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Research Article

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Optimization of stable genetic transformation protocol in castor (*Ricinus communis* L. cv. TMV 5) using beta glucuronidase reporter gene for pioneer of desirable genes

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ABSTRACT

The simple and stable protocol was standardised for castor (*Ricinus communis* L. cv. TMV 5) genetic transformation using *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary plasmid pBAL2 (18.8 kb). Cotyledonary nodes from ten days old, *in vivo* seedlings were utilized as target cells for *Agrobacterium* mediated transformation. Explant pre-culture studies were carried out at 2, 4, 6, 8, 10 and 12 day intervals. The 4th day old explants cultivated on mMS medium (MS medium+B5 Vitamins) using plant growth regulators had the highest response percentage (50.6%). Kanamycin (0-175 mg/L) and Hygromycin (0-13 mg/L) sensitivity in well-developed shoots was investigated. Of the two antibiotics, Kanamycin 50 mg/L and Hygromycin 3 mg/L was found optimum. Different levels of acetosyringone (0-200 mg/L) were used in the co-cultivation medium to study the transformation efficiency of castor. Among the different concentrations, maximum number of explants showed GUS expression at 100 mg/L of acetosyringone in the co-cultivation medium at 2 days of co-cultivation period and the Cotyledonary node produced multiple shoots development and plantlet establishment in 0.3 mg/L TDZ, 0.6 mg/L PF-68, kanamycin 50 mg/L, 0.3 mg/L GA_3, 1.5 mg/L IBA and 0.6 mg/L $AgNO_3$. The rooted shoots were successfully acclimatized. Histochemical GUS assay was used to monitor T-DNA delivery into the target cells. PCR and Southern hybridization were used to confirm the transformants with the NPT II and GUS gene. A very high frequency (29.3%) of β-glucuronidase (GUS) gene expression was obtained through *Agrobacterium*-mediated gene transfer into cotyledonary node explants of Castor. The standardized protocol would be useful for *Agrobacterium*-mediated genetic transformation of Cator with desirable gene of agronomic importance.

KEYWORDS: Histochemical GUS assay, *Ricinus communis*, Southern hybridization, *Agrobacterium tumefaciens*, β-Glucuronidase, Polymerase Chain reaction

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INTRODUCTION

Plant breeding has produced a wide range of commercial plants and commodities with a number of significant agronomic features using traditional methods. Plant genetic engineering is a sophisticated tool for developing abiotic and biotic stress resistance. The transfer of desirable gene(s) into the plant genome has been made possible thanks to recent breakthroughs in gene transfer technology. Many tools for studying genetic change have been developed over the last two decades. Long-lived perennials have unique challenges when it comes to reproduction which can be overcome by employing gene modification (De Cleene & De Ley, 1976). In India and the United States, studies on the need for genetic transformation of castor to improve its agronomical properties have been conducted. The direct gene approach employing a particle is the most often utilised transformation method.

Because to the fact that both strategies have advantages and disadvantages (Potrykus, 1991; Sharma *et al.*, 2005), efforts have been made to combine the two ways to improve the conditions for economic transformation. In order to produce genetically altered plants, McKeon and Chen (2003) used the *Agrobacterium*-mediated transformation method of vacuum

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infiltration of wounded flower buds in *Ricinus communis* L. (US Patent No 6,620,986). The convenience, low cost, and simplicity of transgenic integration patterns are some of its benefits. Its main flaw is that it may have a limited host range. Tissue culture procedures as well as other selection approaches and *Agrobacterium* helper stains have to be improved to overcome this difficulty (Songstad *et al.*, 1990).

Agrobacterium tumefaciens, a pathogenic bacterial vector in plants, is used to transform plants (-proteobacterium of the Rhizobiaceae family). Over 600 plant species have been discovered to be infected with this disease (De Cleene & De Ley, 1976; Romano *et al.*, 1995; Zoina & Raio, 1999). When wounded plant tissue is subjected to *Agrobacterium* cells carrying a plasmid encoding the gene of interest and a selectable marker gene placed inside the transferred DNA (T-DNA) region, *Agrobacterium*-mediated transformation happens. Only a small number of cells are infected with T-DNA. *Agrobacterium* T-DNA permeates plant cells, in which it gradually merges with the nuclear genome and expresses, resulting in herbicide resistance, disease resistance as well as self-resistance (Liu & Binns, 2003).

Many sophisticated plant transformation vectors have previously been developed based on this naturally occurring gene transfer technique and are used in genetic engineering (Fraley *et al.*, 1986). A small vector bearing a false T-DNA and a helper Ti plasmid which supplies the essential pathogenic activities for transfer make up the extensively used binary system (Bevan, 1984; An *et al.*, 1988). The presence of binary vectors in A. tumefaciens makes it easy to clone desirable genes across T-DNA boundaries. The transformed cells acquire a unique edge over the proportionally large population of non-cells by being exposed to a selective agent in an *in vitro* transformation system.

Plant transformation vectors and techniques have been improved in terms of improving plant genetic transformation performance and create uniform transgene expression. Gene transformation which is *Agrobacterium* - mediated is influenced by a number of parameters, including temperature, phenols, cell damage, host cell division etc. (Wu *et al.*, 2003; Hiei & Komari, 2006). *Agrobacterium* does have numerous benefits over direct gene delivery like, it eliminates unwanted gene silence, transformants have few genomic alterations, and the copy number is low (Kohli *et al.*, 1998; Sudhakar *et al.*, 2006).

Employing reporter gene applications such as green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), red fluorescent protein (DsRed), β-glucuronidase (GUS), chloramphenicol acetyltransferase (CAT) in *Discosoma* sp., assessment of plants that are transformed can be proven (Lu & Kang, 2008; Wang & Xu, 2008). GUS and GFP are the most often utilised visual reporter genes (Jefferson *et al.*, 1987; Davis & Vierstra, 1998; Taylor & Fuquet, 2002). Anatomically and histochemically, the transformed transgenic plants are assessed. Typically reporting proteins are detected by enzymatically producing coloured fluorescent products, which may subsequently be easily quantified or localised. Schrammeijer *et al.* (1990), Alibert *et al.* (1999) and Müller *et al.* (2001) investigated stable, chimeric and transgenic gene expression in different dicot plants, and Sujatha and Reddy (2005) investigated these in castor. The tests for glucuronidase are particularly interesting because they do not use radioactivity. The degrees of transgenic expression, on the other hand, are usually unpredictably variable among separate transformants (Finnegan & McElroy, 1994).

Castor is not just a crop that can be genetically modified. A genotype-independent and extremely efficient transformation process is required for designing a castor transformation protocol. Castor transformation procedures were largely based on the multiplication of meristematic tissues due to the reluctance of castor tissues to *in vitro* treatments (Sujatha & Sailaja, 2005; Malathi *et al.*, 2006). *Agrobacterium*-mediated transformation using many castor explants was effective in transforming castor meristematic tissues, according to McKeon and Chen (2003) and Sujatha and Sailaja (2005) (US Patent No. US 6620986 B1). The advantages of *Agrobacterium*-mediated transformation include not only its ease and consistency, but also the capacity to create genetically homogenous transformed progeny by reducing somaclonal variance associated with tissue culture and regeneration (Sujatha & Tarakeswari, 2018). Using cotyledonary node explants as a starting point, an effective preliminary genetic transformation technique was constructed, and will be particularly beneficial during the intended gene transformation in the coming days to meet our needs.

MATERIALS AND METHODS

Plant Material

The castor seeds cv. TMV 5 was received from Tapioca and Castor Research Station, Yethapur, P. G. Palayam (PO), Salem district, Tamil Nadu, India. The cotyledonary node (CN) explant was selected as the optimal explant for castor transformation research. The CN explants were dissected from a 10-day old castor cv. TMV 5 seedling and performed the sterilising method based on the Kulathuran and Narayanasamy (2015). Depending on the outcomes of the cotyledonary node culture, more transformation studies were conducted out.

Pre-culture of Explants

In *Agrobacterium*-mediated transformation research, pre-culture is a crucial stage. The pre-incubation phase prepares the explant tissue to tolerate bacterial invasion and other stressors encountered during the *in vitro* pre-culture period. The preculture of the CN explants was done based on the Kulathuran and Narayanasamy (2015) before being selected on the Kanamycin (Kan) containing medium.

Determination of Antibiotic Sensitivity

By cultivating the explants in multiple shoot induction medium with Kanamycin (25, 50, 75, 100, 125, 150 mg/L)

and Hygromycin (2, 4, 6, 8, 10, 12 mg/L), the sensitivity of the CN explants to selection marker was assessed. The antibiotics were prepared in 1 g/10 mL of autoclaved double distilled water before being filter-sterilized and added to the autoclaved medium. The absolute death of the explants on a specific concentration of antibiotic-containing media was used to determine the antibiotic's minimum inhibitory concentration (MIC).

Agrobacterium Strain and Plasmid

The vector system for transformation was *Agrobacterium* strain LBA4404, which carried the binary plasmid pBAL2 (18.8 kb). The terminator sequences and 35S promoter were used to trigger the reporter gene uidA/GUS (β-glucuronidase). The selectable marker gene was the neomycin phophotranferase II (NPT II) gene, which was controlled by the nopaline synthase (Nos) promoter and terminator sequences (LBA4404 strains) (Figures 1 & 2).

Co-Cultivation and Selection of Stable Transformants

Agrobacterium tumefaciens strain LBA4404 with pBAL2 was cultivated on Luria Bertaini (LB) Medium (HiMedia) containing 50 mg/L Kanamycin (contains 10 g/L Bacto Tryptone, Bacto, 5 g/L Yeast extract, and 10 g/L NaCl). One bacterial colony was injected into 50 mL of liquid LB, and it was grown there with the same antibiotic overnight at 28 °C and 180 rpm. The following day, this overnight culture was reinoculated into 50 mL of brand-new LB medium with 50 mg/L Kanamycin. The volume fraction of the bacteria was adjusted to 0.8-1.2 OD600 (5×108 cells/mL). Bacterial cells were suspended in 25 mL of hormone-free liquid mMS medium with 3% sucrose after being pelleted at 5,000 rpm for 10 minutes. Even before to infection with *Agrobacterium*, a cotyledonary node obtained from a 10-day-old seedling was precultured on medium supplemented with 0.3 mg/L TDZ for 3-5 days.

The explants in the meristematic area, which is characterised by its typical swelling, were damaged with two hypodermic needle strokes (Dispovan India Ltd. 0.63x25). The treated explants were shaken manually for 10, 15, and 30 minutes in an *Agrobacterium* suspension. The explants were then blotted dried on sterile filter paper and co-cultured for 2 days in culture bottles comprising full-strength mMS basal salts containing the multiple shoot induction medium as well as various concentrations of Kanamycin and Hygromycin, and kept in the light at 26±2 °C under a 16:8 h light-dark cycle with a light intensity of 70 mol/m^2/s.

Explants were shaken (180 rpm) for 10-30 minutes even during bacterial incubation, solubilized using glass beads, and washed with acetosyringone (0-200 mg/L) to improve the uptake of *Agrobacterium* vector into target tissues. The infected explants were cultivated on the co-cultivation medium for two days to see if the co-cultivation period had an impact on the frequency of transformation. After co-cultivation, the explants were rinsed three times with sterile distilled water for five minutes each time with constant stirring, and they were then blotted dry on sterile filter paper. The explants were then washed twice with 250 mg/L cefotaxime for two minutes each. The technique for explant preparation, co-cultivation, three selection cycles (10 days each), and complete plantlet recovery is shown in Figure 3. The rooted shoots were acclimatised for 15 days in a plastic cup containing 1:1:1 sand, soil, and vermiculite and kept in an environmental growth chamber. Established plantlets were moved to a greenhouse environment and allowed to mature in earthen pots.

Putative Transformant Analysis

Uid A gene expression by histochemical assay

Some modifications to the GUS histochemical assay were made in accordance with Jefferson *et al.* (1987) instructions. The experiments made use of purportedly changed leaves, shoots, and shoot cultures that were taken from media that had been pre-selected using Kanamycin. The samples were incubated in a substrate solution comprising 0.05 M phosphate buffer (pH 7.0), 30% Triton X-100, and 1 mM X-Gluc (5-bromo-4chloro-3indolyl β-D, glucuronide) overnight at 37 °C is being pursued. After Gluc staining, the shoots were bleached in 95 percent (v/v) ethanol and examined under a stereo binocular microscope. The changed shoots to blue colour validated the expression of GUSA (D'Aoust *et al.*, 1999).

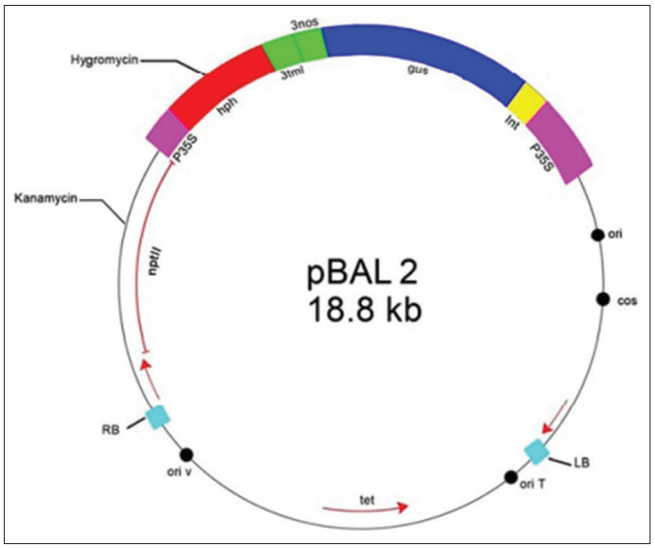

Figure 1: Complete Map of *Agrobacterium tumefaciens* pBAL2 transforming vector

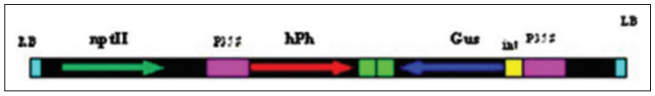

Figure 2: T – DNA region of pBAL2

Figure 3: Agrobacterium mediated genetic transformation of *Ricinus communis* L. using pBAL2 Vector. a) Preculture of explants (1.0 x), b, c, d) Shoot bud initiation from cotyledonary node explants in selection medium (1.0 x, 1.0 x & 1.5 x), e, f) Multiple shoot initiation & proliferation (2.0 x), g) Rooting (0.5 x), h, i) Hardening (0.1 x & 0.2 x), j) Well grown plant (0.1 x), k) Control shoot & leaf and l and m) Histochemical localization of Gus activity in node, shoots and leaves

Molecular Investigation

DNA amplification and PCR evaluation

Isolation of DNA from leaf tissues (Cetyl trimethyl ammonium bromide (CTAB) method)

Young leaves and shoots of putative transformants and untransformed (negative control) plants were utilised to obtain total genomic DNA using the CTAB method (Doyle, 1990).

Isolation of plasmid DNA from E.coli

The plasmid DNA extraction from *E. coli* was performed using the method described by Sambrook and Russell (2001) without modification.

Estimation of DNA concentrations

Fluorometry (Hoechst Dye-33258) was used to calculate the amount of extracted DNA (Cesarone *et al.*, 1979). A calf thymus DNA standard was employed.

PCR reaction

The PCR analysis techniques described by Edwards *et al.* (1991) were used. For PCR analysis, genomic DNA was collected from young leaves of putatively transgenic plants, positive and negative controls, and untransformed plants. The pair of unique uidA (GUS) primers (F) 5′- TTT CCA GTC GAG CAT CTC TTC AGC GT - 3′, (R) 5′- CCA GTC GAG CAT CTC TTC AGC GT - 3′, and pair of NPT II - 3′ The presence of the GUS (1.9 kb) and NPT II (680 bp) genes in putative transgenic plants was checked using specific primers (F) 5' – AAT CTC GTG ATG GCA GGT TGA – 3' and (R) 5' – GAG GCT ATT CGG GAT ATG ACT – 3'.PCR reaction master mix was made up of 12.5 of PCR Master mix (enzyme, buffer, dNTP combination) and 25 µL of total volume (Genei, Bangalore) 1.0 µL of each primer, 1.0 µL of Template/DNA (100 ng), and sterile distilled water to make up the final amount. 1X Taq buffer with 1.5 mM $MgCl_2$, 200 M of each nucleotide, 0.4 M of each primer, 1.0 unit of Taq DNA polymerase, and 100 ng of template in a total reaction volume of 25.0 L were used to make the final quantity of PCR components. The PCR sample was stored in a PTC 100 Programmed Thermal Cycler (Eppendorf, Germany) with the cycling temperature set to initial denaturation at 94 ºC for 5.0 min, followed by 30 cycles of denaturation at 94 ºC for 1.0 min, and annealing at 95 ºC for 1.0 min with extension at 72 ºC for 1.0 min. The reaction was held at 4 ºC for short-term storage after a final extension of 10 minutes at 72 ºC.

Agarose gel electrophoresis

PCR products were separated on a 0.8 per cent agarose gel that had been prepared with 1.0X TBE buffer by loading 20 µL of each sample and 3 µL of loading buffer. The gel was made by boiling the requisite proportion of agarose in distilled water in the proper amounts. After cooling the molten agarose to about 60 °C, 10 X TBE or 50 X TAE buffer was administered to an ultimate concentration of 1X and mixed thoroughly. To make wells, the gel was cast in a platform secured with a comb. For solidification, the gel was left to cool down temperature (normally left for 30 min after casting). 1/10 volume of loading dye (10 X) was mixed with 1/10 volume of DNA sample and loaded into the wells. Once electrophoresis was completed at 4-8 v/cm in 1 X TBE or TAE buffer, DNA in the gel was stained with ethidium bromide (0.5 g/mL) and viewed under UV illumination (PD Quest - BioRad). Castor is genetically transformed employing the GUS gene.

Elution of DNA from agarose gels

Electroelution of DNA

The electroelution of DNA samples was carried out according to Maniatis *et al.* (1982).

DNA Purification using glass matrix

The prepA-gene DNA purification kit (Bio Rad Laboratories, USA) was used to purify DNA from glass matrix, following the directions provided by the manufacturer.

Ligation of DNA fragments

DNA samples digested with restriction endonuclease were extracted twice with neutral chloroform/phenol and subsequently with water saturated ether, then precipitated with ethanol and utilised for ligation. For cloning studies, a concentration ratio of 1:3 of vector meant to be inserted was maintained. For ligation, approximately 50 ng of vector DNA was employed. Ligation reactions were carried out at 14 °C for 10 to 12 hours in the vicinity of 1 mM ATP and the manufacturer's buffer.

Southern hybridization analysis

For DNA blotting and hybridization investigation, the Southern (1975) technique was followed. On a rocker platform, for 45 minutes, the gel containing the DNA moved to a nylon membrane was soaked in 250 mL of denaturation solution. The gel was properly cleaned four times in sterile distilled water. After that, it was immersed in 250 mL of neutralising solution and rocked for 45 minutes. The gel was piled on a glass plate upside down on three numbers of Whatman No.3 sheets cut to the size of the gel and soaked in 20X SSC. The membrane was covered with a nylon membrane and then a dry sheet (Zeta-Probe, Bio-Rad, USA) tailored to the dimensions of the gel, wetted in distilled water and submerged in 20X SSC. On top of that, a glass plate was placed and a mass of roughly 250 g was placed over it. After 10 to 12 hours, the membrane was taken, rinsed briefly in 2X SSC, air dried, and baked for 30 minutes in a vaccum oven at 80 °C. When plasmid DNA was transferred, the semi-dry blotting procedure indicated above was used. To transmit genomic DNA from plants and total DNA from bacteria, a wet blot approach was used. The process is nearly identical to semi-dry blotting, with the following exceptions. 1) The longest Whatman No.3 sheet was dip in a reservoir containing 20X SSC, and the projecting corners of the sheet was immersed in a reservoir carrying 20X SSC. 2) The transfer was completed for duration of 16 hrs.

Solution for denaturation

0.5 M NaOH and 1 M NaCl.

Solution for neuralization

0.5 M Tris HCl, 1.5 M NaCl at pH 7.0SSC 20X.

In 750 mL distilled water, trisodium citrate (88.2 g) and sodium chloride (175.3 g) were dissolved. The solution's pH was raised to 7.0 using HCl, and volume was increased to 1000 mL with distilled water before autoclaving.

Radio labelling on DNA labelling

The probe DNA fragments utilised in the southern hybridization study were radio-labelled with a random primer provided by Amersham International Plc. UK. According to the producer's instructions, reactions were carried out in a final volume of 50 L using 20 to 30 ng of labelled DNA and 30 Ci of (-32P) dCTP.

The reaction was halted by introducing an equal volume of dye mix (6 mg blue dextran and 1 mg orange G in one mL of 0.5 M EDTA, pH 7.0) and the dye was separated in a Sephadex G - 50 column using the column buffer (0.1 M NaCl, 12 mM Tris HCl, pH 7.0, 2.5 mM EDTA). Separately, the blue portion containing the tagged probe was retrieved used for hybridization.

Radiolabeling of probe DNA

DNA fragment was radiolabeled using an Amersham International Plc, UK, random primer Oligolabeling Kit. The randomly chosen primers from the kit and the electroeluted DNA (25 ng) were mixed to a final volume of 35 L in sterile distilled water in an Eppendorf tube. The tube was heated for five minutes in a boiling water bath to denature the DNA, and then it was cooled to room temperature. 10 µL of the labelling mix, 30 µci of (-p32) dCTP (specific activity-1.48 x 1014 Bq/mmole), and 2 µL of Klenow fragment were supplied to denatured annealed DNA. The tube was gently mixed, microfuged for five seconds, then incubated for 20 minutes at a temperature of 37 °C.

Hybridization

The nylon membrane supplier's suggestions for hybridization were followed (Bio-Rad, USA). For pre-hybridization, hybridization, and post-hybridization washing, a hybridization oven was employed (Bachofer, Germany). Membrane was inserted in the hybridization bottle, along with 10 mL of pre-hybridization solution (which contains 7% SDS for blocking). If there were any air bubbles, they were carefully removed. Pre-hybridization was carried out at 65 °C for 30 minutes. The solution was removed after prehybridization and 10 mL of fresh prehybridization solution was administered. After 5 minutes of probe denaturation in boiling water, the sample was promptly chilled on ice. Hybridization was performed at 65 °C for 12 to 24 hours after adding the denatured probe DNA to the bottle.

Post hybridization washes

The nylon membrane manufacturers (Bio-Rad, USA) suggested that the post-hybridization washing be performed at lower stringency circumstances (for heterologous probes) or at higher stringency circumstances (for homologous probes) (for homologous probes). The blots were subjected to Kodak Biomax MS film at a temperature of -80 °C after hybridization and washout.

Wash at low stringency conditions

The membrane was washed with 2X SSC/0.1 per cent SDS solution at 65 °C after hybridization was completed. After that, three washes at 65 °C with 2X SSC/0.1 per cent SDS were conducted (each wash for around 30 min). The membrane was then air dried before being exposed to Konica X-ray film at a temperature of -70 °C with an amplifying screen.

RESULTS AND DISCUSSION

This work performed preliminary castor transformation utilising pBAL2 containing the GUS gene. Physical and biological elements were optimised to improve the frequency of transient GUS expression. Bacterial cell density, incubation length, explant preculturing, acetosyringone, which is a phenolic chemical that acts as a vir gene inducer, glass beads, and hypodermic needle prickling were all relevant. As the bacterial transformation is meristem-based, it proved challenging to get rid of the bacterium at greater concentrations. After 15 minutes of incubation and cocultivation on complete mMS media, all tests were conducted using Agrobacterium tumefaciens strain LBA4404 carrying the pBAL2 plasmid at a bacterial density of 5 x 108 cells per mL.

Plant Materials

For meristem-based transformation, it is necessary to have access to a prolific system of shoot proliferation from bud explants. Because of their exceptional proliferative abilities, cotyledonary node explants from 10-day-old seedlings were found to be the best of the various explants. Many methods for multiplying castor shoots from different meristematic explants (Athma & Reddy, 1983; Reddy *et al.*, 1987; Sangduen *et al.*, 1987; Molina & Schobert, 1995; Sujatha & Reddy, 1998) was recorded. Regarding the enormous proliferative power of the meristems, the approach developed in our lab performs better than the others. In this study, the effects of several cytokinins on shoot proliferation from bud explants were investigated, and PF - 68's effectiveness for castor multiplication was shown for the first time. With the remarkable capacity of the CN's meristematic zone to proliferate on medium enriched with PF - 68, the current work attempted to modify castor by A. tumefaciens-mediated gene transfer. Due to the improved responsiveness of the meristematic zones with PF-68 pre-treatment prior to transformation, PF-68 is becoming a crucial component in the genetic enhancement of woody species (Vanjildorj *et al.*, 2006; Deguchi *et al.*, 2020; Jogam *et al.*, 2022).

Preculture of Explants

In Agrobacterium-mediated transformation research, pre-culture is a crucial stage. Alteration from either the explant or the bacteria can improve transformation efficiency by increasing virulence. Pre-culturing explants to enhance the number of competent cells for transformation is the basis for such treatments (McHughen *et al.*, 1989; Burrus *et al.*, 1996). Preculture trials of explants were performed at frequent intervals of 2, 4, 6, 8, 10, as well as 12 days in this investigation. The 4-day-precultured explants cultivated on mMS medium using plant growth regulators showed highest percentage of response (50.6%) of the group (Figures 3a & 4). As the explants were not able to tolerate the robust reactive power of *Agrobacterium*, 2 day precultured explants exhibited the lowest survival rate in the medium. Likewise, due to extensive explant growth, the explants pre-cultured for 6, 8, 10, 12, and 14th days were unable to receive the *Agrobacterium*. Many workers have also mentioned

the role of pre-culture in transformation research (Veluthambi *et al.*, 1989, Vasudevan *et al.*, 2002). The researchers found that pre-culturing explants minimises wound stress and increases the number of competent cells at the injured location through direct organogenesis (Bautista-Montes *et al.*, 2022).

Impact of the Selection Process and Antibiotics

The sensitivity of well-developed shoots to medium treated with Kanamycin (0-175 mg/L) and Hygromycin (0-13 mg/L) was examined. Shoots bleached with larger concentrations of the antibiotic on medium with 100 mg/L, with 100% bleaching identified after one week, and rising concentrations of Kanamycin also indicated a dramatic decline in the mortality rate of the explants on the medium with 100 mg/L. The results of the experiment using higher Kanamycin concentrations showed a significant decrease in the survival frequency of explants on media containing more than 50 mg/L and higher concentrations of the antibiotic, with some shoot cultures failing to survive during the second shortlisting cycle. On the third selection cycle, shoots started to grow on medium containing 50 mg/L Kanamycin. Mortality on medium was less abrupt with initial antibiotic treatment of 50 mg/L compared to greater doses. Thus, Kanamycin 50 mg/L was found to be the best concentration because it was not harmful to changed shoots and had a slower effect than the reduced concentration, which might result in escape recovery. The elimination of untransformed shoots while maintaining the growth of putative transformants was demonstrated to be balanced by the use of kanamycin 50 mg/L. The control plants did not survive the first selection cycle on Kanamycin 50 mg/L media (Figures 3b, c & 5a).

In this context, one of the most crucial components in the transgenic plant screening process is the right concentration of antibiotics (Bibi *et al.*, 2013). The results of the experiment with increasing hygromycin concentrations showed a significant decrease in the retention frequency of explants in combination with 3 mg/L and higher concentrations of the antibiotic, and the shoot cultures was unable to flourish during the second selection phase. In media containing an initial concentration of 1 mg/L of Hygromycin, shoots continued to grow during the third selection phase. On medium, mortality wasn't quite as sudden with an initial antibiotic treatment of 3 mg/L as it was with greater dosage, but only a small percentage of shoots were able to survive the third selection. As a result, Hygromycin 3 mg/L was shown to be the most effective and is now considered the minimal inhibitory dose that can lead to escape recovery (Figures 3d & 5b).

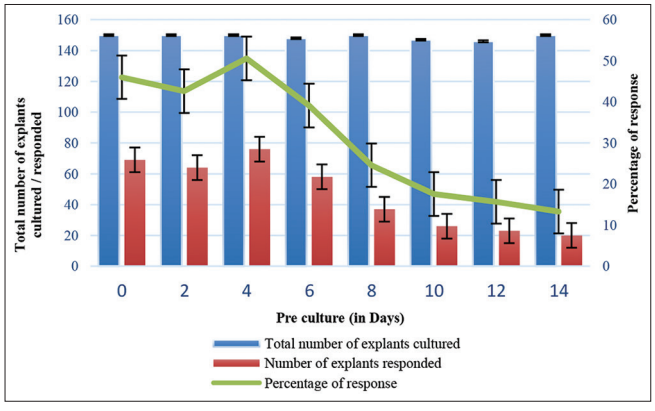

Figure 4: The effect of pre culture on growth response of cotyledonary node explants after co-cultivation

Effect of Acetosyringone on Transformation

Acetosyringone is a recognised activator of the Ti plasmid's vir genes, which would assist in the effective transfer of T-DNA. The efficiency of castor transformation was studied using significant ratios of acetosyringone (0-200 mg/L) in the co-cultivation medium. GUS expression was observed in certain explants in the absence of acetosyringone. However, the majority of the explants that expressed GUS accomplished so with a concentration of 100 mg/L of acetosyringone in the co-cultivation medium. Acetosyringone is a phenolic compound released by the injured tissues of dicotyledons. The current findings were consistent with those disclosed for Castor (Sujatha & Sailaja, 2005), Camellia (Lopez *et al.*, 2004), Sorghum (Pandey *et al.*, 2010), *Passiflora edulis* f. *edulis* × *Passiflora edulis* f. *flavicarpa* (Asande *et al.*, 2020) and Moroccan Duram Wheat (Ahansal *et al.*, 2022) wherein the adding of acetosyringone to co-cultivation media ultimately results in the highest genetic transformation frequency (100 per cent) (Figure 6). Glass beads utilised for cocultivation showed no differences in transformation frequency, hence they were excluded from future trials.

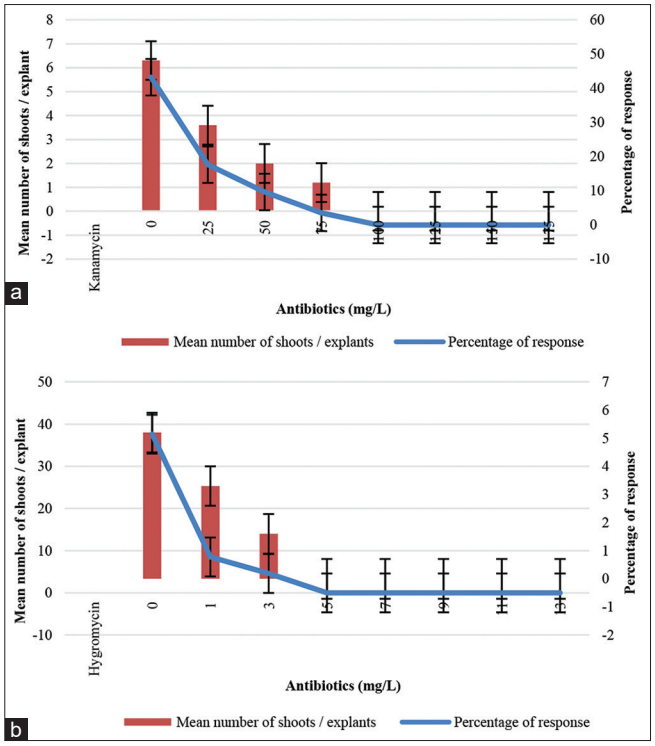

Figure 5: a) The effect of Kanamycin sensitivity with growth regulators TDZ (0.3 mg/L) and PF-68 (0.6 mg/L) and b) The effect of Hygromycin sensitivity with growth regulators TDZ (0.3 mg/L) and PF-68 (0.6 mg/L)

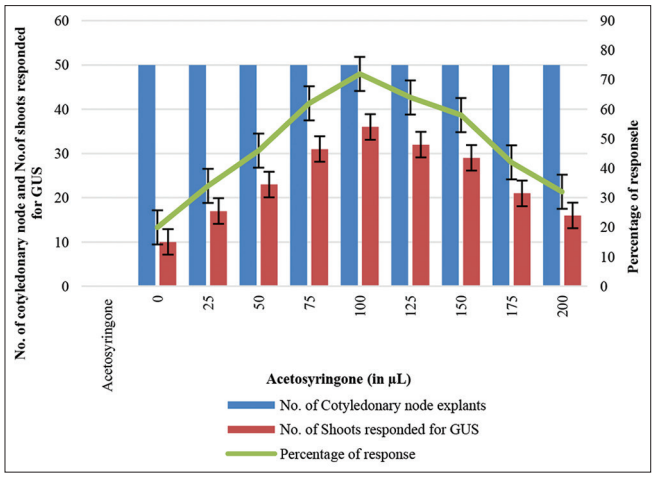

Figure 6: Effect of acetosyringone concentration on transformation efficiency using GUS

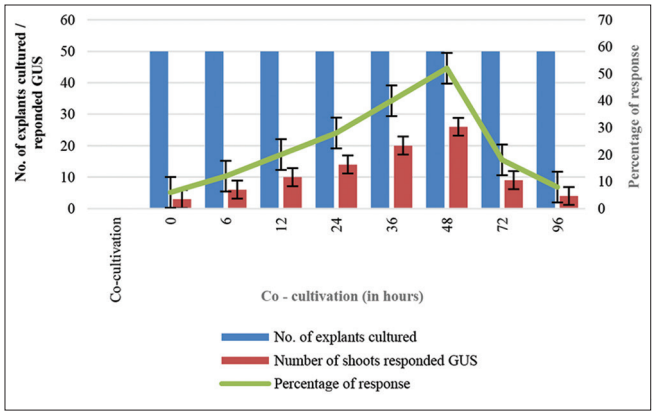

Figure 7: Effect of co-culture duration on transformation efficiency of cotyledonary node explants

Period of Co - Cultivation

Throughout the third selection cycle, differences brought on by the co-cultivation time were discernible (Figure 7). The duration of co-cultivation varied from 0 to 10 days. The considerable survival rate during the 10-day co-cultivation of cotyledonary node explants resulted in a high rate of transformation. In all stages of selection, shoot development was modest and accompanied by bacterial overgrowth, which was uncontrollable even after the third cycle of selection. For most plant species, an *Agrobacterium*-mediated transformation delay of two to seven days is advised (Sujatha & Sailaja, 2005). As a result, in this study, a two-day co-cultivation interval was favoured (Figure 7 (48 hours-two days)) which was also reported by Naing *et al.* (2016) in *Chrysanthemum* cv. *'Shinma* and Haider *et al.* (2020) in *Chrysanthemum* cv. *Jinba*. After two days of co-cultivation, the explants were washed in sterile half-strength mMS liquid media with 250 mg/L Cefotaxime to stop *Agrobacterium* overgrowth in the infected explants (data not shown). The diseased explants were then put into the selection medium. *Agrobacterium* overgrowth was entirely prevented by Cefotaxime, resulting in the destruction of the explants. Contrary to our findings, Cefotaxime, an *Agrobacterium* suppressor, was found to significantly slow down shoot regeneration from *Arabidopsis* root explants (Valvekens *et al.*, 1988). Cefotaxime (250 mg/L) had no effect on callus induction or shoot regeneration ability in *J. curcas* cotyledon explants, confirming our observations. As a result, after co-cultivation with cotyledon explants, Cefotaxime was employed to limit *Agrobacterium* (Li *et al.*, 2006, 2008). Outgrowth of *Agrobacterium* was reduced by 250 mg/L Cefotaxime in time of transformation of shoot apexes as well as embryo axes with the GUS gene (Sujatha & Sailaja, 2005). Cefotaxime at 150 mg/L was determined to be an environmentally safe dose for reducing *Agrobacterium tumefaciens* growth and creating a healthy Sugarcane shoot (Tiwari *et al.*, 2018). Kazemi *et al.* (2014) discovered that Cefotaxime 400mg/L had a bacteriostatic effect in tomato throughout their experiments.

In a great number of investigations on establishing the conditions for enhancing transformation efficiency via A. *tumefaciens*-mediated gene transfer, a transient GUS assay is utilised as a marker of T-DNA transfer. In the current investigation, results were drawn based simply on the likelihood of shoot survival during three selection cycles. The findings supporting this approach are based on stable integration of the introduced gene.

Shoot Development and Plantlet Establishment

On media containing 0.3 mg/L TDZ, the cotyledonary node generated numerous shoots with zero base callusing. The CN elongated and developed normal thickening at the meristematic area after one week in the medium with 0.1 mg/L BA prior to co-cultivation. The meristematic region expanded significantly as a result of subculture, co-cultivation, and CN culture on medium supplemented with 0.3 mg/L TDZ and 0.6 mg/L PF-68, in addition to a significant number of microscopic green invaginations. The protuberances diversified into shoot-like structures while these cultures were introduced to selection media having 0.6 mg/L PF-68, 0.3 mg/L TDZ, 50 mg/L Kanamycin and 0.6 mg/L PF-68. Non-transformed cultures become necrotic on the second and third selection rounds, although potential transformants continued to proliferate. Shoot elongation was enhanced by transferring cultures subjected to 3 selection cycles to media comprising 0.3 mg/L GA_3 and 0.6 mg/L PF-68. All the elongated shoots with more than two independent nodes formed roots on media supplemented with 0.6 mg/L AgNO3 and 1.5 mg/L IBA. The rooted shoots acclimatised well (Figures 3e-k & 8).

Histochemical Gus Analysis

The GUS assay was performed on the plant acquired after transformation. For the study, shoots and leaves were gathered. These specimens were placed in X–Glu solution then cultured at 37 °C overnight. The obtained samples were then processed with a 2:1 mixture of acetic acid and ethanol. The pre-culture of explants, co-cultivation duration, acetosyringone level, and bacterial cell density all had a substantial impact on the GUS expression in the meristematic region. For approximately four

months, the GUS expressed of transgenic plants was investigated. The shoots that were recovered after three selection cycles had undergone histochemical GUS screening, which showed the presence of GUS expression (Figures 3l & m). GUS staining was more intense in shoot primordia as well as in young leaves. The strain of the bacteria *Agrobacterium* is yet another critical element that may have an impact on the effectiveness of genetic modification. Agrobacterium-mediated gene transfer has been effectively employed in various dicots, and this technology has been extensively utilized in a large number of genotypes (Su *et al.*, 2023). Depending on how susceptible each *Agrobacterium* strain is, different plant species may be able to use different *Agrobacterium* strains. LBA4404 was better and more efficient than the other castor (McKeon & Chen, 2003; Sujatha & Sailaja, 2005), *Jatropha* (Li *et al.*, 2008) and in *Eucalyptus urophylla x Eucalyptus grandis* DH32 – 29 (Wang *et al.*, 2023).

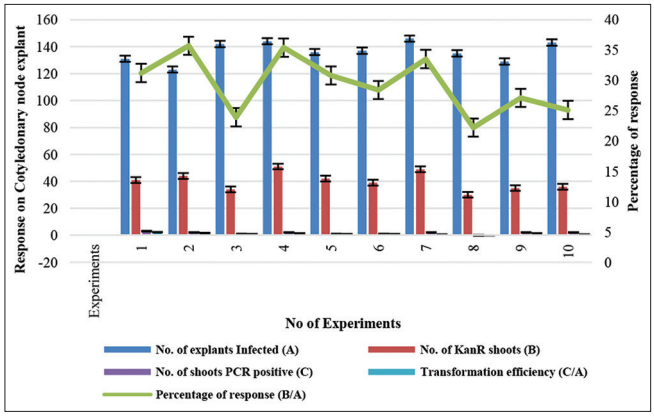

Figure 8: Preliminary gene transfer with Agrobacterium strain pBAL 2 and Kanamycin 50 mg/L (Selection Marker) using 2 days preculture explants with 100 mg/L acetosyringone on their response on cotyledonary node explants

Molecular Confirmation

PCR analysis

In this study, the molecular study was performed using PCR. The detection of the NPT II and GUS genes in kanamycin-resistant putative transformed plants obtained following 3 cycles of selection in LBA4404 Agrobacterium strains bearing the binary plasmid pBAL2 supported the amplification. Gene specified primer sequences for NPT II and GUS were used for PCR amplification, generating 680 bp and 1.9 kb amplified fragments, accordingly. Shoots that had not been altered (control) produced no increased product (Figures 9a & b). The transformation percentage of Agrobacterium tumefaciens was estimated using the proportion of PCR positive plants and the overall number of co-cultivated explants. The frequency of putative transformants retrieved after three selection phases was determined to be 16 in 1,366 (1.17 percent) CN cultured cells (Table 1, Figures 9a & b).

Southern hybridization

The stability of the amplified gene segment was confirmed using Southern blot hybridization upon the PCR gels. Hind

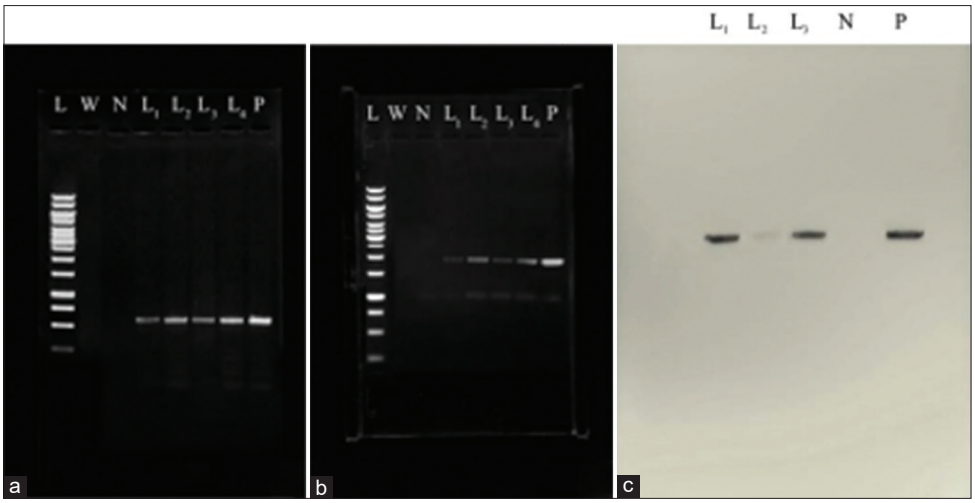

Figure 9: Confirmation of transformants using PCR and Southern hybridization techniques. a) PCR analysis of putatively transformed plants using NPT II (L-Ladder W-Water Control, N-Untransformed plant DNA (negative control) L1 to L4-Transformed plant DNA, P-Plasmid DNA (positive control), b) PCR analysis of putatively transformed plants using Gus (L-Ladder W-Water Control, N-Untransformed plant DNA (negative control), L1 to L4-Transformed plant DNA, P-lasmid DNA (positive control) and c) Southern hybridization of putatively transformed plants (P-Plasmid DNA (positive control), L1 to L3-Transformed plant DNA, N-Untransformed plant DNA (negative control)

Table 1: Summary of transformation using Agrobacterium strain with pBAL2, Kanamycin selection regime and response of cotyledonary node explants

No. of explants Infected (A)	Number of Kanamycin^R shoots (B)	Percentage of Response (B/A)	No. of shoots PCR positive (C)	Transformation Efficiency (%) (C/A)
1366	401	29.3	16	1.17

III and Eco RI were used to digest the genomic DNAs. One duplicate of the NPT II gene was found on a Southern blot assay of genomic DNA. The digested DNA of the control plant did not contain any hybridization signals. While genomic DNA digested with Hind III produced DNA fragments that hybridised at 1.9 kb, PCR-amplified molecules produced a signal at 690 bp when they hybridised with the probe. The results of Southern hybridization revealed that perhaps the transgenic plants had a steady incorporation of foreign DNA (Figure 9c).

CONCLUSION

This research is the first successful attempt to develop a stable transformation system for castor through *A. tumefaciens* (strain LBA4404) mediated transfer using CN from 10-day-old *in vivo* seedlings. With this protocol, a primary transformant can be developed within 4 months from culture initiation. At present, there are no described protocols for the genetic transformation of castor using vegetative explants. In this study, a reliable system of plant regeneration has been developed for castor, a meristem-based transformation system was developed using a reliable system of plant regeneration which will be useful for the introduction of desirable genes into this commercially important crop.

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