CHAPTER -III

MATERIALS AND METHODS

3.1. Plant material

The best performing genotypes of toxic cultivar (CSMCRI-JC-1, IC 56557 13), (CSMCRI-JC-2, IC 56557 16) and (CSMCRI-JC-3, IC 56557 17) and a non-toxic cultivar of *J.curcas* identified in the field experiments established over 35 hectares in CSMCRI experimental field, Chorvadla, India (21° 75'N, 72° 14'E) on wastelands (Plate 2) were selected for the present study (Plate 3A, B, C and D). Nodal explant of 4.0 -5.0 cm (Plate 3E) was used not only for establishment of stock culture for generating experimental materials (leaf and petiole explant) but also in all experiments involving direct organogenesis and genetic transformation. Similarly seeds (Plate 3F) from selected genotypes were used for generating seedlings to obtain cotyledonary leaf and cotyledonary petiole as a source of explant for their eventual use in all experiment involving direct organogenesis.

PART A

3.2. OPTIMIZATION OF REGENERATION PROTOCOL

3.2.1. Establishment of aseptic cultures

In vitro aseptic shoot cultures of above mentioned genotypes/cultivar were established by culturing nodal explant collected from 3-4 years old plants. The excised nodal explant of 3-4 cm with axillary buds was surface sterilized by 0.1% mercuric chloride ($HgCl_2$) for 15 min and rinsed five times in sterile distilled water under sterile conditions. The sterilized nodal explant was cultured on MS solid medium (Murashige and Skoog, 1962, Appendix-1) supplemented with 3% sucrose, 6 mg/L 6-benzyl aminopurine (BAP) and 1 mg/L indole-3-butyric acid (IBA) for sprouting of axillary bud and formation of shoots (Plate 3I) . After 4 weeks of culture, leaf and petiole explant was collected from these axillary shoots and used as *in vitro* explant (Plate 3N & O). Leaf and petiole close to apical buds

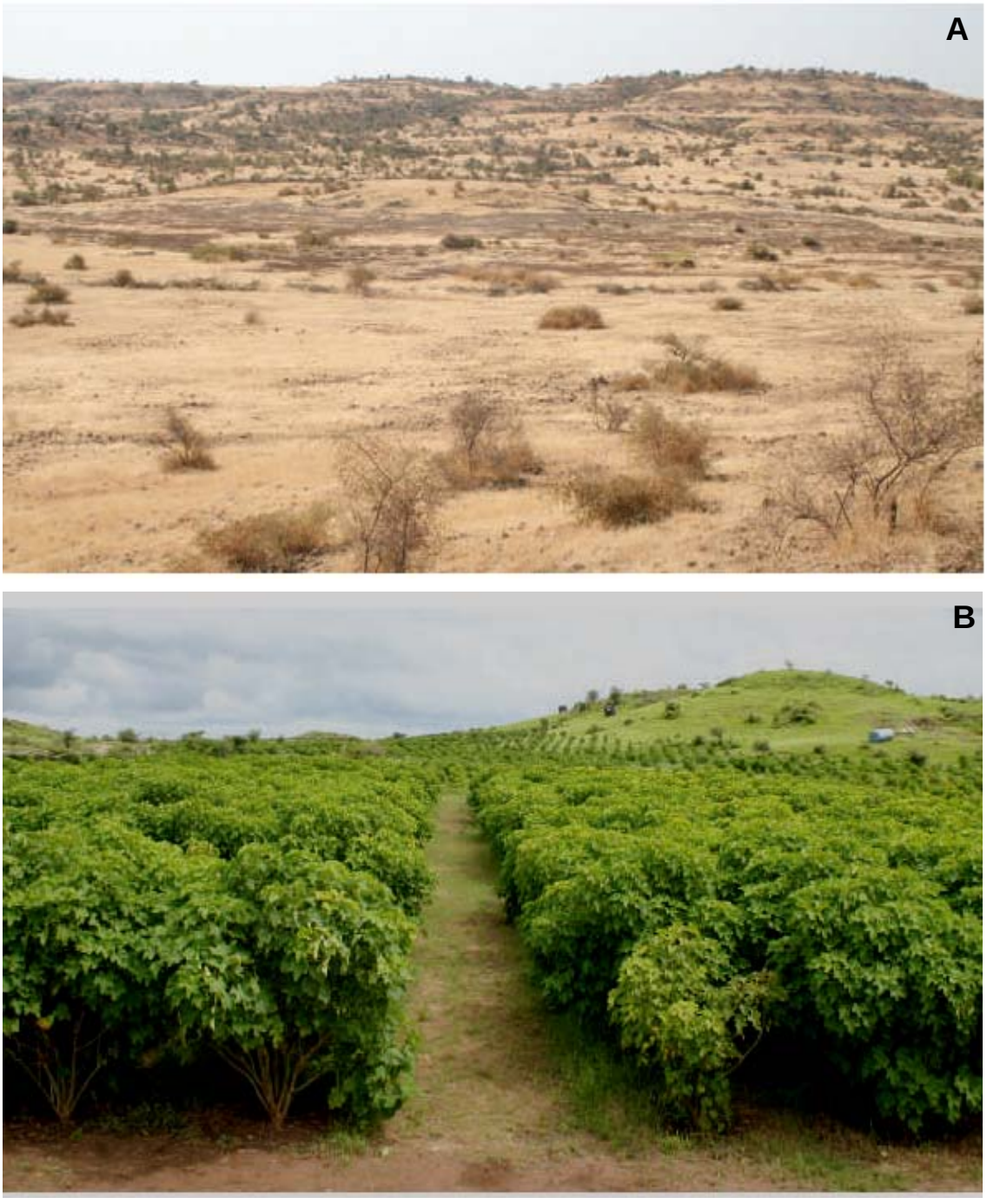

Plate 2. Cultivation of *J.curcas* on wasteland.

(A) Wasteland (CSMCRI experimental field, Chorvadala, Bhavnagar) before plantation

(B) After plantation with *J.curcas*

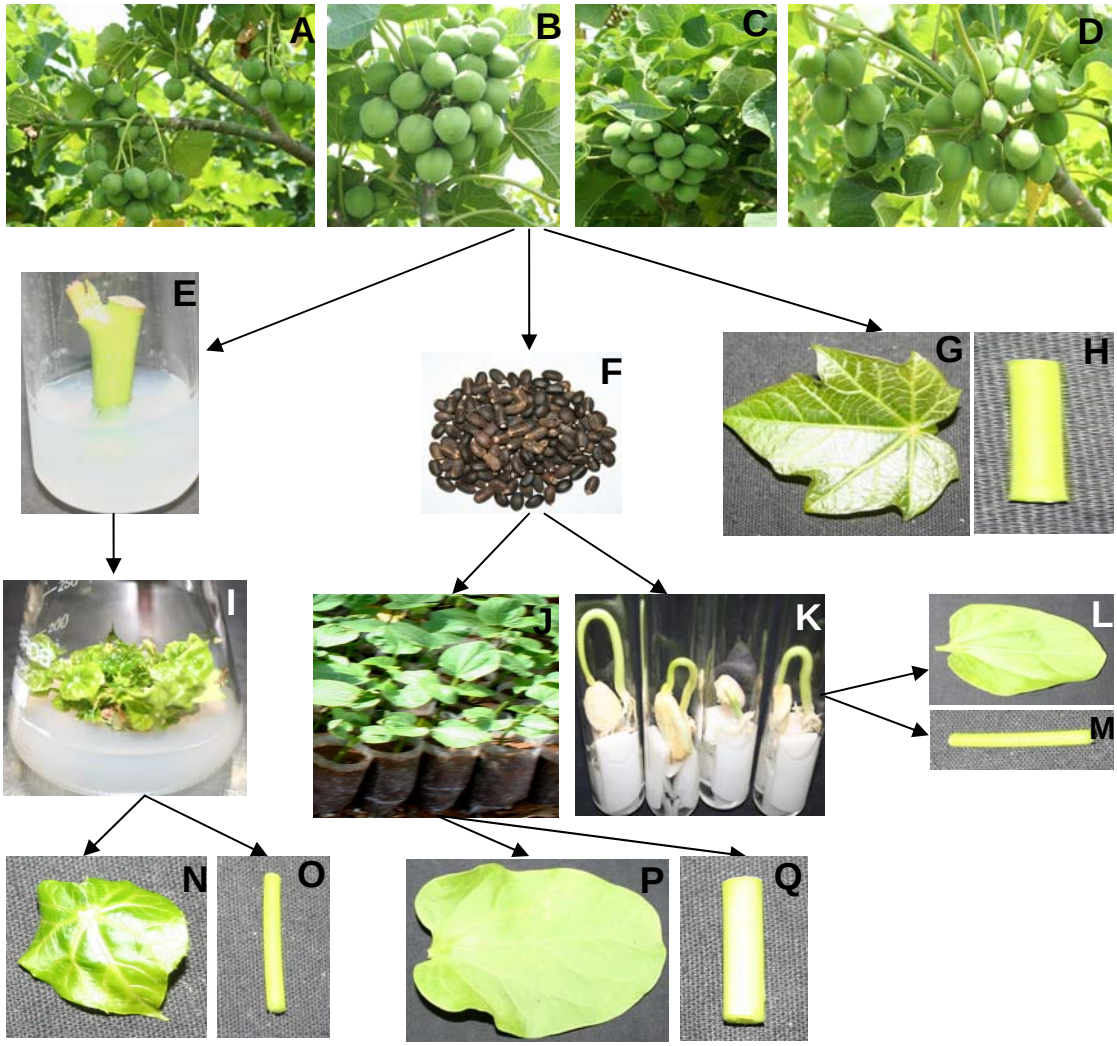

Plate 3. Establishment of experimental materials of *J.curcas*

(A), (B) & (C) CSMCRI-JC-1, CSMCRI-JC-2 and CSMCRI-JC-3 genotypes of toxic cultivar. (D) Non-toxic cultivar. (E) Aseptic nodal explant. (F) Seeds (G) *In vivo* leaf explant. (H) *In vivo* petiole explant. (I) Established aseptic culture from nodal explant. (J) *In vivo* germinated seedling. (K) *In vitro* germinated seedling. (L) *In vitro* cotyledonary leaf explant. (M) *In vitro* cotyledonary petiole explant. (N) *In vitro* leaf explant from shoot culture. (O) *In vitro* petiole explant from shoot culture. (P) *In vivo* cotyledonary leaf explant. (Q) *In vivo* cotyledonary petiole explant.

were excised from 3-4 years old plants, surface disinfected by 0.1% mercuric chloride ($HgCl_2$) for 7 min separately served as *in vivo* explant (Plate 3G & H).

For cotyledonary leaf and cotyledonary petiole explant, seedcoat of the genotypes/cultivars were removed and then surface sterilized by 0.1% mercuric chloride ($HgCl_2$) for 7 min and rinsed five times in sterile distilled water. The sterilized decoated seeds were germinated on hormone free MS liquid medium (Murashige and Skoog 1962) supplemented with 3% sucrose with the support of filter paper boat (Plate K). After two weeks of germination, cotyledonary leaf and cotyledonary petiole explant was excised and used as *in vitro* explant (Plate 3L & M). For *in vivo* explant, seedlings were raised in the nursery and cotyledonary leaf and cotyledonary petiole explant were collected from two week old seedlings (Plate 3J), and sterilized by 0.1% mercuric chloride ($HgCl_2$) for 7 min and rinsed five times in sterile distilled water and used as *in vivo* explant (Plate 3P & Q).

3.2.2. Shoot bud induction

Both *in vitro* and *in vivo* leaf and petiole explant of toxic and non-toxic cultivars of *J.curcas* were cultured separately on MS solid medium supplemented with 3% sucrose, 0.7% agar and various concentrations of thidiazuron (TDZ) or BAP alone and in the presence of IBA to find an optimum hormone concentration for inducing shoot buds regeneration. Intact leaf explant was inoculated horizontally on medium surface in 200 X 38 mm culture tubes (Borosil) whereas, petiole explant was inoculated on medium both in horizontal and vertical position. The percentage of shoot buds induction and the number of shoot buds per explant were recorded after 6 weeks of culture.

3.2.3. Proliferation and elongation of shoot buds

The induced shoot buds of both toxic and non-toxic cultivar were transferred on MS medium supplemented with 3% sucrose, 2 mg/L kinetin (Kn), 1 mg/L BAP and 1 mg/L α-naphthaleneacetic acid (NAA) for 4 weeks for shoot proliferation (Reddy et al., 2008). Shoots were individually separated and further tested for their elongation on MS medium supplemented with 3% sucrose and different concentrations and combinations of BAP, indole-3-acetic acid (IAA), NAA and IBA. The length of elongated shoots was recorded after 6 weeks of culture.

3.2.4. Rooting and acclimatization

Green and healthy elongated shoots with three to four leaves were excised and cultured on full or half strength MS medium supplemented with 2% sucrose and different concentrations and combinations of auxins like IBA (1-3 mg/L), IAA (1-3 mg/L), NAA (1-3 mg/L) and 0.25 mg/L activated charcoal for rooting. In separate study green and healthy elongated shoots were cultured on root induction medium containing half or full strength of basal MS supplemented with 2% sucrose and different concentrations and combinations of IBA (1-3 mg/L), IAA (1-3 mg/L), NAA (1-3 mg/L) for 4 days as pulse treatment with the support of whatman No. 44 filter paper boat. Pulse treated elongated shoots were cultured to a medium for rooting, containing hormone free full or half strength of basal MS medium with 2% sucrose and 0.25 mg/L activated charcoal for 4-6 weeks. The frequency of root induction was scored after 4 weeks. Rooted shoots were carefully taken out of the medium, washed thoroughly in autoclaved distilled water to remove adhering medium attached to the roots and transferred to polythene bags containing sterilized sand and soil in the ratio 1:1. Further the soil mixture was wetted with 0.02% w/v carbendazim and finally covered with transparent plastic bags to maintain humidity. After 3-4 weeks, the established plants were transplanted to polybags containing garden soil and farmyard manure and transferred to a green house for further growth and the numbers of surviving plants were recorded after 6-8 weeks.

3.2.5. Culture conditions and data analysis

Uniform culture conditions were applied in all experiments. The pH of the medium was adjusted to 5.7 using 1 N KOH or HCl, prior to autoclaving at 1.05 kg /cm^2 pressure at 121°C for 20 min. The cultures were maintained at 25 ± 2° C under a 16 hour photo-period with light intensity of 35-40 µmolm^{-2} s^{-1} (cool white fluorescent tubes).

All the experiments were set up in completely randomized design (CRD) and repeated three times with 25 replicates per treatment and one explant was cultured per test tube. Statistical difference among the means was analysed by Duncan's multiple range test using the SPSS (version 7.5). The results are expressed as the means ± SD of three experiments.

PART B

3.3. GENETIC TRANSFORMATION

3.3.1. *Agrobacterium* strain and plasmid vector

The disarmed *A. tumefaciens* strain LBA 4404 harbouring DREB2A, *hpt* and GUS genes as reporter gene under the control of CaMV35S promoter and. pCAMBIA 1301 vector was used for all the genetic transformation studies.The T-DNA region of the p35S:DREB2A based plasmid used in our experiments is shown in Plate 4. For routine use, above mentioned strain of *Agrobacterium* was grown in the dark at 28°C in agar solidified YMB medium (Appendix-2) supplemented with 25 µg/ml hygromycin monophospahte (Sigma).

3.3.2. Growth media and conditions for *Agrobacterium* culture

The above mentioned strain of *Agrobacterium* was grown in YEB medium supplemented with 25 µg/ml hygromycin. Culture was grown at 200 rpm on a rotary shaker at 28 °C for 24 h. Aliquots of *Agrobacterium* culture at hourly intervals were pipetted out and the optical density of the culture was measured at 600nm. The optical density was plotted against time to give the characteristic growth curve. The culture after 18 h starts showing a decline in the optical density and thereby 18 hour grown culture was used for all the experiments.

3.3.3. Determination of phytotoxic level of selective and bactericidal antibiotics

Leaf explant and *in vitro* derived shoot was inoculated separately on MS medium supplemented with different levels of hygromycin monosulfate (0, 5, 10, 15, 20, 25, 50 and100 mg/L) for selection of transformants. In a separate study, three bactericidal antibiotics, namely cephalexin (Sporidex; Ranbaxy India.), cefotaxime (Claforan; Russel India) and carbenicillin (Hi-media, India), were tested each at different levels (250, 500, 750 and 1000 mg/L) for controlling the *Agrobacterium* after transformation.

3.3.4. Genetic transformation

Single cell colonies of *Agrobacterium* strain LBA 4404 harbouring p35S:DREB2A was grown in liquid 20 ml YMB medium containing 25 µg/ml hygromycin as mentioned above and cells corresponding to OD_{600nm} = 0.6 were pelleted by centrifugation at 6000 rpm for 10 min.

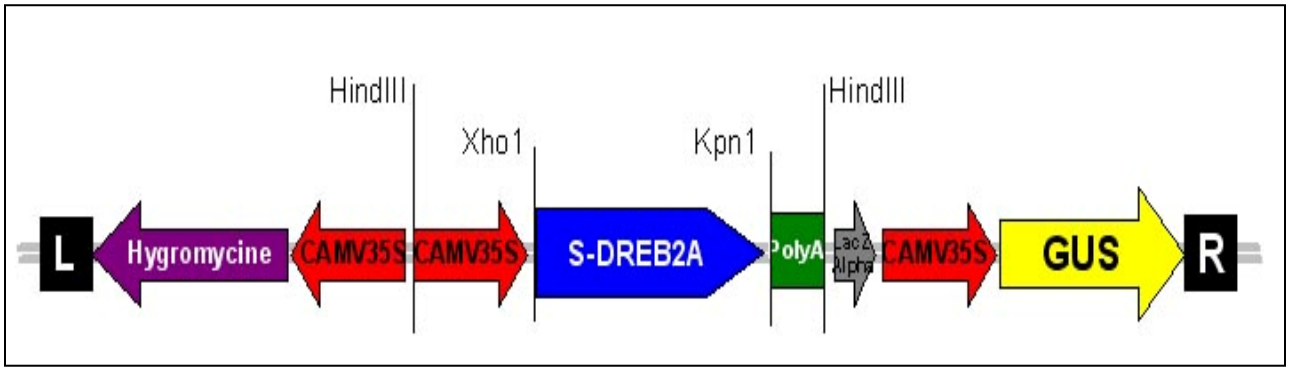

Plate 4. *Agrobacterium* LBA 4404 strain containing *Oriza sativa* Indiaca-Pokalli variety sense-DREB2A Gene: 35S in pCAMBIA 1301 vector.

The bacterial pellet was suspended in calculated amount of liquid YEMB (10^9 cells/ml density) as given below:

a X OD obtained X 10^9 cells/ml X 3 = 1 X 10^9 cells/ml X y ml

[a = amount of bacterial solution to be taken from the overnight grown bacterial culture in order to obtain an optimal density of 10^9 cells/ml; 3 = correction factor; y = the total volume of culture required for genetic transformation]

The bacterial pellet was suspended in liquid YMB medium (10^9 cells/ml density) and precultured leaf explant was submerged in it for 20 min, blot dried on sterile filter paper and finally transferred to regeneration medium supplemented with 100 µM acetosyringone for co-cultivation for different durations. Leaf explant submerged in bacteria-free YMB prior to co-cultivation served as control. After co-cultivation, the infected leaf explant was first washed with liquid regeneration medium containing 500 mg/L cefataxime to remove *Agrobacterium*, blot-dried on sterile filter paper and transferred to regeneration medium containing 0.5 mg/L TDZ and 500 mg/L cefotaxime. Following 4-5 week culture period, explant was sub-cultured on fresh medium as above with cefotaxime as well as the phytotoxic antibiotic hygromycin (5 mg/L). Hygromycin resistant shoots were subcultured at 15 day intervals.

3.3.5. Evaluation of factors influencing transformation

A range of parameters was evaluated, and each experiment included three replicates of ten leaf explant each. These parameters included the length of the pre-culture period (0, 1, 2, 3, 4, 5, 6, 7 days) of leaf explant in regeneration medium prior to infection, bacterial growth phase (OD values of 0.4, 0.6, 0.8, 1.0 at 600 nm), bacterial cell density (10^7, 10^8, 10^9, 10^{10} cells/ml), method of wounding of leaf explant (glass beads, pricking with hypodermic needle), infection time (5, 10, 15, 20, 30 min), length of co-cultivation period (0, 1, 2, 3, 4, 5, 6, 7 days), pH of the co-cultivation medium (5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0) and acetosyringone concentration (50, 100, 200,400 µM). The phenolic compound acetosyringone (Sigma- Aldrich) was dissolved in ethanol, and the stock volume made up in autoclaved distilled water. The appropriate aliquot of the filter- sterilized stock solution was added to autoclaved regeneration medium

to make the required final concentration. All of the parameters were evaluated and optimized on the basis of regeneration of resistant shoots on regeneration medium containing 5 mg/L hygromycin and PCR.

3.3.6. Analysis of putative transformants

3.3.6.1. Histochemical GUS staining

The histochemical assay of GUS activity was carried out as described by Jefferson et al. (1987). Young leaf tissue from putative transformed (hygromycin resistant) shoots were incubated in GUS assay buffer (Appendix-3) in the wells of ELISA plates (Hi-media) and subjected to vacuum infiltration (1–2 min) in a vacuum desiccator until bubbles appeared. The ELISA plates were then sealed with parafilm and incubated overnight at 37°C. Leaf material was treated with 70% ethanol for 1 h. The process was repeated until all the traces of chlorophyll were removed. The possibility of endogenous GUS expression was tested by subjecting uninfected leaf explant to the histochemical GUS assay. GUS expression was visually observed and photographed under a light stereo-zoom microscope (Nikon HF II).

3.3.6.2. Molecular characterization of putative transformants

3.3.6.2.1. Genomic DNA isolation

Six weeks after transferring the transformed shoots to the selection medium, total genomic DNA was extracted from randomly selected transformed leaf as described by Pamidimarri et al. (2008).

Steps for genomic DNA extraction

1. Fresh young plant leaf tissue was plucked from genetically transformed plants.
2. 0.1 gm of this tissue was ground in a precooled mortar with pestle to a fine powder using liquid nitrogen along with 10 mg (2% of extraction buffer) of PVP (Sigma). The powdered tissue was scraped into a 2.0 ml microcentrifuge tube containing preheated (65°C) extraction buffer (Appendix-4) in a 1:5 ratio (0.5 ml). β-mercaptoethanol was then added to the final concentration of 0.2M and mixed well. The mixture was incubated in water bath at 65°C for 90 min and cooled for 5 min.

3. An equal volume of chloroform: isoamyl alcohol mixture (24:1) was added to the extract and mixed by gentle inversion for 5 to 10 min to form a uniform emulsion. The mixture was centrifuged at 8000 rpm for 8 min at RT. chloroform: isoamyl alcohol extraction step was repeated again. The aqueous phase was pipetted out gently, avoiding the interface.
4. To the above solution 5M NaCl (to final concentration 2M) and 0.6 volume of isopropanol to the total solution was added to it and incubated at RT for 1 h.
5. To the above solution two volumes of 80% ethanol was added and incubated again for 10 min at RT for DNA precipitation. After incubation the mixture was centrifuged at 10,000 rpm for 15 min at RT. The white/translucent pellet was washed with 70% ethanol, dried and resuspended in 200µl of TE buffer (Appendix-5).
6. The sample was then treated with RNase (20µl of 10mg/ml of RNase) and incubated at 37^0 C for 60 min.
7. After incubation with RNase, one volume of tris saturated phenol (pH 8.0) was added and mixed gently by inverting the microcentrifuge tube till it formed a milky white emulsion. The emulsion was then centrifuged at 10,000 rpm for 5 min at RT. The supernatant was pipetted out into a fresh tube.
8. The sample was then extracted with equal volume of chloroform: isoamyl alcohol (24:1) twice. The DNA was reprecipitated with 0.6 volumes of isopropanol, 2.0 M NaCl (final concentration) and incubated for 10 min.
9. To the above 20 µl of sodium acetate(3 M, pH 5.2) and 1 volume of 80% ethanol were added, incubated at RT for 30 min, and centrifuged at 10,000 rpm for 15 min to pellet the DNA. The pellet was then washed with 70% ethanol twice; air dried and finally suspended in 40-50 µl of TE buffer.

3.3.6.2.2. Plasmid DNA isolation

Plasmid DNA isolated from *Agrobacterium* following the method developed by Sambrook et al. (1989).

Steps for Plasmid DNA isolation

1. Fill a microcentrifuge tube with saturated bacterial culture grown in YMB medium and 50 µg/ml hygromycin. Spin tube in microcentrifuge for 1 min, and make sure tubes are balanced in microcentrifuge. Discard supernatant and drain tube briefly on paper towel.
2. Repeat step1 in the same tube, filling the tube again with more bacterial culture. The purpose of this step is to increase the starting volume of cells so that more plasmid DNA can be isolated per prep. Spin tube in microcentrifuge for 1 min. Pour off supernatant and drain tube on paper towel.
3. Add 0.2 ml ice-cold resuspension buffer (Appendix-6) to cell pellet and resuspend cells as much as possible using disposable transfer pipet.
4. Add 0.4 ml lysis buffer (Appendix-7), cap tubes and invert five times gently. Let tubes sit at room temperature for 5 min.
5. Add 0.3 ml ice-cold neutralization buffer (Appendix-8), cap tubes and invert five times gently. Incubate tubes on ice for 10 min.
6. Centrifuge tubes for 5 min. Transfer supernatant to fresh microcentrifuge tube using clean disposable transfer pipet. Avoid taking any white precipitate during the transfer..
7. Add equal amount of isopropanol to the supernatant and allow tube at room temperature for 20 min.
8. Centrifuge tubes for 5 min. and pour off supernatant without dumping out the pellet. A milky pellet should be at the bottom of the tube Drain tube on paper towel.
9. Add 1 ml of ice-cold 70% ethanol. Cap tube and mix by inverting several times. Spin tubes for 1 min. Pour off supernatant (be careful not to dump out pellet) and drain tube on paper towel.
10. Allow tube to dry for ~5 min. Add 50 µl milli Q water to tube and allow the pellet to be dissolved.

3.3.6.2.3. Quantification of genomic and plasmid DNA:

Extracted genomic and plasmid DNA was quantified separetly according to the method suggested by Sambrook et al. (1989). Purified DNA sample, 15µl,

was dissolved in 735µl of TE buffer and Optical Density (OD) was recorded at 260 and 280 nm. Quantity of DNA was calculated by using the following formula:

Amount of DNA in ng = OD at 260 nm x dilution factor x 50 (extension coefficient)

Ratio of amount of DNA at OD 260 and 280 nm was calculated. It varied from 1.25 to 1.80. Samples having the ratio of OD from 1.6 to 1.8 were selected for further study.

3.3.6.2.4. PCR characterization

Extracted genomic DNA from putative transformed leaf tissues were analyzed for the presence of the DREB2A and GUS transgene by PCR amplification. The forward and reverse primers used for amplification of the DREB2A sequences were 5' GATCCC-TCGAGATGGAGCGGGGGGAGGG 3' and 5' TCCGAGGTACCCTAATAGGAGAAAAGGCTA 3' respectively and forward and reverse primers used for amplification of the GUS gene were 5' GGTGGGAAAGCGCGTTACAAG 3' and 5' TGGATCCCGGCATAGTTAAA 3' respectively and they were designed as to amplify a 866-bp fragment of the T-DNA of DREB2A and 400 bp fragment of the T-DNA of GUS gene. A total of 25-50 ng of genomic DNA was used as template in a 15-µl PCR reaction mixture containing final concentration of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each dNTP, 3.0 mM MgCl2, 0.4 µM primer and 1unit Taq DNA polymerase. As positive control, 50 pg of DREB2A and GUS plasmid DNA used. Amplification was done using thermal cycler (master cycle epgradient S, Eppendorf, Germany) with following program: initial denaturation at 94^{o}C for 4 min, 35 cycles were run with denaturation at 94^{o}C for 30 seconds, primer annealing at 55^{o}C for 1min, extension at 72^{o}C for 1 min, and final extension at 72^{o}C for 4 min. PCR products were run on 1.5% agarose gel in 1 X TBE buffer (Appendix-9). The gel was stained with ethidium bromide and photographed by using gel documentation system (Syngene, USA)

3.3.6.2.5. Dot blot analysis

DNA of transformed and un-transformed (control) leaf explants was isolated and subjected to dot blot analysis:

3.3.6.2.5.1. Blot preparation

1. Hybond positively charged nylon membrane (Amersham, UK) of appropriate size was cut and spots were marked at equal distance with the help of a pencil.
2. DNA (15, 30 and 45 ng) was taken and dilutions were made to make a total volume of 6 µl each for all the cultivars in the present study. DNA was denatured by heating in boiled water for 10 min, snap cooled on ice and centrifuged briefly. First 3 µl of each dilution from each DNA samples were taken and dotted on the marked membrane. After the drying of the first dot, another 3 µl placed on the same spot.
3. The membrane was then UV cross-linked for 2 min in a UV cross-linker.

3.3.6.2.5.2. Probe preparation and labeling

Isolated plasmid DNA containing DREB2A fragment was amplified using DREB2A primer and amplified fragment was isolated and purified by Qiaex II agarose gel extraction method (QUIAGEN Gel Extraction Kit, Germany). The fragment was labeled non-radioactive by Alkphos direct labeling and detection system (Amersham Gene Images Alkphos Direct Labeling and detection Kit, UK).

3.3.6.2.5.3. Hybridization and detection of blot

Prepared blot was hybridized with labeled probe and detected by Alkphos direct labeling and detection system (Amersham Gene Images Alkphos Direct Labeling and detection Kit, UK).

3.3.6.2.6. Southern hybridization

DNA of transformed and un-transformed (control) leaves were isolated and subjected to Southern hybridization:

3.3.6.2.6.1. Blot preparation

DNA fractions (15 µg) from negative control (Non transformed leaf explant), putatively transformed plant, and positive control (Plasmid DNA) were digested with *Hind*III (10 units/mg of DNA) for overnight at 37 °C. Digested DNA samples were electrophoresed on a 1% agarose gel for a period of 6 hrs at 50 milli amps in 1 x TAE buffer. The gel was stained with ethidium bromide (0.5 mg/ml) for 30 min and destained in double distilled water for 15 min. The gel was blotted onto hybond positively charged nylon membrane (Amersham, UK) and

DNA was capillary transferred to membrane by standard alkali transfer method where 0.4 M NaOH was used as transfer reagent. DNA was fixed by baking the membrane for 2 h at 80 °C.

3.3.6.2.6.2. Hybridization and detection of blot

Prepared blot was hybridized with labeled probe and detected by Alkphos direct labeling and detection system (Amersham Gene Images Alkphos Direct Labeling and detection Kit, UK).

3.4. Multiplication and establishment of transformed plants

Regenerated transformed shoot buds on regenerating medium supplemented with 5 mg/L hygromycin were transferred on MS medium supplemented with 3% sucrose, 2 mg/L Kn, 1 mg/L BAP, 1 mg/L NAA and 5 mg/L hygromycin for 4 weeks for shoot proliferation (Reddy et al., 2008). Shoots were individually separated and further tested for their elongation on MS medium supplemented with 3% sucrose and 1.5 mg/L IAA and 0.5 mg/L BAP. Green and healthy elongated shoots with three to four leaves were excised and cultured half strength MS medium supplemented with 2% sucrose and 3 mg/L IBA, 1 mg/L IAA, 1 mg/L NAA and 0.25 mg/L activated charcoal for rooting. Rooted shoots were carefully taken out of the medium and washed thoroughly in autoclaved distilled water to remove basal MS medium attached to the roots. The plants were transferred to polythene bags containing sterilized sand and soil in the ratio 1:1 and wetted with 0.02% w/v carbendazim and covered with transparent plastic bags to maintain humidity. After 3-4 weeks, the established plants were transplanted to polybags containing garden soil and farmyard manure and transferred to a green house for further growth.