; CPM - callus proliferation and regeneration medium; GUS - β-glucuronidase; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - 1-napthaleneacetic acid; RIM - root induction medium; TDZ - thidiazuron [1-phenyl-3-(1,2,3-thiadiazol-5-YL) urea].

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[1] Present address: Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Marg, New Delhi-110021, India

* Corresponding author: fax: (+91) 11 26742316, e-mail: suresh@icgeb.res.in

the frequency of recovery of rooted transgenic plants ranged only from 1.3 - 3.1 %, and transgenic plant were obtained by a prolonged period of tissue culture, which could lead to morphological variation in the cultured plants. Senthil *et al*. (2004) showed transformation efficiency that ranged from 2.0 - 13.3 %. However, due to inconsistent performance of *in vitro* rooted plants in the glasshouse, grafting of shoots was performed, which was very laborious (Jayanand *et al*. 2003).

Keeping this in mind, our ongoing efforts were concentrated on establishing a protocol for high-efficiency *Agrobacterium*-mediated transformation of different cultivars of chickpea.

Materials and methods

Plants and cultivation: Immature seeds (20 - 25 d after flowering) of chickpea (*Cicer arietinum* L.) cultivars Pusa-256, KWR-108, Pusa-1003 (obtained from Indian Agricultural Research Institute, New Delhi) and a local line (from market) were used as experimental materials. To obtain a continuous supply of immature seeds we grew chickpea plants in the greenhouse from the mature seeds of all the four genotypes used.

Surface-sterilized (with 10 % bleach for 20 min on shaker) immature seeds were allowed to imbibe water for 2 - 3 h and plated on Murashige and Skoog (1962; MS) medium without growth regulators and kept in dark for germination (at 16 - 18 °C). On the fourth day of culture, the seed coats of germinated seeds was removed and the embryo axes devoid of all other parts were used directly for *Agrobacterium*-mediated transformation. At least fifty embryo axes slices were used for a single experiment and all the experiments were repeated thrice.

Both, the cotyledons and the cotyledonary-nodes were cultured on callus induction medium (CIM; Table 1). All the cultures were incubated at 25 ± 2 °C under white fluorescent light (irradiance of 37.5 μmol m^{-2} s^{-1} with 16-h photoperiod). After 15 d of culture, callus that started appearing from cotyledons and cotyledonary nodes were transferred to fresh CIM and allowed to grow for another 10 d. Next, the calli were sub-cultured on

Table 1. Composition of different media used for *Agrobacteruim*-mediated transformation of callus obtained from cotyledon and cotyledonary-nodes of germinated seeds of four genotypes of chickpea.

Medium	Composition
CIM	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 2.0 mg dm^{-3} BAP, 1.0 mg dm^{-3} NAA
CPM	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 1.0 mg dm^{-3} zeatin, 0.2 mg dm^{-3} IAA
Co-cultivation	CPM medium supplemented with 22 mg dm^{-3} acetosyringone
Cefotaxime-selection	CPM medium supplemented with 250 mg dm^{-3} cefotaxime
Hygromycin-selection	CPM medium supplemented with 250 mg dm^{-3} cefotaxime and 10 - 30 mg dm^{-3} hygromycin
RIM	½ MS, ½ B5 vitamins, 1.5 % sucrose, 0.25 % agarose, 0.1 mg dm^{-3} IBA, 250 mg dm^{-3} cefotaxime and 10 - 30 mg dm^{-3} hygromycin
RPM	½ MS, 1.5 % sucrose, 0.25 % agarose, 0.001 mg dm^{-3} IBA, 250 mg dm^{-3} cefotaxime and 10 - 30 mg dm^{-3} hygromycin

Table 2. Composition of different media used for *Agrobacteruim*-mediated transformation of embryo axes obtained from germinated seeds of four genotypes of chickpea.

Medium	Composition
Co-cultivation I	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 2.0 mg dm^{-3} BAP, 0.05 mg dm^{-3} NAA, 22 mg dm^{-3} acetosyringone
Co-cultivation II	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 1.1 mg dm^{-3} TDZ, 22 mg dm^{-3} acetosyringone
Cefotaxime-selection I	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 2.0 mg dm^{-3} BAP, 0.05 mg dm^{-3} NAA, 250 mg dm^{-3} cefotaxime
Cefotaxime-selection II	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 1.1 mg dm^{-3} TDZ, 250 mg dm^{-3} cefotaxime
Hygromycin-selection I	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 2.0 mg dm^{-3} BAP, 0.05 mg dm^{-3} NAA, 250 mg dm^{-3} cefotaxime, 10 - 30 mg dm^{-3} hygromycin
Hygromycin-selection II	MS salts, B5 vitamins, 0.3 % sucrose, 4 % agarose, 1.1 mg dm^{-3} TDZ, 250 mg dm^{-3} cefotaxime, 10 - 30 mg dm^{-3} hygromycin
RIM	½ MS, ½ B5 vitamins, 1.5 % sucrose, 0.25 % agarose, 0.1 mg dm^{-3} IBA, 250 mg dm^{-3} cefotaxime, 10 - 30 mg dm^{-3} hygromycin
RPM	½ MS, 1.5 % sucrose, 0.25 % agarose, 0.001 mg dm^{-3} IBA, 250 mg dm^{-3} cefotaxime, 10 - 30 mg dm^{-3} hygromycin

callus proliferation medium (CPM; Table 1) and incubated at the same conditions. On the fourth day of culture, the very dark green portions of the calli were used for *Agrobacterium*-mediated transformation.

***Agrobacterium* strains and transformation vectors:** Plasmid pCAMBIA 1301 (GeneBank accession number AF234297) is a binary vector (obtained from the Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia) having *hpt* and *gus-int* genes in the T-DNA region and carries an extra set of *vir* gene. The construct was mobilized into three *Agrobacterium* host strain LBA4404 (Hoekema *et al*. 1983), EHA105 (Hood *et al*. 1986) and AGL1 (Lazo *et al*. 1991) through freeze-thaw method.

To prepare the inocula, *Agrobacterium* culture was streaked out on AB medium (Chilton *et al*. 1974) supplemented with 50 mg dm^{-3} kanamycin and 50 mg dm^{-3} hygromycin and incubated at 28 °C. A 50 cm^3 culture was grown overnight to achieve an absorbance (A_{600}) of 0.6 - 0.8 at 28 °C and 200 rpm. Bacteria were collected by centrifugation, re-suspended in 50 cm^3 of half-strength B5 medium (Gamborg *et al*. 1968) with 22 mg dm^{-3} acetosyringone for 15 min to induce the *vir* gene. The induced *Agrobacterium* suspension was used for co-cultivation of embryo axis as well as callus.

Co-cultivation of embryo axis and plant regeneration: *Agrobacterium*-infected embryo axes slices were cultured on co-cultivated medium (I and II) supplemented with 22 mg dm^{-3} acetosyringone (As), incubated in the dark for 3 d, transferred to cefotaxime-selection medium (I and II, Table 2) and grew for 2 d at above mentioned conditions. The growing embryo axes were subjected to a second selection on the above medium for 4 additional days, and were sub-cultured on hygromycin selection medium (I and II) for further growth. After 2 weeks of the sub-culture, the clumps of multiple shoots were transferred to fresh hygromycin selection medium (I and II, Table 2) and allowed to grow further in the same medium for 2 - 3 weeks and then they were subjected to a third selection on fresh hygromycin selection medium (I and II) where they grew into 2 - 3 cm long green healthy putative transgenic shoots.

Co-cultivation of callus and plant regeneration: 3 to 4-week-old, dark-green calli were directly immersed in the bacterial suspension for 10 min and then excess bacterial suspension was removed from the explants by placing them on sterile filter paper. *Agrobacterium*-infected calli were cultured on co-cultivation medium for 2 d in dark at 25 ± 2 °C. Then calli were cultured on cefotaxime-selection medium at 25 ± 2 °C, 16-h photoperiod and irradiance of 37.5 μmol m^{-2} s^{-1} for 3 d. The growing explants were subjected to a second selection on the above medium for further 3 d. Then the putative transformed calli were transferred to hygromycin selection medium and allowed to grow for three weeks. Calli that produced shoot-like structures were sub-cultured on fresh medium with 20 mg dm^{-3} hygromycin and allowed to grow further. Green shoots (2 - 3 cm long) were separated from each other carefully and cultured on fresh hygromycin selection medium supplemented with 250 mg dm^{-3} cefotaxime and 30 mg dm^{-3} hygromycin and allowed to grow further in jam bottles.

Rooting of putative transgenic plants: Transgenic shoots (5 - 6 cm) were transferred to root induction medium (RIM) supplemented with 250 mg dm^{-3} cefotaxime and 10 mg dm^{-3} hygromycin (Tables 1 and 2). Shoots that produced roots were then carefully transferred to fresh RIM medium with higher concentration of hygromycin (20 mg dm^{-3}). The plants that survived for at least 20 d were sub-cultured on root proliferation medium (RPM) supplemented with 250 mg dm^{-3} cefotaxime and 30 mg dm^{-3} hygromycin (Tables 1 and 2) to achieve vigorous and strong root growth. Plants were allowed to grow in 30 mg dm^{-3} hygromycin for 2 - 3 weeks and subsequently were transferred to pots containing autoclaved *Vermiculite* as described below.

Hardening and transplantation of the rooted plants: The regenerated plantlets with vigorous roots were taken out carefully from the jam bottle and kept in liquid MS medium supplemented with 0.001 mg dm^{-3} IBA for half an hour to one hour in the culture room. Healthy plantlets with good rooting systems were transferred to small sized pots (plastic) containing autoclaved vermiculite and kept at the same conditions. The young plants were covered with transparent polythene bags to maintain high humidity for the first two weeks. Then the plants were carefully shifted to pots containing a mixture of soil:*Vermiculite* (50:50) and gradually exposed to the ambient conditions. Next, the plants were shifted to pots containing a mixture of soil:compost (50:50) and kept in the greenhouse for further growth. The plants were watered every second day with 10 - 25 cm^3 of water. Plants that rooted poorly were transferred to Hoagland solution for initiation of rooting following the method developed by Jayanand *et al*. (2003).

Gus assays: GUS activity was assayed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) following the method described by Jefferson *et al*. (1987). Histochemical assays were performed on 15 - 20 *Agrobacterium*-infected calli/embryo-axis slices per treatment, every 5 d after inoculation, to monitor transient GUS activity, and on calli/embryoaxis slices 28 d after inoculation with *Agrobacterium* to estimate stable transformation. Tissues of leaves, stems, floral organs of putative transgenic plants (T_0 generation) and from their seed-derived T_1 and T_2 plants were also assayed for GUS activity. Visual quantification was done to check the localization and intensity of blue staining in the tissue.

A comparative study was done on the effect of interaction of three different strains of *Agrobacterium* (LBA4404, AGL1 and EHA105) on the efficiency of transformation of four different chickpea genotypes.

Efficiency of the transformation was scored on the basis of growing the infected plant cells in selection medium with 30 mg dm^{-3} hygromycin followed by GUS staining of the hygromycin-resistant plant cells. Putative transformed plant tissues and cells were scored as GUS-negative (GUS–) if no blue-stained tissue was visible and were unable to grow in hygromycin, or as GUS positive (GUS+) if any blue spot or streak was present in the tissue. All experiments were carried out in a completely randomized design. Each experiment was replicated twice and each set within a replicate consisted of 20 explants. Data were analyzed statistically and standard error values calculated for each replicated set.

Genomic DNA PCR and Southern analysis of T_0 transgenic plants: Before transplanting, genomic DNA was isolated from 3 - 4 leaflets of 20 independent GUS positive T_0 chickpea transgenic lines and wild type plants following rapid DNA extraction method (Edwards *et al*. 1991). PCR was performed by using forward and reverse primers of *hpt* gene to confirm the presence of the gene in the transgenic lines. pCAMBIA 1301 was used as positive control. Wild type plant DNA was used as negative control. Primers used for amplification of *hpt* gene were Hph1F 5'-GAACTCACCGCGACG TCTGTCGAG-3' and Hph1R 5'-TCCGTCAGGACA TTGTTGGAGCCG-3'.

Southern hybridization analysis was only performed on PCR positive transgenic chickpea plants that established in the greenhouse. Genomic DNA was isolated from leaves of 10 randomly selected green and healthy T_0 transgenic plants and wild type plants of chickpea using the CTAB method (Dellaporta *et al*. 1983). For Southern analysis, to confirm the presence of *hpt* gene and to estimate the copy number of the transgene in the transgenic chickpea plants, 20 µg of genomic DNA were digested with *Xho*I (cuts at the ends of the *hpt* gene releasing a 1.1 kb fragment) and *Hin*dIII (cuts within the T-DNA but outside the *hpt* gene), respectively. Digested samples were electrophoresed on 0.8 % agarose gel, and transferred on to nylon membranes, as described by Sambrook *et al*. (1989). Blots were probed with [α^{32}P] dCTP-labelled 1.1 kb fragment with the *hpt* gene (obtained by digesting pCAMBIA1301 with *Xho*I) fragment. Hybridization and stringency washes were done at 65 °C. The blot was subsequently autoradiographed with X-ray films or using a phosphor imager (*Typhoon, GE Healthcare*, Piscataway, USA).

Northern analysis of T_0 transgenic plants: RNA was extracted using TRIZOL method according to the manufacturer's protocol (*Invitrogen*, Carlsbad, USA). Approximately 30 µg of total RNA was fractioned on 1 % formaldehyde-denaturating agarose gel and transferred to nylon membranes. Pre-hybridization, hybridization and washing were done according to the methods described by Sambrook *et al*. (1989).

Analysis of progeny: T_0 seeds of all the four genotypes (10 independent lines from each genotype) were surface sterilized and germinated on semi-solid half strength MS medium supplemented with hygromycin (20 mg dm^{-3}). Cultures were maintained under the same conditions as for plant regeneration. The hygromycin resistance of seedlings was scored 15 d after seed germination. Seedlings were scored for their antibiotic-resistance (green, healthy) or antibiotic-sensitivity (bleached, dead) to confirm 3:1 segregation in the progeny. Ten randomly selected independent T_1 transgenic lines were analyzed for GUS expression. The GUS-positive plants were PCR-analyzed to confirm the presence of *hpt* gene. Inheritance of the *hpt* gene was further confirmed by Southern analyses of T_1 and T_2 progeny of PCR positive transgenic plants (5 each of T_1 and T_2 that were growing in the greenhouse).

Hygromycin leaf assay: Hygromycin assay was performed with leaf pieces of T_1, T_2 and T_3 generation of transformed plants that was placed on MS basal medium supplemented with 20 mg dm^{-3} hygromycin (Wang and Waterhouse 1997). The leaf pieces were incubated in the light for 48 h before being scored for bleaching of chlorophyll.

Callus induction assay: Seeds harvested from T_0, T_1 and T_2 transgenic and non-transformed plants of all chickpea genotypes were dehusked and surface sterilized with 0.1 % $HgCl_2$ and washed thoroughly with sterile water. Sterilized seeds were plated on CIM medium, supplemented with 20 mg dm^{-3} hygromycin for callus induction. Cultures were maintained in the dark (27 ± 1 °C) and scored for the presence or absence of callusing after 20 d. Calli were histochemically assayed for GUS activity.

Results

Regeneration of putative transgenic shoot from *Agrobacterium*-infected calli and embryo axes: In the present study, rapid and efficient shoot regeneration was achieved from putative transformed callus obtained from both cotyledons and cotyledonary nodes of four different genotypes of chickpea. Growth of the infected dark green calli was continued on the initial culture medium supplemented with 22 mg dm^{-3} acetosyringone and 250 mg dm^{-3} cefotaxime. In 7 - 10 d, globular structures appeared on the upper surface of the callus and visible morphological changes were observed on the callus-selection medium (Fig. 1*A*). These structures developed into dark-green shoot-like structures (Fig. 1*B*) and eventually formed complete shoots in the hygromycin selection medium

with 10 mg dm^{-3} hygromycin (Table 1). These shoot-like structures developed into normal shoots (Fig. 1*E*) when sub-cultured on fresh hygromycin selection medium with higher concentration of hygromycin (20 and 30 mg dm^{-3}) (Table 1), and they formed healthy shoots (2 - 3 cm) after another 3 - 4 weeks (Fig. 1*F*).

Multiple shoots started appearing from the *Agrobacterium*-infected embryo-axis slices after 10 - 12 d of culture in hygromycin selection medium (I and II). Initially, small protuberances emerged on the epidermal cell layer at the basal portion of each embryo axis (Fig. 1*C*) on cefotaxime selection medium (I and II) and eventually these structures developed to be clump of shoots (Fig. 1*D*) on transfer to hygromycin selection medium (I and II) supplemented with 10 mg dm^{-3} hygromycin. With gradual increase in hygromycin concentration in fresh hygromycin selection medium (I and II), growth and elongation of the regenerated shoot continued to result in 2 - 3 cm long dark green shoots in about 30 d (Fig. 1*F*). Concentration of antibiotics was increased gradually to reduce the sudden antibiotic-shock to the regenerated shoot.

Inclusion of the synthetic phenolic compound acetosyringone (As) in the co-cultivation medium used either for callus or embryo axis transformation was essential for successful transformation (data not shown). The maximum transient expression of GUS (containing an intron) was enhanced in all the genotypes used here when As was added at a final concentration of 22 mg dm^{-3} to the co-cultivation medium.

Rooting of regenerated shoots and establishment of the shoots in the greenhouse: Once the shoots elongated to a reasonable length (4 - 5 cm), they were transferred to RIM medium supplemented with antibiotics. IBA proved to be critical for the vigorous rooting. Immediate transfer of the regenerated green and healthy transgenic shoots on to RIM increased the efficiency of rooting in all the genotypes. It was observed that roots developed best on shoots that had been separated by breaking the clumps of shoots at their natural point of weakness rather than pulling them apart by mechanical means. On transfer to RIM supplemented with 10 mg dm^{-3} hygromycin, shoot proliferation continued and root initiation started

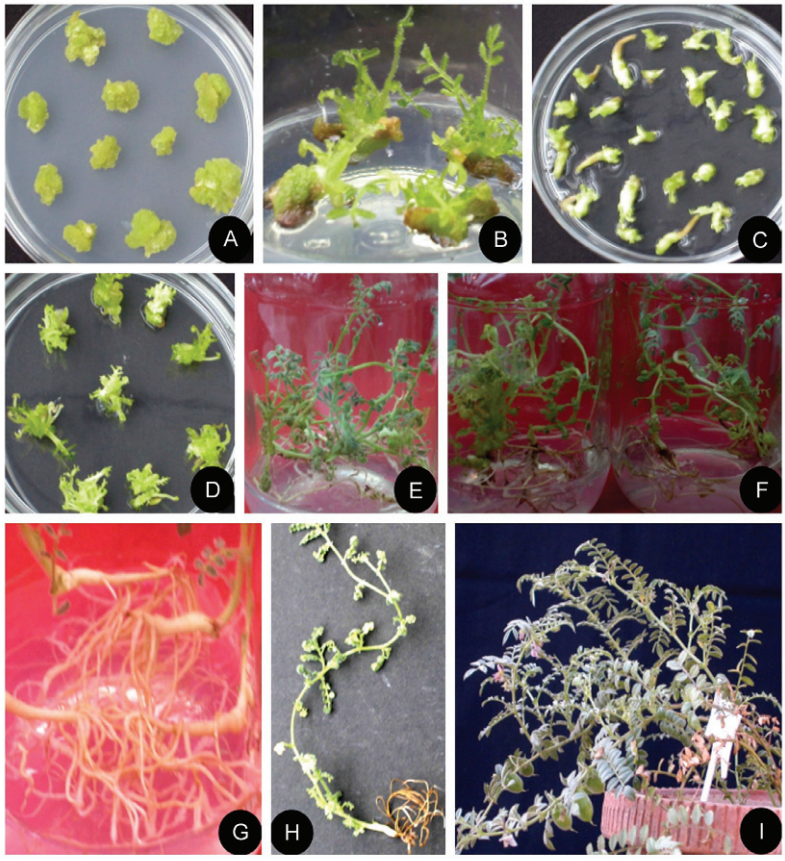

Fig. 1. Plant regeneration from chickpea after *Agrobacterium*-mediated transformation. *A* - *Agrobacterium*-infected calli growing in hygromycin selection medium (see Table 1). *B* - Plant regeneration from *Agrobacterium*-infected calli on hygromycin-selection medium. *C* - *Agrobacterium*-infected embryo axis after preliminary proliferation on hygromycin selection medium I (see Table 2). *D* - Vigorously growing multiple shoots on hygromycin selection medium I. *E* - Elongated shoot growing on hygromycin selection medium in jam bottle. *F* - Complete plantlet with roots on RIM medium (Table 1) in jam bottle. *G* - Vigorous rooting of transgenic plants on RPM medium (Table 1). *H* - Complete transgenic plant of chickpea from pot. I - Mature transgenic plants with pods.

soon. But in some cases it took as many as 3 changes of fresh RIM supplemented with IBA and higher concentration of hygromycin (20 - 30 mg dm^{-3}) in 15-d intervals to achieve good rooting. The differentiation of roots occurred at a period of 3 - 4 weeks in four different lines of chickpea (Fig. 1*F*). Randomly 10 healthy shoots were selected from each explant (cotyledon- and cotyledonary node-derived-callus and embryo axis) and genotype and were allowed to grow in RIM. On transfer to RPM the shoots from all genotypes achieved vigorous and strong root growth (Fig. 1*G*). It was observed that half-strength B5 vitamins in the RIM and reduced concentration of gelling agent (0.25 % agarose) was essential for obtaining vigorous root growth (Fig. 1*G*).

Hardening and transplanting of putative transgenic plants: Healthy plantlets with efficient rooting (Fig. 1*H*) on transfer to small sized plastic pots containing autoclaved *Vermiculite* in the tissue culture-room grew very well. At the initial stages of hardening, high humidity (90 %) was maintained and on subsequent transfer to soil:*Vermiculite* (50:50) they grew well in the greenhouse. The plants were then transferred to a mixture of soil:compost (50:50) in earthen pots and these plants exhibited normal growth in the greenhouse and produced morphologically normal flowers and pods with viable seeds (Fig. 1*I*). However, using hydroponics, we were unable to induce a rooting in any of the regenerated shoots of chickpea, and the transformed plant subsequently resulted in premature death.

Effect of genotype and source of explant on percent shoot regeneration and rooting of the transgenic shoot: Number of shoots regenerated and rooting of the regenerated shoots from the *Agrobacterium*-infected plant tissue was influenced by the source of genotype and choice of explants used for raising the transformed shoots, and that was irrespective of the *Agrobacterium* strain used for infection. A comparative study shows that transgenic plant regeneration percentage range of four different genotypes was 38.95 - 73.00, 23.67 - 59.07 and 13.45 - 55.23 % from cotyledonary nodes-derived callus, cotyledon-derived callus and embryo axis, respectively (Table 3). Putative transgenic calli that originated from the cotyledonary nodes of Pusa-256 and KWR-108 showed the best regeneration and rooting percentage compared with any other genotypes used for *Agrobacterium*-mediated transformation. About 40 % of the regenerated shoots of Pusa-256 showed root formation within 3 - 4 d and another 55 % produced roots by 7 d after transfer to fresh culture medium. The remaining 5 % did not produce any roots even after maintaining the shoots on fresh rooting medium for extended periods and eventually these shoots died (data not shown). The lowest regeneration and root production was observed in the regenerated shoots obtained from embryo-axes-derived multiple shoots of Pusa-1003 (Table 3). Since it was very hard to establish rooting in these plants, we maintained these shoots in hydroponic culture using Hoagland's solution. However, survival rate of the plants were poor and the few that did root took a longer period to establish on subsequent transfer to soil (data not shown).

Effect of cytokinin on regeneration of transformed shoots and subsequent rooting of these shoots: Choice of cytokinins in the initial culture medium was found to play an important role in complete plant regeneration from the calli as well as embryo axis after *Agrobacterium*-mediated transformation. Although multiple shoot induction was almost the same when *Agrobacterium*-infected embryo axis were cultured in

Table 3. Effect of genotype and three different sources of explants on transformation efficiency, regeneration percentage (on hygromycin selection medium), rooting of regenerated shoots (on RIM medium) and survival percentage of complete plants of four genotypes (Pusa-256, KWR-108, Pusa-1003 and local line) of chickpea after *Agrobacterium* mediated transformation. Means ± SE, $n = 10$ (cot. - cotyledon or cotyledonary).

Genotype	Target tissue	Transformation efficiency [%]	Plant regeneration [%]	Number of regenerated plants	Number of plants producing roots	Rooting [%]	Survival in soil [%]
Pusa-256	cot.-derived callus	19.35±0.43	56.78±0.19	78	9.34±0.45	93.40	80.67
	cot. node-derived callus	25.56±0.78	73.00±0.08	100	10.00±0.00	100.00	100.00
	embryo axis	13.68±0.56	55.23±0.67	65	7.61±0.09	76.10	34.67
KWR-108	cot.-derived callus	17.89±0.34	59.07±0.15	67	8.46±0.01	84.60	79.67
	cot. node-derived callus	23.45±0.45	63.12±0.89	95	10.00±0.00	100.00	100.00
	embryo axis	11.78±0.41	53.78±0.45	62	7.83±0.6	78.30	42.89
Pusa-1003	cot.-derived callus	11.67±0.98	23.67±0.21	35	5.52±0.0	55.20	61.83
	cot. node-derived callus	14.68±0.23	38.95±0.56	52	7.46±0.11	74.60	95.78
	embryo axis	9.34±0.01	13.45±0.24	24	4.03±0.12	40.30	22.89
Local line	cot.-derived callus	12.37±0.67	27.72±0.67	45	7.03±0.34	70.30	53.89
	cot. node-derived callus	16.56±0.23	43.45±0.98	87	10.00±0.00	100.00	86.34
	embryo axis	7.56±0.54	15.13±0.03	37	5.44±0.56	54.40	25.78

Table 4. Effect of genotype and two different selection media (hygromycin selection medium I and II) on shoot elongation, rooting percentage and survival in soil of plantlets derived from embryo axes of four different genotypes (Pusa-256, KWR-108, Pusa-1003 and local line) of chickpea after *Agrobacterium*-mediated transformation (total number of infected explants = 50, * - average of 3 experiments with 10 plants, ** - from 15 plants transferred to soil).

Genotype	Selection medium	Shoot induction [%]	Shoot elongation [%]	Number of plants producing roots*	Rooting [%]	Number of plants survived in soil**	Survival in soil [%]
Pusa-256	I	98.19±0.23	100.00±0.00	9.73±0.41	97.30	12	80.00
	II	82.00±0.01	54.98±0.56	3.61±0.78	36.10	2	13.33
KWR-108	I	85.78±0.27	100.00±0.00	7.89±0.23	78.90	8	53.34
	II	81.45±0.56	43.78±0.45	4.21±0.12	22.10	3	20.00
Pusa-1003	I	54.67±0.12	65.89±0.22	6.73±0.09	67.30	5	33.34
	II	43.08±0.12	10.89±0.23	1.20±0.67	12.00	1	6.67
Local line	I	59.02±0.03	58.23±0.34	4.86±0.45	48.60	6	40.00
	II	51.67±0.67	11.01±0.34	2.02±0.34	20.20	2	13.33

hygromycin selection medium (I and II), only 50 % of the shoots obtained from TDZ supplemented medium were able to elongate further on in hygromycin selection medium II and on subsequent transfer to RIM medium only 10 % of the shoots produced roots. Moreover, maintaining these shoots longer on either hygromycin selection medium II or RIM media (supplemented either with antibiotics or without antibiotics), resulted in yellowing of the shoots. Shoot tip damage was observed in these plants when maintained for a long time on the rooting medium with hygromycin (data not shown). On the other hand, multiple shoots obtained from BAP-supplemented-hygromycin-selection medium I grew fast and resulted in over 20 - 25 healthy and dark green shoots per explant on transfer to fresh hygromycin selection medium I (data not shown). A significant variation was observed in rooting and recovery of complete plants from the regenerated shoots obtained from two different hygromycin selection medium used. Rooting percentage was 12.00 - 36.10 and 48.60 - 97.30 % in hygromycin selection medium I and II, respectively, from different genotypes (Table 4). Number of complete plants recovered from hygromycin selection medium II containing TDZ and that survived in the soil was low and survival percentage 6.67 - 20.00 % (Table 4). However, number of plants recovered from hygromycin selection medium I supplemented with BAP was the highest and survival percentage was 33.34 - 80.00 % in the four different genotypes of chickpea (Table 4).

GUS expression and interaction of three different *Agrobacterium* strains with four genotypes of chickpea: Histochemical analysis (16-h incubation in X-Gluc) revealed GUS activity in leaves, stems and inflorescences of *Agrobacterium*-infected tissue/cells (data not shown). A comparative study was performed on interaction of three different *Agrobacterium* strains (AGL1, LBA4404 and EHA105) with four genotypes of chickpea (Pusa-256, KWR-108, Pusa-1003 and local line). Transformation efficiency and percentage of plant regeneration was determined by visual transient GUS expression of hygromycin resistant putative transgenic cells/tissue and PCR analysis of transgenic plants (Table 5). Transient GUS activity ranged from 0.11 to 25.56 % in three different sources of target tissue transformed with three different *Agrobacterium* strains (Table 5). Highest number of blue-stained tissue (GUS activity) was obtained from the putative transgenic tissue infected by LBA4404 strain (see Table 5). Interestingly, we also observed large variation in the frequency of GUS activity in the putative transgenic tissue from different sources of explant derived from the four genotypes. Maximum GUS activity was observed in cotyledonary-node derived putative transgenic calli of Pusa-256 (see Table 5) and highest number of transgenic plants was obtained when medium was supplemented with 30 mg dm^{-3} hygromycin. Minimum GUS activity was observed in putative transgenic embryo axis slices of Pusa-1003 and the number of transgenic plants recovered was also less (Table 5). GUS expression in the putative transgenic plants confirmed that there was no correlation between the duration of culture and level of transient expression of GUS in different chickpea genotypes (data not shown).

Molecular characterization of putatively transformed T_0 plants: Genomic DNAs from 20 independent putative transformed lines were amplified with *hpt* gene-specific primers. All of them showed an amplification of approx. 608 bp fragment of the *hpt* gene, which confirmed that these lines were transformants (Fig. 2). Genomic DNA digested with *Xho*I released a 1.1 kb fragment of *hpt* gene in all transgenic lines of chickpea used for Southern analyses (Fig. 3*A*). Northern blots of T_0 transgenic plants also showed a single 1.1 kb transcript in all the transgenic lines (Fig. 3*B*,*C*). Restriction of the genomic DNAs with *Hin*dIII exhibited presence of bands of molecular mass of different sizes (10 - 4 kb) indicating single and multiple insertions (Fig. 4) of the gene. However, plant No, 4, 5, 7 and 17 showed the same size of fragment after Southern hybridization.

Progeny analysis: T_0 seeds germinated on hygromycin-containing medium showed a 3:1 segregation pattern. A total of 39 lines showed a ratio of 3:1 (Hyg^R and Hyg^S, hygromycin-resistant and -susceptible) as was expected for integration of the transgene at a single locus (Table 6). Transmission of the transgene in subsequent progenies was investigated by following the inheritance of the GUS and *hpt* genes in the T_1 and T_2 generations. Leaves and stems of T_1 and T_2 plants showed high GUS expression in four different genotypes (data not shown), which confirmed that GUS activity was retained in the next generation as well. Southern blot analysis of genomic DNA isolated from of T_1 and T_2 transgenic plant Pusa-256 (plant No. 4 in Fig. 3*A*) digested with *Xho*I showed a 1.1 kb fragment of *hpt* gene confirming inheritance of *hpt* gene in the progeny (Fig. 5).

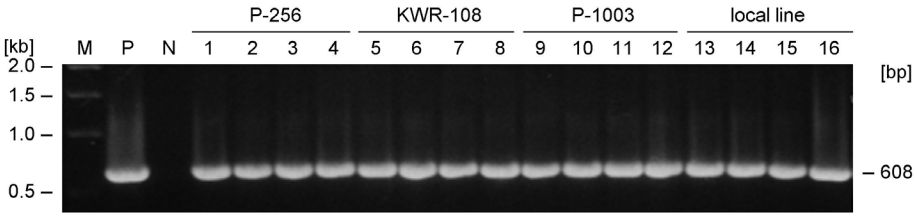

Fig. 2. PCR analysis of transgenic plants (T_0) obtained from four different genotypes of chickpea Pusa-256 (P-256), KWR-108, Pusa-1003 (P-1003) and local line. A 608 bp *hpt* gene fragment was amplified using gene-specific primers (see Materials and methods). Figures on the left indicate molecular mass. P - positive control, N - negative control.

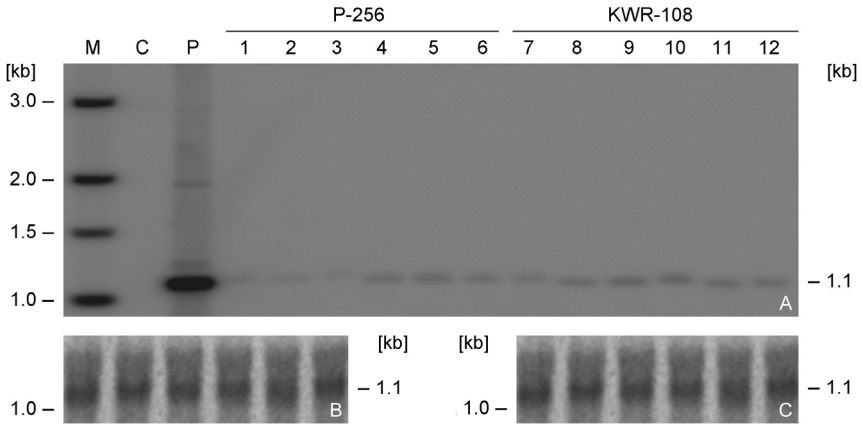

Fig. 3. Molecular analysis of transgenic plants (T_0) of chickpea. *A* - Southern analysis of *Xho*I-digested DNA from transgenic plants, obtained from two different genotypes of chickpea Pusa-256 and KWR-108, and probed with the 1.1 kb fragment (with the *hpt* gene), obtained after *Xho*I digestion of pCAMBIA 1301, confirmed the presence of *hpt* gene in all the plants tested. C - negative control, P - pCAMBIA 1301 restricted with *Xho*I. *B* and *C* - Northern analysis of transgenic plants (T_0) obtained from chickpea genotypes Pusa-256 (*B*) and KWR-108 (*C*) showing a 1.1 kb *hpt* transcript. Probe was prepared as in *A*. Figures on the left of each panel indicate molecular masses.

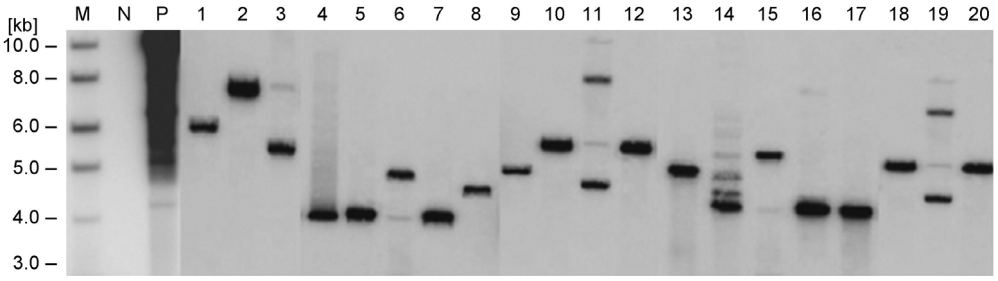

Fig. 4. Southern analysis of DNA of transgenic plants (T_0), obtained from one genotype of chickpea Pusa-256, restricted with *Hin*dIII showing single and multiple insertion of *hpt* gene. Probe prepared as given in figure legend 3*A*. Figures on the left indicate molecular mass. N - negative control, P - positive control, pCAMBIA1301 restricted with *Hin*dIII.

Hygromycin leaf assay: Leaves obtained from putative transgenic plants growing in the greenhouse did not show any bleaching when kept for hygromycin assay. However, control leaf was completely bleached when cultured on hygromycin (20 mg dm^{-3}) plate (data not shown).

Table 5. Effect of different *Agrobacterium* strains and explant type on transformation efficiency and transgenic shoot regeneration in four genotypes (Pusa-256, KWR-108, Pusa-1003 and local line) of chickpea. Means ± SE, $n = 40$.

Genotype	Explant	Transformation efficiency [%]			Shoot regeneration [%]		
		LBA4404	EHA105	AGL1	LBA4404	EHA105	AGL1
Pusa-256	cotyledon	19.35±0.43	9.02±0.23	2.42±0.06	56.78±0.19	12.78±0.65	7.89±0.45
	cotyledonary node	25.56±0.78	11.63±0.46	4.31±0.23	73.00±0.08	17.89±1.23	9.23±0.34
	embryo axis	13.68±0.56	4.46±0.39	1.02±0.27	55.23±0.67	10.03±0.67	6.56±0.38
KWR-108	cotyledon	17.89±0.34	5.43±0.21	1.02±0.36	59.07±0.15	13.76±0.56	8.90±0.45
	cotyledonary node	23.45±0.45	7.63±0.12	5.61±0.48	63.12±0.89	19.45±0.34	11.78±0.56
	embryo axis	11.78±0.41	2.12±0.02	0.99±0.23	53.78±0.45	8.98±0.56	7.89±0.34
Pusa-1003	cotyledon	11.67±0.98	3.12±0.56	0.23±0.98	23.67±0.21	5.56±0.23	3.78±0.12
	cotyledonary node	14.68±0.23	4.61±0.92	0.98±0.20	38.95±0.56	7.45±0.11	7.67±0.32
	embryo axis	9.34±0.01	1.02±0.48	0.11±0.42	13.45±0.24	2.89±0.23	5.61±0.12
Local line	cotyledon	12.37±0.67	3.04±0.12	0.98±0.23	27.72±0.67	3.78±0.54	3.78±0.67
	cotyledonary node	16.56±0.23	5.69±0.56	2.63±0.12	43.45±0.98	6.78±0.45	5.65±0.23
	embryo axis	7.56±0.54	1.02±0.91	0.43±0.21	15.13±0.03	2.34±0.98	4.56±0.34

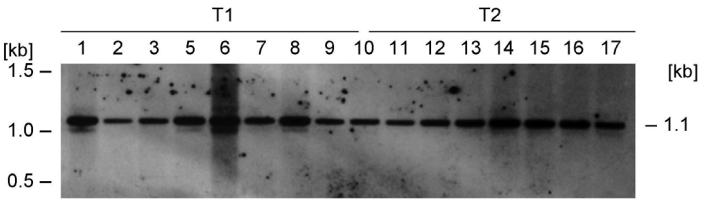

Fig. 5. Southern analysis of *Xho*I-digested DNA of transgenic plants (T_1 and T_2), obtained from chickpea genotype Pusa-256 (Plant No. 4 in Fig. 3*A*), showing a 1.1 kb fragment confirming the presence of *hpt* gene. Probe prepared as given in figure legend 3*A*. Figures on the left indicate molecular mass.

Callus and multiple shoot induction assay: Callus and multiple shoot induction in selection medium were used as an additional test to confirm the transgenic status of the T_1 and T_2 plants (data not shown). Hygromycin resistant calli obtained from T_1 and T_2 seeds and the embryo axes derived from germinated T_1 and T_2 seeds showed dark blue histochemical staining (data not shown). Some seeds failed to germinate and others germinated but did not produce either callus of embryo axes (data not shown). Results that were obtained from the callus induction assay correlated well with the data obtained from segregation analysis of hygromycin resistance progeny derived from T_0 seeds (see Table 6).

Discussion

A large number of transgenic chickpea plants were recovered after *Agrobacterium*-mediated transformation of calli as well as embryo axes of four different genotypes of chickpea. From the present study it is evident that various factors such as choice of genotypes, suitability of explants (target plant cell), efficient interaction of *Agrobacterium* with the target plant cell and a reproducible complete plant regeneration protocol are crucial for successful transformation of chickpea. In addition, age of explants and culture duration of the explants in antibiotic selection medium also play important role in part in successful *Agrobacterium*-mediated transformation.

For grain legumes that have been regarded as recalcitrant to transformation, regeneration *in vitro* is highly genotype-specific and only a few of the genotypes are amenable to regeneration after *Agrobacterium*-mediated transformation. Regeneration and the number of transformed plant generated from each explants is often low and it is also previously reported that regeneration potential of transgenic plant is influenced by various factors, such as, origin and age of explant, culture media composition and the *Agrobacterium* strain used for transformation (Senthil *et al*. 2004, Sharma *et al*. 1990). Our results showed that immature-seed-derived explants provided the best target tissue for *Agrobacterium*-mediated-transformation. Differentiation of shoots is greatly affected by age of the target tissue/plant cell used for transformation. Three- to 4-week-old calli and excised embryo-axes from 3 to 4-d-old germinated immature seeds exhibited great regeneration potential, which subsequently declines with increasing age of the target cells. We have clearly demonstrated that tissues which respond well in tissue culture, also tended to respond well

Table 6. Segregation analysis of four genotypes (Pusa-256, KWR-108, Pusa-1003 and local line) of chickpea with respect to hygromycin resistance (R) or susceptibility (S). Expected R/S ratio was 3:1.

Genotype		T_1 seed number	Hygromycin R	S	χ^2	*P*
Pusa-256	1	65	48	16	0.00	1.00
	2	60	45	15	0.00	1.00
	3	46	35	11	0.03	0.90-0.75
	4	70	51	17	0.00	1.00
	5	50	36	12	0.00	1.00
KWR-108	1	60	44	15	0.01	0.95-0.90
	2	50	37	12	0.01	0.95-0.90
	3	65	48	15	0.05	0.90-0.75
	4	60	46	14	0.09	0.90-0.75
	5	61	47	14	0.14	0.75-0.50
Pusa-1003	1	50	36	12	0.00	1.00-0.99
	2	30	21	7	0.00	1.00-0.99
	3	40	29	9	0.04	0.90-0.75
	4	40	30	9	0.08	0.90-0.75
	5	30	21	4	1.08	0.50-0.25
Local line	1	35	25	8	0.01	0.95-0.90
	2	48	36	12	0.00	1.00
	3	45	34	11	0.01	0.95-0.90
	4	35	26	9	0.01	0.95-0.90
	5	40	27	8	0.09	0.90-0.75

with reference to transformation and subsequent regeneration of transgenic plants. Our results indicate that in the better responsive genotypes (such as Pusa-256 and KWR-108), it was possible to use any source of explant, including callus and embryo-axis, for a high-efficiency transformation yielding the highest recovery of transgenic plants, further proving that transformation of chickpea is highly genotype dependent.

In most of the previous reports on *Agrobacterium*-mediated transformation of chickpea, either embryo axes or slices of embryo axes were used as the target tissue (Fontana *et al*. 1993, Kar *et al*. 1996, 1997, Krishnamurthy *et al*. 2000, Polowick *et al*. 2004, Senthil *et al*. 2004) and two of the recent reports were based on the decapitated embryo explant attached with one half of the cotyledon (Sarmah *et al*. 2004, Tewari-Singh *et al*. 2004). However, in the present study, one-month-old, dark-green cotyledonary-node-derived calli showed the highest transformation efficiency and was found to be the best source of explant for regeneration of complete plant from all the four genotypes compared to any other source of explants used. When using embryo axes or slices of embryo axis as the target tissue for transformation, efficiency of transformation as well as recovery of their rooted transgenic plants were less than those of the cotyledon- and cotyledonary-node-derived calli in all the genotypes (Table 3). In addition, establishment of rooting in the transgenic shoots in antibiotics containing medium was found to be very laborious. Using the other source *i.e.* decapitated embryo explants attached with one half of the cotyledon (Jayanand *et al*. 2003, Sarmah *et al*. 2004, Tewari-Singh *et al*. 2004) produced a lot of false transgenic shoots after *Agrobacterium*-mediated transformation that drastically reduced the transformation efficiency.

Cytokinins in general are required to induce regeneration from cultured tissue. In the present study, BAP + NAA combination in the CIM medium produced efficient callus from cotyledon as well as cotyledonary node of all the genotypes. TDZ has been effectively used for multiple shoots induction from embryo axis (Jayanand *et al*. 2003, Krishnamurthy *et al*. 2000, Senthil *et al*. 2004, Singh *et al*. 2002) and is also reported to be more active then other cytokinins such as BAP and kinetin when tested for grain legumes such as chickpea (Jayanand *et al*. 2003, Malik and Saxena 1992, Senthil *et al*. 2004) and other plants (Mok *et al*. 1987). In contrast to the previous report, we observed BAP and NAA combination in the culture medium (hygromycin selection medium I) was the best for multiple shoot induction and shoot elongation from the embryo axis (Table 4) after *Agrobacterium*-mediated transformation (Fig 1*C*). There was not much variation in efficiency of multiple shoot induction from embryo axes obtained either from hygromycin selection medium I or II. However, shoots obtained from hygromycin selection medium I, proliferated (Fig. 1*D*) and elongated faster in contrast to ones on hygromycin selection medium II and these shoots rooted vigorously in the presence of hygromycin in RIM medium and the plants easily established in the greenhouse. We are unable to achieve efficient rooting in the transformed shoots that originated from hygromycin selection medium II in any of the genotypes used.

Other alternative methods for rooting used by some laboratories include *in vitro* grafting by utilizing scions from pre-existing seedlings (Krishnamurthy *et al*. 2000, Senthil *et al*. 2004, Singh *et al*. 2002) and hydroponics cultures (Jayanand *et al*. 2003). We find that such methods are very much technique-specific, laborious, time-consuming and sometime the success rate of such technology varies among different laboratories (Jayanand *et al*. 2003). Although in previous reports it is stated that BAP alone or in a combination with another cytokinin or auxin was successfully used for regeneration and transformation of legumes (Dayal *et al*. 2003, Polowick *et al*. 2004, Saini *et al*. 2003, Satyavathi *et al*. 2002, Thu *et al*. 2003), the success rate varied among different legumes. Besides, in some cases the method used was too lengthy and the initial stages of transformation were very laborious (Polowick *et al*. 2004).

Presence of acetosyringone in the culture medium was essential for maximum transgenic expression in the genotypes assessed and this observation is supported by a recent study done by Polowick *et al*. (2004) in chickpea.

In most of the earlier report on chickpea transformation, selection of transformed plant tissue was done using either kanamycin or phosphonothricin (PPT) as a selective agent. However, in the present study, presence of 30 mg dm^{-3} hygromycin in the culture media

is found to be beneficial for recovery of the highest number of transgenic shoots of chickpea after *Agrobacterium*-transformation, besides resulting in 100 % reduction in the chances of getting any false transgenic shoots. Lower concentration of antibiotics in the initial culture medium was found to be beneficial for the plant cells to get established in the medium. However, according to Polowick *et al*. (2004), transgenic chickpea plants are able to survive at 150 mg dm^{-3} kanamycin, the frequency of recovery of transgenic plants was 3.1 %, and the duration of tissue culture phase for obtaining transgenic plants reported was 133 -384 d. We observed that, increasing the duration of tissue culture period resulted in abnormalities such as shoot tip damage, yellowing of leaves of transgenic shoots and at a later stage, when these plants were shifted to greenhouse, height of the plant was less compared with normal seed-derived plants; the number of pods/transgenic plant was also very low (only 5 or 6 per plant) compared with normal seed-derived plants. A gradual increase in hygromycin from 10 to 30 mg dm^{-3} in the rooting medium did not interfere with the initiation of rooting and subsequent growth of the plant.

Efficient interaction of bacterium with the target plant cell used for transformation was also found to play a major role in transformation (Cho *et al*. 2008, Kant *et al*. 2007, Tiwari *et al*. 2008). *Agrobacterium* strain, LBA4404 carrying the binary vector pCAMBIA1301 was the most effective in transforming any line of chickpea used here (Table 5). In contrast, other *Agrobacterium* strains EHA105 and AGL1 could not enhance efficiency of chickpea transformation confirmed by variation in the frequencies of GUS blue-staining observed in the *Agrobacterium*-infected plant cells after co-cultivation with four genotypes (Table 5). Interestingly, a similar observation was also made following inoculation of embryo axes slices of chickpea with different strains of *Agrobacterium* (Senthil *et al*. 2004).

We also observed that efficiency of GUS expression was higher in both infected calli compared with that of embryo axis irrespective of the *Agrobacterium* strains used for transformation thereby showing a specific interaction of *Agrobacterium* strain with the source of explants i.e. the target plant cell. Over 99 % of the GUS positive transgenic lines obtained from different genotypes was *hpt* positive (confirmed by PCR and the Southern blot) in the T_0 generation (Figs. 2 and 3*A*) and produced T_1 seeds from each line of chickpea.

In conclusion, the protocol reported here for *Agrobacterium*-mediated transformation of chickpea is very efficient for high frequency adventitious shoot regeneration in four different genotypes of chickpea from two source explants (callus and embryo axis) and importantly whole plant regeneration was obtained in less than 100 d. Using cotyledonary-node derived callus devoid of pre-existing meristem as target tissue for *Agrobacterium*-mediated transformation we achieved the highest transformation efficiency that resulted in large number of transgenic plant production from four different genotypes of chickpea than ever reported before. The present protocol also describes an efficient and reproducible method for vigorous rooting of the transgenic plants. In the present study, due to a gradual increase in hygromycin concentration in the culture medium, we were able to eliminate most of the non-transformed shoots and in practical terms that resulted in very high efficiency of transformation and regeneration of transformed shoots.

References

Chilton, M.-D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P., Nester, E.W.: *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. - Proc. nat. Acad. Sci. USA **71**: 3672-3676, 1974.

Cho, M.-A., Moon, C.-Y., Liu, J.-R., Choi, P.-S.: *Agrobacterium*-mediated transformation in *Citrullus lanatus*. - Biol. Plant. **52**: 365-369, 2008.

Dayal, S., Lavanya, M., Devi, P., Sharma, K.K.: An efficient protocol for shoot regeneration and genetic transformation of pigeon pea [*Cajanus cajan* (L.) Millsp.] using leaf explants. - Plant Cell Rep. **21**: 1072-1079, 2003.

Dellaporta, S.L., Wood, J., Hicks, J.B.: A plant DNA mini preparation: version II. - Plant mol. Biol. Rep. **1**: 19-21, 1983.

Edwards, K., Johnstone, C., Thompson, C.: A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. - Nucl. Acids Res. **19**: 1349, 1991.

Fontana, G.S., Santini, L., Caretto, S., Frugis, G., Mariotti, D.: Genetic transformation in the grain legume *Cicer arietinum* L (chickpea). - Plant Cell Rep. **12**: 194-198, 1993.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., Schilperoort, R.A.: A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. - Nature **303**:179-180, 1983.

Hood, E.E., Fraley, R.T., Chilton, M-D.: The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBO542 outside of the T-DNA. - J. Bacteriol. **168**: 1291-1301, 1986.

Huda, S., Islam, R., Bari, M.A., Asaduzzaman, M.: Shoot differentiation from cotyledon derived callus of chickpea (*Cicer arietinum* L.). - Plant Tissue Cult. **13**: 53-59, 2003.

Jayanand, B., Sudarsanam, G., Sharma, K.K.: An efficient protocol for the regeneration of whole plants of chickpea (*Cicer arietinum* L.) by using axilary meristem explants derived from *in vitro*-germinated seeds. - *In Vitro* cell. dev. Biol. Plant **39**: 171-179, 2003.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W.: GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. - EMBO J. **6**: 3901-3907, 1987.

Kant, P., Kant, S., Jain, R.K., Chaudhury, V.K.: *Agrobacterium*-mediated high frequency transformation in dwarf recalcitrant rice cultivars. - Biol. Plant. **51**: 61-68, 2007.

Kar, S., Basu, D., Das, S., Ramakrishnan, N.A., Mukherjee, P., Nayak, P., Sen, S.K.: Expression of *Cry 1A(c)* gene of *Bacillus thuringiensis* in transgenic chickpea plants inhibits development of pod borer (*Heliothis armigera*) larvae. - Transgenic Res. **6**: 177-185, 1997.

Kar, S., Johnson, T.M., Nayak, P., Sen, S.K.: Efficient transgenic plant regeneration through *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L). - Plant Cell Rep. **16**: 32-37, 1996.

Krishnamurthy, K.V., Suhasini, K., Sagare, A.P., Meixner, M., Kathen, A. De Pickardt, T., Schieder, O.: *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) embryo axes. - Plant Cell Rep. **19**: 235-240, 2000.

Lazo, G.R., Stein, P.A., Ludwig, R.A.: A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. - BioTechnology **9**: 963-967, 1991.

Malik, K.A., Saxena, P.K.: Thidiazuron induces high frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicer arietinum*), and lentil (*Lens culinaris*). - Aust. J. Plant Physiol. **19**: 731-740, 1992.

Mok, M.C., Mok, D.W.S., Turner, J.E.: Biological and biochemical effects of cytokinin-active phenylurea derivatives in tissue culture systems. - HortScience **22**: 1194-1197, 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Olhoft, P.M., Flagel, L.E., Donovan, C.M., Somers, D.A.: Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. - Planta **216**: 723-735, 2003.

Polowick, P.L., Baliski, D.S., Mahon, J.D.: *Agrobacterium tumefaciens*-mediated transformation of chickpea (*Cicer arietinum* L.): gene integration, expression and inheritance. - Plant Cell Rep. **23**: 485-491, 2004.

Polowick, P.L., Quandt, J., Mahon, J.D.: The ability of pea transformation technology to transfer genes into peas adapted to Western Canadian growing conditions. - Plant Sci. **153**: 161-170, 2000.

Rohini, V.K., Rao, K.S.: Transformation of peanut (*Arachis hypogaea* L.): a non-tissue culture based approach for generating transgenic plants. - Plant Sci. **150**: 41-49, 2000.

Sagare, A.P., Suhasini, K., Krishnamurthy, K.V.: Plant regeneration *via* somatic embryogenesis in chickpea (*Cicer arietinum* L.). - Plant Cell Rep. **12**: 652-655, 1993.

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

Saini, R., Jaiwal, S., Jaiwal, P.K.: Stable genetic transformation of *Vigna mungo* L. Hepper *via Agrobacterium tumefaciens*. - Plant Cell Rep. **21**: 701-705, 2003.

Sambrook, J., Fritsch, E.F., Maniatis, T.: Molecular cloning: A Laboratory Manual. 2nd Ed. Vol. 3. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989.

Sarmah, B.K., Moore, A., Tate, W., Molvig, L., Morton, R.L., Rees, D.P., Chiaiese, P., Chrispeels, M.J., Tabe, L.M., Higgins, T.J.V.: Transgenic chickpea plants expressing high levels of a bean α-amylase inhibitor. - Mol. Breed. **14**: 73-82, 2004.

Satyavati, V.V., Prasad, V., Khandelwal, A., Shailia, M.S., Lakshmi Sita, G.: Expression of hemagglutinin protein of Rinderpest virus in transgenic pigeon pea [*Cajanus cajan* (L.) Millsp] plants. - Plant Cell Rep. **21**: 651-658, 2002.

Senthil, G., Williamson, B., Dinkins, R.D., Ramsay, G.: An efficient transformation system for chickpea (*Cicer areitinum* L.). - Plant Cell Rep. **23**: 297-303, 2004.

Sharma, K.K., Anjaiah, V.: An efficient method for the production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens*-mediated genetic transformation. - Plant Sci. **159**: 7-19, 2000.

Sharma, K.K., Bhojwani, S.S., Thorpe, T.A.: Factors effecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. - Plant Sci. **66**: 247-253, 1990.

Singh, R., Jat, R.S., Sahoo, P.D., Srinivasan: Thidiazuron induced multiple shoot formation in chickpea (*Cicer arietinum* L.). - J. Plant Biochem. Biotechnol. **11**: 129-131, 2002.

Somers, D.A., Samac, D.A., Olhoft, P.M.: Recent advances in legume transformation. - Plant Physiol. **131**: 892-899, 2003.

Tewari-Singh, N., Sen, J., Kiesecker, H., Reddy, V.S., Jacobsen, H.J., Guha-Mukherjee, S.: Use of a herbicide or lysine plus threonine for non-antibiotic selection of transgenic chickpea. - Plant Cell Rep. **22**: 576-583, 2004.

Tiwari, R.K., Trivedi, M., Guang, Z.-C., Guo, G.-Q., Zheng, G.-C.: *Agrobacterium rhizogenes* mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots. - Biol. Plant. **52**: 26-35, 2008.

Thu, T.T., Mai, T.T.X., Dewaele, E., Farsi, S., Tadesse, Y., Angenon, G., Jacobs, M.: *In vitro* regeneration and transformation of pigeonpea [*Cajanus cajan* (L.) Millsp]. - Mol. Breed. **11**: 159-168, 2003.

Wang, M., Waterhouse, P.M.: A rapid and simple method of assaying plants transformed with hygromycin or PPT resistance gene. - Plant mol. Biol. Rep. **15**: 209-215, 1997.

Zhang J., Xing A., Staswick P., Clemente T.E. The use of glufosinate as selective agent in *Agrobacterium*-mediated transformation of soybean. - Plant Cell Tissue Organ Cult. **56**: 37-46, 1999.