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Full Length Research Paper

Study on the development of salt tolerant kenaf varieties through *Agrobacterium*-mediated genetic transformation

S. K. Bhajan[1], N. Biswas[1, 2*], N. M. R. B. Rahman[1], M. M. Hasan[1], S. K. Saha[1], and A. Khatun[3]

[1]Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh.
[2]Department of Chemical and Biological Sciences, School of Applied Sciences, University of Huddersfield, United Kingdom.
[3]Bangladesh Jute Research Institute, Manik Mia Avenue, Dhaka, Bangladesh.

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Cotyledons with attached petioles of two kenaf varieties were cultured on MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l IAA after co-cultivation with salt and drought tolerant *Agrobacterium* vector LBA-4404 (pBI121ClPKsense). Variety HC-2 showed higher percentage of shoot regeneration (80%) than variety HC-95. The regenerated shoots of both of the kenaf varieties showed resistance against kanamycin (50 mg/l). Non-transformed shoots became albino and died within 6 weeks. GUS histochemical assay was performed for the kanamycin resistant shoots. Maximum of 90.0% GUS positive explants were found from the variety HC-2 and variety HC-95 showed 80.0% GUS positive response. In both varieties, the stable GUS gene integration was proved by PCR amplified DNA band using GUS specific primers set. MS medium was supplemented with different concentrations of NaCl e.g. 25, 50, 75, 100 and 125 mM. Transformed shoots were able to survive upto 100 mM NaCl. Non transformed shoots died on salt containing medium. Salt tolerant transgenic shoots were rooted on hormone free MS medium and transferred to soil. After maturity these plants produced flowers and seeds.

Keywords: Kenaf, Transformation, Kanamycin resistance, GUS expression, salt tolerance.

INTRODUCTION

Kenaf is one of the most important fibre crops in the world next to cotton (IJSG, 2004). Currently, kenaf shows promise as a source of pulp for the manufacture of various grades of paper and card board. In Bangladesh, 963.00 thousand tons of kenaf were produced from 499.80 thousand hectares of land in 2003-2004 (FAO, 2004). Much research developed to meet the demands of high fibre yielding and disease resistant kenaf in the recent decades (Dempsey, 1975; Bitzer, 2000).

Many land area in Bangladesh are under salinity and drought condition and this abiotic factor is a major problem to cultivate kenaf varieties. To overcome this problem, traditional breeding is employed in different countries including Bangladesh. However, traditional breeding is time consuming and has a risk of assimilation of undesired genes. Therefore, development of salt tolerant kenaf through *Agrobacterium* mediated gene transfer might be a shorter and alternative method for kenaf varieties against salinity stress. Recently, several approaches were attempted to improve the stress tolerance of plants by gene transfer (Holmberg and Bulow, 1998).

Tissue culture of a crop is a prerequisite for the improvement of a crop through genetic transformation. Recently, plant regeneration has been reported from the explants of kenaf (Khatun *et al.,* 2003). Both callus induction and plant regeneration from the explants require the presence of appropriate combinations and concentrations of plant growth regulators in the culture media (Ahn *et al*., 2001; Ehsanpour and Jones, 2000). The aims of this study were to develop a reproducible and efficient

*Corresponding Author E-mail: nirupamgene@gmail.com
Tel: +447745738807

protocol for the insertion of foreign gene into two kenaf varieties through *Agrobacterium tumefaciens* strains. Another objective of this study was to develop a salt tolerant kenaf variety to be useful for the saline zone.

MATERIALS AND METHOD

The seeds used for seedling production in this experiment were collected from Bangladesh Jute Research Institute (BJRI), Dhaka. The strains of *Agrobacterium tumefaciens* LBA 4404 (pBI121CIPKsense) used in this study were obtained through ICGEB New Delhi India. Seeds of *H. cannabinus* (vars. HC-2 and HC-95) were surface sterilized by immersing in absolute alcohol for 1 min. Then immersed in 0.1% Mercuric Chloride ($HgCl_2$) for 20 min. Seeds were thoroughly washed with autoclaved water 6 times. The sterilized seeds were transferred in a 500 ml conical flask containing 50 ml of hormone free cotton supported liquid MS (Murashige and Skoog, 1962) medium contained in a 500 ml capacity of conical flasks. Each flask contained 10 seeds and was placed in a growth room with 28±2°C under 1500 lux fluorescent illumination with 12 h alternate dark and light condition. Cotyledons with attached petioles of kenaf were used as explants. It was made sure that the emerging shoots were not remained attached with the petioles. For shoot regeneration BAP 3 mg/l and IAA 0.5 mg/l were used. The cultures were maintained in a growth room with 28±2 °C temperature under 1500 lux fluorescent illumination of 12 h photoperiod.

Luria Broth (LB) medium with kanamycin was used to grow genetically engineered *Agrobacterium tumefaciens* strain. Agar solidified LB medium was used for the maintenance of the *Agrobacterium* and liquid LB medium was used for co-cultivation experiment. A single colony was taken in an inoculation loop from the stock and inoculated in a 100 ml conical flask containing 20 ml liquid LB medium with 50 mg/l kanamycin .The culture was allowed to grow at 14 hrs in a shaker (100 rpm) at 28±2°C to get optimum population of *Agrobacterinm* for infection and co-cultivation with explants.

Cotyledons with attached petioles were excised from the *in vitro* grown seedlings and placed in a Petri dish containing liquid bacterial solution for one minute. Prior to transfer of the explants to co-cultivation medium explants were blotted dry with sterile tissue papers to remove excess of bacterial suspension. Following infection, the explants were co-cultured in regeneration medium containing growth regulators BAP 3 mg/l and IAA 0.5mg/l. All the explants were kept in a growth room with 28 ± 2 ^{0}C for co-cultivation. After 24 h co-cultivation, the explants were transferred to regeneration medium consisting of MS medium supplemented with 3.0 mg/l BAP, 0.5 mg/l IAA and 500 µg/ml cefotaxime. Amount of cefotaxime was gradually reduced in every sub culture. Infected explants were then placed under fluorescent illumination for 12 hours alternate light and dark condition at 28 ± 2 ^{0}C. The intensity of light was maintained at 1500 lux.

Following four week of post-cultivation, the explants were transferred to a selection medium consisting of MS medium without hormone, 50 mg/l kanamycin and 300 µg/ml cefotaxime.

Randomly selected tissues regenerated on selection media were examined for GUS histochemical assay (Jeffereson *et al*., 1987). For this experiment, regenerated shoot tissues were immersed in X-*gluc* (5-bromo-4-chloro-3-indolyl glucuronide) solution and were incubated at 37 ^{0}C overnight. A characteristic blue colour would be the expression of GUS *(β-glucuronidase*) gene in the plant tissue. Proper control for GUS histochemical assay was done with the explants having no *Agrobacterium* infection.

Plants regenerated from GUS positive explants were used for DNA isolation and polymerase chain reaction (PCR) analysis confirmed that GUS$^+$ shoots contained T-DNA. Primer pairs Forward 5' TTTGCAAGTGGTGAATCCCGACCT 3' and Reverse 5' AGTTTACGCGTTGCTTCCGCCAGT 3' were used. PCR amplification was done in an oil-free thermal cycler (master cycler Gradient, Eppendorf) following the PCR profile of 95 ^{0}C for 3 minutes (initial denaturation) followed by 35 cycles of 1 minute denaturation at 95 ^{0}C, 1 minute annealing at 54 ^{0}C and elongation or extension at 72 ^{0}C for 1 minute. After the last cycle, a final step of 5 minutes at 72 ^{0}C was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4^0 C. PCR reactions were performed on each DNA sample I a 10 Ul reaction mixture containing 1.0 uM of 10XA Taq buffer (tris with 15 mM $MgCl_2$), 1.0 ul of 2.5 nM dNTPs, 2.25 ul of 1.0 uM each of forward and reverse primer, 0.3 ul of 3 U/ul Ampli Taq DNA polymerase (Bangaloare Genei Pvt. Ltd., India), 3 ul of 25 ng/ul genomic DNA and a suitable amount (0.2 ul) of sterile deionized distilled water. After amplification 2 ul loading dye was added to the amplification product for separation using 1.4% agarose gel (containing Ethidium Bromide 0.8 ugm^{-1}) electrophoresis. Electrophoresis was performed at 100 V for 2.5 hours. DNA ladder 100 bp (Bangaloare Genei Pvt. Ltd., India) was run alongside the reactions. Expected amplified DNA fragment was observed on UV-transilluminator in Gel Documentation system (Unitech, DBT-2000LS), printed and soft copy for lateral use.

For testing salt tolerance in putative transgenic kenaf plants, explants were cultured on hormone free MS medium supplemented with different concentrations of NaCl (25, 50, 75, 100 and 125 mM).

Figure 1. Shoot regeneration from LBA 4404 (pBI121CIPKsense) infected cotyledons with attached petioles of kenaf (var. HC -2) 25 days after culture on MS solidified medium containing IAA (0.5mg/l) and BAP (3.0 mg/l) (× 6.5).

Figure 2. Shoot regeneration from LBA 4404 (pBI121CIPKsense) infected cotyledons with attached petioles of kenaf (var. HC -2) 45 days after culture on MS solidified medium containing IAA (0.5mg/l) and BAP (3.0 mg/l) (× 5.5).

Figure 3. Regeneration shoots from LBA 4404 (pBI121CIPKsense) infected cotyledons with attached petioles of kenaf (var. HC -2). 40 days after culture on MS solidified medium containing 50 mg/l kanamycin showing kanamycin resistance (× 4.2).

Figure 4. Regenerated shoot from cotyledons with attached petioles of kenaf (var. HC -2) (control). 40 days after culture on MS solidified medium containing 50 mg/l kanamycin showing albino (× 4.5).

Figure 5. Stereomicroscopic view of expression of GUS gene from the regenerated kanamycin resistant shoots of cotyledons with attached petioles of kenaf varieties HC-2 showing the blue coloured cells (× 100).

Figure 6. Stereomicroscopic view of expression of GUS gene from the regenerated kanamycin resistant shoots of cotyledons with attached petioles of kenaf varieties HC-95 showing the blue coloured cells (× 100).

Table 1. Effect of varieties on days for initiation of shoot regeneration, number of shoot per flask, per cent shoot regeneration at MS medium supplemented with 3 mg/l BAP concentration and 0.5 mg/l IAA after infection with *Agrobacterium* strains.

Agrobacterium strains used	Varieties	Days for initiation of shoot regeneration	Number of shoot/flask after 30 days of culture	Percentage of shoot regeneration
LBA4404 (pBI121CIPKsense)	HC-2	13.8 ± 1.09	3.2 ± 0.83	80.0
	HC-95	12.4 ± 1.14	3.0 ± 0.70	75.0

Values represent mean ± SE of 10 replicates per treatment in three repeated experiments.

Table 2. Selection of explants of two kenaf varieties transformed with LBA 4404 (pBI121 CIPKsense) using kanamycin.

Varieties	Explants	Number of explants set for selection	Number of explants survived and regenerated	Percentage of regeneration
HC-2	Cotyledons	100	85	85
	Hypocotyls	100	75	75
HC-95	Cotyledons	100	80	80
	Hypocotyls	100	70	70

The transformed shoots were transferred to hormone free MS agar solidified medium for rooting. The cultures were maintained at 28±2 °C with a day length of 12 hour (1.0 Wm^2 of daylight fluorescent illumination).

The rooted transgenic kenaf plantlets were then transferred to pots containing mixed soil. As peat soil was not available, 70% dairy soil (Savar Dairy, 70%) was mixed with 30% commercial sand. The mixture was sterilized before use. The idea of mixture was to make soil pours for good aeration. Plastic pots (6 cm dia and 7 cm height) with a small whole at the bottom were used for transfer purpose. Pots were placed on a 9 cm Petri dishes each containing 20 ml of water. The plantlets were washed with sterilized tap water to remove agar and then transferred into the pots. The plantlets were then covered with a cellophane paper bag and placed in a well ventilated place. After one week, two wholes were made in each bag to allow some fresh air. In the second week, more wholes were made in the bags and during the third week the bags were removed. During the fourth week the plants were finally transferred to 30 cm pots containing dairy soil mixed with chemical fertilizers. Survival rate of the plants was recorded.

RESULTS AND DISCUSSION

Excised *Agrobacterium* infected cotyledonary attached petioles of kenaf varieties HC-2 and HC-95 transformed with LBA4404 (pBI121CIPKsense) strain produced shoots directly or via limited amount of callus (Figure 1 and 2). The target cells of these experiments were the cut surfaces of the cotyledonary petioles. The cotyledonary petioles developed higher per cent (80%) of shoot from HC-2 kenaf varieties compared to HC-95 (75%) (Table 1). The present finding indicates that the cotyledons of kenaf will respond to shoot regeneration provided the petioles remained attached to the cotyledons. This finding is comparable to jute, *Corchorus capsularis* (Khatun *et al.,* 1993), *Brassica spp*. (Sharma *et al*., 1991) and apple (Kouider *et al.,* 1984). Like kenaf, these species similarly require an attached petiole to undergo morphogenesis. Successful transformation were reported using cotyledonary petioles of jute (Khatun *et al.,* 2003), kenaf (Hoque, 2005), and mesta (Saha, 2008).

Agrobacterium-infected regenerated shoots survived in MS agar solidified medium containing kanamycin 50 mg/l (Figure 3). This result was found consistent with the result reported (Haque, 2005). Higher percentages (85%) of kanamycin resistant shoots were found from the variety HC-2 (Table 2). Non transgenic shoots became albino in the presence of kanamycin 50 mg/l and died within 6 weeks (Figure 4).

Transient GUS assay was performed from the regenerated shoots survived in kanamycin selection medium. Conspicuous GUS positive (blue color) regions were detected in the thick section of regenerated shoots resistant to kanamycin (Figure 5 and 6). Eighty to 90 % of explants showed GUS positive response (Table 3). Between of the varieties, HC-2 showed maximum GUS positive response (90%) compared to HC-95 (80%). This result was comparable to the findings of Haque (2005) and Barman (2005) on kenaf and jute respectively. Similar response was reported by Sarker *et al.,* (2008) in jute varieties.

Finally, transformation was confirmed at DNA level through PCR using GUS specific primer (Figure 7). From the gel it was observed that single band formed in one transformed plantlet. The result indicated that GUS gene inserted in the genomic DNA of transformed plantlet.

Transformed plants were able to survive on 25 mM to 100 mM salt concentrations while the control plants became albino on 75 mM onwards NaCl concentration

Table 3. GUS assay of cotyledons of two kenaf varieties transformed with LBA 4404 (pBI121CIPKsense).

Agrobacterium strains used	Varieties	Explants	Number of explants assayed for GUS	Number of explants positive for GUS	Percentage of GUS positive explants
LBA4404 (pBI121CIPKsense)	HC-2	Cotyledons	10	9	90
	HC-95	Cotyledons	10	8	80

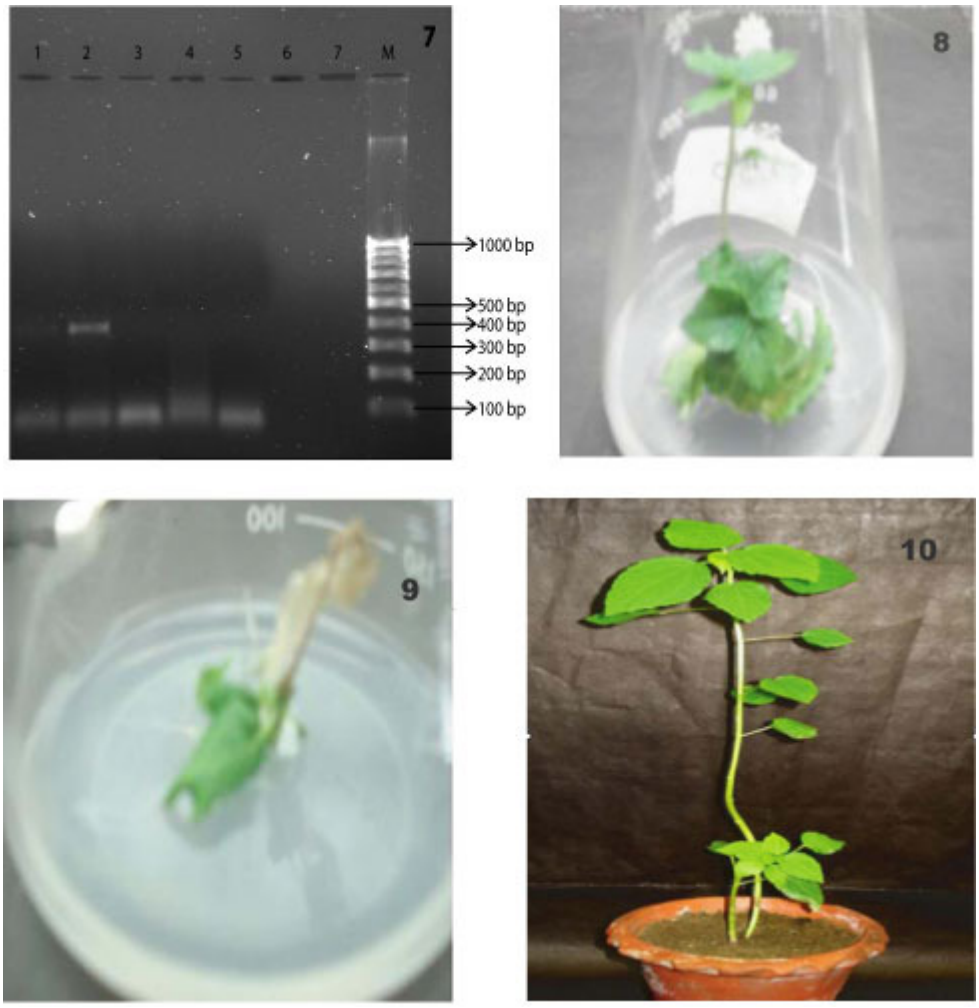

Figure 7. Detection of GUS gene by PCR of genomic DNA isolated from transgenic kenaf variety HC-2 Lane-2 Genomic DNA of transformed plant, lane-7 control, Lane -M 100 bp ladder.

Figure 8. Transgenic plantlets survived on 100 mM NaCl (× 3).

Figure 9. Non transgenic plantlets (control) became albino on 100 mM NaCl (× 3).

Figure 10. Kanamycin resistant, GUS positive and salt tolerant transgenic kenaf plants (variety HC-2) 40 days after transfer

(Figure 8 and 9). In the case of 100 mM salt, the survival rate was 70 % (Table 4). The transformed plants survived on 100 mM of NaCl in MS agar solidified medium indicated the transformation and expression of salt tolerant gene in the kenaf varieties (Figure 10). At 125 mM of NaCl, the transformed plants were found albino. Kenaf can be grown, with 0.8 m of good quality irrigation water, on a saline soil with the salinity level of 10.2 dS/m. The soils with salinity levels greater than 4.5 dS/m do not appear to be conducive for kenaf production even when irrigated with good quality water **(**Bhangoo *et al.,* 1994). For salt tolerance analysis, Hossain *et al*., (2005)

Table 4. Selection of explants under various salt concentrations in variety HC-2 transformed with LBA4404 (pBI121CIPKsense).

Treatments (salt concentrations)	Number of explants assayed	Number of explants survived	Percentage of survived plants
25 mM	10	10	100
50 mM	10	10	100
75 mM	12	12	100
100 mM	10	7	70
125 mM	8	1	12.5

reported the similar response in sugarcane varietis supplemented with NaCl of different concentrations (50, 100 and 150 mM for 13.11, 17.10 and 21.66 dS/m, respectively). Sugarcane transformed plants were able to survive on supplemented media up to 150 mM (21.66 dS/m) NaCl concentration and 7.5% of PEG concentration while controlled plants died.

The transgenic shoots produced roots on MS medium without growth regulators. Root production from transgenic kenaf was found to be quite easy on hormone free MS medium. The 95% transgenic kenaf plantlets survived after transfer to soil and grew into maturity. The matured plants produced flower and fruits without showing any morphological abnormality.

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