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Research Article

Optimization of different factors for an *Agrobacterium*-mediated genetic transformation system using embryo axis explants of chickpea (*Cicer arietinum* L.)

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Abstract In this study, we developed a reliable and efficient *Agrobacterium*-mediated genetic transformation system by applying sonication and vacuum infiltration to six chickpea cultivars (ICCV2, ICCV10, ICCV92944, ICCV37, JAKI9218, and JG11) using embryo axis explants. Wounded explants were precultured for 3 days in shoot induction medium (SIM) before sonication and vacuum infiltration with an *Agrobacterium* suspension and co-cultivated for 3 days in co-cultivation medium containing 100 μM/l of acetosyringone and 200 mg/l of L-cysteine. Responsive explants with putatively transformed shoots were selected using a gradual increase in kanamycin from 25 mg/l to 100 mg/l in selection medium to eliminate escapes. Results showed optimal transformation efficiency at a bacterial density of 1.0, an optical density at 600 nm wavelength (OD_{600}), and an infection duration of 30 min. The presence and stable integration of the β-glucuronidase (*gusA*) gene into the chickpea genome were confirmed using GUS histochemical assay and polymerase chain reaction. A high transformation efficiency was achieved among the different factors tested using embryo axis explants of cv. JAKI 9218. Of the six chickpea cultivars tested, JAKI9218 showed the highest transformation efficiency of 8.6%, followed by JG11 (7.2%), ICCV92944 (6.8%), ICCV37 (5.4%), ICCV2 (4.8%), and ICCV10 (4.6%). These findings showed that the *Agrobacterium*-mediated genetic transformation system will help transfer novel candidate genes into chickpea.

Keywords *Agrobacterium*, chickpea, embryo axis, sonication, transformation efficiency, vacuum infiltration

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Introduction

Chickpea is one of the most significant dietary sources of protein for the vegetarian population of India (Gaur et al. 2014; Jacob et al. 2016). Chickpea is considered the third most crucial edible grain legume, with about 17.2 million metric tonnes production worldwide in 2018 (FAO 2020). It is cultivated in more than fifty countries, covering all parts of the globe (Muehlbauer and Sarker 2017). India is the prominent producer of chickpea, with around 66% of global production with 11.38 million tonnes per annum (FAO 2020). Chickpea production is severely impeded by several biotic and abiotic stresses, notably fungal diseases like fusarium wilt (caused by *Fusarium oxysporum* f. sp. *ciceri*), Aschochyta blight [caused by *Ascochyta rabiei* (Pass.) Labr.], and insect pests predominantly pod borer (*Helicoverpa armigera*) (Nene et al. 2012).

The development of biotic (fungal and insect pest) stress-resistant chickpea varieties using conventional breeding methods is not feasible due to constricted genetic variability in its germplasm (Gatti et al. 2016) and the existence of strong sexual incompatibility for genetic hybridization in chickpea (van Rheenen et al. 1993). Therefore, introducing biotic stress-resistant candidate genes into chickpea through genetic engineering could be a good choice for developing transgenic plants. A proficient and

repeatable plant regeneration protocol amenable to gene transfer must produce transgenic chickpea plants (Leonetti et al. 2018; Pandey et al. 2021; Yadav et al. 2017). In general, chickpea is considered one of the highly recalcitrant species of legumes to manipulate through plant regeneration and genetic engineering (Atif et al. 2013). This problem could also be because of the unavailability of regenerable target tissue for efficient delivery and integration of transgene from *Agrobacterium*. The success of transformation in chickpea depends upon several factors like explant type, age of target tissue, genotype, enzymatic browning, tissue necrosis, and death of target tissue due to wounding and/or plant-pathogen defense response (Yadav et al. 2017).

Due to genotype specificity in chickpea, *Agrobacterium*-mediated transformation methods were applied only for a few genotypes (Atif et al. 2013; Leonetti et al. 2018). The explant with meristematic cells are frequently targeted in chickpea transformation like embryo axis (Ignacimuthu and Prakash 2006), decapitated embryo axis (Pathak and Hamzah 2008), embryo axis slices (Kaur and Chawla 2014), axillary meristem (Das et al. 2017; Srivastava et al. 2017), cotyledonary node (Pandey et al. 2021; Yadav et al. 2017), and half-embryo with cotyledon (Chakraborty et al. 2016; Das Bhowmik et al. 2019). Many successful transformation protocols have been reported from different explants of chickpea (Leonetti et al. 2018). Most of those reports showed the different transformation efficiencies (Atif et al. 2013), the poor rooting ability of putative transgenic shoots, and recovery of transgenic plants (Das et al. 2017). Considerable efforts have also been made to improve the transformation frequencies in chickpea by applying various parameters such as identification and selection of responsive genotypes (Tripathi et al. 2013), improving selection and plant regeneration (Das Bhowmik et al. 2019), precondition of explants, cocultivation duration, bacterial cell density and infection time (Srivastava et al. 2017; Tripathi et al. 2013), an addition of amino acids to cocultivation medium (Das et al. 2017; Srivastava et al. 2017), different gene constructs (Das Bhowmik et al. 2019; Yadav et al. 2017), addition acetosyringone to induce the expression of *vir* genes (Srivastava et al. 2017), sonication and vacuum infiltration treatments (Pandey et al. 2021; Srivastava et al. 2017), etc.

Therefore, the present study was undertaken to explore the conditions influencing *Agrobacterium*-mediated chickpea transformation. The experiments were designed to determine various factors such as suitable genotype, kanamycin concentration for selection transformants, pricking (wounding), the preculture duration, bacterial cell density and infection time, cocultivation duration, acetosyringone concentration, L-cysteine concentration, sonication, and vacuum infiltration treatments on chickpea genetic transformation with *A. tumefaciens* strain LBA4404 harbouring pCAMBIA2301 (*nptII* and *gusA* genes) using the embryo axis explants.

Materials and Methods

Seed source, seed sterilization, and explant preparation

Chickpea genotypes JAKI9218, JG11, ICCC37, ICCV10, and ICCV92944 (JG14) (desi) and ICCV2 (Swetha) (Kabuli) seeds were procured from Germplasm Resource Unit, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, Telangana State, India. Uniform and healthy seeds of the JAKI9218 genotype were chosen for explant preparation. The seeds were sterilized with 70% (v/v) ethanol for 2 min, followed by 0.1% mercuric chloride ($HgCl_2$) for 10 min. The sterilized seeds were rinsed with sterile distilled water 5 to 6 times. The sterile seeds were soaked overnight in water, and the seeds were blotted dry. Then the seed coat was removed and germinated on medium amended with 1.0 mg l^{-1} BAP (15 per plate) at 24 ± 2°C in dark condition for one day. The embryo axis explants were prepared by removing radicle (root apex), shoot (plumule), and cotyledons. These embryo axes were used as explants for transformation experiments (Pathak and Hamzah 2008; Sadhu et al. 2020).

Culture media and conditions

Decapitate embryo axis explants were inoculated on MSB5 medium augmented with 6-Benzyl amino purine (BAP) (2.0 mg l^{-1}) and Indole-3-butyric acid (IBA) (0.05 mg l^{-1}) designated as shoot induction medium (SIM) (Sadhu et al. 2020). Shoot induction medium (SIM) added with different kanamycin concentrations (25, 50, 75, and 100 mg l^{-1}) and 300 mg l^{-1} cefotaxime designated as selection medium. Responsive explants with a bunch shoots were inoculated onto shoot elongation medium (SEM) amended with BAP (1.0 mg l^{-1}), Indole-3-acetic acid (IAA) (0.05 mg l^{-1}), Gibberellic acid (GA_3) (1.0 mg l^{-1}), 50 mg l^{-1} kanamycin and 100 mg l^{-1} cefotaxime. Putative transgenic shoots (~3.0 cm length) were transferred into the root induction medium (RIM) fortified with 2.0 mg l^{-1} IBA, 50 mg l^{-1} kanamycin, and 100 mg l^{-1} cefotaxime. The pH of the media was adjusted to 5.6 using 0.1N NaOH /0.1N HCl,

then gelled with 0.8% agar before autoclaving at 121°C for 20 min. All cultures were maintained at 24 ± 2°C with a 16/8 h photoperiod using cool fluorescent lights (50 μMol m^2 S^{-1}).

The sensitivity of embryo axis explants to kanamycin

An initial study was conducted to check the influence of kanamycin (selection agent) on decapitated embryo axis explants were cultured in SIM along with different concentrations (0, 25, 50, 75, 100, and 150 mg l^{-1}) of kanamycin. The explants were subcultured three times into a fresh SIM medium with respective concentrations of kanamycin (at ten days intervals) after ten days of subculture. Kanamycin (50 mg/ml) stocks were prepared; filter-sterilized (0.22 μm) and stored as aliquots at -20°C. The aliquots were added to the warm autoclaved medium to obtain the needed concentration of kanamycin. A control without kanamycin was also maintained for the explants.

Agrobacterium strain, plasmid vector and culture preparation

Agrobacterium tumefaciens strain LBA4404 harbouring the binary vector pCAMBIA2301 was employed to evaluate various parameters. The plasmid vector contains both selectable and reporter (*nptII* and *uidA*) genes under the control of CaMV35S promoter and CaMV35S poly-A and nos terminators. A colony of *Agrobacterium* was streaked on LB medium fortified with kanamycin (50 mg l^{-1}) and rifampicin (10 mg l^{-1}) gelled with 0.8 % Difco-bacto agar. A loopful *Agrobacterium* was inoculated into LB broth supplemented with kanamycin (50 mg l^{-1}) and rifampicin (20 mg l^{-1}) and placed in an orbital shaker at 180 rpm overnight at 28°C. Then, 200 μl of the *Agrobacterium* culture was added to 30 ml of liquid LB medium supplemented with two antibiotics [rifampicin (10 mg l^{-1}) and kanamycin (50 mg l^{-1})]. The cultures were incubated in an orbital shaker at 200 rpm at 28°C until the bacterial cell density reached 1.0 at OD_{600} (~5 × 10^8 cells/ml). Approximately 30 ml of bacterial culture was centrifuged at 6,000 rpm for 10 min to obtain a pellet. The bacterial pellet was re-suspended in 30 ml of liquid infection medium (LIM) comprising MSB5 medium, sucrose (30%), MES buffer (3 mM) [pH 5.8], 200 mg l^{-1} L-cysteine and acetosyringone (100 μM). The embryo axis explants were infected with *Agrobacterium* cell suspension.

Agrobacterium-mediated transformation of embryo axis explants

The embryo axis explants were subjected to infection in batches of 50 explants/30 ml of *Agrobacterium* suspension for 30 min with occasional shaking for every parameter except bacterial infection duration parameter. For optimizing different treatments affecting GUS expression in the explants, 100 explants were infected in each treatment, and each experiment was repeated three times. Thirty explants were picked randomly from 100 treated explants, from each replicate and measured for transient GUS expression after (i) without pricking (wounding) and pricking, (ii) preculture (preconditioning) of explants for 1, 2, 3, 4 and 5 days (iii) bacterial cell density (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 OD_{600}) and bacterial infection time period (5, 10, 15, 20, 30 and 40 min) (iv) co-cultivation time period for 1, 2, 3, 4, and 5 days (v) diverse aceto-syringone concentrations (0, 50, 100, 150, 200 and 250 μM/l) and various concentrations of L-cysteine (0, 50, 100, 200, 300 and 400 mg l^{-1}) (vi) sonication treatments (0, 30, 60, 90, 120 and 150 sec) and vacuum infiltration treatments (0, 5, 10, 15, 20 and 25 min) for each parameter.

For standardization of transformation efficiency, hundred embryo axis explants were infected with *Agrobacterium*-suspension for each treatment and repeated thrice. Thirty randomly selected explants that responded in each replicate were considered *GUS* assay after 30 days of culture (after three successive subcultures, each subculture with 8-10 days) on the selection medium (with kanamycin). The percentage of explants that responded in the selection medium (SIM + 100 mg l^{-1} kanamycin) [after a fourth cycle or 60 days of selection] and mean numbers of *GUS* positive shoots/explants were presented in tabular form.

Induction and elongation of putative transgenic shoots

After 3 days of cocultivation, embryo axis explants were thoroughly washed with the sterile liquid washing medium (LWM) comprising MS salts and B5 vitamins, sucrose (30%), MES buffer (3 mM), along with cefotaxime (200 mg l^{-1}) twice to eliminate the excess of *Agrobacterium* then blot-dried with sterile tissue paper and transferred onto SIM supplemented with cefotaxime (200 mg l^{-1}) to initiate shoot induction for the first 7-10 days under the same culture conditions without kanamycin. After 7-10 days, the explants were inoculated onto the SIM fortified with cefotaxime (250 mg l^{-1}) and kanamycin (25 mg l^{-1}) incubated for 7-10 days. After the first selection cycle, the

explants with multiple shoot bud initiation were transferred onto the second cycle of choice with relatively higher selection pressure (kanamycin at 50 mg l^{-1}) and incubated for 7-10 days. After the second subculture, the explants were transferred onto the third cycle of selection medium with a higher selection pressure (kanamycin at 75 mg l^{-1}) and incubated for another 7-10 days. After this, the embryo axis explants containing the newly developed shoot buds were subcultured 3 times with a ten-day interval into fresh SIM augmented with kanamycin (100 mg l^{-1}) and cefotaxime (250 mg l^{-1}) for selection of transformed shoots. Further proliferation explants with multiple shoots were moved onto SEM amended with MES buffer (3 mM), cefotaxime (100 mg l^{-1}), and kanamycin (50 mg l^{-1}) for shoot elongation. After 15 days, the shoot buds were again transferred onto fresh SEM fortified with kanamycin (50 mg l^{-1}) for 15 days. To obtain complete putative transgenic plantlets from putative transgenic shoots (> 3 cm) were isolated and shifted onto the root induction medium.

Rooting of putative transgenic shoots and acclimatization

The elongated putative transgenic shoots (> 3 cm) were separated from a bunch of multiple shoots then inoculated onto RIM amended with cefotaxime (100 mg l^{-1}) and kanamycin (50 mg l^{-1}) incubated 4 weeks. The complete plantlets were obtained after rooting of putative transgenic shoots. The complete plantlets were carefully removed from culture tubes and washed with sterile distilled to eliminate the traces of agar sticking to roots. The complete plantlets were shifted to plastic cups filled with an equal ratio (1 : 1 : 1) of sterile sand, soil, and vermiculite. The plantlets were enclosed with polythene bags with the slightest puncture, then placed culture for two to three weeks and wetted once in two days. After three weeks, plantlets were shifted to pots and grown in the greenhouse.

GUS histochemical assay

Histochemical GUS assay was carried out to verify in embryo axis explants after each treatment and T0 putative plantlets using the substrate X-GlucA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) (Jefferson 1987). The plant material was washed with methanol for two h to remove chlorophyll pigments. The explants with positive transformants showed blue staining, and the explant with staining was recorded as the GUS positive. The non-transformed explants (control), cultured under similar conditions, served as control.

Polymerase chain reaction (PCR) analysis

Genomic DNA from leaflets of the putative T0 transformants regenerated from the embryo axis explants of chickpea was isolated (Doyle and Doyle 1990). Wild-type (untransformed control) plant genomic DNA and plasmid pCAMBIA2301 (positive control) were also used for amplification. The polymerase chain reaction (PCR) was performed with specific primer sets to *gusA* gene with forward primer 5'-AACGTATCCACGCCGTATTC-3' and reverse primer 5'-CGTACCTCGCATTACCCTTAC-3' to amplify 551 bp fragments and confirm the stable integration of *gusA* gene into the putative transformants of chickpea. The amplified products were separated using electrophoresis on 0.8% agarose gel and analyzed with GelDoc (Bio-Rad, USA).

Data analysis

The data were scored for each treatment, and each experiment was repeated three times. Each replicates with 100 embryo axis explants to optimize different factors on transformation efficiency. The data were scored for each treatment to evaluate various factors using 30 randomly selected embryo axis explants for *gus* histochemical assay after 15 days of culture. Data were presented as means ± standard error. The mean separations were carried out using Duncan's multiple range test (DMRT) and determined the significance at a 5% level.

Results and Discussion

Explant type

Explant type is also one of the highly imperative factors affecting plant genetic transformation. The *Agrobacterium*-mediated genetic transformation response was evaluated using the embryo axis explants. The study showed considerable variations in the transformation capability within the explants. Several genetic transformation protocols have been developed from different explants such as an embryo axis, cotyledonary node, epicotyl, axillary meristems, a half embryo with cotyledon (half-seed), and embryo axis slices using *Agrobacterium*-mediated transformation in chickpea (Atif et al. 2013). All these reports were showed different transformation efficiencies, transgenic plant recovery, and rooting efficiency (Atif et al. 2013). The differences in the transformation efficiencies might be

associated with variations in the regenerative potential of the explant and genotype.

The sensitivity of embryo axes explants to kanamycin

The identification and selection of transformants and non-transformants generally depend on the growth difference of transformed against non-transformed tissues in the presence of a selection agent. Several selectable marker genes encode resistance to an antibiotic which has been used in genetic transformation experiments. Legumes' genetic improvement programmes were deterred due to the lack of an efficient transformation protocol and reliable selection strategy (Nyaboga et al. 2014). Kanamycin resistance is one the most commonly used markers in legumes, including chickpea (Chakraborty et al. 2016; Das et al. 2017; Srivastava et al. 2017). For the selection of transformed shoots, different kanamycin concentrations ranging from 50-150 mg l^{-1} were used (Anbazhagan et al. 2015; Chakraborty et al. 2016; Das et al. 2017; Srivastava et al. 2017). Based on these studies, to decide the most favourable dose of kanamycin on the survival and regeneration response of the embryo axes were evaluated in chickpea. The survival and regeneration response of the explants was tested on different concentrations of kanamycin (25, 50, 75, and 100 mg l^{-1}), and the explant browning was recorded periodically (7, 14, and 21 days after culture initiation). The increase in kanamycin concentration severely decreased the survival and regeneration response of the embryo axis explants. Our study has used four to five selection cycles with diverse selection regimes of kanamycin (25-100 mg l^{-1}). The different selection regimes were helpful in the efficient recovery of putative transformants and successful prevention escapes during selection. The step-wise selection pressure permits transformed explants to express the antibiotic-resistance gene efficiently and induce cell division, thus recovering the regeneration of explants to produce plants (Bull et al. 2009). The low antibiotic concentration at early stages promotes transformed cell recovery, and a gradual improvement of antibiotic concentration efficiently eliminates non-transformed cells in the selection medium (Anbazhagan et al. 2015; Burgos and Alburquerque 2003). The final selection of putatively transformed shoots on a higher concentration of antibiotics eliminates chimeras (escapes) and generates positive plants. Finally, a low to stringent selection (25-100 mg l^{-1}) regime of kanamycin was adopted for all the transformation experiments. The kanamycin was one of the most efficiently used selectable markers for generating transgenic plants of a variety of legumes like *Arachis hypogaea* (Bhauso et al. 2014), *Vigna mungo* (Sainger et al. 2015), *Vigna radiata* (Mekala et al. 2016), *Glycine max* (Hada et al. 2018), and *Vigna unguiculata* (Bett et al. 2019; Che et al. 2021).

Effect of pricking (wounding)

For optimum delivery of T-DNA from *Agrobacterium* to explants requires efficient exposure of regenerable cells. The explants pricking before transformation offers accessibility for *Agrobacterium* cells to transform the deeply situated cells in the tissue (Hada et al. 2018). Pricking the embryo axis explants with a hypodermic needle resulted in more efficient transient expression than unwounded explants. The regenerating region of explants showed the highest transient expression of *GUS* compared to unwounded explants infected with *Agrobacterium*. The pricking of the embryo axis explants facilitates the accessibility of *Agrobacterium* infection to deliver T-DNA. The embryo axis explants were wounded by gentle pricking in the axillary and apical meristematic areas increased the transformation efficiency (18.6%) in explants (~2 fold). Similar observations of mechanical wounding significantly improved transformation efficiency in other plant species, including recalcitrant grain legumes such as green gram (Yadav et al. 2012), black gram (Sainger et al. 2015), soybean (Hada et al. 2018), and including chickpea (Srivastava et al. 2017).

Effect of preculture (preconditioning) period

The influence of the preculture period on transformation efficiency was determined by culturing the explants for different time durations. The explants were precultured before *Agrobacterium* infection improved the transformation efficiency (Srivastava et al. 2017). The preculture medium supplemented with growth regulators generally promotes cell division, and it is well known that actively dividing cells are more suitable for the delivery and integration of transgene (Yadav et al. 2017). The preculture period has played a significant role in improving transformation efficiency in the embryo axis explants of chickpea. The transformation efficiency progressively improved, increasing up to a 3 d preculture duration. The 3-day precultured explants showed maximum transformation frequency in the embryo axis (26.8%). Preculture of explants before *Agrobacterium* infection has been successfully applied to increase the transformation efficiency in leguminous plants like soybean (Arun et al. 2015), peanut (Tiwari et al.

2015), green gram (Mekala et al. 2016) and chickpea (Srivastava et al. 2017).

Effect of bacterial cell density and bacterial infection period

The influence of different bacterial cell densities was evaluated on transformation efficiency with the embryo axis explants of chickpea. The histochemical *GUS* assay performed after three days of cocultivation with various bacterial cell densities showed *GUS* expression response in 36.6% of embryo axis explants at OD_{600} 1.0 without any critical effect on regeneration compared to other cell densities. At OD_{600} 1.2, *GUS* expression response of explants was significantly decreased by 32.5% in the embryo axis. Different transformation efficiencies were observed in various crop plants with diverse bacterial cell densities ranging from 0.1 to 2.0 OD (Wang et al. 2009). Our results revealed the influence of bacterial densities on transformation efficiency up to 1.0 OD, which decreased considerably after that. Such bacterial cell density has been reported as an essential factor to improve transformation efficiency in chickpea (Das Bhowmik et al. 2019; Srivastava et al. 2017) and other plant species such as ramie (An et al. 2014), peanut (Karthik et al. 2018; Tiwari et al. 2015), and soybean (Hada et al. 2018).
The embryo axes were infected in *Agrobacterium* suspension (OD_{600} = 1.0) for diverse time durations (5, 10, 15, 20, 30, and 40 min) influence of infection duration on transformation efficiency. The embryo axis explants incubated with *Agrobacterium* cells at OD_{600} = 1.0 showed considerably improved transformation frequency than those infected (transformed) for different time durations. The infection time for 30 was observed optimum in embryo axis explants, while exposure to *Agrobacterium* for more than 30 min resulted in decreased transformation frequency. Longer infection time negatively affected the explant because of excessive bacterial cells. Different studies have shown a vast difference in the infection time of explants with bacterial suspension. In the present study, an infection time of 30 minutes with gentle shaking was optimal for the survival of explant selection media. This time showed conformity with previous observations in chickpea (Ignacimuthu and Prakash 2006).

Effect of cocultivation period

The embryo axis explants were co-cultivated for 1, 2, 3, 4, and 5 days after infection with *Agrobacterium* (Table 1) to determine the transformation efficiency. Co-cultivation of explants with *Agrobacterium* is an essential factor in the plant genetic transformation, and the duration of cocultivation significantly influences the transformation efficiency. Different cocultivation periods such as two days (Tripathi et al. 2013), three days (Das et al. 2017; Srivastava et al. 2017), four days (Senthil et al. 2004), and five days (Das Bhowmik et al. 2019) were reported to enhance the transformation efficiency in chickpea. The co-culture of the embryo axis explants for 3-days with the *Agrobacterium* resulted in maximum frequency with 46.4% of *GUS* expression in embryo axis explants (Table 1). More than three days of cocultivation resulted in excess growth of *Agrobacterium*, which declined regeneration and transformation efficiency in embryo axis explants. In the present study, a cocultivation period of 3 days was optimum for the efficient transformation of chickpea. Similar observations were made that three days of cocultivation duration was optimum in chickpea (Das et al. 2017; Ignacimuthu and Prakash 2006) and other leguminous plant species like *Vigna radiata* (Mekala et al. 2016), *Vigna mungo* (Kapildev et al. 2016), and *Glycine max* (Hada et al. 2018).

Effect of acetosyringone concentration

Acetosyringone (AS), one of the plant phenolic compounds produced during plant cell wounding, stimulates bacterial attachment and triggers the transcription of virulence genes that control the processing and transfer of T-DNA from *Agrobacterium* to plant cells (Gelvin, 2003). The effect of acetosyringone (50, 100, 150, 200, and 250 μM) on transformation efficiency was studied by subjecting the embryo axis explants for transformation with a cocultivation duration of 3 days. Acetosyringone has been shown to enhance the transient expression of *GUS* in different species due to the activation of *vir* genes (Atkinson and Gardner 1991). Our results convincingly showed that the addition of acetosyringone improved the transformation efficiency in embryo axis explants compared to control. A concentration of 100 μM acetosyringone showed the maximum *GUS* expression (Table 1). Our results suggest that acetosyringone augmentation in the cocultivation medium considerably increased the transformation efficiency and is necessary for the successful transformation of chickpea. Our results are in agreement with others on the successful application of acetosyringone concentration of 100 μM to enhance the transformation efficiency in many plant species, including legumes like peanut (Karthik et al. 2018), black gram (Kapildev et al. 2016; Sainger et al. 2015), cowpea (Bett et al. 2019), green gram (Mekala et

Table 1 Influence of co-cultivation duration and acetosyringone and cysteine concentrations on transformation efficiency in embryo axis explants of chickpea cv. JAKI9218[1]

Co-cultivation duration (days)	Acetosyringone concentration (μM/l)	Cysteine concentration (mg/l)	Explants infected, *n*	*gus*[2]-Positive explants, mean ± SE[3,4]
0	-	-	100	36.6 ± 1.47^{op}
1	-	-	100	38.2 ± 1.58^{op}
2	-	-	100	42.6 ± 1.63^{lm}
3	-	-	100	46.4 ± 1.74^{kl}
4	-	-	100	38.8 ± 1.68^{no}
5	-	-	100	36.4 ± 1.83^{op}
3	0	-	100	46.4 ± 1.36^{kl}
3	50	-	100	48.2 ± 1.65^{jk}
3	100	-	100	66.2 ± 1.82^{cd}
3	150	-	100	58.4 ± 1.68^{fg}
3	200	-	100	56.2 ± 1.72^{gh}
3	250	-	100	54.6 ± 1.66^{hi}
3	100	0	100	66.2 ± 1.81^{cd}
3	100	50	100	68.4 ± 1.65^{cd}
3	100	100	100	70.6 ± 1.48^{cb}
3	100	200	100	76.5 ± 1.60^{a}
3	100	300	100	62.2 ± 1.78^{ef}
3	100	400	100	60.8 ± 1.64^{ef}

[1]The control group consisted of wounded explants precultured for 3 days at a bacterial cell density 1.0 at OD_{600}[5] and an infection duration of 30 min. Transformation efficiency = (GUS explants/total explants infected) × 100. We used 100 embryo axis explants per treatment, and each treatment was repeated thrice.
[2]*gus*: β-glucuronidase.
[3]SE: standard error.
[4]Means ± SEs of values of three independent experiments. Values with the same letter within columns are not significantly different according to the DMRT[6] at a 5% level.
[5]OD_{600}: optical density at 600 nm wavelength.
[6]DMRT: Duncan's multiple range test.

al. 2016), soybean (Hada et al. 2018), and chickpea (Chakraborty et al. 2016; Srivastava et al. 2017).

Effect of L-cysteine concentration

In the present investigation, we have noticed that the embryo axis explants exhibited enzymatic browning and tissue necrosis at the wounded sites following cocultivation, which were likely to affect the efficient transformation. L-cysteine is being considered one of the most useful anti-necrotic agents. Therefore, to reduce *Agrobacterium* induced tissue browning and necrosis, several concentrations of L-cysteine were employed to optimize the transformation efficiency in chickpea. The wounding and pathogen-defense responses of explant tissue trigger the production of PPO-like compounds, which decreases the *Agrobacterium*-mediated transformation success rate (Olhoft and Somers 2001; Olhoft et al. 2003; Yadav et al. 2017). The amendment of L-cysteine (50-400 mg l^{-1}) to the CCM supplemented with acetosyringone (100 μM) controlled enzymatic browning and tissue necrosis when compared to control (CCM without L-cysteine). Among the diverse concentrations of L-cysteine tested, 200 mg l^{-1} was found optimal, and at this concentration, embryo axis explants showed maximum transformation efficiency. Further, an increase in the concentration of L-cysteine (> 200 mg l^{-1}) reduced transformation efficiency (Table 1). Our results are in accord with earlier reports in chickpea, showing that L-cysteine supplementation during cocultivation increases the transformation efficiency (Srivastava et al. 2017). Similar to the results obtained in the present study, various concentrations of L-cysteine was supplemented to enhance the transformation efficiency in several leguminous species such as *Arachis hypogaea* (Tiwari et al. 2015), *Glycine max* (Hada et al. 2018), *Vigna mungo* (Sainger et al. 2015), and *Vigna unguiculata* (Bett et al. 2019).

Table 2 Influence of sonication and vacuum infiltration on transformation efficiency in embryo axis explants of chickpea cv. JAKI9218[1]

Sonication duration (s)	Vacuum infiltration duration (min)	Explants infected, *n*	*gus*[2]-Positive explants, mean ± SE[3,4]
0	-	100	76.5 ± 1.38[ef]
30	-	100	78.6 ± 1.62[de]
60	-	100	81.4 ± 1.64[cd]
90	-	100	73.2 ± 1.68[gh]
120	-	100	70.4 ± 1.82[hi]
150	-	100	73.2 ± 1.56[gh]
90	0	100	81.4 ± 1.62[cd]
90	5	100	82.6 ± 1.86[bc]
90	10	100	82.8 ± 1.46[bc]
90	15	100	84.8 ± 1.58[ab]
90	20	100	78.8 ± 1.66[de]
90	25	100	74.6 ± 1.62[fg]

[1]The control group consisted of wounded explants precultured for 3 days at a bacterial cell density of 1.0 at OD_{600}[5] and an infection duration of 30 min, with 100 μM/l of acetosyringone and 200 mg/l of L-cysteine. Transformation efficiency = (GUS explants/total explants infected) × 100. We used 100 embryo axis explants per treatment, and each treatment was repeated thrice.
[2]*gus*: β-glucuronidase.
[3]SE: standard error.
[4]Means ± SEs of values of three independent experiments. Values with the same letter within columns are not significantly different according to the DMRT[6] at a 5% level.
[5]OD_{600}: optical density at 600 nm wavelength.
[6]DMRT: Duncan's multiple range test.

Effect of sonication

In chickpea, explants with meristematic cells are the primary target for *Agrobacterium*-mediated genetic transformation. Sonication provides the effortless penetration of *Agrobacterium* into meristematic cells by creating micro-wounds (Teixeira da Silva and Dobranszki 2014). The transformation efficiency progressively improved with increasing sonication treatments up to 90 sec. The transformation efficiencies were 81.4% in the embryo axis explants (Table 2). In the present investigation, transformation efficiency was approximately 2.8 fold in embryo axis explants increased up to 90-sec treatment than non-sonicated (0 s) tissues. Sonication treatment for a more extended period (120 and 150 sec) showed decreased transformation efficiency (Table 2). The sonication treatments were significantly increased the genetic transformation efficiency in several plants species (Teixeira da Silva and Dobranszki 2014). The micro-wounds generated during sonication may secrete more phenolic compounds, thus facilitating *Agrobacterium* to penetrate deeply throughout the tissue and improve plant transformation efficiency (Bett et al. 2019; Che et al. 2021).

The sonication treatment increased the *GUS* expression in the embryo axis explants. At an optimal time of 90sec, the embryo axis explants showed a drastic increase in the percentage of explants showing transient *GUS* expression (Table 2). Similar to the present study, SAAT was successfully adopted to increase transformation efficiency in several plants, including leguminous crop plants such as soybean (Hada et al. 2018), cowpea (Bett et al. 2019; Che et al. 2021), black gram (Kapildev et al. 2016), and peanut (Karthik et al. 2018). Our observations are similar to earlier reports on the sonication treatment that successfully improved the transformation efficiency in chickpea (Pandey et al. 2021; Srivastava et al. 2017).

Effect of vacuum infiltration

To further evaluate the transformation efficiency in chickpea, the *Agrobacterium*- suspension was employed to vacuum infiltration for different lengths of time from 5-25 min. The embryo axis explants were immersed in a liquid suspension of *Agrobacterium* then subjected to decreased pressure followed by rapid re-pressurization. The vacuum infiltration treatment helps to take out the gases from the inside of the explants through micro-cavities produced by pricking with the help of negative pressure (Tague and

Mantis 2006). Hence, explants pricked at the meristematic region followed by the vacuum infiltrating with *Agrobacterium*-suspension at 750 mm Hg for 15 min was optimum and improved the transformation efficiency of 84.8 % in the embryo axis explants (Table 2). Vacuum infiltration was significantly enhanced the transformation efficiency in the embryo axis explants up to 15-min treatment and then decreased the transformation efficiency (Table 2). The *Agrobacterium* infiltration method is helpful in the delivery of different agronomically functional genes for stable integration in plant tissues (Tague and Mantis 2006). The vacuum pressure played a pivotal role in improving the transformation efficiency and survival of explants. The vacuum infiltration treatment has been successfully employed for the generation of transgenic plants in several crop plants, including *Glycine max* (Hada et al. 2018), *Vigna mungo* (Kapildev et al. 2016), and *Arachis hypogaea* (Karthik et al. 2018). The successful utilization of vacuum infiltration treatment enhanced transformation efficiency, as reported in earlier studies on chickpea (Srivastava et al. 2017).

Regeneration of shoots, elongation, rooting, and hardening

An efficient selection approach is highly significant for developing a reproducible plant transformation protocol. The co-cultivated explants were washed to remove the excess *A. tumefaciens* and transferred to SIM containing kanamycin showed relentless tissue necrosis and no-shoot formation. The co-cultivated explants transferred to SIM without a selection agent (kanamycin) for 5-7 days significantly encouraged the induction of transformed shoot buds from the embryo axis explants (Fig. 1a). The resting step was frequently used to alleviate *Agrobacterium* and selection agent stress (Hada et al. 2018). After one week, the explants were transferred onto SIM containing cefotaxime (250 mg l^{-1}) and kanamycin (25 mg l^{-1}). Then the explants with multiple shoot buds were further subcultured onto 50 mg l^{-1} kanamycin (relatively higher selection pressure) for 7-10 days. After the second selection cycle, the explants with putatively transformed shoot buds were placed on SIM supplemented with 75 mg l^{-1} kanamycin (higher selection pressure) for another ten days in the third selection cycle. After the third selection cycle, explants with many putatively transformed and untransformed shoots were inoculated onto the medium containing 100 mg l^{-1} kanamycin (severe selection pressure) for two to three passages at a ten-day interval to eliminate escapes and chimeras. Our observations are in agreement with a previous report on the *Agrobacterium*-mediated genetic transformation in chickpea (Anbazhagan et al. 2015). In the present investigation, repeated transfer of the embryo axis explants to a medium containing kanamycin facilitated uniformly *GUS* expressing shoots. The non-transformed kanamycin-sensitive shoots of the embryo axis explants were completely bleached and showed retarded growth. The observation suggested that incremental kanamycin (25 to 100 mg l^{-1}) resulted in the efficient selection of transformed cells from non-transformed cells (Fig. 1b). The kanamycin induced the rapid death of non-transformed cells (sensitive cells) throughout the cycles of the selection period. The selection period improved the growth and development of healthy shoots resistant to kanamycin and avoided the problem of chimerism. The comparable selection regime in the presence of kanamycin was reported in chickpea as an earlier study (Anbazhagan et al. 2015; Srivastava et al. 2017). The complete plants were obtained after rooting the putatively transformed shoots on a rooting medium augmented with IBA 2.0 mg l^{-1}, 100 mg l^{-1} cefotaxime, and 50 mg l^{-1} kanamycin (Fig. 1d). The

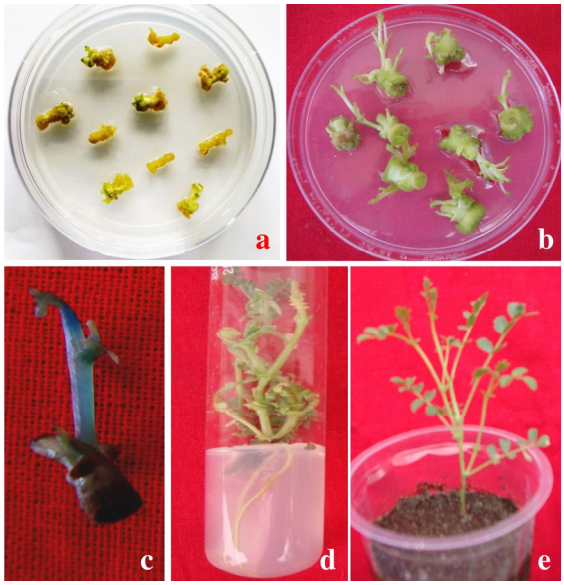

Fig. 1 *Agrobacterium*-mediated genetic transformation of embryo axis explants of chickpea cv. JAKI9218 with the LBA4404 strain harboring pCAMBIA 2301 with *nptII* and *gusA* genes. (a) Shoot bud induction from explants in SIM supplemented with cefotaxime (250 mg/l) and kanamycin (25 mg/l) after 1 week, (b) multiple shoot bud induction from explants after 4 weeks in SIM supplemented with cefotaxime (250 mg/l) and kanamycin (100 mg/l), (c) transient GUS expression in putative transgenic shoot after 3 weeks, (d) putative transgenic plants after root formation on RIM supplemented with cefotaxime (100 mg/l) and kanamycin (50 mg/l), and (e) Acclimatization of putative transgenic plant in a pot under greenhouse conditions. GUS: β -glucuronidase; RIM: root induction medium; SIM: shoot induction medium

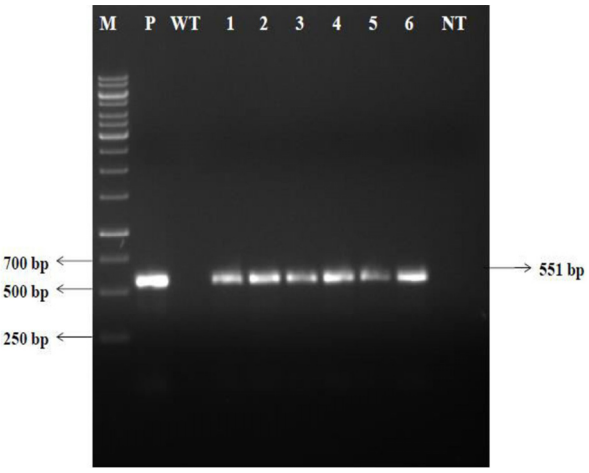

Fig. 2 Molecular confirmation of chickpea cv. JAKI9218 transgenic plants and analysis for the presence of the *gusA* gene in putative transgenic plants in the T0 generation using *gusA*-specific primers. *gus*: β-glucuronidase; lanes 1-6: putative transgenic plants in the T0 generation; M: marker DNA ladder (1 kb ladder, Thermo Fisher Scientific); NT: no template (negative control); P: positive control (plasmid DNA); WT: wild type (control plant DNA)

putatively transformed plantlets were successfully acclimatized under greenhouse conditions (Fig. 1e). The regenerated plants established under greenhouse for morphologically similar and flowered normally.

Gus histochemical analysis

The visual or selectable markers are required for detection due to the relatively small number of cells in which integration of the foreign DNA occurs during the development of gene transfer protocols in plant species (Xing et al. 2000). After three successive subcultures, the explants with shoots were examined for *GUS* expression using histochemical assay (Fig. 1c). Srivastava et al. (2017) reported *GUS* expression with the CaMV35S promoter in the shoots of axillary meristem explants in chickpea. In the present study, intense *GUS* expression was observed in putative T0 transformants derived from embryo axis explants.

Molecular analysis of putative transformants

The PCR analysis confirmed the existence of the *gusA* gene in the putative T0 transformants regenerated from embryo axis explants and using the PCR amplified *gusA* gene with 551 bp (Fig. 2) gene fragment showed the incorporation of the transgene into the chickpea genome of cv. JAKI9218. The histochemical GUS assay in putative transgenic shoots and PCR amplification of *gusA* in T0 transgenic plants have shown stable integration of the *gusA* gene. Our observations are similar to earlier reports on stable integration of *gusA* gene from pCAMBIA 2301 in chickpea genotype C-235 using axillary meristem explants (Srivastava et al. 2017; Tripathi et al. 2013).

Effect of chickpea genotype on transformation efficiency

The transformation efficiency strongly depends upon genotypes, and transformation efficiency may be different in various genotypes. Similarly, different transformation efficiencies have been reported with multiple explants from different chickpea genotypes (Tripathi et al. 2013). Hence, the *Agrobacterium*-mediated transformation technique

Table 3 Influence of different cultivars of chickpea on *Agrobacterium*-mediated transformation using embryo axis explants of chickpea cv. JAKI9218[1]

Chickpea genotype	Explants infected, *n*	Explants that responded (after 3 subcultures), mean ± SE[2,3]	*gus*[4]-Positive explants, mean ± SE	Transformation efficiency (%)[5]
JAKI9218	100	12.8 ± 0.76[a]	8.6 ± 0.84[a]	8.6
JG11	100	10.4 ± 0.68[b]	7.2 ± 0.78[b]	7.2
ICCC37	100	9.6 ± 0.86[d]	5.4 ± 0.92[d]	5.4
ICCV10	100	7.3 ± 0.82[e]	4.6 ± 0.83[e]	4.6
ICCV92944 (JG14)	100	10.2 ± 0.92[bc]	6.8 ± 0.76[bc]	6.8
ICCV2 (Swetha)	100	6.3 ± 0.88[f]	3.8 ± 0.78[ef]	4.8

[1]We used 100 embryo axis explants per treatment, and each treatment was repeated thrice.
[2]SE: standard error.
[3]Means ± SEs of values of three independent experiments. Values with the same letter within columns are not significantly different according to the DMRT[5] at a 5% level.
[4]*gus*: β-glucuronidase.
[5]Transformation efficiency = (GUS explants/total explants infected) × 100.
[6]DMRT: Duncan's multiple range test.

was adopted in the present investigation using chickpea cv. JAKI9218 was extended to assess other cultivars, such as JG11, ICCV10, ICCC37, ICCV92944 (JG14), and ICCV2 (Swetha). Among the different genotypes evaluated, JAKI9218 was an efficient genotype with a higher transformation efficiency of 8.6%, followed by JG11 (7.2%), ICCV92944 (6.8%), ICCC37 (6.4%), ICCV10 (4.6%), and ICCV2 (3.8%) (Table 3). The protocol developed in this investigation might be valuable to transform novel candidate genes into diverse chickpea genotypes.

Conclusions

In the present investigation, we have standardized several factors for efficient *Agrobacterium*-mediated transformation protocol using the embryo axis explants of different chickpea genotypes. All the evaluated genotypes reacted for transformation and showed superior transformation efficiencies. Several factors like pricking, preculture, bacterial cell density, bacterial infection duration, cocultivation duration, acetosyringone, L-cysteine supplementation, sonication, and vacuum infiltration treatments were shown to have enhanced transformation frequency using embryo axis explants. Among six chickpea cultivars evaluated, the JAKI9218 cultivar has shown the maximum transformation efficiency of 8.6%, followed by JG11 (7.2%), ICCV92944 (6.8%), ICCV37 (5.4%), ICCV2 (4.8%), and ICCV10 (4.6%). These results showed that embryo axis explants showed better transformation efficiency, which will be used in experiments to overexpress different candidate genes to improve chickpea genotypes.

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