

GENETIC TRANSFORMATION IN WHITE JUTE THROUGH *AGROBACTERIUM* AND SALINITY SCREENING OF TRANSGENIC PLANT

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Abstract

The experiment was conducted to establish an efficient and reproducible protocol for the plant regeneration and genetic transformation in white Jute (*Corchorus capsularis* L.). The regeneration and transformation processes depend on optimum growth conditions, suitable explants and varieties. An attempt was made for *Agrobacterium* mediated genetic transformation in white jute varieties using gene construct conferring both salt and drought tolerance (CIPK and Gly-1) along with the marker genes. Interestingly the two varieties (CVL-1 and Tricap-1) showed the response of both callus induction and plant regeneration on a single formulation i.e. MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA. Explants were dipped to liquid culture of bacteria for one minute and then transferred to co-cultivation media for 24 hours. Shoot regeneration from *Agrobacterium* infected cotyledon was found highest in variety CVL-1 (43%) than Tri cap (38%). After co-cultivation and selection histochemical *GUS* assay was performed in different varieties (vars. Tricap-1, CVE-3 & CVL-1). In the transformed explants, *GUS* reporter gene was expressed showing blue colour in the explants tissues. Among the varieties CVE-3 showed the highest expression blue colour in the explants tissues. Those transgenic plants are transferred to salt medium and soil for evaluation.

Keywords: Media, *Agrobacterium tumefaciens*, *Corchorus olitorius*, transformation.

Abbreviations: *GUS*- β -glucuronidase; LB-Luria-Bertani; NPT-neomycine phosphotransferase; Ti-tumor inducing; YMB- yeast extract mannitol broth,

Introduction

Agrobacterium tumefaciens-mediated transformation is generally used for genetic transformation of higher plants. Genetic transformation of crop plants has been evolved, which offers the ability to introduce single new character into a plant cultivar without altering of its existing traits (Gardner, 1993). *Agrobacterium*-mediated transformation has many advantages, such as

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low copy number of transgene, and stable inheritance of transgenes in a Mendelian fashion (Gelvin, 2003). Most *Agrobacterium*-mediated transformations are carried out using in vitro tissue culture. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-induction (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing crown gall. Thus transformation efficiency highly depends on the regeneration abilities of genotypes and explants. Transformation efficiency can be increased by the manipulation of either the plant or bacteria for enhancing competency of plant tissue and *vir* gene expression, respectively (Mondal *et al.*, 2001; Chakrabarty *et al.*, 2002). It is now possible to transform a wide range of plants, including agronomically and horticulturally important crops, flowers and trees have been genetically modified using this method (Ko and Korban, 2004; Lopez *et al.*, 2004).

The worldwide awareness on environment and health is likely to provide new opportunities on jute due to its environment-friendly characteristics is biodegradable and its products can be easily disposed without causing environmental hazards. Considering the importance of jute in Bangladesh and limitation in improving its yield and quality, it is necessary to incorporate genes of desired traits in to the local varieties. However, there have been limited responses of different varieties towards shoot regeneration. *In vitro* plant regeneration and genetic transformation systems in white jute via *Agrobacterium tumefaciens* which would further be used for the introduction of important genes such as salt, drought tolerance gene for improvement of jute plant quality.

Materials and Method

Germination of seeds on culture media

Seeds of white Jute (vars. Tri cap, CVL-1, CVE-3) were surface sterilized by immersion in 0.1% (w/v) Mercuric chloride for 20 min, followed by 4-5 washes with deionized water. All seeds were placed on the surface of the 50 ml aliquots of hormone free agar solidified (0.8%, w/v) MS basal medium (Murashige and skoog, 1962) in 100 ml conical flasks. In another set of experiment, surgical cotton (1 gm approx. in each flask) was used instead of agar in association with MS basal liquid medium to obtained optimum seedling production. Each flask contained 20 ml of hormone free MS liquid medium. Cultures were placed in a growth room with 20°C under 1.0 Wm² of daylight fluorescent tubes with 12 h photoperiod. Seed germination percent and number of healthy seedling was recorded.

Callus induction, shoot and root regeneration

Cotyledons (with attached petiole) of varieties of *C. capsularis* were cultured on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l IAA. The ultimate goal of *in vitro* technique was to production of free-living plantlets via shoot and root formation from the explants. The responses of different varieties towards shoot regeneration were found varied. When the shoot was 2-3 cm length, they were rescued aseptically from the cultured flasks and was separated from each other and again cultured individually on 250 ml conical flask with freshly prepared MSO (hormone free MS medium) medium for root production. Conical flask containing plantlets was incubated at 28°C under a 1 Wm² of daylight florescent tube with a 12 h photoperiod.

***Agrobacterium tumefaciens* strain and binary vector**

Agrobacterium tumefaciens LBA4404 harbouring the binary vector pBI121 containing selectable marker *CIPK* gene and screenable marker *GUS* gene were grown on YMB (Yeast Mannitol Broth) medium (1.0% Mannitol, 0.04% Yeast extract, 0.01% NaCl, 0.02% MgSO₄ · 7H₂O, 0.05% K₂HPO₄) containing Kanamycin as the selective agent at 200 rpm in a shaker at 28°C for overnight. Bacterial concentration was determined by visual observation. *Agrobacterium* from these cultures were used for infection of cotyledonary petioles of young jute plants.

Explants preparation

Cotyledonary petioles from germinated seedlings were used as explants. After seven days, cotyledons were excised from the seedlings. This was carried out by gently holding the hypocotyls with forceps, and cutting between the joint just below the shoot tip using sterilized surgical blades.

***Agrobacterium* culture**

Two kinds of culture media were needed for the *Agrobacterium* strain. One for maintaining *Agrobacterium* stock and the other for the infection of explants. For maintenance, one single colony from *Agrobacterium* stocks was streaked into freshly prepared Petri dish containing YMB medium having Kanamycin. The Petri dish was sealed with parafilm and kept in room temperature for 48 h. For infection, *Agrobacterium* stock single streak was taken in an inoculation loop and was inoculated in a conical flask containing LB medium with 50 mg/L Kanamycin. The cultures were allowed to grow at 28° C to get optimum population of *Agrobacterium* for infection and co cultivation of explants.

Infection and incubation

The *Agrobacterium* grown in liquid LB media was used for infection and incubation. To get suitable and sufficient infection of the explants, freshly excised explants were dipped into bacterial suspension for 1 min before transferring them to co-cultivation medium.

Co-cultivation

Following infection and incubation, the explants were co-cultured on plant regeneration medium in Petri dishes containing 2mg BAP and 0.5mg IAA. Prior to transfer of all explants to regeneration medium, they were blotted dry with sterile filter papers for a short period to remove excess bacterial suspension. All the explants were maintained in co-cultivation medium for 24 hours. Petri dishes containing explants were placed under fluorescent illumination with 12 hours dark cycle at 28^oC. The intensity of light was maintained at 1000 lux.

Transfer to the nutrient medium and sub culturing

Following 24 hours co-cultivation, the explants were transferred to regeneration medium consisting of MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l IAA and 500 µg/ml Cefotaxime. After 6-7 weeks, the regenerated shoots were transferred to hormone free MS medium with 250µg/ml Cefotaxime. Amount of Cefotaxime was gradually reduce in every sub culturing.

Transfer to salt media

Putatively transformed shoots were transferred to 25mM/L, 50mM/L, 75mM/L, 100mM/L; 125mM/L salt containing MS media.

Transfer to soil

Plantlet was rescued aseptically from the cultured flask and washed to remove the chemical associated with it and placed normal environment for hardening. Seedlings were transferred to plastic pots containing autoclaved sterilized soil mix (peat moss, perlite, and vermiculite, 5:3:2, v/v/v. Young, pampered seedlings that were grown either indoors or in a greenhouse need a period to adjust and acclimate to outdoor conditions, prior to planting in the garden. After hardening, regenerated shoots were transferred to normal soil.

***GUS* Histochemical assay**

GUS activity was detected as described (Jefferson *et al.*, 1998). Randomly selected co-cultivated cotyledons cultured on selective medium were used for *GUS* assays. Immediately after inoculation on selection medium, cotyledons were incubated in *GUS* staining solution at 37oC for 24 h in dark. The X-glu

was broken down by the activity of *GUS* gene, which was transferred with T-DNA in the cotyledonary tissue and produced a characteristic blue colour.

Statistical Analysis

The data for the characters under present study were statistically analyzed wherever applicable. The experiments were conducted in growth room and arranged in Completely Randomized Design (CRD) with five replications. The analysis of variance for different characters was performed and means were compared by Duncan's Multiple Test (DMRT).

Table 1. Varietal performance on seed germination.

Variety	No. of seeds germinated	Percent seed germination
CVE -3	9.33	93.3
CVL-1	9.13	91.3
Tricap-1	7.99	79.9
CV (%)	3.93	-
LSD	0.78	7.86

Figures followed by same letter in a column do not differ significantly at 5% level of probability by DMRT.

Results and Discussion

Effect of media on seed germination

Healthy seedling production was one of the major criteria for plant regeneration from jute explants. Seeds of *C. capsularis* varieties (CVE-3, CVL-1, and Tricap-1) were germinated on both agar solidified medium and surgical cotton supported liquid medium. The highest percentage of seed germination was found in variety CVE-3 (93.30%) and the lowest was found in Tricap-1 (79.97%) (Table.1). Percent seed germination was found to be higher on cotton supported liquid MS medium (85%) than agar solidified MS medium (62%) (Table 2). This finding is supported by the findings of Naher *et al.* (2003) who also found the highest seed germination in the variety CVE-3 (97.33%) in cotton supported medium. Germination of jute seed and seedling growth in cotton supported liquid medium were higher and healthier than the agar solidified medium .

Table 2. Effect of media on seed germination.

Media Support	No. of seeds germinated	Percent seed germination
Agar	12.0	62.0
Cotton	17.0	85.0

Figures followed by same letter in a column do not differ significantly at 5% level of probability by DMRT.

Table 3. Performance of varieties of *C. capsularis* on shoot regeneration.

Varieties	No. of explants showing shoot regeneration	Percent shoot regeneration
CVE 3	5.80 a	64.2 a
CVL-1	4.80 b	53.3 b
Tricap 1	3.80 c	50.3 b

Figures followed by same letter in a column do not differ significantly at 5% level of probability by DMRT

Shoot regeneration on media

Shoot regeneration was the highest in CVE-3 (64.27%) followed by CVL-1 (53.30%) and Tricap-1 (50%) (Table 3). High responsive genotype CVE-3 for callus induction had high regeneration capacity, indicating that callus induction capacity is related to regeneration of shoot. This result is similar to the findings of Khatun (2001) (plate-1).

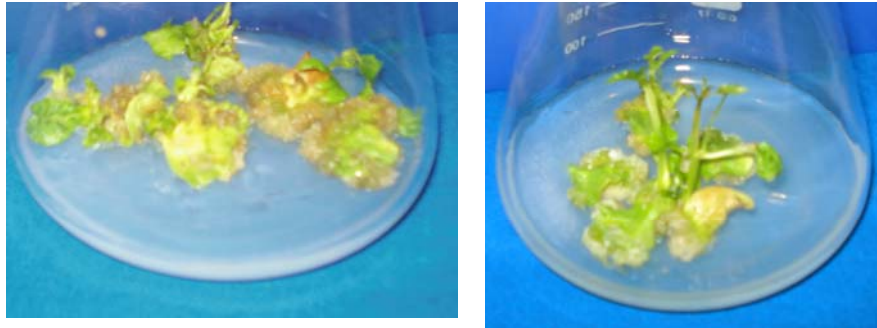
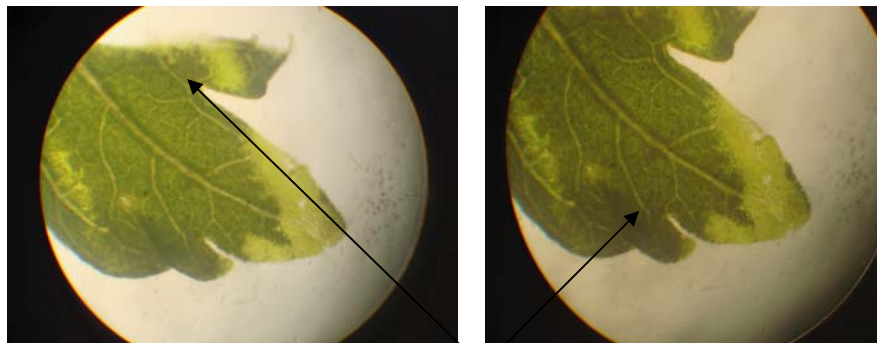
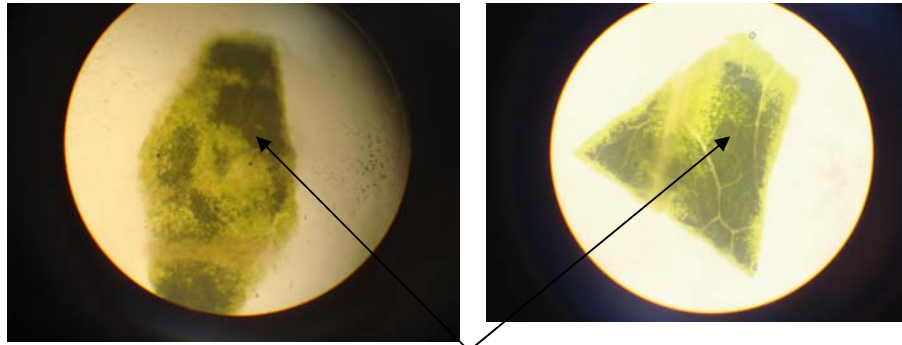


Plate 1. Shoot regeneration of CVE-3 explants on MS + 2 mg/l BAP + 0.5 mg/l IAA medium



Blue zone

Plate 2. GUS positive tissues showing in leaf section of variety CVL-1-(cipk)1



Blue zone

Plate 3. *GUS* positive tissues showing in leaf section of Tri cap-1.



A. Plantlet regeneration in 25 mM NaCl salt concentration



B. Plantlet regeneration in 50 mM NaCl salt concentration

Plate 4. Salt tolerance profile of Ps GLY-I gene transformed regenerating plants at different salt concentration (variety CVL-1)



C. Plantlet regeneration in 75 mM NaCl salt concentration



D. Plantlet regeneration in 100 mM NaCl salt concentration

Plate 4. Salt tolerance profile of Ps GLY-I gene transformed regenerating plants at different salt concentration (variety CVL-1)

***Agrobacterium* culture and incubation of explants**

The *Agrobacterium* strain grown in liquid YMB medium was used for infection and incubation. Shoot regeneration from *Agrobacterium*-infected cotyledons was found to be the highest in CVL-1 (43.00 %) followed by CVE-3 (38.67%). Shoot regeneration from *Agrobacterium*-infected cotyledons was found to be the lowest in Tricap-1(33%) (Table 4).

Table 4. Performance of varieties of *C. capsularis* on percent shoot regeneration and average number of shoots from *Agrobacterium* infected cotyledon.

Variety	No. of cotyledons infected	Percent shoot regeneration	Average number of shoots produced by each cotyledon
CVE-3	60	38.6 ab	11.3 b
CVL-1	60	43.0 a	14.3 a
Tri cap-1	60	33.0 b	8.66 c
CV(%)		6.83	4.17
LSD		6.045	1.77

Figures followed by same letter in a column do not differ significantly at 5% level of probability by DMRT

Co-cultivation and histochemical *GUS* assay

After infection of the explants in *Agrobacterium* suspension culture, the explants were transferred to co-cultivation medium. Following incubation and co-cultivation with *Agrobacterium*, transformation ability was monitored through histochemical assay of *GUS* reporter gene in explants tissue. Transient *GUS* assay was done at the end of co-cultivation with randomly selected 20% inoculated explants tissue. In the *GUS* assay, conspicuous *GUS* positive (blue color) region were detected in the explant surface (Plate 2 and Plate 3).

Following *GUS* histochemical assay, it was found that all the three varieties showed positive responses towards transformation. Following infection and co-cultivation with the strain LBA4404, the cotyledons attached with petioles were cultured on plant regeneration medium containing Cefotaxime 500 µg/ml for shoot development. The average number of shoots produced by each cotyledon was also the highest in CVL-1 (14.33) and the lowest in Tri cap (8.66). Control explants did not show response to the assay. For selection of transformed cells and tissues, the callus proliferating shoots were transferred to selection and regeneration media containing 50 mg/L Kanakycin and 500 µg/mL Cefotaxime. In presence of Kanamycin (100 mg /l) in the selection media greatly influenced

by the emergence of transgenic shoot from the transformed callus. A few of the calli continued to grow and differentiate into shoots. Among the varieties CVE-3 shows the highest response in *GUS* test. Those transgenic plants are transferred to salt medium and soil for evaluation (Table 5).

Table 5. Influence of explants on transformation as assessed through *GUS* histochemical assay.

Variety	No. of explants infected	No. of explants assayed for <i>GUS</i>	No. of explants+ve for <i>GUS</i>	% of <i>GUS</i> +ve explants
CVE-3	60	10	9	90.00
Tricap-1	60	10	8	80.00
CVL1	60	10	8	80.00

Figures followed by same letter in a column do not differ significantly at 5% level of probability by DMRT.

Selection of explants under various salt concentrations

For the evaluation of salt tolerant genes transformation in the jute varieties was done using *in vitro* method under controlled conditions. MS media supplemented with NaCl salt concentrations (25, 50, 75, 100 and 125 mM) was used for salt tolerance testing of transformed plants. Transformed plants of the three varieties were able to survive up to 100 mM salt concentration while the controlled plants died out at 50mM (Plate-4). All of the varieties survived at (50 %) up to 75 mM salt concentrations. However, in case of 100 mM, the survival rate was 20 % (Table 6).The explants were albino at 125 mM. The survival of transformed plants at 75 mM indicates the transformation and expression of the genes in the varieties. The above mention research showed the possibility of the salt tolerant variety development using recombinant DNA technology.

Table 6. Selection of explants under various salt concentrations.

Treatments (Salt concentration)	No. of explants assayed	No. of explants survived	Percentage of survived plants	Days of survival
25 mM	10	10	100	60
50 mM	10	9	90	30
75 mM	10	5	50	15
100 mM	10	2	20	8
125 mM	10	0	00	5

Regenerated plantlets transfer to soil

After root production, transgenic plantlets were subjected to hardening (Plate 5) and then transferred to sterilize soil successfully and after shoot and root development it was transferred to normal soil. Among the transgenics only three plants survived up to maturity (Plate 6).

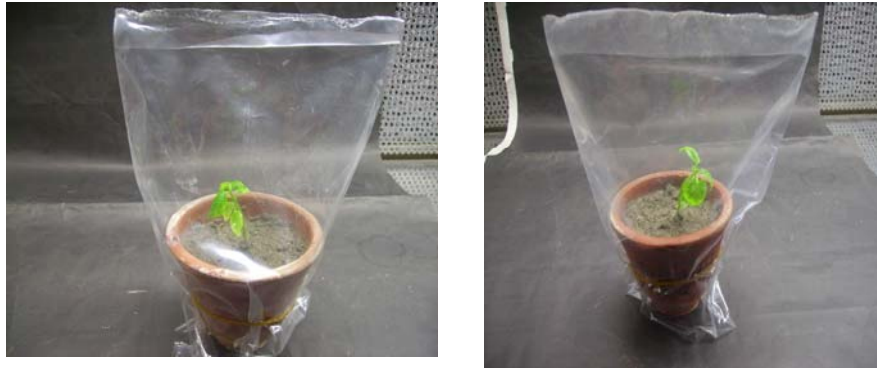


Plate 5. Hardening of regenerated plantlets (CVL-1)

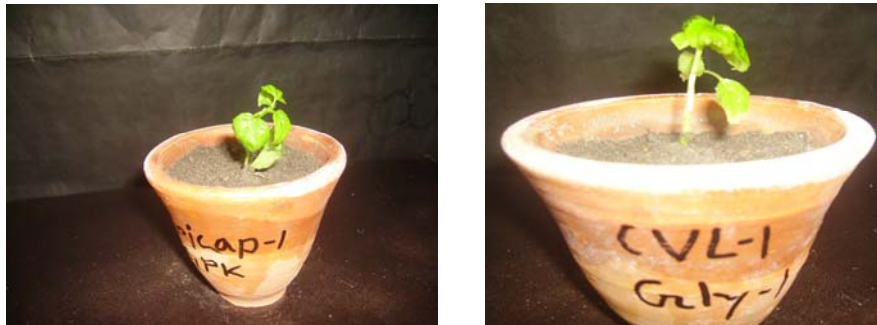


Plate 6: Transgenic seedlings transferred to sterilized soil

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