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Regeneration and genetic transformation of Tossa Jute (*Corchorus olitorius* L.)

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Abstract

The experiment was conducted to establish an efficient and reproducible protocol for the plant regeneration and genetic transformation, which can be used for the improvement of tossa Jute (*Corchorus olitorius* L.). It was demonstrated that the percent of seeds germination in agar based medium found to be very low (43.1%) , as compared to clinical cotton supported MS liquid medium, which was found to be very high (97.6%) and the highest percentage of root initiation was recorded for the variety O-9897 (53.6%) which was statistically identical by variety O-72 (49.4%) .The performance of varieties in *A. tumefaciens* showed significant difference in respect of number of explants produced shoot and percent shoot regeneration. Variety O-9897 gave the best performance as compared to variety O-72. In transformed explants, GUS reporter gene was expressed showing blue color in the explant tissues. Non-transformed explants did not show any color. Variety O-9897 showed the highest response to GUS assay (86.6%) as compared to O-72 (80.0%). This efficent regeneration and transformation system can be further used for the improvement of Jute quality using other useful genes.

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

Regenerated plants from seedling explants of *C. olitorius*, and somatic embryos were developed for the first time from protoplasts of *C. capsularis* (Saha and Sen, 1992). However, plant regeneration could not be achieved as the somatic embryos failed to germinate. Plant regeneration potentiality from tissue culture of cultivated jute species (*Corchorus* spp.), a major fibre crop of the Indian subcontinent, is exceedingly low (Saha and Sen, 1992). This has restricted the application of gene transfer techniques in this species. *Agrobacterium tumefaciens*-mediated transformation is generally used for genetic transformation of higher plants. Plant transformation was initiated when *Agrobacterium tumefaciens*-mediated gene delivery was reported for the production of transgenic plants (Horsch *et al*., 1985). *Agrobacterium*-mediated transformation has many advantages, such as low copy number of transgene, and stable inheritance of transgenes in a Mendelian fasion (Gelvin, 2003). 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).

Table 1. Combined effect of variety and culture media on seed germination in tossa jute (*Chorchorus olitorius* L.)

Variety	Culture media	No. of seed germinated	Seed germination (%)
O-9897	Cotton	46.2b	92.4b
	Agar	21.7c	43.1c
	Cotton	48.8a	97.6a
O-72	Agar	22.9d	45.8d
Level of significance		**	**
Lsd 0.05		0.916	1.83

In the same column, significant differences according to LSD at the P≤ 0.05 level are indicated by different letters.

Most *Agrobacterium*-mediated transformations are carried out using *in vitro* tissue culture. Thus, transformation efficiency highly depends on the regeneration abilities of genotypes and explants. Reports presented early attempts to transform the Gramineae with *A. tumefaciens*, including *A. tumefaciens*-mediated infection of plants with vira1 genomes (Chan *et al*., 1993; Shen *et al*., 1993). The production of transgenic rice plants by inoculating immature embryos with an *A. tumefaciens* strain and proved the transformation by molecular and genetic analysis (Chan *et al.*, 1993). *A. tumefaciens*-mediated transformation of rice and maize were regenerated and characterized (Lin and Zhang, 2005; Ishida *et al*., 1996). However, transformation in rice remains difficult but there have been few reports of successful transformation in rice by *Agrobacterium* (Khanna and Raina, 2002; Mohanty *et al.,* 2002). 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).

Jute is the most important and environment-friendly natural fibre that produces diversified economic important product worldwide. It falls into the bast fibre category along with kenaf, industrial hemp. Though there are over 100 species of *Corchorus*, only two, *Corchorus capsularis* L. and *C. olitorius* L., are cultivated widely (Sarker and Al-Amin 2007). There is only one established transformation protocol for jute (Ghosh et al., 2002) involving *Corchorus capsularis*. There have been limited studies on *A. tumefaciens*-mediated transformation of Tossa Jute (*Corchorus olitorius* L.). Here we report on in vitro plant regeneration and genetic transformation systems in Tossa Jute via *Agrobacterium tumefaciences* which would further be used for the introduction of important genes for improvement of Jute plant quality.

Materials and methods

Germination of seeds on culture media

Seeds of Tossa Jute (vars. O-9897 and O-72) were surface sterilized by immersion in 0.1% (w/v) Mercuric chloride for 20 min, followed by 4-5 washes with deionized water. All the 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.

Root regeneration

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 Wm-2 of daylight florescent tube with a 12 h photoperiod. Day to day observation was carried out to note the responses.

Acclimatization/Hardening

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

Table 2. Percentage of root regeneration from the regenerated shoots and success rate of regenerated plantlet in the soil

Variety	No. of shoot induced roots	Percentage of root regeneration	Days to root initiation	No. of plantlet sowing	No. of healthy seedling	Success rate (%)
Var. O-9897	19.3	53.6	8.2	40.0	37.0	92.5
Var. O-72	17.8	49.4	9.5	40.0	38.0	95.0
Var. OM-1	14.5	40.3	10.4	40.0	30.0	75.0
Level of Significance	**	**	**			
LSD 0.05	1.834	5.096	0.774			

Each value represents the mean of three replicates per treatment with 20 explants per treatment. The data were statistically analyzed using LSD. In the same column, significant differences according to LSD at the $P \leq 0.05$ level.

autoclaved sterilized soil mix (peat moss, perlite, and vermiculite, 5:3:2, v/v/v).

Sources of plant materials and plant culture

The seeds of *C. olitorius* (var. O-9897 and O-72), a popular and high-yielding jute variety cultivated in Asia were taken for study. Sterilized seeds were placed onto seed germination medium MS medium (MS salts with MS vitamins, 3% sucrose, 0.8% agar, pH 5.8) in 100 mL conical flask. In each flask 25-30 seeds were incubated at 25°C in a growth room, with a 16/8 h day/night cycle and with a fluorescent light intensity of 70 µmol photons $m^{-2}s^{-1}$. Seven days old seedlings were used as source of disease free explants.

Agrobacterium tumefaciens strain and binary Vector

Agrobactarium tumefaciens LBA4404 harbouring the binary vector pBI121 containing selectable marker nptII 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. $7H_2O$, 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 spectrophotometer at a wave-length of 600 nm. 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 petridish 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 mgL^{-1} kanamycin. The culture was 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 were used for infection and incubation. Prior to this "optical density'' of the bacterial suspension was determined at 600 nm with the help of a spectro-photometer. Following the determination of density, to get suitable and sufficient infection of the explants, freshly excised explants were dipped into bacterial suspension (OD_{600}= 0.6) for 1 min before transferring them to co-cultivation medium.

Co-cultivation

Following infection and incubation, the explants were co-cultured on MS media with kanamycin (50 µg/ml) and acetosyringone(50 µg/ml) . All the explants were maintained in co-cultivation media for 2 days. Co-cultured petri dishes containing explants were placed under fluorescent illumination with 16/8 h light/dark cycle at 28°C.

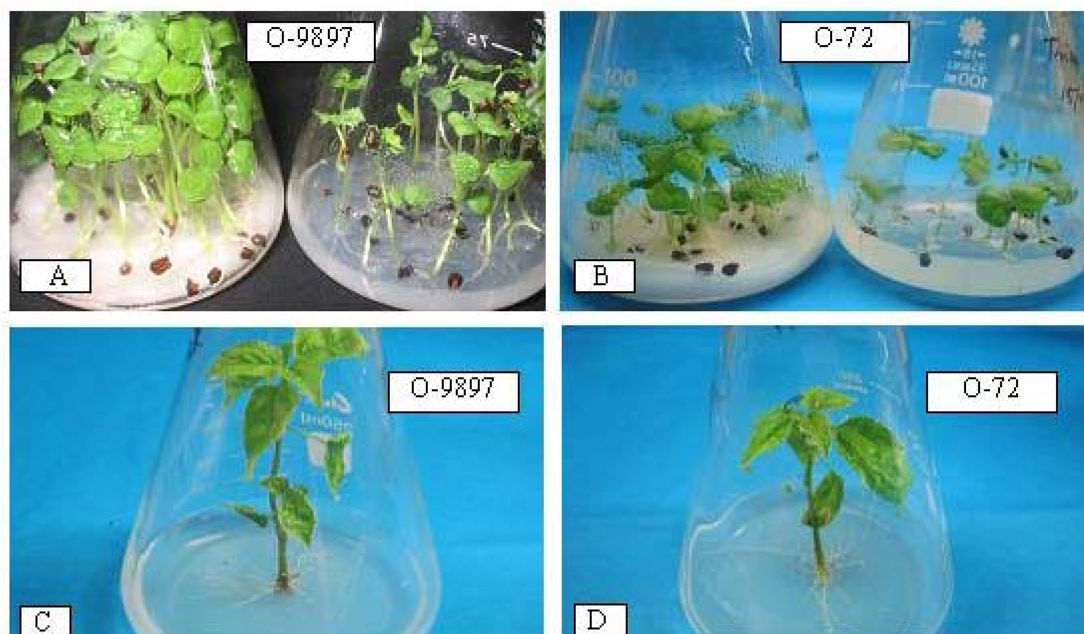

Fig 1. Seed germination on clinical cotton and agar based media (A) (left, 0-9897) and (B) (right, 0-72). Initiation of roots from regenerated shoots of O-9897 (C) and O-72 (D) on MSO medium

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 37°C 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 color.

Statistical analysis

Data were analyzed Least Significance Difference (LSD). In the same column, significant differences according to LSD at the $P \leq 0.05$ level are indicated by different letters using statistical package software SPSS (2003).

Results and discussion

Effect of media on seed germination

Seeds were germinated from the varieties of *C. olitorius* (O-9797 and O-72) on both clinical cotton and agar supported MS solidified medium. The percent of seeds germination in agar medium found to be very low (43.1%) , as compared to clinical cotton supported MS liquid medium, which was found to be very high (97.6%) (Table 1). The seedlings of Tossa Jute (*C. olitorius*) varieties grown on clinical cotton supported medium were found to much healthier than the seedlings grown on agar supported medium (Fig. 1 A-D).

Root regeneration on media

Highest percentage of root initiation was recorded for the variety O-9897 (53.6%) which was statistically identical by variety O-72 (49.4%) (Table 2). On the contrary the lowest percentage root initiations were recorded in variety OM-1 (40.3%). Regenerated plantlets of all varieties of *C. olitorius* started to produce roots on hormone free agar solidified MS medium within a week. Plantlets of all varieties successfully transferred to soil, grew well into maturity and produce fruits. No morphological changes were noticed. The success rate of plantlets survival was recorded above 90% for var. O-72 and O-9897 and 75% for var. OM-1 (Table 2).

Genetic transformation of Tossa Jute

Genetic transformation is a powerful and important tool which can be used in Jute improvement program. Efficient and reproducible transformation protocol is required for successful genetic transformation. Therefore, in the present study, investigations were made to generate transgenic plant from two varieties of *C. olitorius* (vars. O-9897 and O-72) through

Table 3. Performance of varieties in *A. Tumefaciens and* Performance of varieties in GUS histochemical assay

Varieties	No. of shoot producing cotyledons	shoot producing cotyledons (%)	Average no. of shoots/ cotyledon	No. of infected cotyledons	No. of assayed cotyledons for GUS	No. of cotyledons +ve for GUS	GUS +ve cotyledons (%)
O-9897	3.6 a	60.0 a	8.8 a	60	15	13	86.6
O-72	3.5 a	58.3 a	8.5 b	60	15	12	80.0
$LSD_{0.05}$	0.5276	8.799	0.1319				
CV	14.70	14.70	1.48				

Each value represents the mean of three replicates per treatment with 20 explants per treatment. The data were statistically analyzed using LSD. In the same column, significant differences according to LSD at the P≤ 0.05 level are indicated by different letters.

Agrobacterium-mediated transformation using cotyledons (with attached petiole) as explants.

The performance of varieties in *A. tumefaciens* showed a statistically significant difference in respect of number of explants produced shoot and percent shoot regeneration (Table 3). The maximum numbers of shoot producing cotyledons (3.6) were recorded in the var. O-9897 with *A. tumefaciens* which was statistically identical (3.5) with the var. O-72. In case of percent shoot regeneration var. O-9897 produced highest cotyledons (60.0%) with *A. tumefaciens* which was statistically identical (58.3%) with the var. O-72. The average numbers of shoots produced by each cotyledon were count in the present trial in laboratory condition. The highest number of shoots (8.8) produced by each cotyledon were recorded in the var. O-9897 and that was 100 mg/l kanamycine resistant which was statistically matching with the var. O-72 (8.5) (Table 3). The lowest number of shoots (8.0) produced by each cotyledon was recorded in the var. O-72.

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 (Fig. 2). Following GUS histochemical assay, it was found that the two varieties showed positive responses towards transformation. Among the vars. O-9897 showed the highest response (86.6%) to GUS assay and O-72 showed the lowest response (80.0%) to GUS assay (Table 3). Control explants did not show in 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 (Fig. 3).

Plants have been engineered to be resistant to herbicides (Cao *et al.,* 1992) viruses (Harrison *et al.,* 1987) and insects (Vaeck *et al.,* 1987). *A. tumefaciens* is the causative agent for crown gall disease, whilst *A. rhizogenes* causes hairy root disease of the host plant. This tumor inducing and hairy root-forming ability is based on a plasmid for *A. tumefaciens* the Ti plasmid and for *A. rhizogenes* the Ri plasmid. Vectors carrying a genetically manipulated T-region can be introduced directly into an *Agrobacterium* that has a separate *vir* plasmid. Successful transfer of genes using *Agrobacterium* has been achieved in a number of crop plants e.g. potato (Sheerman and Bevan, 1988), *Brassica* (Moloney *et al.,* 1989), tobacco, (Hatamoto *et al.,* 1990) strawberry (Nehra *et al.,* 1990). Like the Ti plasmid an Ri plasmid can be engineered as a vector for gene transfer (Tempe' and Casse-Delbart, 1989) and has been used successfully for the transformation of crop plants including apple (James *et al.,* 1989), potato (Sheerman and Bevan, 1988) and tobacco (Hatamoto *et al.,* 1990). Discovery of insecticidal proteins from *Bacillus thuringiensis* and Chitinase genes against fungal diseases made it possible to introduce genes against fungal and insects into crop plants. Bt toxin genes have been introduced into many crop plants (Sharma and Kumar, 1995).

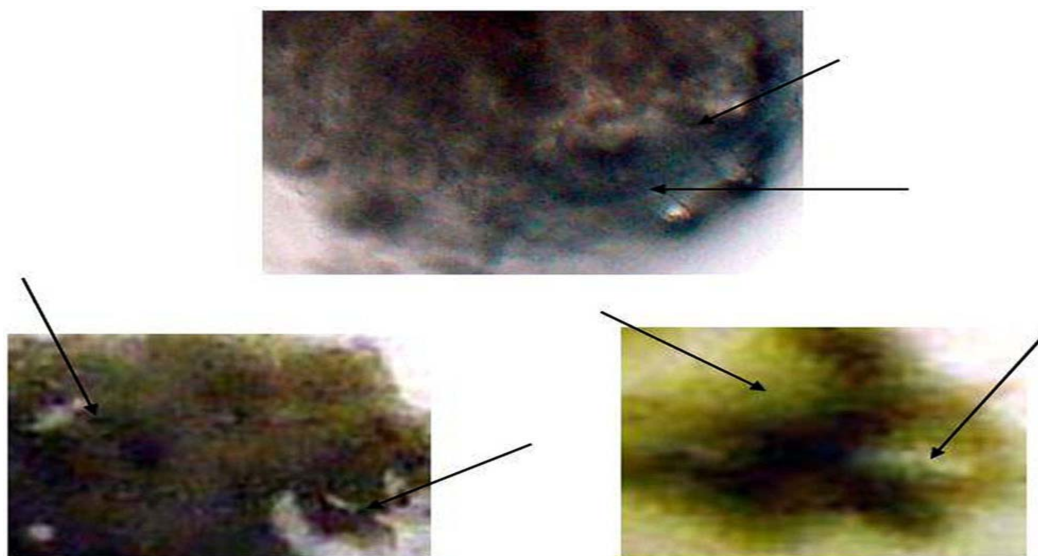

Fig 2. Histochemical localization of GUS activity (blue zone) at the infected callus (upper) and cotyledonal tissue (left) with control explant (right), which is indicated by arrow.

Transformation could indirectly provide opportunity for the improvement of the two cultivated Jute species, *Corchorus capsularis* and *C. olitorius* by the use of *Agrobacterium* based vectors carrying important desirable genes like resistance to diseases and pests with the introduction of selectable marker as a basis for a selection. Hypocotyls and cotyledons of *C. capsularis* and *C. olitorius* were inoculated with *A. rhizogenes* strains 8196 and A4T carrying wild type Ri plasmids and with strains pBI121, R1601 and LBA9402pBin19 carrying engineered plasmids (Khatun *et al.,* 1992). Explants were found to be very susceptible to *A. rhizogenes* strains and produced hairy roots from the infected regions. These roots were confirmed as transformed with positive opines and *npt*II assays (Khatun, 1992). Additionally, the engineered strains showed resistance to 100 µg/mL of kanamycin sulfate whereas the non-transformed seedling roots could only survive upto 25 µg/mL of kanamycin. Somatic embryos were obtained via callus formation from hairy roots.

The cut ends of the cotyledonary petioles have been found highly susceptible to *Agrobacterium*-mediated gene transfer and displayed a high plant regeneration rates, often with numerous shoot production per explants. Multiple shoot-buds were reported to be regenerated from the *Agrobacterium-infected* cotyledonary petioles of *C. capsularis.* The target cells of these experiments were at the cut surfaces of cotyledonary petioles. The plantlets showed abnormal morphology including adventitious root formation from the leaves and stems. These adventitious roots appeared like hairy roots and could be excised and maintained on hormone free MS medium, which indicated the transformed nature of those plants. These plants continued to grow and transferred into soil in pots. However, no further report was available on the survivability of the putative transgenic Jute plants and in all these cases transformation of Jute was not confirmed by the DNA hybridization analysis. Further experiment for genetic transformation will be open a new window in the biotech crops.

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