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Research Article

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# Development of an efficient Micro propagation based Agrobacterium-mediated Genetic Transformation Protocol in Commercial cultivar of Jute (*Corchorus capsularis* L.)

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**Abstract**

The present study describes an efficient *in vitro* culture protocol for direct plantlet regeneration and *Agrobacterium*- mediated genetic transformation of *Corchorus capsularis* L. cultivar JRC 517. *In vitro* morphogenetic capacity of different explants was evaluated. Nodal explants with immature axillary buds showed maximum *in vitro* culture response (95%) with plantlets induction when cultured on MS with 0.1 mg $l^{-1}$ IAA and 0.1 mg $l^{-1}$ Kin. *A. tumefaciens* strain LBA4404 harbouring a binary vector pBI121, containing *gus*A reporter gene under the transcriptional control of Cauliflower Mosaic Virus (CaMV) 35S promoter and NOS terminator was used in addition with neomycin phosphotransferase (*npt-II*) as plant selection marker gene. Different parameters viz. $O.D_{600}$.- of *Agrobacterium* cell suspension: 0.3; one day preculture; one min explants dipping, vacuum infiltration for 10 min at 600 mm Hg pressure; 0.001 ml $l^{-1}$ concentration of non-ionic surfactant (Tween 20) and two days co-cultivation with 100 µM acetosyringone (AS) were found to be optimum treatment to achieve maximum number of stable genetic transformants (~3.6%). The putative transformants were screened on MS medium supplemented with 50 mg $l^{-1}$ kanamycin (Kan50) and their transient expression was confirmed through GUS histochemical assay of the reporter gene and PCR analysis. The survivor plants were grown under Kan50 selection pressure, and rooted successfully. Regenerated plantlets were acclimatized, hardened and transplanted to green house. Stable integration of the transgene into the recipient genome was confirmed by PCR using compatible primers of *gusA* and *nptII*, and through Southern hybridization. The transgenic plants showed normal morphology and most of them followed 3:1 ratio of Mendelian inheritance for a single dominant locus. *In vitro* direct shoot regeneration protocol from nodal explants with concurrent transgenic development deemed to be successfully involving economically important gene/s and trait enrichment in jute.

**Keywords**

Jute, immature axillary buds, transformation, micropropagation, direct shootlet regeneration

## Introduction

Jute is commonly known as the 'golden fiber' and it is the second important cash crop in terms of fibre production next to cotton (Kundu 1956, Kirby 1963). Nearly 90% of the total world production of the raw jute fibre comes from Indo-Bangladesh subcontinent (Hossain *et al*. 1994). The plants are annual or short-lived perennials, distributed accross tropical, sub-tropical and warm regions of the world. Among the 100 species of the jute plant genus *Corchorus*, only *C. capsularis* L. and *C. olitorius* L. are cultivated as fiber crop and gained commercial importance due to its diversified value-added manufacturing products. However, the jute crop demands immediate attention of plant breeders to improve agronomically desirable traits. Sexual incompatibility and strong transexual un-relatedness between two cultivated species are serious bottlenecks to harness the benefits of *cis*genics for its genetic improvement (Patel *et al*. 1960, Hossain *et al*. 2002, Saha *et al*. 2014a, Begum *et al*. 2013) and limited development of better quality of jute fibers through conventional breeding methods (Islam and Rashid 1960, Patel and Datta 1960, Swaminathan *et al*. 1961, Sarker and Hoque 1992, 1994).

Although *in vitro* tissue culture technique coupled with genetic transformation has created new possibilities for the step up of different crop plants its contribution seems to be inadequate in jute. One of the most important prerequisite of gene transfer technique is a robust, reproducible and efficient *in vitro* regeneration protocol. However, jute is reported to be *in vitro* recalcitrant in their response to plantlet regeneration and genetic transformation (Sarker *et al*. 2007, Saha *et al*. 2014), the grounds might be physiological grade of explants, exogenous as well as endogenous plant growth regulators (PGRs), nutrient media composition, solidifying agents, physico-chemical environments etc. There are a number of reports on *in vitro* culture and plantlets regeneration of jute (Islam *et al*. 1982, Rahman *et al*. 1985, Das *et al*. 1986, Ahmed *et al*. 1989, Saha and Sen 1992, Seraj *et al*. 1992, Khatun *et al*. 1993, Hossain *et al*. 1994, Abbas *et al*. 1997, Saha *et al*. 1999), yet needs improvement with respect to their reproducibility. Islam *et al*. (1992) reported protocols with limited regeneration in a few varieties of *C. capsularis*, whereas Saha and Sen (1992) reported somatic embryogenesis from cotyledon and hypocotyl protoplast derived calluses in *C. capsularis* without any regenerate. However, an efficient clonal propagation protocol was reported, which regenerated the plantlets till flowering in jute (Abbas *et al*. 1997, Sarker *et al*. 2007).

Micropropagation is a technique that manipulates small quantities of axenic plant material *en masse*, to form new plantlets. This technique has been proved to be the most efficient and cost-effective. General clonal propagation techniques (cuttings, grafting, and division of parental stock materials) are highly vulnerable to seasonal variations and development of large number of plantlets*in*

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*natura* is tedious. However, micropropagation offers advantages of year-round production of new plants at rates significantly higher than other conventional methods. The plants produced are genetically uniform, vigorous, and free from disease, which can be rooted and the plantlets can be planted by reducing the time requirement for the grower when seeds or cuttings are found to be slow to establish. The technique is preferred over the conventional asexual propagation methods since *(a)* it requires small amount of tissue as the initial explant for producing of microclones year around, *(b)* it is a possible alternative for developing resistance in many species; *(c)* provides a mean for international exchange of safe planning materials, hence the problem for introduction of disease does not arise which is quarantine offensive; *(d) in vitro* stock could be quickly proliferated as it is not season dependent, and *(e)* precious germplasm can be stored for extended period (Hu and Want 1983, Mascarenhas and Muralidharan 1989). The technology is gaining importance in many valuable horticultural crop husbandry, tree species, orchids and plants propagated asexually very fast due to their high economic importance, owing to phytosanitary safety.

In order to harness the advantage of *in vitro* micropropagation technique in jute, different explants were cultured in this series of experiments to evolve most efficient protocols for shooting of jute and rooting of micrpropagted shootlets for development of healthy whole plants.

## Materials and Methods

### Surface sterilization and explant preparation

Mature seeds of *C. capsularis* var.JRC 517, were collected from the Gene Bank of ICAR-Central Research Institute for Jute and Allied Fibres (CRIJAF), Barrackpore, Kolkata 700120, India. Seeds were washed with tap water three times and surface-sterilized by snap wash in 70 % ethanol for 1 min followed by immersing in freshly prepared 0.2 % (w/v) $HgCl_2$ solution for 10 min with a drop of Tween 20 (Sigma, USA) as non-ionic surfactant under vertical Laminar Air Flow (LAF, Klenzaids). Subsequently the seeds were rinsed thoroughly with sterile $ddH_2O$ for 3-5 times for removing traces of detergent and $HgCl_2$. Sterilized seeds were soaked in sterile $ddH_2O$ in dark (~100 seeds/ 20 ml), until those sprouted to a length of ~0.5-1.0 cm. At every 8 h interval the water was replaced with fresh sterile $ddH_2O$. Finally, the germinating seeds were blot dried on sterile Whatman filter paper and transferred onto solid medium containing MS salts with vitamins (Murashige and Skoog 1962), 3 % (w/v) sucrose, pH adjusted to 5.8 ± 0.02 and 3.5g $L^{-1}$ Gelzan$^{TM}$ CM (Sigma Aldrich USA) as solidifying agent followed by autoclaving at 121$^0$C, 15 psi for 21 min.

### Micro-shoots regeneration protocol and culture condition:

In this experiment three separate explants were used for *in vitro* culture to assess maximum response and to find out the most efficient plantlet regeneration system for development of transgenic plants**.**

### A. *In vitro* micropropagation using shoot meristem-tip explant

Explants (meristem-tip: less than 1 mm in length) were excised from 3-days old germinating seedlings and cultured on MS salts and vitamins with 3 % (w/v) sucrose, supplemented with kinetin (Kin 0.15, 0.5, 1.0, 1.5 and 2.0 mg $l^{-1}$) and Indole-3-acetic acid (IAA 0.03, 0.05, 0.1 and 0.3 mg $l^{-1}$). Initially the Petri dishes (90 mm dia, Tarson, India) (five explants per dish) were kept in dark for 3 days, subsequently transferred to culture trolleys illuminated with cool white fluorescent lamps at 70-mol $\mu m^{-2}$ $s^{-1}$ (Philips, India). Cultures were subcultured at every 21 days interval and maintained for 16 h light and 8 h dark photoperiod at 28$^0$ ± 1$^0$C. Observation was recorded periodically to assess the morphological changes during the course of *in vitro* culture.

### B. *In vitro* micropropagation using epicotyl explant

Epicotyls portion (about 1 cm) from *in vitro* seedlings (5 days-old) were excised and cultured on MS medium containing different concentrations of auxin (IAA, 0.03- 0.5 mg $l^{-1}$) and Kin (0.15-2.0 mg $l^{-1}$) for induction of multiple shootlets. The culture vessels with explants and maintained as stated above.

### C. *In vitro* micropropagation using nodal explant with immature axillary buds

The nodal explant with immature axillary buds were excised aseptically (using a surgical blade) from 2-3 months old *in vitro* grown mature plantlets. Explants were cultured in upright position slightly embedded in the MS medium supplemented with IAA (0.03 - 0.2 mg $l^{-1}$), NAA (0.03 - 0.2 mg $l^{-1}$) BAP (0.05 - 1.0 mg $l^{-1}$), Kin (0.5 - 0.75 mg $l^{-1}$) in different combinations for induction of microshoots. The cultures were incubated in dark initially for 3 days and subsequently transferred to same light and temperature as practiced earlier, and subcultured at every 21 days interval.

### Plant genetic transformation

### Bacterial strain, plasmid construct and culture conditions

A single colony of the *Agrobacterium tumefaciens* (strain LBA4404) harbouring the recombinant pBI121::CaMV35S:*gusA*: NOS binary vector was inoculated in 50 ml YMB medium supplemented with 50 mg/l kanamycin (Kan 50). The bacterial culture was grown at 26 C for overnight in an orbital shaker a 150 rpm. The overnight grown culture was centrifuged at 7,000 rpm for 5 min and the bacterial pellet was re-suspended in liquid MS medium without antibiotics, supplemented with (50, 100, 150 and 200 µM) acetosyringone (AS), at pH 5.4 was maintained and kept about 5 h for *vir* gene activation. Prior to infection different concentrations of Tween-20 (0.001–1%) were

added to the *Agrobacteriun*-suspension (O.D. $_{600}$ – 0.3 + 50 µM of AS), to increase the transformation frequency of the explants.

**Transformation**

Based on the *in vitro* tissue culture response of different explants, axillary node with immature bud were chosen for transformation experiments. To synchronise and increase competence of explants as well as efficient T -DNA delivery by the *A. tumefaciens,* different preculture duration (12, 24, 36, 48 and 72 h) and period of co-cultivation (1, 2, 3, 4 days) were evaluated. After preculture meristematic regions of those axillary buds (~ 1 cm size) were injured slightly by a sterile blade and submerged in *Agrobacterium* suspension (for 1, 2, 3 and 4 min) followed by exposure to vacuum infiltration for 0, 2, 5, 10, and 15 min at 600 mm Hg pressure. The infected explants were blot dried on sterile Whatman filter paper and inoculated on MS medium without any antibiotics for co-cultivation under continuous dark for 2 days. To remove the adhered *Agrobacterium* cells from the infected surface of the explants after co-cultivation, the explants were washed thrice with sterile dd$H_2O$ followed by a wash with (100. 150, 200, 250, 300 mg $l^{-1}$) cefotaxime (Duce, India), not more than for 10 min. Co-cultivated explants were blot dried and transferred on to Kan50 supplemented micro shoot induction medium. The cultures were maintained under 16 h light and 8 h dark photoperiod cycle at 28° C.

**Induction and multiplication of microshoot from transforming explants**

Co-cultivated axillary buds were cultured on MS medium containing kanamycin (20, 30, 40, 50, 60, 70 mg $l^{-1}$) and maintained under 16 h / 8 h (l/d) photoperiod at 28° C for 14 days. Under the same media-light-temperature regime and selection pressure, explants were regularly sub cultured at 21-day interval and the morphogenetic response of the cultures were documented. The dead and necrotic tissues from the explants were trimmed during each subculture to eliminate escapes and chimeras. After 3–4 weeks of microshoot proliferation followed by shoot multiplication, the cultures were further subjected to three separate experimental systems: (a) the mother transformed stock separated out from the mature shoot bunches and transferred to fresh medium containing same composition; (b) to further enhance the rate of shoot multiplication, the immature shoot clumps (2–3 shoots) were again cultured on optimized medium; (c) excised shoots were individually cultured on MS medium with 1 mg $l^{-1}$ $GA_3$ to assess their response in respect of elongation.

**Root induction in regenerated plants**

Healthy elongated shoots (2–3 cm) derived from shoolets bunches were transferred to full or half strength MS medium supplemented with different concentrations of IBA or IAA and 1 % sucrose for root induction.

**Acclimatization and hardening**

*In vitro* rooted plantlets were taken out from culture tubes carefully and washed with sterile dd$H_2O$ to remove adhered nutrient agar. The well-rooted plantlets were kept in autoclaved Hoagland solution for acclimatization for 1 wk in the culture room at 28° C under 16 h light (80-100 mol $\mu m^{-2}$ $s^{-1}$) and 8 h dark cycle. Acclimatized plantlets with well developed roots were transferred to small pots containing a mixture of vermiculite and sterile sand in 1:1 ratio for hardening in glass house. To maintain high humidity, initially each pot was covered with a polyethylene bag for a few days (14-20 days). Subsequently, the humidity was reduced by making a few holes in the polythene bags to facilitate smooth gaseous exchange to harden the plants. The plantlets were watered at every alternate day with d$H_2O$ and once a week with modified Hoagland's solution (5 ml per plantlet). After 3 months, plantlets were evaluated for their survival, rooting and performance.

All experiments were carried out independently and each experiment was consisted of three replications per treatment (each with 100 explants) with corresponding control set (medium without hormone). The percentage of multiple shoots, average number of shoots per explant, percentage of rooting, and average number of roots per shootlets were recorded after 4 weeks. The photographs were captured by using Sony NEX-3 E5 camera.

**Anatomical and GUS-Histochemical study:**

Plantlet regeneration capacity from the nodal axillary explants was confirmed by a couple of histological analysis, where different stages of tissue were collected during the culture process and fixed in 2.5 % (v/v) glutaraldehyde and 4.0 % (v/v) formaldehyde buffered with 50 mM phosphate (pH 7.0) and stored at 4° C. The samples were dehydrated in a series of ethanol [30 – 100 % (v/v) in water] and embedded in paraffin followed by polymerization at 65° C overnight. Serial sections of 5–11 µm thick were made using a rotary microtome (Leica RM 2025), mounted onto glass slides with a drop of water and fixed on a hot plate (~ 70° C). For general histological observation, hematoxylin-eosin blue O [0 .5 % (w/v) in 0.1 M phosphate buffer] was used. The images were captured using the image capture system Power Short A80 (Canon, PC1059) camera attached to Axioskop 40, Carl Zeiss (Germany) microscope.

To confirm the transgene (*gusA)* expression in putative tranformants histo-chemical assay was carried out using histochemical procedure as described by Jefferson et al. (1987) with minor modifications. Tissues were incubated overnight at 37° C in 100 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM $K_3[Fe(CN)_6]$ and 0.5 mM $K_4[Fe(CN)_6]$, 10 mM $Na_2EDTA$, 0.5% (v/v) Triton X-100 and 0.5 mg $l^{-1}$ 15-bromo-4-chloro-3-indolyl-β-D-glucuronide (Sigma, USA) and the reaction mixture was kept under vacuum pressure for 5 min.

Following the incubation, chlorophylls were removed and the samples were fixed in a mixture of 95% (v/v) ethanol and 1% (v/v) glacial acetic acid.

**Molecular analysis for transgene integration**

Genomic DNA from putative transgenic plants developed through *Agrobacterium*-medeated micropropagation routewas isolated and purified in bulk from putative transgenic plants following the protocol of Kundu *et al*. (2011) for PCR and Southern analysis.

**Polymerase Chain Reaction (PCR)**

To confirm the putative transformants for transgene integration, the genome DNA was subjected to PCR using custom synthesized gene (*nptII* and *gusA)* specific forward and reverse primers. The oligonucleotide primer pair sequence used for the amplification of *nptII* gene was: Forward: 5' AGA TGG ATT GCA CGC AGG 3' and Reverse: 5' AGA AGA ACT CGT CAA GAA GGC GA 3'. The primer pair sequence for the amplification of *gus* gene : F: 5' ATG TTA CGT CCT GTG GAA ACC 3'and R: 5' TTC ATT GTT TGC CTC CCT GCT 3'. The PCR cycle profile followed had initial denaturation at 94 $^0$C for 5 min and then 35 cycles of denaturation at 94 $^0$C for 1 min, 1 min annealing at 58 $^0$C (for *nptII*) or 58.5 $^0$C, 1 min elongation at 72 $^0$C, followed by 15 min at 72 $^0$C for the final product extension. Amplified products were stored at 4 $^0$C until further use. The PCR product (amplicom) was size fractionated on horizontal electrophoresis system (Bio Rad) on 1.0% (w/v) agarose (Sigma, USA) gel with ethidium bromide (EtBr) and visualized under UV light and photographed with gel documentation system (Alphaimager®, USA).

**Southern hybridization**

Southern hybridization was performed, with 10µg of purified genomic DNA from each PCR-positive transgenic plants (assumed to have different transgenic events) and non-transgenic plants were digested with restriction endonucleases- *Hind*III and *Eco*RI. The double digested sample was size-fractionated by electrophoresis on 0.8% (w/v) agarose gel. Following the standard procedure (Sambrool *et al.*1989) of depurination, denaturation and neutralization, the DNA fragments were transferred to a Hybond-N$^+$ nylon membrane (Amersham, UK) by capillary transfer method in a neutral buffer (Roche, Switzerland) and cross-linked to the membrane under UV irradiation. The DNA fixed on membranes were prehybridized with salmon sperm DNA (Invitrogen, USA) at 68° C for 8 h to avoid non specific binding followed by hybridization with the probe at 68° C for overnight. The double restriction digestion (*HindIII-EcoRI*) released a fragment of ~ 3.0 kb (*gusA* gene) from plasmid vector pBI121, which was labelled with α-dCTD using the Random Prime DNA Labeling Kit (Roche Applied Science, IN, USA) following manufacturer's instructions, and was used as radio labelled probe. Finally, the membranes were exposed to autoradiographic film for 4 h (Indu Make) and were developed with an automatic film processor.

**Inheritance study of the transgene:**

To study segregation pattern of the transgene in the progeny of putative transgenic plants, analysis was carried out at $T_2$ generation. Seeds from $T_1$ and control plants were germinated on MS supplemented with 50 mg l$^{-1}$ Kan50 sulphate to select the true transgenic progeny. To continue the selection pressure, survived seedlings were subcultured at an interval of 7 days to fresh media containing 60 mg l$^{-1}$ Kan50. After 4 weeks, segregation ratio of the *nptII* transgene (no. of surviving seedlings**:** no. of non- surviving seedlings) was taken for all the sets. Survived seedlings were grown in soil at greenhouse and were further used for genomic DNA isolation for PCR using *nptII* gene specific primers.

**Statistical analysis**

Data were analyzed using Duncan's Multiple Range Tests (DMRT). All statistical analysis were performed ( $P < 0.05$) using SigmaPlot 10.0.

## Results and Discussion

**Shoot induction and multiplication**

In the present study, three different explants, i.e. 7 days old seedling meristematic tip, seedling epicotyle and nodal immature axillary bud from young plant of *C. capsularis* (var. JRC 517) were used for plantlet regeneration through micropropagation. Different morphogenic responses were observed during *in vitro* culture (Fig 1 A-C) with variable numbers of microshoot per explants. Meristem tips responded most effectively to produce microshoots (2.92 ± 0.17) on MS medium supplemented with 0.03 mg l$^{-1}$ IAA and 0.5 mg l$^{-1}$ Kin after 7 days of meristem culture in dark (Table 1). The maximum response of shoot multiplication (11.8 ± 0.52) was observed during 3$^{rd}$ passaging (Fig. a3). Microshoots induction from epicotyl explants also took place after 7-10 days of dark incubation with maximum microshoot (2.30 ± 0.15) on MS medium fortified with 0.1 mg l$^{-1}$ IAA and 1.0 mg l$^{-1}$ Kin (Table 2). In subsequent passaging to enhance the rate of shoot maturation, the microshoot clumps (2–3 shoots) were cultured on medium without any PGRs.The nodal explants showed bud breaking on MS medium containing cytokinin (BAP or Kin) and auxin (NAA or IAA) after 1 week (Fig 1C). MS medium devoid of PGRs showed induction of bud (maximum ~2 / explants), which were permanently arrested at immature condition (~0.48 - 0.64 cm in length). Between two cytokinin tested along with auxin (IAA or NAA), Kin was found most appropriate for the production of maximum shootlets viz. 5.67 ± 0.30 cm (Table 3). The combination of 6BAP and IAA was found to be less effective in promoting bud induction (2.78 ± 0.22) compared with 6BAP and NAA (3.89 ± 0.23) in Table 3. Multiplication of shoots along with some callus induction was also observed from explants during the process of subculture. After excision of the multiple shootslets, when the mother explants were cultured on the fresh shoot multiplication MS medium (0.1 mg l$^{-1}$ Kin + 0.1 mg l$^{-1}$ IAA), the shoot

**Table 1.** Effect of different concentrations of PGRs in microshoots induction from shoot meristem-tip culture.

| Plant growth regulator (mg $l^{-1}$) | | In vitro culture response (%) | No. of shootlets induction per explants (mean ± S.E.) | Height (cm) (mean ± S.E.) |
|---|---|---|---|---|
| IAA | Kin | | | |
| 0.00 | 0.0 | 68 | $1.17 \pm 0.12^{e}$ | $0.92 \pm 0.04^{d}$ |
| 0.03 | 0.15 | 86 | $1.28 \pm 0.13^{d}$ | $0.88 \pm 0.04^{e}$ |
| 0.05 | 0.5 | 84 | $2.86 \pm 0.16^{a}$ | $0.93 \pm 0.02^{d}$ |
| 0.10 | 1.0 | 64 | $2.76 \pm 0.13^{b}$ | $0.96 \pm 0.03^{c}$ |
| 0.30 | 1.5 | 72 | $0.72 \pm 0.04^{f}$ | $0.94 \pm 0.07^{d}$ |
| 0.50 | 2.0 | 58 | $1.11 \pm 0.13^{ef}$ | $0.88 \pm 0.04^{e}$ |
| 0.03 | 0.5 | 86 | $2.92 \pm 0.17^{a}$ | $1.94 \pm 0.04^{a}$ |
| 0.07 | 0.5 | 83 | $2.56 \pm 0.12^{b}$ | $1.08 \pm 0.07^{b}$ |
| 0.10 | 0.5 | 84 | $2.19 \pm 0.15^{c}$ | $0.62 \pm 0.02^{f}$ |
| 0.30 | 0.5 | 86 | $0.70 \pm 0.04^{f}$ | $1.08 \pm 0.07^{b}$ |

Mean ± s.e. in each column followed by same upper letters are not significantly different (P = 0.05) as per DMRT analysis.

**Table 2.** Effect of different concentrations of PGRs in microshoots induction from seedling epicotyle.

| Plant growth regulator (mg $l^{-1}$) | | | | |
|---|---|---|---|---|
| IAA | Kin | In vitro culture response (%) | Induction of shoot-lets per explants (mean ± S.E.) | Height (cm) (mean ± S.E.) |
| 0.00 | 0.00 | 45 | $0.08 \pm 0.02^{h}$ | $0.60 \pm 0.01^{g}$ |
| 0.03 | 0.15 | 48 | $0.56 \pm 0.01^{g}$ | $0.65 \pm 0.03^{e}$ |
| 0.05 | 0.50 | 52 | $0.94 \pm 0.06^{f}$ | $0.87 \pm 0.08^{f}$ |
| 0.10 | 1.00 | 62 | $2.30 \pm 0.15^{a}$ | $1.45 \pm 0.01^{a}$ |
| 0.30 | 1.50 | 42 | $1.44 \pm 0.09^{d}$ | $0.90 \pm 0.08^{d}$ |
| 0.50 | 2.00 | 45 | $1.08 \pm 0.06^{e}$ | $0.79 \pm 0.07^{e}$ |
| 0.03 | 0.50 | 43 | $1.56 \pm 0.12^{c}$ | $1.10 \pm 0.06^{c}$ |
| 0.07 | 0.50 | 42 | $1.68 \pm 0.11^{b}$ | $1.14 \pm 0.09^{a}$ |
| 0.10 | 0.50 | 38 | $1.54 \pm 0.11^{c}$ | $1.12 \pm 0.11^{b}$ |
| 0.30 | 0.50 | 32 | $0.90 \pm 0.05^{f}$ | $1.14 \pm 0.11^{a}$ |

Mean ± S.E. in each column followed by same upper letters are not significantly different (P = 0.05) as per DMRT analysis.

**Table 3.** Effect of different PGRs on bud breaking and shoot growth of nodal explants of JRC 517

| Plant growth regulator (mg $l^{-1}$) | | | | In vitro culture response (%) | No. of shootlet induction per explant (mean ± s.e.) | Height (cm) (mean ± s.e.) |
|---|---|---|---|---|---|---|
| IAA | NAA | 6BAP | Kin | | | |
| 0.00 | 0.00 | 0.00 | 0.00 | $57^{f}$ | $0.71 \pm 0.10^{j}$ | $0.64 \pm 0.07^{hi}$ |
| 0.03 | - | - | 0.05 | $62^{ef}$ | $1.22 \pm 0.14^{gh}$ | $0.64 \pm 0.11^{hi}$ |
| 0.05 | - | - | 0.07 | $72^{d}$ | $2.88 \pm 0.31^{c}$ | $2.73 \pm 0.29^{b}$ |
| 0.10 | - | - | 0.10 | $95^{a}$ | $5.67 \pm 0.30^{a}$ | $2.98 \pm 0.29^{a}$ |
| 0.15 | - | - | 0.50 | $84^{bc}$ | $5.50 \pm 0.26^{a}$ | $2.64 \pm 0.28^{c}$ |
| 0.20 | - | - | 0.75 | $67^{e}$ | $3.35 \pm 0.36^{b}$ | $2.65 \pm 0.38^{c}$ |
| | | | | | | |
| - | 0.03 | 0.05 | - | $72^{d}$ | $1.06 \pm 0.09^{h}$ | $1.06 \pm 0.10^{e}$ |
| - | 0.05 | 0.10 | - | $81^{c}$ | $1.43 \pm 0.11^{g}$ | $1.46 \pm 0.07^{ef}$ |
| - | 0.10 | 0.30 | - | $95^{a}$ | $2.69 \pm 0.11^{d}$ | $1.88 \pm 0.06^{d}$ |
| - | 0.15 | 0.50 | - | $94^{a}$ | $3.89 \pm 0.23^{ab}$ | $1.63 \pm 0.05^{e}$ |
| - | 0.20 | 1.00 | - | $65^{e}$ | $2.86 \pm 0.22^{c}$ | $1.40 \pm 0.06^{f}$ |
| | | | | | | |
| - | 0.03 | - | 0.05 | $52^{g}$ | $0.85 \pm 0.08^{i}$ | $0.56 \pm 0.02^{i}$ |
| - | 0.05 | - | 0.07 | $78^{c}$ | $1.76 \pm 0.16^{f}$ | $0.78 \pm 0.03^{h}$ |
| - | 0.10 | - | 0.10 | $90^{b}$ | $2.33 \pm 0.11^{e}$ | $1.56 \pm 0.05^{e}$ |
| - | 0.15 | - | 0.50 | $95^{a}$ | $3.55 \pm 0.15^{ab}$ | $1.64 \pm 0.06^{e}$ |
| - | 0.20 | - | 0.75 | $92^{b}$ | $3.38 \pm 0.13^{b}$ | $0.95 \pm 0.03^{gh}$ |
| | | | | | | |
| 0.03 | - | 0.05 | - | $66^{e}$ | $0.73 \pm 0.07^{j}$ | $0.96 \pm 0.09^{d}$ |
| 0.05 | - | 0.10 | - | $75^{d}$ | $1.25 \pm 0.08^{gh}$ | $0.58 \pm 0.01^{i}$ |
| 0.10 | - | 0.30 | - | $80^{c}$ | $2.87 \pm 0.22^{c}$ | $1.21 \pm 0.08^{fg}$ |
| 0.15 | - | 0.50 | - | $74^{d}$ | $1.73 \pm 0.15^{f}$ | $0.97 \pm 0.04^{g}$ |
| 0.20 | - | 1.00 | - | $56^{f}$ | $0.84 \pm 0.07^{i}$ | $0.90 \pm 0.08^{gh}$ |

Mean ± s.e. in each column followed by same upper letters are not significantly different (P = 0.05) according to DMRT analysis.

number increased significantly for the next three repeated transfers and reduced thereafter (Fig 2).

Among the different media tested, the media with half-strength MS was found to be best for the rooting of shoots (Table 4). Half-strength MS supplemented with 1.5 mg $l^{-1}$ IBA showed significant rooting response (11.4 ± 0.2) than other combinations.

**Fig 1**. Different phases of plantlet regeneration from different explants in *C. capsularis* var. JRC 517. Protocol A: Meristamatic tip cultured on MS medium supplemented with PGRs showing different morphogenic responses viz. callus induction (a1), proliferation (a2), microshoot induction and multiplication (a3) . Protocol B: Epicotyl (shoot tip with attached coteledons) explants showing micro-shoot induction from the meristematic zone of tip portion (b1) and their maturation (b2). Limiting numbers (2-3) of microshoot obtained without callus phase (b3). Protocol C: Nodal explant culture showed bud induction (c1), bud braking (c2) and sustainable multiplication rate with healthy plantlets (c3).

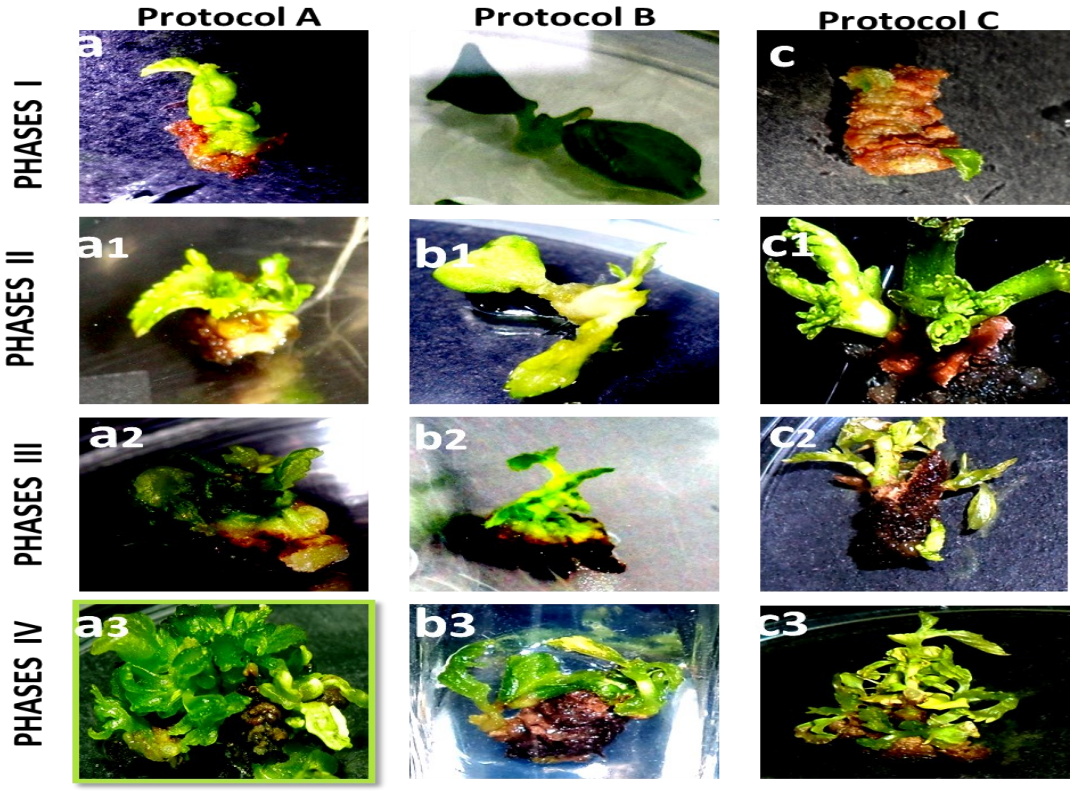



**Fig 2**. Effect of successive passaging of starter mother explants on shoot multiplication media. Mean ± s.e. values followed by the same letter for shoot number (no.) and shoot length (in cm) are not significantly (P > 0.05) different from each other as confirmed by DMRT analysis.

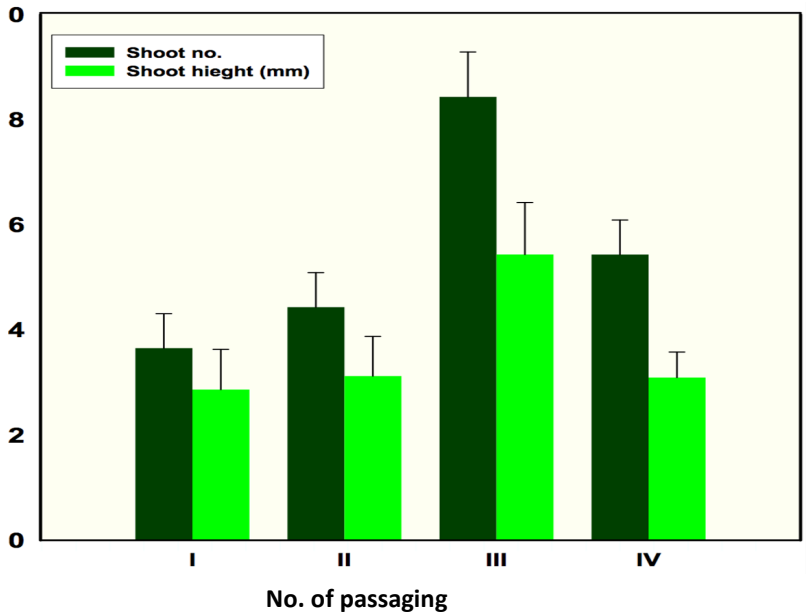


**Fig 3**. T-DNA region of the binary vector pBI121::CaMV35S:*gus*A:NOS showing restriction sites and the *gus*A gene use in *Agarobecterium* -mediated genetic transformation in *C. capsularis* var. JRC 517. RB- right border, NOS promoter- nopaline synthase promoter, $Kan^R$/$Neo^R$- neomycin phosphotransferase or *nptII* gene, NOS-terminator nopaline synthasetranscription terminator, CaMV35S promoter- Cauliflower Mosaic Virus 35S promoter, GUS intron containing β-glucuronidase or *gusA* gene, and LB left border of T-DNA.

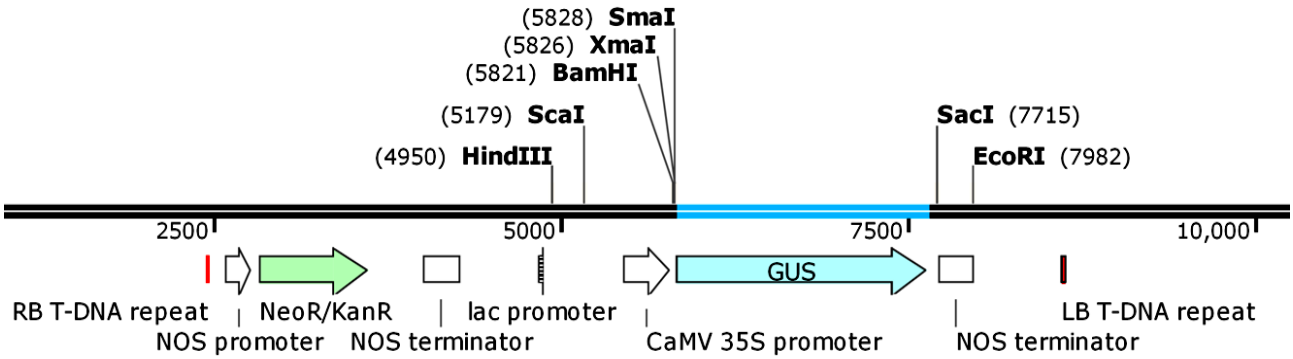
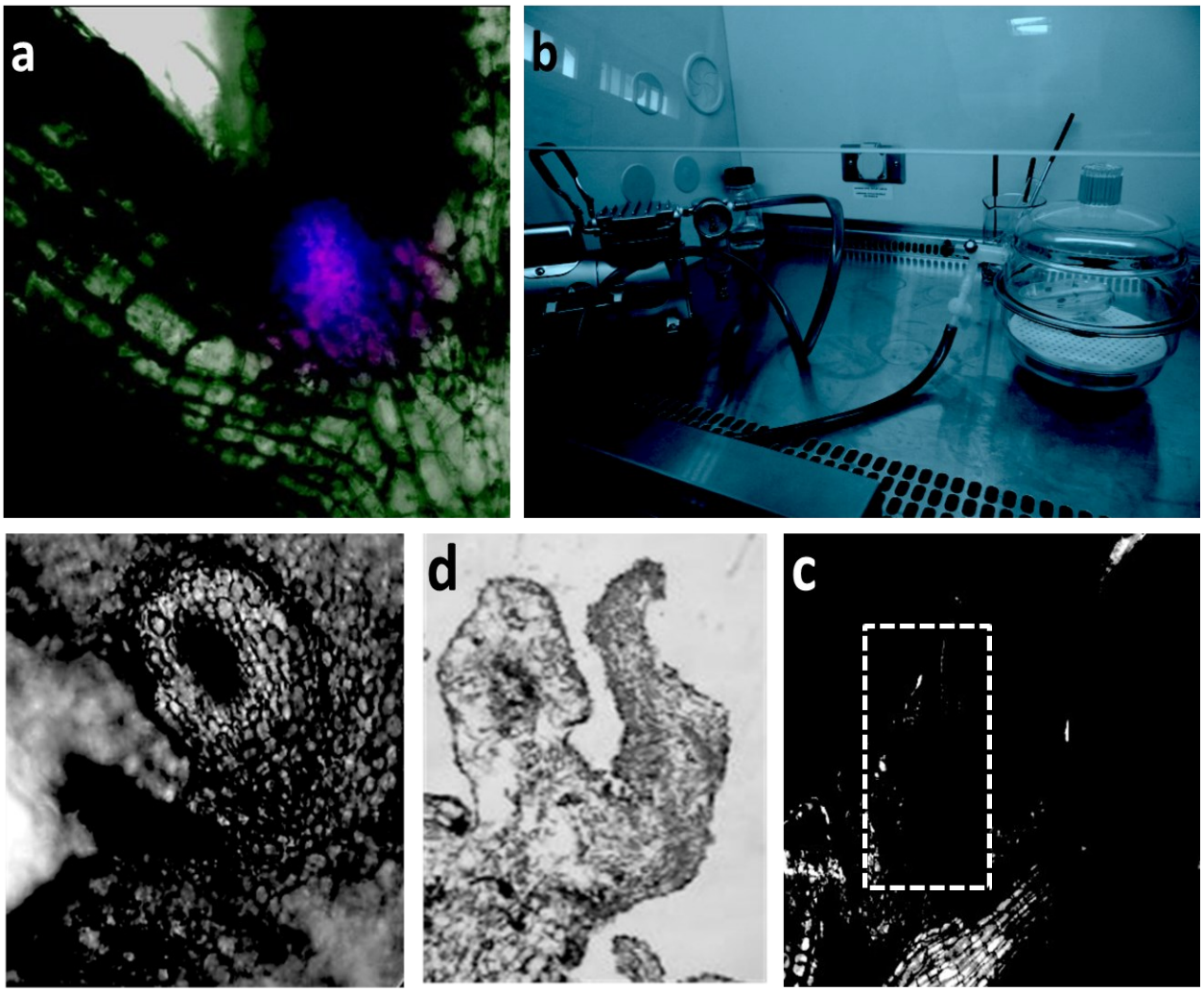



**Fig 4**. Detailed view of protocol C, i.e. Agro-infection set up and histological -study of developmental cascades of nodal explants derived putative transgenic plantlets. (a) LS of nodal explants showing immature bud induction, arrow indicates the slit-site (about 1 mm) where infections were done. (b) Vacuum infiltration of slited nodal explants of in *Agrobacterium* suspension using vacuum pump and desiccators for 10 min at 600 mmHg pressure under the Biological Safety Cabinet (Thermo Fisher 1300 SERISE A2). (c-e) Growth of transformed explant after recovery period.



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**Response of regenerated micro shootlets to Kan50**

To optimize the most suitable concentration of selective marker *nptII* (neomycin phosphotransferase *II*) transformed microshoots induced from axillary nodal portion of *C. capsularis* (var. JRC 517) primary and secondary shoots were subjected to different concentration of kanamycin. Among all concentrations of kanamycin tested on the primary shoot formation, kanamycin at 60 mg $l^{-1}$, halted complete primary shoot formation. Secondary shoot multiplication in MS liquid medium was found to be rapid and clump containing about 4-5 shoots or even more than seven shoots within 15 days in the successive cycles of multiplication was produced. Because of this rapid multiplication, the sensitivity of secondary shootlets to kanamycin varied as compared to the primary shoots. The secondary shootlets survived in medium supplemented with kanamycin at 50 mg $l^{-1}$.

**Effect of culture condition and *Agrobacterium* infection**

*A. tumefaciens* (strain LBA4404) harbouring the genetic construct pBI121::CaMV35S:*gusA*: NOS (Fig 3) was used to transform the nodal axillary immature bud of *C. capsularis* ( var. JRC 517). The Agro-infection setup and detailed microscopy view of the nodal explants are shown in Fig 4. Addition of phenolic compounds such as acetosyringone (AS) have been amply report in many dicot plants to enhance genetic transformation efficiencies by activating *vir* genes, which governs the *Agro*- infection (Van Wordragen and Dons 1992). In the present study, 100 axillary buds were infected with *Agrobacterium* solution containing different concentrations of AS (50, 100 and

**Table 4.** Effect of full and half strength MS medium and different concentrations of PGRs on in vitro root induction from shootlets after 4 weeks of culture

| Basal medium | Plant growth regulator (mg $l^{-1}$) | | In vitro culture response (%) | No. of root per explant | Root length (cm) (mean ± S.E.) |
|---|---|---|---|---|---|
| | IAA | IBA | | | |
| MS | 0.0 | 0.0 | $65^{g}$ | $2.13 \pm 0.32^{j}$ | $1.45 \pm 0.21^{i}$ |
| MS | 0.5 | - | $71^{f}$ | $3.25 \pm 0.21^{i}$ | $2.21 \pm 0.32^{h}$ |
| MS | 1.0 | - | $83^{d}$ | $3.74 \pm 0.24^{h}$ | $2.89 \pm 0.13^{g}$ |
| MS | 1.5 | - | $81^{e}$ | $3.98 \pm 0.36^{g}$ | $3.12 \pm 0.32^{f}$ |
| MS | - | 0.5 | $84^{d}$ | $3.96 \pm 0.37^{g}$ | $3.10 \pm 0.43^{e}$ |
| MS | - | 1.0 | $81^{e}$ | $6.49 \pm 0.12^{c}$ | $3.78 \pm 0.52^{cd}$ |
| MS | - | 1.5 | $89^{c}$ | $6.51 \pm 0.13^{c}$ | $3.98 \pm 0.61^{bc}$ |
| 1/2MS | 0.0 | 0.0 | $95^{b}$ | $2.83 \pm 0.15^{ij}$ | $1.63 \pm 0.31^{i}$ |
| 1/2MS | 0.5 | - | $100^{a}$ | $5.72 \pm 0.31^{e}$ | $2.95 \pm 0.56^{fg}$ |
| 1/2MS | 1.0 | - | $100^{a}$ | $5.96 \pm 0.22^{d}$ | $3.08 \pm 0.43^{e}$ |
| 1/2MS | 1.5 | - | $100^{a}$ | $4.04 \pm 0.21^{f}$ | $3.54 \pm 0.64^{c}$ |
| 1/2MS | - | 0.5 | $100^{a}$ | $9.21 \pm 0.18^{b}$ | $3.36 \pm 0.25^{d}$ |
| 1/2MS | - | 1.0 | $100^{a}$ | $11.37 \pm 1.02^{a}$ | $5.97 \pm 0.11^{b}$ |
| 1/2MS | - | 1.5 | $100^{a}$ | $11.40 \pm 0.20^{a}$ | $6.22 \pm 0.27^{a}$ |

Mean ± S.E. in each column followed by same upper letters are not significantly (P =0.05) different according to DMRT analysis.

150 µM) during pre culture and (50, 100, 150 and 200 µM) co-cultivation (Fig 6b and 6f).

To optimize maximum capability and developmental synchrony the explants were subjected to varying durations of pre-culture (0, 1, 2 or 3 days), prior to inoculation was evaluated. The effect of different durations of pre-culture on percentage of regenerating explants following the recovery period after 3-4 days of co-cultivation with *A. tumefaciens* (Fig 6c). Regeneration dataindicated that, with the increase in duration time of pre-culture, regeneration percentage was turn down. Moreover, the percentage of regenerating explants without pre-culture, and following 1 day pre-culture, did not differ significantly with respect to control. However, prolonged pre-culture (more than 1 day), decreased the percentage of regenerating explants significantly.

To established standard culture conditions for stable genetic transformation of *C. capsularis* var. JRC 517, type of explants, pre-culture and *A. tumefaciens* co-cultivation schedules were tested. After 15$^{th}$ days of infection from the GUS assay it was observed that the highest percentage of GUS expressing shoot tips were observed from the set, which were pre-cultured for 1 day followed by 3 days co-cultivation. Moreover, co-cultivation of 3 days produced the most promising results pertaining to their respective pre-culture sets. Taking into consideration the effect of pre-culture period as a whole, it was observed that 1 day pre-culture produced the maximum number of regeneration, while the sets without pre-culture and 3 days pre-culture recovered the least number of micro- shoot. The results suggest that 1 day pre -culture period and subsequent by 3 days co-cultivation emerged as be the optimum conditions for genetic transformation of *C. capsularis* var. JRC 517 that concurrent with the result of Saha *et al*., 2014.

**Selection and regeneration of transgenic shoots**

The co-cultivated axillary bud explants produced single primary shoots on MS medium supplemented with 50 mg $l^{-1}$ kanamycin within 3 weeks (Fig 5), which later produced a clump of 5–7 secondary shoots on semi-solid MS medium containing 50 mg $l^{-1}$kanamycin. The transformed secondary shootlets not survive at a higher dose of kanamycin 60 mg $l^{-1}$ during selection. To eliminate possibility of chimeric micro-plants and escapes, the secondary shoots produced from the primary shoots were subjected to five succeeding generation improvement with the same level of selection stress (kanamycin 50 mg $l^{-1}$).After five passages, the chimeric shoots and escapes turned brown and died while the transgenic shoots continued to survive and grown to maturity like normal plants (Fig. 5).

**Confirmation of transgenic plants through GUS assay**

Putative transgenic plants were confirmed through histochemical GUS analysis. A strong GUS activity was detected in leaf, stem, and root tissue of putative transgenic plants, while no activity was observed in the control plants in the primary infected explants ($T_0$ generation) (Fig 6a). It has been reported that number of chimeras are generally reduce at second and third passaging (Peña *et al*. 1995b, 1997; Gutiérrez-E *et al*.1997; Luth and Moore 1999). In our study, GUS assay also revealed no chimeras interestingly among all the putative transgenic plants, indicating that regeneration of transgenic plants via indirect organogenesis from nodal axillary bud is an appropriate route for transgenic development.

**Verification of stable integration of transgene through PCR and Southern hybridization**

Successful and stable integration of the transgene into the recipient jute genomes of the putative transgenic plants was confirmed by PCR and Southern hybridization which are strong confirmative techniques. Genomic DNA isolated from the putative transformed lines ( viz. JCP1-8), and non-transformed control plants, and plasmid (pBI121::CaMV35S:*gusA*:NOS) was used as template DNA for PCR amplification of the *gusA* gene using gus specific primers**,** which produce an amplicon of 1.8 kb in transformed shootlets of (Fig 8a). As expected, this DNA was not amplified in non-transformed control plants. Amplified product of 0.78 kb obtained with *nptII* gene specific primers in $T_1$ (Fig 7b) confirmed the integration of the *npt II* gene as well. The present protocol of micropropagation –based *A grobacterium* mediated genetic transform was found to have a transformation efficiency of ~3.6% (Table 6). PCR product of ~0.78 kb also obtained with *nptII* gene specific primers in $T_2$ (Fig. 7c) confirmed the integration and subsequent segregated of the *npt II* gene as well.

To confirm stable T-DNA integration into transgenic plants genome, Southern hybridization was performed using total genomic DNA isolated from the leaves of Kan50 resistant shootlets from putative transgenic plants ($T_1$) and from non-transformed control plants. Probe was prepared by digesting plasmid pBI121::121CaMV35S:*gusA*:NOS with *Eco*RI and *Hind*III (Fig 3), hybridized with the transgenic DNA samples, which were digested with the same restriction enzymes. To detect the stable insertion in the plant genome, samples were digested with *Eco*RI -*Hind*III and hybridized with the same probe. A band of ~3.0 kb was detected at lanes loaded with DNA sample (Fig 7c), with no signal in lanes loaded with DNA samples isolated from control samples.

**Segregation analysis of $T_2$ plants**

Seeds from control plants when germinated on selective MS medium supplemented with Kan50, they became yellow or pink and died after 3 days. Selfed seeds from each $T_1$ lines showed that most of seedlings grew normally on the same selective medium with well-developed cotyledons and roots, which were classified as positive true transgenic plants, whereas other abnormal seedlings (such as the control) were grouped as negative or non-transgenic plants. In those progenies JCP 1, JCP 3 and JCP 8 plants showed 3:1 segregation ratio, line JCP 2 showed a 15:1 ratio, while line JCP 6 was 1:1 for

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**Fig 5**. Regeneration of transgenic jute plants through micropropagation of nodal explants. (a) Immature axillary nodal buds as source of the explants (box); (b) bud induction from transformed explant; non-transformed plant tissue died under Kan50 selection; (c) secondary shoot induction from transformed nodal explants; (d) putative transgenic shoos transferred in elongation media; (e) mature healthy transformed shoots; (f) side view of image "e" shown root induction in shoot clump; (g) healthy transgenic plant.

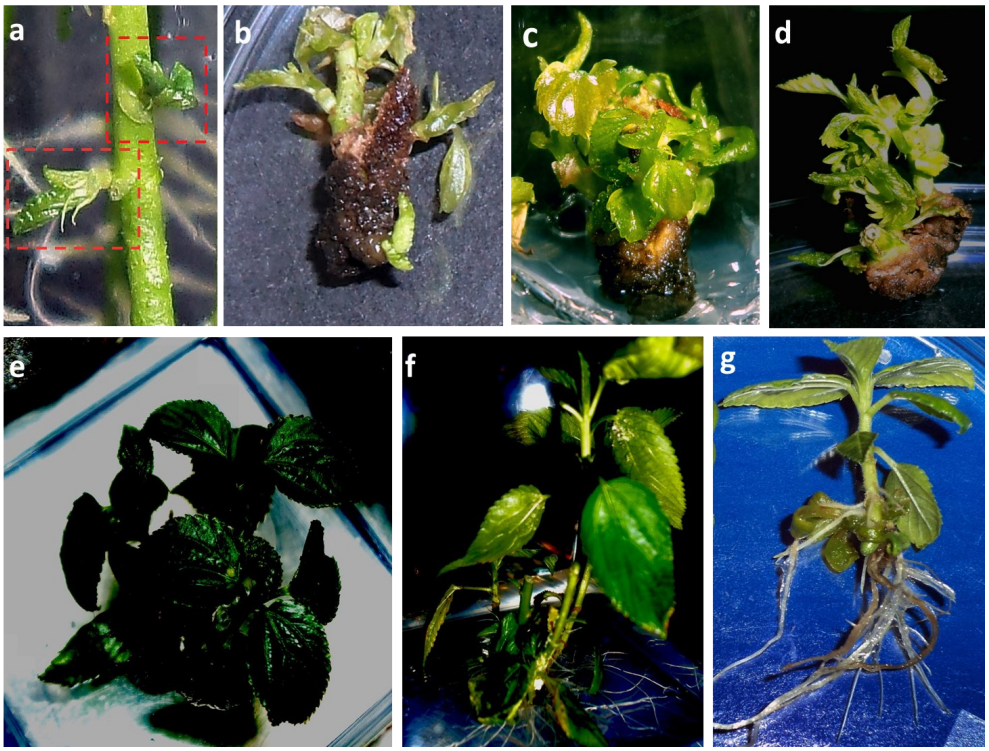


**Fig 6.** Effect of different treatments on *Agrobacterium* –mediated genetic transformation efficiency and regeneration in *C. capsularis* var. JRC 517. (a) Optical density (O.D.); (b) Effect of acetosyringone (AS); (c) Pre-culture duration; (d) Duration of explant deeping in Agro-suspension; (e) Duration of vacuum infiltration; (f) Effect of AS during co-culture; (g) Effect of co-culture duration; (h) Effect of non-ionic surfactant Tween-20.

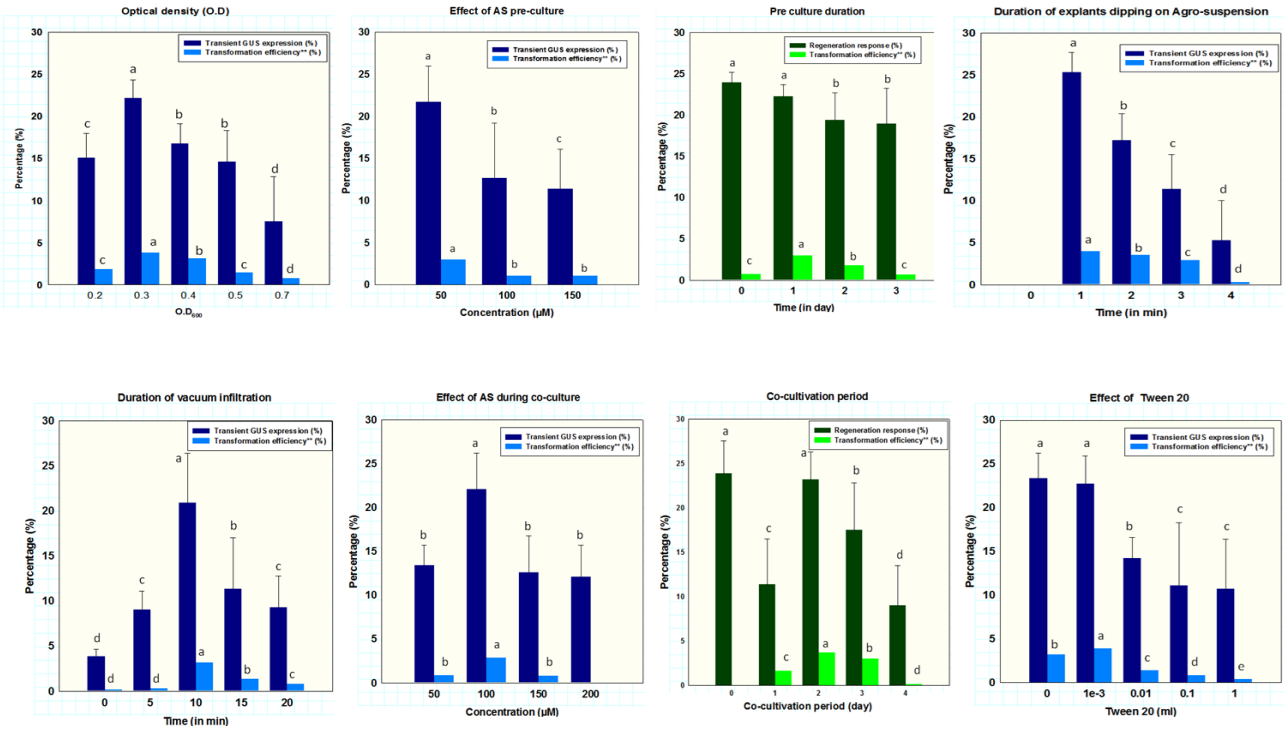

**Fig 7**. GUS histochemical assay (a) Uniform *gus*A expression in regenerated whole plant after third passaging (a' before GUS assay); (b) no *gus*A expression in leaf of control plant; (c) uniform gus expression and activity in leaf of transformed T1plant. (d) Comparison of GUS assay of leaf of control and $T_2$ plants.

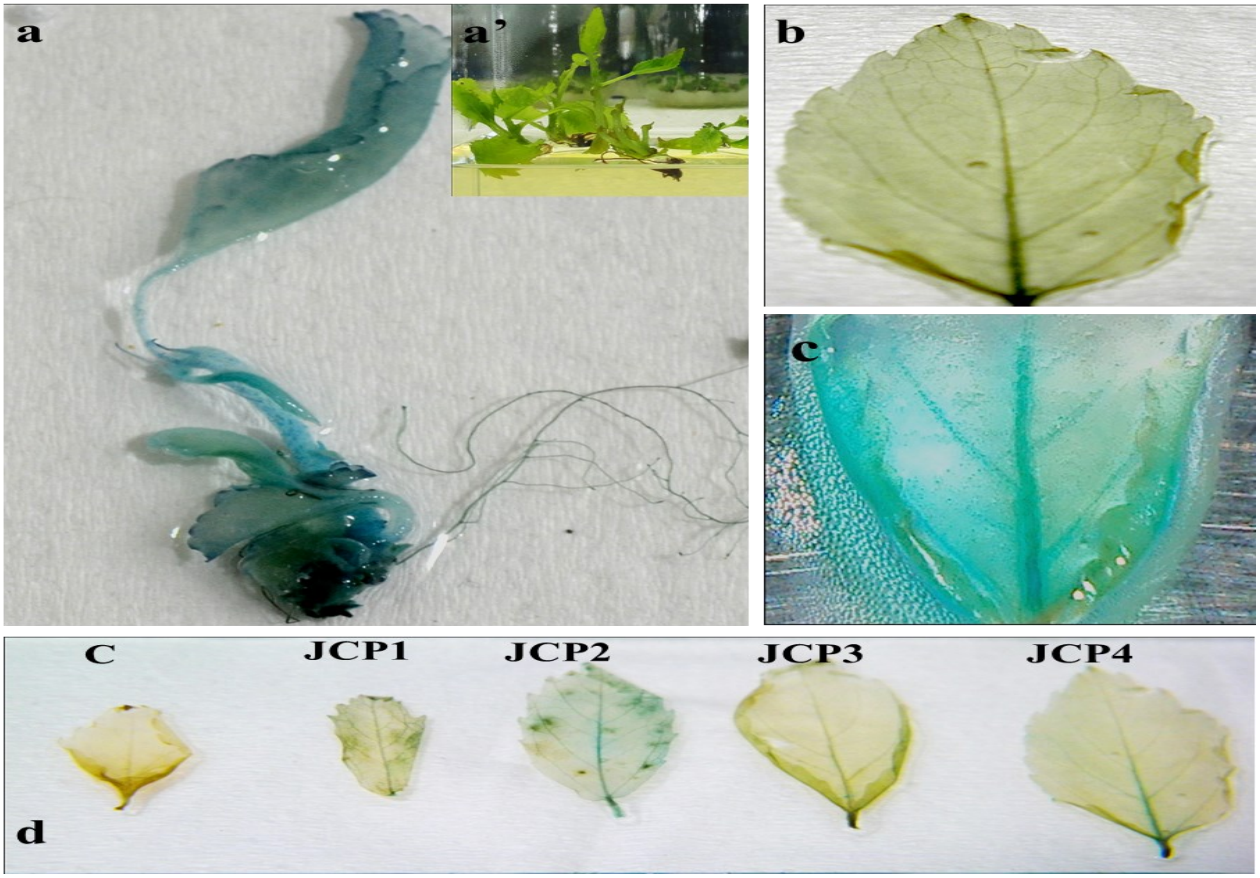


**Fig 8**. PCR screening and Southern hybridization for transgenic conformation. (a) PCR of putative transformants showing 1.8 kb fragment of *gus*A amplification; (b) PCR amplification of *nptII* gene (750 bp). (c) PCR amplification of *nptII* gene (750 bp) of T2. (d) Southern hybridization of four transgenic jute lines (T2) showing ~ 3.0 kb band, where no such signal was in control plant.

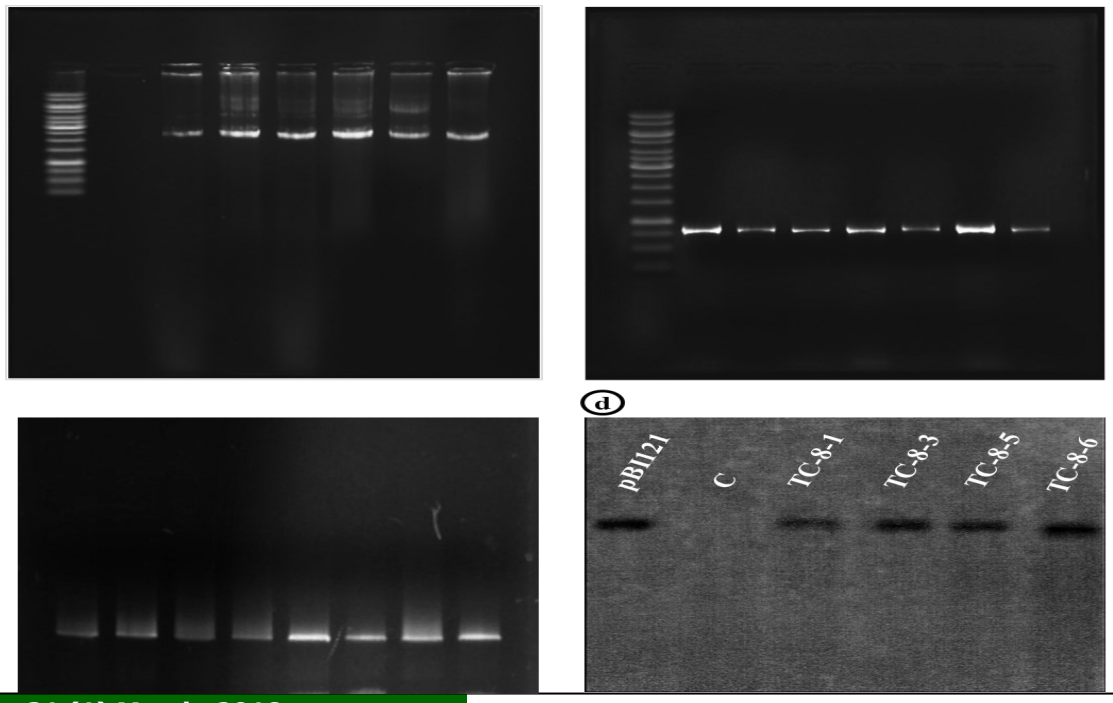

**Table 5.** Synopsis of the genetic transformation experiments using nodal bud explants of *C. capsularis* var. JRC 517, co-cultured with *A. tumefaciens* strain LBA4404 harbouring binary vector pBI121.

| Replication | No. of explants | No. of total survived plants | No of npt II positive plants (PCR based) | No of GUS positive plants | Genetic Transformation efficiency (%) |
|---|---|---|---|---|---|
| Total | 300 | 16 | 11 | 11 | 3.66 |

**Table 6.** Inheritance of stable transformed nptII gene through segregation analysis of $T_1$ generation progeny plants.

| Transgenic plant Designation (Plant ID) | No. of seeds plated on MS medium with 50 mg $l^{-1}$ kanamycin | nptII positive | nptII negative | $nptII^+$: $nptII^-$ | Best fitted segregation ratio | $\chi^2$ value* | P value |
|---|---|---|---|---|---|---|---|
| JCP1 | 44 | 30 | 14 | 2.14 | 3:1 | 1.08 | 0.29 |
| JCP2 | 45 | 40 | 05 | 8.00 | 15:1 | 1.81 | 0.17 |
| JCP3 | 42 | 28 | 14 | 2.00 | 3:1 | 1.56 | 0.21 |
| JCP6 | 45 | 24 | 21 | 1.14 | 1:1 | 0.20 | 0.65 |
| JPC8 | 42 | 30 | 12 | 2.50 | 3:1 | 0.28 | 0.59 |

*$\chi^2$ value, *chi*-square values.

resistance to Kan50. The segregation ratio of positive and negative progenies is in contingency with the Mendelian ratios of each $T_1$ lines expect numbers deviation (Table 6). P Values indicate values significantly fit to the tested ratios. Thus, data gleaned from this experiment confirmed stable genetic transformation, integration and inheritance of the transgene in jute. However the ratios which deviated from Mendelian ratio in a few cases perhaps may be due to extremely small sample size, epistatic interaction or might be due to linkage drag. This needs further examination of the segregation pattern of the progenies in future generation.

## Conclusion

Moreover, stable integration, expression and the transgene segregation within the progeny plants without exhibiting any morphological aberrations signifying an efficient micropropagation –based *Agrobacterium*-mediated genetic transformation protocol in *Corchorus capsularis* L. var. JRC 517. Our findings will help in jute crop improvement with desirable genes/traits in future.

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