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Development of an Efficient and Reproducible Embryonic Axis-targeted Tissue Culture-based Transformation Protocol for Pigeonpea (*Cajanus cajan*)

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ABSTRACT

Background: Tissue culture-based transformation approach poses significant challenges for genetic modification of recalcitrant pigeonpea due to poor regeneration efficiency. In this study, we present a comprehensive high-frequency regeneration and transformation protocol using embryonic axesas explants.

Methods: Optimization of transformation parameters and culture conditions for different stages of adventitious shoot regeneration, including shoot induction, elongation and rooting, significantly enhanced transformation efficiency. *Agrobacterium*-mediated transformation was carried out using embryonic axes explants with pCAMBIA 2301 vector. Culturing of explants on MS basal media supplemented with BAP 2.5 in Preculture, BAP 2.5 in Selection Medium I, IAA 0.5, GA 0.5 and Zeatin 1 in Selection Medium II resulted in formation of numerous multiple shoots from primordia-forming calli at axillary bud regions.

Result: Shooting efficiency was 82% and 77%, for cultivars, Pusa 992 and Asha respectively. Rooting efficiency was nearly 100% for both when cultured on half-strength MScontaining 15 g/l sucrose and 0.5 mg/l IAA. Regenerated plants exhibited a high level ofsurvival of 90% upon transplantation into pots. Successful gene integration and expression were confirmed through GUS histochemical and molecular analyses, with approximately 57% and 65% transformation efficiencies for cultivars Pusa 992 and Asha, respectively. This study presents a robust and reproducible tissue culture-based transformation system for pigeonpea, applicable across diverse genotypes, offering new possibilities for genetic improvement in this crucial legume crop.

Key words: Embryonic axes, Pigeonpea, Tissue culture, Transformation.

INTRODUCTION

Pigeonpea, an important legume in semi-arid tropics, is essential for protein needs in Indian subcontinent. Enhancing pigeonpea with pest, disease resistance and abiotic stress tolerance is crucial. Traditional breeding methods face challenges due to their limited genetic diversity and self-pollination. Genetic transformation is essential for introducing desirable traits,but pigeonpea's tissue culture difficulties hinder progress. Researchers are trying to establish reliable regeneration methods for efficient transformation (Thu *et al*., 2003; Krishna *et al*., 2011).

Tissue culture, a vital technique for creating transgenic plants, relies on manipulating totipotent properties of plant cells. Among various techniques, organogenesis-mediated plant regeneration has been preferred method for transforming pigeonpea, mainly because of its high success rate in regeneration. Process of regenerating pigeonpea *in vitro* is usually dependent on genotype and not feasible for numerous cultivated varieties. Various types of explants have been employed to trigger organogenesis in pigeonpea. However, when it comes to regeneration, cotyledonary node/ embryonic axes show higher responsiveness compared to other tissues. Out of different types of explant tissues evaluated,shooting frequency varied, ranging from 65.4% for leaves to 93.2% for cotyledonary nodes of pigeonpea (Geetha *et al*., 1998). Although, much progress has been achieved in pigeonpea tissue culture, genetic transformation remains challenging due to several constraints. We urgently need genotype-independent methods for stable regeneration and precise genetic modification. Factors like genotype, seed germination, growth medium and plant growth regulators influence pigeonpea organogenesis. Optimizing transformation parameters would help efforts on development transgenic pigeonpea. In this study, we aimed to develop a protocol for multiple genotypes, emphasizing rapid shoot production from embryonic axes.

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MATERIALS AND METHODS

The current study was performed at ICAR-National Institute for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi from 2021-2023. The specific details of the research materials and methods used in the study are described as follows:

Experimental framework

We investigated factors affecting transformation efficiency, focusing on *Agrobacterium* infection and explant regeneration. We studied key elements influencing *Agrobacterium* infection, including virulence inducer concentrations, explant preparation, *Agrobacterium* suspension medium, co-cultivation time and pigeonpea varieties. Additionally, we explored factors affecting explant regeneration, including shooting and rooting rates, by experimenting with various growth hormone concentrations.

Culture medium and conditions

Seeds germinated on Murashige and Skoog (MS) medium with or without BAP (6-benzylaminopurine). Before transformation, explants were pre-cultured on MS with various BAP concentrations. For selective shoot regeneration, antibiotics {kanamycin (100-120 mg/l), cefotaxime (250 mg/l) and carbenicillin(250 mg/l)} were added.Shoot induction medium/Selection medium I (SMI) was composed of MS with either BAP alone or in combination with indole-3-acetic acid (IAA) and/or gibberellic acid (GA3) at different concentrations. Shoot elongation medium/Selection medium II (SMII) consisted of MS with varying IAA, Zeatin and GA3 concentrations. Root induction medium (RIM) was prepared using Half MS with different compositions and concentrations of sucrose and hormone IAA, indole-3-butyric acid (IBA) or hormone-free B5 media.

Explant preparation and pre-culture

Seeds of two pigeonpea varieties, Pusa 992 and Asha (obtained from IARI, New Delhi), were surface sterilized using tween 20, ethanol, sodium hypochlorite and mercuric chloride and then soaked overnight.Germination was enhanced through various setups: (i) placement on MS basal agar medium without plant growth regulators (PGRs), (ii) moistened cotton with MS liquid, (iii) moistened filter paper with MS liquid and (iv) moistened filter paper in MS liquid supplemented with 2.5 mg/l BAP. After germination, cotyledons and apical meristems were removed, leaving each explant with two prominent axillary buds/meristems. These explants were then pre-cultured in darkness for two days.

Agrobacterium infection and co-cultivation

For optimization of transformation, explants were infected with *Agrobacterium tumefaciens* strain EHA105 harbouring pCAMBIA 2301 plant binary vector, which has reporter gene *β-glucuronidase* (*GUS)* and *neomycin phosphotransferase II* (*nptII,* kanamycin) as selectable marker. *Agrobacterium* culture was prepared on AB minimal medium by incubation for 16 hrs at 28°C (180 rpm). After this incubation period, 2 different concentrations of acetosyringone (100 mM or 200mM) were added to create culture density ranging from OD_{600} of 0.2-1 and further incubated at 28°C (180 rpm) for 2 hrs. Explants immersed in *Agrobacterium* suspension and subjected to vacuum infiltration for 15-25 minutes at 500 psi units to optimize cell density and infection time. Infected explants were placed on co-cultivation medium for 48 to 96 hours to maximize transformation efficiency.

Plant tissue culture and transplantation

Co-cultivated explants were washed, dried and placed on SMI for 10-12 days for shoot induction.Explants with numerous shoot buds were moved to SMII for two or three subsequent sub-cultures at 7-10-day intervals. Elongated Shoots (4-5 cm) were transferred to RIM for rooting process.Plants were transferred to soilrite and kept in a culture room for hardening. After 14-15 days, plants were carefully transplanted to soil and shifted to greenhouse.

Molecular analysis

Molecular confirmation involved genomic DNA extraction from fresh leaves using CTAB method. Presence of *nptII* gene was analyzed through PCR amplification with *nptII* gene-specific primers (5′-ATGATTGAACAAG ATGGATTGC ACGCAGGT-3′, 5′-TCAGAAGAACTCGTCAA GAAGGCGAT AGAA-3′). Total RNA was extracted using TRIzol and cDNA was synthesized using a first-strand cDNA synthesis kit. RT-PCR was then conducted using *GUS* gene-specific primers (5′-GCCAAAAGCCAGACAGAGTC-3′, 5′-TGCGAG GTACGGTAGGAGTT-3′).

GUS assay

Transformed and untransformed samples were immersed in GUS staining solution and incubated at 37°C in dark for different periods of time. For both varieties, optimal GUS treatment duration was determined using *in planta* method by following a similar transformation protocol. Overnight-soaked seeds were used for transformation and after 3 days of cocultivation, seeds were treated with GUS buffer for 4 hrs, 6 hrs and 12 hrs. Explants utilized in tissue culture-based transformation underwent GUS analysis after a 3-day co-cultivation period followed by 2 weeks of growth under selection. After staining, materials were treated with 95% ethanol for visual assessment.

Determination of transformation efficiency

Transformation efficiency was determined by evaluating *GUS* gene expression employing RT-PCR analysis. It was calculated as a percentage, with number of positive transgenic plants divided by number of infected explants, multiplied by 100.

Statistical analysis

Experiments were replicated three times and results are presented as a mean value along with the corresponding standard error (SE), calculated from averaged data.

RESULTS AND DISCUSSION

Development of an efficient regeneration system

Expedite seed germination process

To enhance germination rates and expedite germination process, various methods were employed.When seeds were directly cultured on MS basal agar medium in petri plate without any PGRs, germination ranged from 60-70% and took 5-7 days. However, placing seeds on sterile, moistened cotton (MS liquid) in jam bottles, germination reached 90-100% within 4-5 days period. Even more rapid germination (95-100% within 3-4 days) occurred when seeds were placed on sterile, moist filter paper soaked in MS liquid. Similar high germination results have been observed in other studies with various crop species using sterile, moistened filter paper or cotton in Petri plates or test tubes (Geetha *et al*., 1998). Fastest germination (100%) was seen when seed was incubated in dark for 2-3 days on moistened filter paper in MS liquid with 2.5 mg/l BAP (Fig 1a).

Use of embryonic axes as explants

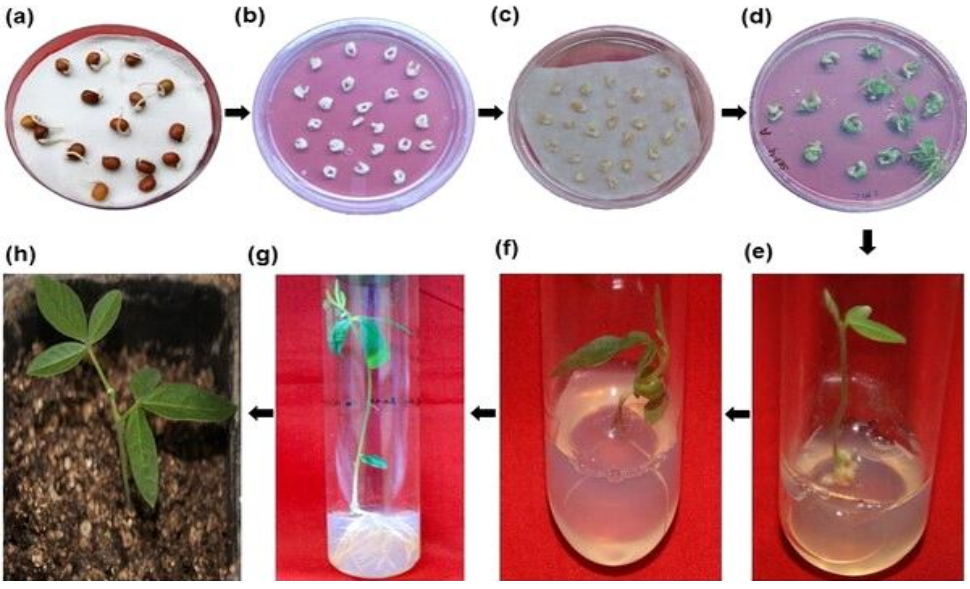
Axillary buds present at junction of cotyledon and embryonic axes contain meristem cells necessary for regeneration and are primary targets for gene delivery. After seed germination, embryonic axes were excised and decapitated by removing root and shoot meristem. Decapitated embryonic axes were placed on a preculturing medium (Fig 1b).

Optimized transformation process

Acetosyringone released in response to injuries in dicot plants, acts as a signaling molecule triggering transfer of T-DNA from *Agrobacterium* to plant. Although acetosyringone and AB media have been commonly used in *in planta* transformation technique, their use in *in vitro* transformation of pigeonpea was not extensively documented (Karmakar *et al*., 2019). Refinement of agro-infection parameters, such as bacterial optical density (OD), acetosyringone concentration, inoculation time and co-cultivation duration, would prove advantageous in enhancing *in vitro* transformation efficiency. Eleven different treatment combinations were evaluated, each varying in bacterial OD_{600} (0.2, 0.5, 1.0), acetosyringone (100, 200 µM/ml), inoculation (15, 25 min) and co-cultivation (48, 72, 96 hrs) (Table 1). Optimal treatment combination for improved transformation efficiency was determined based on average number of explants recovered during kanamycin selection and average number of plants confirmed positive in *nptII* PCR screening. Each treatment was replicated three times, with 50 explants per treatment. Optimized transformation parameters were found to be as follows: bacterial density in AB media (OD_{600}) of 0.2, acetosyringone concentration at 100 µM, vacuum infiltration for 15 minutes and a 72-hour co-cultivation period, resulting in the highest (58-60%) transformation efficiency (Fig 5a).

Plant regeneration and acclimatization

Explants were rinsed, dried (Fig 1c) and moved to a selection medium that contained hormones and antibiotics. Kanamycin, an antibiotic, was added to facilitate selection and regeneration of transgenic shoots, while carbenicillin and cefotaxime were used to control growth of *Agrobacterium* in plant culture. To minimize any adverse effects, a concentration of 250 mg/l for both carbenicillin and cefotaxime was chosen based on previous research



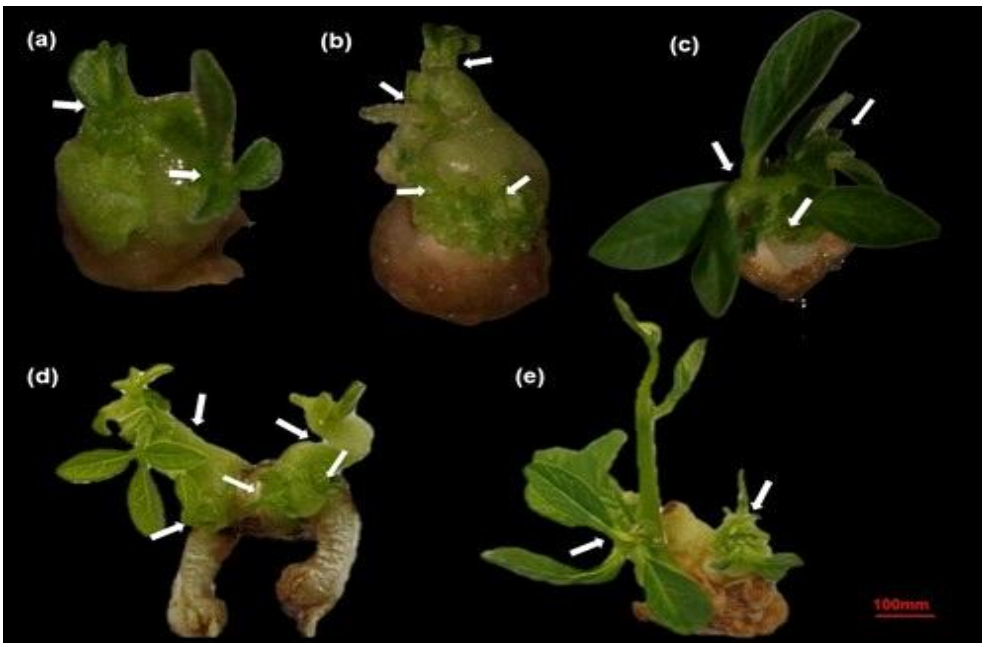
(a) Germination, (b) pre-culturing, (c) co-cultivation, (d) multiple shoot induction, (e) plantlet elongation, (f) root induction (g) rooted plantlets (h) hardening.

Fig 1: Summary of plant transformation and regeneration in pigeonpea.

(Yu *et al*., 2001). Using kanamycin at 100 mg/l has been proven to be effective for selecting transgenic pigeonpea (Ganguly *et al*., 2018). Agar is preferred solidifying agent for selection medium due to its ability to release kanamycin quickly, which reduces chances of escapes compared to using gelrite (Krishna *et al*., 2011).

The ideal ratio of hormones plays a significant role in affecting enzymatic reactions in plants, influencing their growth and morphology. Various PGRs were chosen based on existing literature to develop a standardized protocol for regenerating and transforming two pigeonpea cultivars. Among cytokinins, BAP was preferred over kinetin (KIN) and thidiazuron (TDZ) for its ability to promote shoot bud regeneration. Past research has highlighted significance of BAP in facilitating both formation of callus and promotion of shoot growth, reducing regeneration time (Thu *et al*., 2003). Explants were cultured on MS media (preculture and SMI) with different concentrations of BAP (2, 2.5, 5, 10 mg/l). In axillary bud regions, callus is induced when BAP concentrations were high (5.0 and 10 mg/l), but its growth was hindered. Few primordia-forming calli were detected at concentrations ranging from 2.0-2.5 mg/l. Small shoots, measuring 0.5-0.7 centimeters in length, with callus/explant, were moved to SMI that had varying levels of BAP (2.0, 2.5, 5.0 and 10 mg/l) to promote growth. It was found that BAP at 2-2.5 mg/l, was most effective in inducing shoot growth in about 3-4 weeks (Table 2). Results are consistent with earlier reports that found that cotyledonary node had high shoot bud regeneration at a BAP concentration of 2.0 mg/L (Geetha *et al*., 1998; Thu *et al*., 2003). In most of calli, 70-80% of multiple shoots were produced, with each explant regenerating between 2 to 4 shoots (Fig 2). Excessive cytokinin (5.0 and 10 mg/l) results in intermediate callus formation, affecting regeneration. Addition of auxin along with cytokinin to enable both multiplication and elongation of shoots. Moreover, inclusion of GA3, along with other growth regulators, significantly enhanced occurrence of generating multiple shoots and subsequent generations of axillary nodes. Similar observations were also made in earlier studies (Geetha *et al*., 1998; Sarkar *et al*., 2019). Here, we have prepared SMII with different concentrations of IAA (0.2-0.5 mg/l), GA (0.5-1 mg/l) and Zeatin (0.5-1 mg/l). Shoots-bearing explants were subcultured on SMII for shoot elongation. Most effective media for shoot regeneration were those supplemented with BAP 2.5 in preculture, BAP 2.5 in SMI, IAA 0.5, GA 0.5 and Zeatin 1 in SMII (Fig 1d and e). A maximum shooting frequency of 77-82% was achieved (Fig 5c).

For rooting, elongated shoots (2-4 cm) were removed and cultured on rooting media with various basal media, auxin and sucrose combinations. Sucrose concentrations of 1.5% and 2% were found to be most effective in promoting rooting, while external auxins further enhanced root development. Rooting media composition half MS + 20 g/l sucrose + 0.5 mg/l IBA was used because of its potential to generate 100% roots in pigeonpea variety JKPL (Krishna *et al*., 2011). Whereas, hormone-free B5 medium was selected based on its rooting efficiency (>25%) for pigeonpea genotype ICPL 87 (Thu *et al*., 2003). According to our study, using half MS media with 15 g/l sucrose and 0.5 mg/l IAA



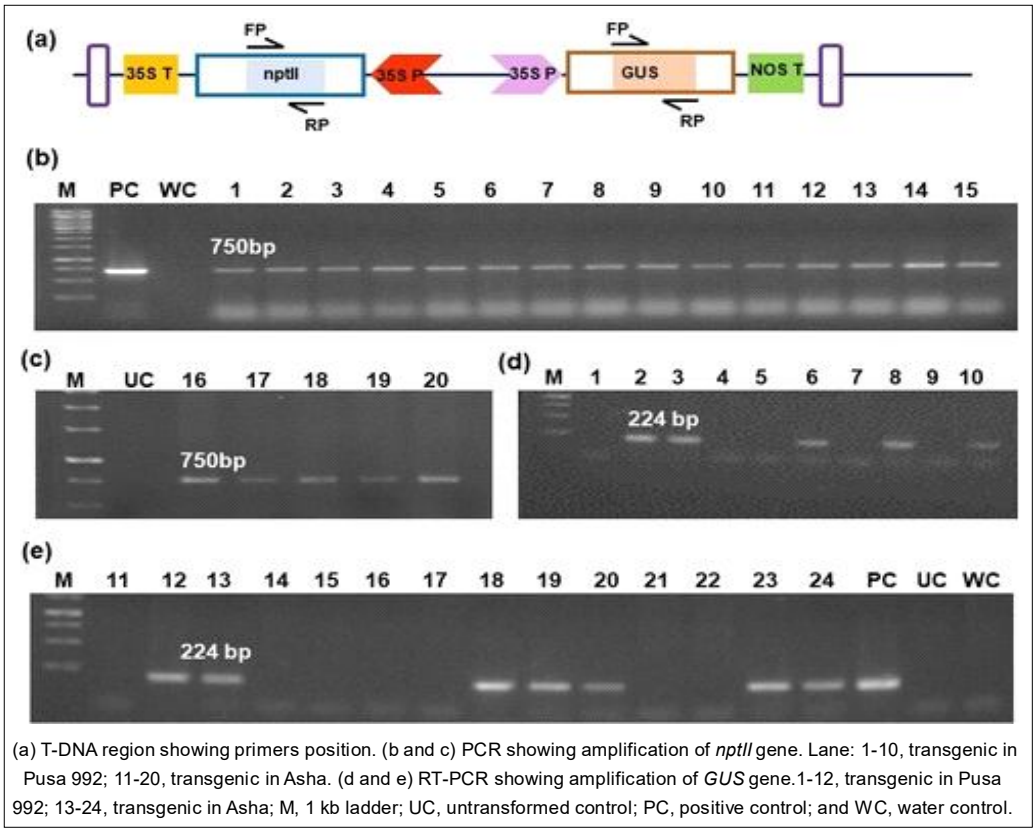
(a), (b) Shoot initials formed at axillary buds. (c), (d), (e) 8 weeks old embryonic axes with adventitious shoots (arrow indicates multiple shoot buds).

Fig 2: Direct Organogenesis of pigeonpea explants.

resulted in better root growth for both genotypes and took around 3 weeks for roots to differentiate on elongated shoots (Fig 1f and g). A maximum rooting frequency of about 100% was achieved (Fig 5d). This finding is consistent with earlier studies on direct rooting (Thu *et al*., 2003; Krishna *et al*., 2011).

Molecular analysis

PCR amplification using primers for *nptII* and RT-PCR for *GUS* was conducted (Fig 3 and Table 3). Out of 858 putative transformants of Pusa992 and 845 plants of Asha regenerated in kanamycin selection, 730 of Pusa 992 and 715 of Asha were positive through RT-PCR. Transformation



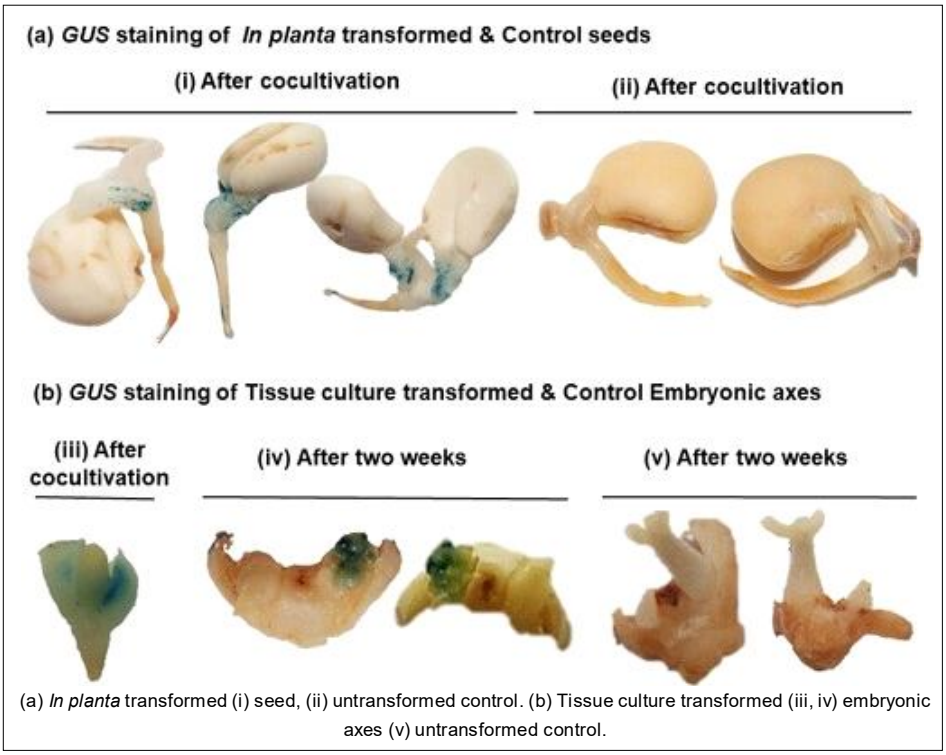
(a) T-DNA region showing primers position. (b and c) PCR showing amplification of *nptII* gene. Lane: 1-10, transgenic in Pusa 992; 11-20, transgenic in Asha. (d and e) RT-PCR showing amplification of *GUS* gene.1-12, transgenic in Pusa 992; 13-24, transgenic in Asha; M, 1 kb ladder; UC, untransformed control; PC, positive control; and WC, water control.

Fig 3: Molecular analysis of the pigeonpea transformants.

Table 1: Optimization of transformation.

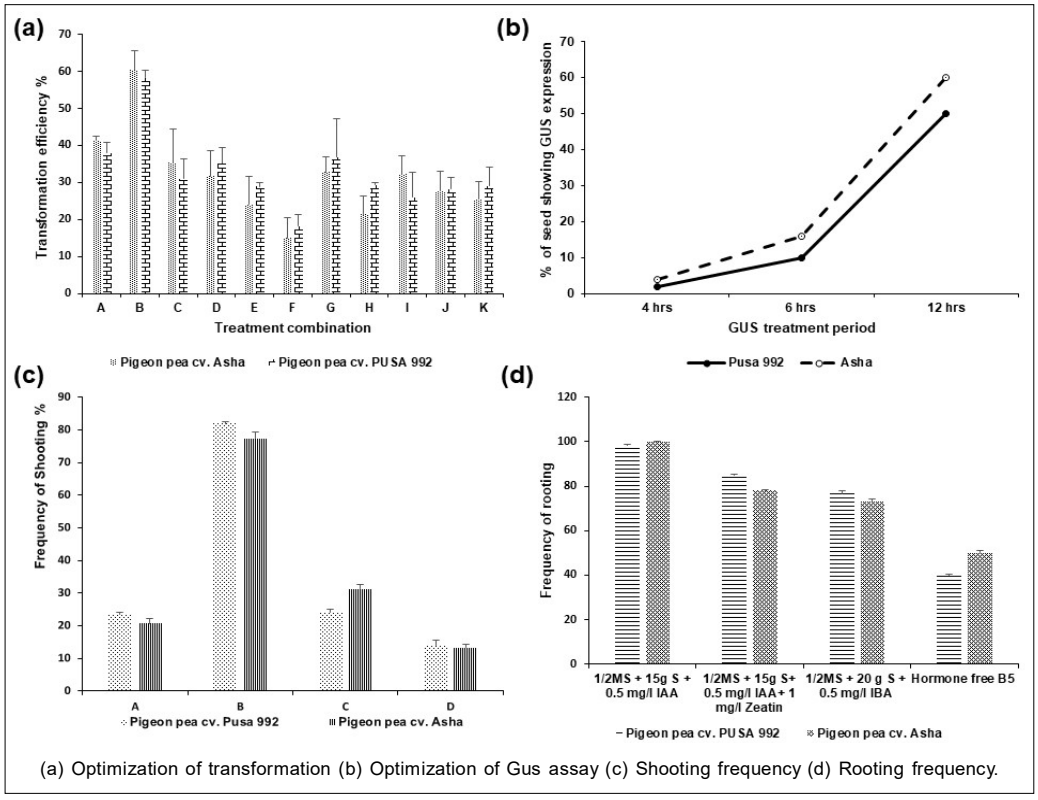
Treatment {OD600 + Acetocyringone (µM) + Inoculation (min) + Cocultivation (hrs)}	Mean no. of explant recovered in kanamycin selection		Mean no. of plants positive in *nptII* PCR		Transformation efficiency (mean with standard error)	
	Asha	Pusa 992	Asha	Pusa 992	Asha	Pusa 992
A- 0.2+100+15+48	46	45.66	19	17.33	41.31±1.14	37.92±2.89
B- 0.2+100+15+72	47.33	46.33	28.67	27	60.54±5.0	58.32±2.04
C- 0.2+200+25+48	33.66	32.33	10	9.66	35.29±9.09	31.00±5.26
D- 0.2+200+25+72	29.67	32.66	9.33	11.33	31.75±6.74	35.3±4.15
E- 0.2+200+15+96	19.67	31.33	4.66	9	24.09±7.58	28.88±0.93
F- 0.5+100+15+48	42	40.33	6.33	7.33	14.98±5.60	18.23±3.10
G- 0.5+100+15+72	36	28.66	16.67	10	32.74±4.10	36.88±10.2
H- 0.5+100+25+96	30.33	22.66	9.33	6.67	21.44±4.77	29.39±0.42
I- 1.0+200+15+48	23.67	21.66	11.67	5.67	32.28±4.93	26.03±6.68
J- 1.0+100+15+96	20.33	23.33	13.33	6.67	27.69±5.39	28.27±3.04
K- 1.0+200+25+48	28.67	25.33	14.33	7.33	25.44 ±4.72	29.11±4.95

Treatment was replicated three times with a set of 50 explants per treatment combination.



(a) *In planta* transformed (i) seed, (ii) untransformed control. (b) Tissue culture transformed (iii, iv) embryonic axes (v) untransformed control.

Fig 4: GUS staining.



(a) Optimization of transformation (b) Optimization of Gus assay (c) Shooting frequency (d) Rooting frequency.

Fig 5: Summary of pigeonpea transformation efficiency.

Table 2: Effect of PGR on shoot regeneration.

PGRs and antibiotics mg/l	Average no. of adventitious shoot buds per explant		Average no. of explants with multiple shoots		Average no. explants with elongated shoots after 4 weeks		Days require for shoots elongation up to 3-5 cm		Shooting frequency (%)	
	Asha	Pusa 992	Asha	Pusa 992	Asha	Pusa 992	Asha	Pusa 992	Asha	Pusa992
A: Preculture-BAP 2, SMI-BAP 2 + K 10 + C 250 + C 250, SMII- IAA 0.2 + GA 0.5+ ZEATIN 0.5+ K 100 + C 250 + C 250	4±0.5	3.6±1.2	18.6±1.8	15±2.8	10.3±1.4	11.6±0.8	36±3.05	40.3±1.2	20.7	23.3
B: Preculture-BAP 2.5, SMI- BAP 2.5 + K 120 + C 250 + C 250, SMII- IAA 0.5+ GA 0.5+ ZEATIN 1+ K 120 + C 250 + C 250	7.3±0.3	6.3±1.2	47.3±1.2	41±2.0	38.7±1.8	41±0.5	15.3±1.4	17±1.5	77.3	82
C: Preculture-BAP 5, SMI-BAP 5 + IAA 0.5 + K 120 + C 250 + C 250, SMII- IAA 0.5+ GA 1 + ZEATIN 1+ K 120 + C 250 + C 250	3.6±0.3	3±1	20±1.2	15.3±2.4	14.6±1.2	12±1.2	54±2.4	52.3±1.4	31.3	24
D: Preculture-BAP 10, SMI-BAP 10 + IAA 0.5 + GA 1 + K 120 + C 250 + C 250, SMII- IAA 0.5+ GA 1 + ZEATIN 1+ K 120 + C 250 + C 250	2.3±0.3	2.6±0.8	9.6±1.2	9.6±0.8	6.6±0.8	7±1.5	64±2.3	61.3±1.8	13.3	18

Table 3: Summary of pigeonpea transformation.

Variety	Number of explants used for transformation	Number of transformed regenerated in kanamycin	*nptII* gene PCR positive putative transformants transformants	*GUS* gene RT-PCR positive putative	Estimated transformation efficiency (%)
Pusa 992	1260	858	825	730	57
Asha	1100	845	812	715	65

Transformation efficiency of two cultivars expressed in percentage of RT-PCR positive T_0 plants/total number of explants used for transformation.

efficiency was determined by dividing total RT-PCR positive transgenic lines by the number of explants used for transformation (Table 3). This resulted in a transformation efficiency of 57% for Pusa 992 variety and 65% for Asha. Similar transformation efficiencies using PCR analysis have been documented when embryonic axes were used as explants for transformation (Thu *et al*., 2003).

GUS analysis

GUS assay was conducted to validate efficacy of transformation protocol. Initially, *in planta* transformation was employed to ascertain optimal GUS treatment duration. After 3 days of co-cultivation, treatment durations of 4 hours, 6 hours and 12 hours were tested. Results demonstrated a progressive increase in GUS expression over time for both the varieties. Peak expression was observed after a 12-hour treatment (Fig 5b). Transformed tissues with GUS gene expression exhibited a prominent blue coloration, primarily concentrated in axillary bud region of *in planta* transformed seeds due to activity of β-glucuronidase (Fig 4a i). In contrast, no GUS activity was detected in untransformed samples (Fig 4a ii). For tissue culture-based transformed explants, analysis was performed following a 3-day co-cultivation and subsequent 2 weeks of growth under selection conditions. Transformed explants displayed a positive reaction in assay (Fig 4b iii and iv). Untransformed exhibited a negative reaction, indicating absence of endogenous GUS-like expression (Fig 4b v). Findings indicated consistent integration and expression of GUS, implying stable integration of T-DNA within genome of primary transformants.

CONCLUSION

This study has optimized and developed an efficient tissue culture-based transformation method for pigeonpea, enhancing its ability to produce numerous adventitious shoots across different genotypes. This system is promising for integrating new genes into pigeonpea genome through *Agrobacterium*-mediated genetic transformation, ensuring high efficiency and reliability. It's a valuable resource for future crop improvement efforts, offering significant potential to enhance pigeonpea's genetic traits and agricultural productivity.

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Conflict of interest

It is declared that all the authors do not have any conflict of interest.

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