Chapter – V

Genetic Transformation of *Tricosanthes cucumerina* using GUS Gene

5.1. INTRODUCTION

Conventional plant breeding has succeeded in producing a wide variety of commercial plants and crops with a range of important agronomic traits. Genetic engineering of plants is an advanced tool for the production of abiotic and biotic stress resistance. Recent advances in gene transfer technology leads to the transfer of desirable gene(s) into the plant genome. Multiple methods for DNA transfer into plants have been developed over the last 20 years. Using such techniques, we can overcome the difficulties associated with the breeding of long lived perennials, which need a long time to produce progeny (DeCleene and Deley, 1976). Keeping in view the need for genetic transformation of *Tricosanthes cucumerina* for genetic improvement of the crop for agronomical characteristics, studies have been undertaken in India and USA for development of transgenic *T. cucumerina* L. . The most widely used methods of transformation are the direct gene method using particle gun and the vector-mediated method using *Agrobacterium tumefaciens*. Since both the methods have their own advantages and limitations (Potrykus, 1991; Sharma *et al.*, 2005), attempts were made to optimize conditions for transformation of *T. cucumerina* L. using the two methods. McKeon and Chen (2003) obtained genetically engineered plants by employing the method of *Agrobacterium*-mediated transformation through vacuum infiltration of wounded flower buds (US Patent No 6,620,986).

Genetic manipulation of plants has been done by plant breeders for years with great success. Gene transfer technologies for plants can be broadly divided into vector mediated methods and direct gene transfer methods (Hooykaus and Schilperoort, 1992; Christou, 1996). Among these methodologies, *Agrobacterium* – mediated transformation was the first system to generate a transgenic plant. Its advantages include ease, low cost, and simplicity of transgene integration patterns. Its major disadvantage is its potential host range limitations. The later problem can often be

overcome by improving tissue culture techniques, and choosing alternate selection methods as well as alternate *Agrobacterium* helper stains (Songstad *et al.*, 1995).

The plant pathogenic bacterial vector *Agrobacterium tumefaciens* (α-proteobacterium of the family Rhizobiaceae) is used for plant transformation. Over 600 plant species are reported as hosts of this disease (DeCleene and Deley, 1976; Zonia and Raio, 1999; Ramon *et al.*, 2000). *Agrobacterium* mediated transformation occurs when wounded plant tissue is exposed to *Agrobacterium* cells containing a plasmid with the gene of interest and a selectable marker gene located within the transferred DNA (T-DNA) region. The T-DNA is transferred to only a small proportion of cells. *Agrobacterium* enter into the plant cells and the T-DNA ultimately integrates with nuclear genome and expresses resulting in the formation of desired trait as disease resistance, self resistance, herbicide resistance (Zhenying and Binns, 2003). Several sophisticated plant transformation vectors based on this naturally occurring gene transfer system have been developed and are employed in genetic engineering (Fraley *et al.*, 1986). The widely used binary system consists of a helper Ti plasmid providing the virulence functions needed for transfer and a small vector carrying an artificial T-DNA (Bevan, 1984; An *et al.*, 1988). The binary vectors replicate in *A. tumefaciens* and allow easy cloning of the genes of interest between the T-DNA borders. In *in vitro* transformation system, the transformed cells are given a selective advantage over the relatively large number of non- transformed cells by exposure to a selection agent.

Plant transformation vectors and methodologies have been improved to increase the efficiency of plant transformation and achieve stable expression of transgenes in plants. *Agrobacterium* mediated transformation in normal conditions is determined by many factors including bacterial strain, temperature, cell wounding, phenols, virulence genes and host cell division (Wu *et al.*, 2003; Yukoh and Toshihiko, 2006). The process of foreign gene transfer from *Agrobacterium* into plant cells during the infection includes several sequential steps: (i) recognition of plant signal molecules by the bacterial *vir*A / *vir*G sensor- transducer system; (ii) binding of *Agrobacterium* to the host cell surface; (iii) activation of the bacterial *vir* genes; (iv) production of the transferable T-strand; (v) formation of the T - complex and its transport into the host

plant cell complex; (vi) nuclear import of the T - complex; and (vii) integration of T-DNA into the plant genome. *Agrobacterium* has significant advantages over direct gene delivery; since it reduces unwanted gene silencing, it has technical simplicity of minimal genome rearrangements in transformants and low copy number (Kohli *et al.*, 1999; Sudhakar *et al.*, 2006) introduced into the plant genome, high coexpression of introduced genes, defines transgene integration, relatively large segments of DNA transfer with little rearrangements within inserts and less fragmentation of the transgene (Hadi *et al.*, 1996; Murray *et al.*, 2004). In addition, *Agrobacterium* is a much more efficient transformation tool in compatible plant species compared to the particle gun protocol (Bidney *et al.*, 1992).

The analysis of transformed plants can be confirmed by several methods using the applications of reporter genes like β-glucuronidase (*gus*), green fluorescent protein (*gfp*), chloramphenicol acetyl transferase (*cat*) and *Discosoma* sp. Red fluorescent protein (*DsRed*) (Gregoria *et al.*, 2006; Geliang and Yinong, 2008; Chaofu and Jinling, 2008). The most widely used visual reporter genes are *gus* and *gfp* (Jefferson *et al.*, 1987; Davis and Vierstra, 1998; Taylor and Fuquet, 2002). The transformed transgenic plants are evaluated histochemically and anatomically. The detection of most reporter proteins such as *gus*, *gfp* and *cat* relies on the enzymatic production of coloured, fluorescent product which then allows an easy quantification or localization. These reporter genes have been used in investigation transient, chimeric and stable gene expression in other dicot plants by Schrammeijer *et al.*, *(*1990); Alibert *et al.*, *(*1999); Muller *et al.*, *(*2001); Koetle *et al.*, (2017); sujatha and Reddy (2005); Gao *et al.*, (2017) and in snake gourd by Subramanyam *et al.*, (2015). The advantage of enzymatic reporters resides in their sensitivity, as a single protein can produce abundant product molecules. Glucuronidase are of special interest since their assays do not involve any radioactivity. However, the levels of transgenic expression are generally unpredictable and vary among the independent transformants (Finnegar and McElroy, 1994).

T. cucumerina L. is not amenable crop to genetic transformation. For developing *T. cucumerina* L transformation protocol, a genotype independent and highly efficient transformation protocol is essential. Owing to the recalcitrance of *T. cucumerina* L tissues to *in vitro* manipulations, transformation protocols in *T.*

cucumerina L were mostly based on proliferation of meristematic tissues (Sujatha and Sailaja, 2005; Malathi *et al.*, 2006). The first success at stable transformation inherited into the T1 progeny plant of snake gourd through *Agrobacterium* mediated transformation has been reported (Subramanyam *et al.*, 2015).

5.2. MATERIALS AND METHODS

5.2.1. Plant Material

The preferred explant or plant material for transformation studies for *T. cucumerina* L was choosen as leaf explant. The leaf explants were dissected from the 10 days old seedling of *T.* cucumerina L. and the sterilization procedure was done as mentioned in the Chapter 2 (2.2.2).

5.2.2. Plant regeneration protocol

Recovery of transgenic plants through any method depends on the following essential factors: 1) an efficient culture system that allows recovery of plants from target tissues, 2) appropriate selectable marker genes and selection conditions and 3) a DNA delivery system (Songstad *et al.*, 1990). Based on the merits observed in previous reports on snake gourd seed culture (Subramanyam *et al.*, 2015), in this present investigation, *T. cucumerina* leaf explant culture methodology (Chapter 2.2) was selected for transformation experiments.

5.2.3. Pre-culture of explants

Pre-culture is an important step involved in *Agrobacterium*-mediated transformation studies. The process of pre-incubation makes the explant tissue competent enough to withstand the bacterial infection and other related stress caused during the pre-culture period *in vitro*. The leaf explants were pre-cultured on the multiple shoot induction medium (Chapter 2.0, Table 2.5), for 0 - 14 days prior to selection on the Kanamycin (Kan) containing medium.

5.2.4. Determination of antibiotic sensitivity

The sensitivity of the leaf explants to Kanamycin was determined by culturing the explants in multiple shoot induction medium (Chapter 2.0) along with Kan (25, 50, 75, 100, 125, 150 mg/l) and Hygromycin (2, 4, 6, 8, 10, 12 mg/l). The antibiotics were prepared in 1g /10 ml of autoclaved double distilled water and were filter-

sterilized and added to the autoclaved medium. The minimum inhibitory concentration (MIC) of the antibiotic was assessed by the complete death of the explants on the particular concentration of the medium with antibiotic.

5.2.5. *Agrobacterium* strain and plasmid

The *Agrobacterium* strain LBA4404 harbouring the binary plasmid pBAL2 (18.8 kb) was used as the vector system for transformation. The *uidA* /*gus* (ß - glucuronidase) gene driven by the CaMV 35S promoter and terminator sequences served as reporter gene. The neomycin phophotranferase II (*npt* II) gene driven by the nopaline synthase (Nos) promoter and terminator sequences were used as the selectable marker gene. LBA4404 strains obtained from Veluthambi, Madurai Kamaraj University, Madurai, Tamil Nadu, India (Plate. 7).

5.2.6. Co-cultivation and selection of stable transformants

Agrobacterium tumefaciens strain LBA4404 harbouring pBAL 2 was grown on Luria Bertaini (LB) Medium (contains 10 g/l Bacto Tryptone, Bacto, 5g/l Yeast extract and 10g/L NaCl) medium containing 50 mg/l of kanamycin. A single bacterial colony was inoculated into 50 ml liquid LB containing the same antibiotic and grown overnight at 28 ^{o}C on a shaker at 180 rpm. Five milliliters of this overnight culture was reinoculated into 50 ml fresh LB medium containing 50 mg/l kanamycin and grown overnight. The bacterial cell density was adjusted to OD_{600} of 0.8 – 1.2 (5 x 10^{8} cells/ml). Bacteria was pelleted at 5,000 rpm for 10 min and resuspended in 25 ml hormone-free liquid mMS medium with 3% sucrose. Leaf isolated from 10 days old seedling was precultured on medium supplemented with 0.3 mg/l TDZ for 3 - 5 days prior to infection with *Agrobacterium*. The explants were injured by two strokes using a hypodermic needle (Dispovan India Ltd. 0.63 x 25 mm) in the meristematic region, which is distinguishable by its characteristic swelling. The processed explants were immersed in bacterial suspension and subjected to manual shaking for 10, 15 and 30 min. Subsequently, the explants were blotted dry on sterile filter paper and co-cultivated for 2 days in culture bottles containing full-strength mMS basal salts containing the multiple shoot induction medium along with the different concentration of Kanamycin and Hygromycin and incubated in the light at 26 ± 2 ^{o}C. To enhance the penetration of the *Agrobacterium* vector into the target tissues, explants were subjected

to shaking (180 rpm) for 10 – 30 min during the bacterial incubation; macerated with glass beads and treated with acetosyringone (0 – 200 mg/l). To assess the effect of the co-cultivation period on the frequency of transformation, the infected explants were cultured for 2 days on the co-cultivation medium. Following co-cultivation, the explants were washed with 250 mg/l cefotaxime for 2 min, rinsed with sterile distilled water 3 times for 5 min each with constant stirring, and blotted dry on sterile filter paper. The procedure optimized for explant preparation, co-cultivation, three cycles of selection (10 days each) and recovery of whole plantlets is presented in Plate-8. The rooted shoots were acclimatized in the plastic cup containing sand, soil and vermiculite in the ratio 1:1:1 and maintained in the environmental growth chamber for 15 days. Established plantlets were transferred to green house condition and allowed to grow in earthen pots for maturity.

5.2.7. Analysis of putative transformants

5.2.7.1. Histochemical assay of Uid A gene expression

The *gus* histochemical assay was carried out according to Jefferson (1987) with few modifications. Putatively transformed leaves, shoots and shoot cultures recovered on Kanamycin - selection media were used in the assays. The materials were incubated overnight at 37 ^{o}C in the substrate solution containing 1 mM X – Gluc (5 – bromo - 4 chloro - 3 indolyl ß - D, glucuronide) in 0.05 M phosphate buffer (pH 7.0) and 30% Triton X-100. Following X - Gluc staining, the shoots were bleached in 95% (v/v) ethanol and viewed under a stereo binocular microscope. The *gusA* expression was identified by the blue coloration of the transformed shoots (Anoust *et al.*, 1999).

5.2.7.2. Molecular analysis

DNA extraction and PCR analysis

a) **Isolation of DNA from leaf tissues**

(*Cetyl trimethyl ammonium bromide (CTAB) method*)

Total genomic DNA was isolated from young leaves / shoots of putative transformants (Positive control) and untransformed (negative control) plants using the CTAB method (Doyle and Doyle, 1990). The leaf tissues were used for the DNA isolation and confirmation of transgene expression. 1.0 g of leaves was taken and ground well by using liquid nitrogen. The powder form of leaves was homogenized in

1.5 ml of extraction buffer (50 mM sorbitol, 100 mM Tris, 5 mM EDTA, 32 mM sodium bisulfate, pH 8.0). The homogenate was centrifuged at 15,000 rpm at -4ºC for 20 minutes. The pellet was collected and re-dissolved in 4 ml of extraction buffer. The nuclei were lysed by addition of 4 ml of nucleus lysis buffer (200 mM Tris aminomethane, 50 mM EDTA, 2M NaCl, 55mM CTAB, pH 7.5), 1.6 ml of 5 % N-laurylsarcosinesodium salt and incubated at 65ºC for 20 min. The mixture was then extracted with chloroform / isoamyl alcohol (24:1) and an equal volume of cold isopropanol was added (the nucleic acids are precipitated). The precipitate was re-dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0. The precipitate form of nucleic acid contains both DNA and RNA. The RNA was removed by RNase solution (0.2 mg/ml) at room temperature for 15 min. DNA was further purified with Phenol / chloroform extraction and ethanol precipitatin methods.

b) **Isolation of plamid DNA from E.coli**

The plasmid DNA was isolated from *E. coli* by the method of Sambrook and Russel (2001) without any modifications.

c) **Estimation of DNA concentrations**

The extracted DNA was estimated by Fluorometry (Hoechst Dye- 33258) method (Cesarone *et al.*, 1979). Calf thymus DNA was used as a standard.

d) **PCR reaction**

PCR analysis was done as per the procedure by Edwards *et al.* (1991). PCR analysis was carried out using genomic DNA were isolated from the young leaves of putative transgenic plants, untransformed plants, positive and negative controls. The pair of *uidA (gus)* specific primers (F) 5′- TTT AAC TAT GCC GGG ATC CAT CGC - 3′, (R) 5′- CCA GTC GAG CAT CTC TTC AGC GT - 3′ and pair of *nptII* Specific primers (F) 5’ – AAT CTC GTG ATG GCA GGT TGA – 3’ (R) 5’ – GAG GCT ATT CGG GAT ATG ACT – 3’ were used to check the presence of *gus* and *nptII* gene in putative transgenic plants, its given a 1.9kb and 680 bp amplified fragment in respectively. The master mix for PCR reaction were carried out of 25µl total volume, containing 12.5µl of PCR Master mix (containing buffer, dNTP and Enzyme mixture) (Genei, Bangalore) 1.0µl of each primer and 1.0µl of Template/

DNA (100ng) and final volume make up with sterile distilled water. The final concentration of PCR components was prepared as 1X Taq buffer with 1.5mM $MgCl_2$, 200µM of each nucleotide, 0.4 µM of each primer, 1.0 units of Taq DNA polymerase and 100ng of template in 25.0 µl of total reaction volume. The PCR sample was kept PTC 100, Programmed Thermal Cycler, (Eppendorf, Germany) and set the cycling temperature as initial denaturation at 94ºC for 5.0 min followed by 30 cycles of denaturation at 94ºC for 1.0 min; annealing at 60ºC for 1.0 min; and extension at 72ºC for 1.0 min. The final extension for 10 min at 72ºC and the reaction was hold at 4 ºC for short term storage of the reaction. The PCR products ware analysed by Agarose gel electrophoresis (0.8%) and gels were stained with Ethidium bromide then photographed under ultra violet light using UVP Gel documentation system (BioRad).

e) **Agarose gel electrophoresis**

PCR products were separated by loading 20µl of each sample and 3µl of loading buffer on a 0.8% agarose gel prepared with 1.0X TBE buffer. The gel was prepared by boiling the required amount of agarose in appropriate volumes of distilled water. Molten agarose was cooled down to about 60ºC and 10 X TBE or 50 X TAE buffer was added to a final concentration of 1X and mixed. Gel was casted in a platform fixed with comb to form wells. The gel was allowed to cool to room temperature for solidification (normally left for 30 min after casting). DNA samples were prepared by mixing 1/10 volume of sample of loading dye (10 X) and loaded into the wells. Electrophoresis was performed at 4-8 v/cm in 1 X TBE or TAE buffer and upon completion of the run; DNA in the gel was stained with ethidium bromide (0.5 µg/ml) and viewed under UV (PD Quest - BioRad).

Elution of DNA from Agarose Gels

a) ***Electroelution of DNA*** **(Maniatis *et al.*, 1982)**

The DNA sample to be eluted was fractionated in agarose gel. At the end of electrophoresis the gel was stained briefly in ethidium bromide solution and electrophoresis solution and viewed under a UV transilluminator. The band corresponding to the fragment to be eluted was sliced out of the gel. The agarose piece was placed inside an activated dialysis tubing containing 200 to 300µl of 0.5X TBE and the ends of the tubing were clamped with dialysis clips. The DNA in the agarose gel

piece was allowed to run out of the piece by eletrophoresis in 0.5X TBE for 1h. The polarity of electrophoresis was reversed for 30 sec. and the contents of the dialysis tubing were transferred to a microfuge tube. The contents were extracted once with neutral phenol/chloroform twice with water saturated ether and DNA was precipitated by adding 1/10 volume of 3M sodium acetate followed by 2.5 volumes of 95% ethanol and left at -20°C overnight. The DNA was pelleted by centrifuging at 12,000 rpm for 10min. at 4°C. The pellet was washed with 70% ethanol, dried and dissolved in an appropriate volume of 0.1 x TE (1mM Tris HCl, pH 8.0, 0.1mM EDTA).

b) DNA Purification using glass matrix

Glass matrix purification of DNA was carried out using prepA-gene DNA purification kit (Bio Rad Laboratories, USA) as per the protocol provided by the manufacturer.

c) Ligation of DNA fragments

Restriction endonuclease digested DNA samples, was extracted once with neutral phenol/chloroform and twice with water saturated ether and precipitated with ethanol, and later was used for ligation. A concentrated ratio of 1:3 of vector designed to be inserted was maintained for cloning experiments. Approximately 50ng of the vector DNA was used for ligation. Ligation reactions were carried out for 10 to 12 hour at 14°C in the presence of 1mM ATP and the buffer provided by the manufacturer.

5.2.7.3 Southern hybridization analysis

The method of Southern (1975) was used for DNA blotting and hybridization analysis. The gel containing DNA to be transferred to a nylon membrane was immersed in 250 ml of denaturation solution for 45 min. on a rocker platform. The gel was thoroughly washed in sterile distilled water for four times. Then soaked in 250 ml of neutralization solution and was kept rocker for 45 min. The gel was placed upside down on three numbers of whatman no.3 sheets cut to the size of the gel, soaked in 20X SSC and stacked on a glass plate. A nylon membrane (Zeta-Probe, Bio-Rad, USA) cut to the size of the gel, wetted in distilled water and soaked in 20X SSC was placed over the membrane followed by a dry sheet. A glass plate was placed on top and a weight of about 250 g was kept over the glass plate. The membrane was

removed after 10 to 12 hour rinsed briefly in 2X SSC air dried and baked in a vacuum oven at 80ºC for 30 min. The semi dry blotting method described above was followed when plasmid DNA was transferred. A wet blot method was followed to transfer genomic DNA from plants and total DNA from bacteria. The procedure is essentially the same as semi-dry blotting but for the following differences. 1) The bottom most whatman No.3 sheet was longer and the protruding ends of the sheet were dipped in a reservoir containing 20X SSC. 2) The transfer was done for a period of 16 hrs.

Denaturation solution

1 M NaCl, 0.5 M NaOH.

Neutralization Solution

1.5 M NaCl, 0.5 M Tris HCl, pH 7.0

20X SSC. NaCl (175.3 g) and trisodium citrate (88.2 g) were dissolved in 750ml distilled water. The pH of the solution was adjusted to 7.0 with HCl and the volume was made up to 1000ml with distilled water and sterilized by autoclaving.

i. **Radio labeling on DNA labeling**

The DNA fragments used as a probe in southern hybridization analysis was radio - labelled using a random primer, labeling system supplied by Amersham International Plc. UK. Reactions were carried out as described by the manufacturer in a final volume of 50 µl with 20 to 30 ng of DNA was labeled and 30 µCi of (α-^{32}P) dCTP. The reaction was stopped by adding an equal volume of dye mix (6 mg blue dextran and 1 mg orange G in one ml of 0.5 M EDTA, pH 7.0) and separated in a Sephadex G - 50 column using the column buffer (0.1 M NaCl, 12 mM Tris HCl, pH 7.0, 2.5 mM EDTA). The blue fraction which contains the labelled probe was collected separately and used for hybridization.

ii. **Radiolabeling of probe DNA**

The random primer oligolabelling kit from Amersham International Plc, UK, performed radiolabelling of DNA fragment. The electroeluted DNA (25 ng) and random primers from the kit were mixed in sterile distilled water in an eppendorf tube to a final volume of 35 µl. The tube was kept over a boiling water bath for 5 min to

denature the DNA and then allowed to cool down to room temperature gradually. To the denatured annealed DNA, 10 µl of labelling mix, 30 µCi of (□-p^{32}) dCTP (specific activity -1.48 x 10^{14} Bq/mmole) and 2 µl of klenow fragment were added. The tube was mixed gently, microfuged for 5 sec and incubated at 37ºC for 20 min. The reaction was stopped by adding 50 µl of dye mix (2 mg of blue dextran and 1 mg of Orange-G in 0.5 M EDTA, pH 7.0). The labelled probe DNA was purified using a Sephadex G-50 column. The column buffer contains 0.1 M NaCl, 12 mM Tris HCl, pH 7.0 and 2.5 mM EDTA. The labelled DNA was denatured in a boiling water bath for 5 min. and placed immediately on ice to prevent annealing.

iii. Hybridization

Hybridization was performed under the conditions recommended by the supplier of the nylon membrane (Bio-Rad, USA). Pre-hybridization, hybridization and post hybridization washes were done in a hybridization oven (Bachofer, Germany). The membrane was placed inside the hybridization bottle and 10 ml of pre-hybridization solution (contains 7 % SDS for blocking) was added. Care was taken to remove air bubbles if any. Pre-hybridization was done for a period of 30 min at 65ºC. At the end of pre- hybridization, the solution was discarded and 10 ml of fresh pre-hybridization solution was added. Probe was denatured in boiling water both for 5 min and chilled on ice immediately. The denatured probe DNA was added to the bottle and hybridization was performed at 65ºC for 12 to 24 h.

iv. Post hybridization washes

Post-hybridization washes were carried out as recommended by the manufacturer of the nylon membrane (Bio-Rad, USA) and washes were done either at low stringency conditions (for heterologous probes) or at high stringency conditions (for homologous probes). After hybridization and washing, the blots were exposed to Kodak Biomax MS film at -80 ºC.

v. Washes at low stringency conditions

At the completion of hybridization the membrane was rinsed briefly with 2X SSC/0.1% SDS solution at 65°C. Following this, three washes were performed with 2X

SSC/0.1% SDS at 65°C (each wash for 30 min). The membrane was air dried and exposed with intensifying screen to Konica X-ray film at -70°C.

vi. **Washes at high stringency condition**

The membrane was rinsed briefly with 2X SSC / 0.1% SDS at 65°C following this, the membrane was washed sequentially once with 2X SSC/0.1% SDS, 0.5X SSC/0.1% SDS and 0.1% SDS. All washes were performed at 65°C for 30 min each. After the washes, the membrane was air dried and exposed with an intensifying screen to Konica X-ray film at -70°C.

5.3. RESULTS AND DISCUSSION

Preliminary transformation of *T. cucumerina* L was carried out using pBAL2 harbouring *gus* gene was studied. Different Physical and biochemical variables that can enhance the frequency of transient *gus* expression were carried out. The variables included bacterial cell density, bacterial incubation period, preculture of explants, acetosyringone – a phenolic compound which acts as an inducer of *vir* genes, glass beads, prickling with hypodermic needle. As the transformation is meristem based, it was difficult to get rid of the bacterium when used at higher density. Hence, all the experiments were carried out with Agrobacterium tumefaciens strain LBA4404 harbouring the pBAL2 plasmid with the bacterial density of 5 x 10^8 cells / ml with 15 minutes incubation and cocultivation on full – strength mMS medium.

5.3.1. Plant materials

Access to a prolific system of shoot proliferation from leaf explants is a prerequisite for meristem-based transformation. Of the various explants, leaf explant from 10 days old seedlings was found to be ideal owing to their extensive proliferative ability. Protocols for shoot proliferation from various meristematic explants of snake gourd are available (Pillai *et al.*, 2008; Rajender abd Reuben, 2017) and Trichosanthus dioica (Saurabh *et al.*, 2017). Among these, the protocol developed in our laboratory is superior interms of the enormous proliferative ability of the meristems. In this particular study, differential effects of various cytokinins on shoot proliferation from leaf explants were assessed and for the first time we demonstrated the usefulness of PF - 68 for *T. cucumerina* L multiplication and also revealed its carryover effect. Taking

into account the enormous expanding ability of the meristematic zone of the leaf explant on medium supplemented with PF - 68, the present investigation was undertaken to transform *T. cucumerina* L via A. tumefaciens - mediated gene transfer. PF - 68 is becoming an integral factor in the genetic improvement of woody species owing to the enhancement of receptivity of the meristematic regions with PF - 68 pre-treatment prior to transformation (Vanjildorj *et al.*, 2006).

5.3.2. Preculture of explants

Pre-culture is an important step involved in *Agrobacterium* mediated transformation studies. The transformation efficiency can be increased by manipulation either by the explant or the bacterium to enhance virulence. Such manipulations are based on increasing the number of component cells for transformation by pre-culturing explants (McHughen *et al.*, 1989; Monique *et al.*, 1996). In this present study, pre-culture trials of explants were conducted at regular intervals 2, 4, 6, 8, 10 and 12 days. Of these, the 4^{th} day old explants cultured on mMS medium with plant growth regulators produced the highest percentage (50.6%) of response than the others (Table 5.1; Plate 9). The present study on pre-culture also observed that the 2 days precultured explants showed the minimum survival rate in the medium, because the explants were unable to withstand the vigorous reactive power of *Agrobacterium.* Similarly the explants pre-cultured, for 6^{th}, 8^{th}, 10^{th}, 12^{th} and 14^{th} days were unable to receive the *Agrobacterium* due to the excessive growth of the explant. Concurrently the importance of pre-culture during transformation studies was reported by many workers (Veluthambi *et al.*, 1989, Vasudavan *et al.*, 2002). Pre-culture of the explants probably serves to reduce wound stress and increase the number of cells at the wound site (Muthukumar *et al.*, 1996).

5.3.3. Effect of antibiotics and selection regime

The sensitivity of well-developed shoots to kanamycin (0 – 150 mg/l) and Hygromycin (0 – 13 mg/l) was tested. On medium with kanamycin, shoots bleached with increasing concentrations of the antibiotic and 100% bleaching were observed within one week on 100 mg/l and increasing concentration of Kanamycin also showed a drastic decline in the survival frequency of the explants on the medium with 100 mg/l (Table 5.2) The results of the experiment using increasing concentrations of kanamycin

showed a drastic decline in the survival frequency of the explants on medium greater than 50 mg/l and higher concentrations of the antibiotic, and some of the shoot cultures failed to survive during the second cycle of selection. On medium with the kanamycin concentration of 50 mg/l, shoots continued to grow during the third cycle of selection. However, on medium with an initial antibiotic concentration of 50 mg/l, death was not as sudden as observed with higher concentrations. Thus, Kanamycin 50 mg/l was found optimum, as it was not detrimental to transformed shoots or as slow in its effect as that of the lower concentration, which could lead to the recovery of escapes. To strike a balance between the elimination of untransformed shoots and proliferation of putative transformants Kanamycin 50 mg/l was proved to be effective. The control plants failed to survive in the first cycle of selection on medium with Kanamycin 50 mg/l (Plate 9).

The results of the experiment using increasing concentrations of hygromycin showed a drastic decline in the survival frequency of the explants on medium with 3 mg/l and higher concentrations of the antibiotic, and the shoot cultures failed to survive during the second cycle of selection (Table 5.2). On medium with an initial hygromycin concentration of 1 mg/l, shoots continued to grow during the third cycle of selection. However, on medium with an initial antibiotic concentration of 3 mg/l, death was not as sudden as observed with higher concentrations but very low percentage of shoots only able to withstand the third selection Thus, hygromycin 3 mg/l was found optimum and considered as minimum inhibitory concentration which could lead to the recovery of escapes.

5.3.4. Effect of acetosyringone on transformation

Acetosyringone is known to be the activator of the *vir* genes of the Ti plasmid that would aid the successful transfer of T-DNA. Different levels of acetosyringone (0 - 200 mg/l) were used in the co-cultivation medium to study the effect of acetosyringone on transformation efficiency of *T. cucumerina*. GUS expression was observed in some explants in the absence of acetosyringone. But maximum number of explants showed GUS expression at 100 mg/l of acetosyringone in the co-cultivation medium (Table 5.3). Acetosyringone is a phenolic compound which is secreted from the wounded tissues of dicotyledons. The results of the present experiment were in accordance with

the results of Sujatha and Sailaja (2005) in castor, Joseph *et al.* (2004) in Camellia, Pandey *et al.* (2010) in Sorghum reported that when acetosyringone was included in the co-cultivation media the maximum genetic transformation frequency (100%) was observed. Glass beads used for coculivation did no show any variations in transformation frequency so the use of glass beads was omitted for further experiments.

5.3.5. Co cultivation period

Differences due to the co-cultivation period were evident during the third selection cycle (Table 5.4; Plate 9). Co- cultivation period was accessed on 0 - 10 days. Co-cultivation of leaf explants for 10 days resulted in a high frequency of transformation as revealed by the high survival frequency. However, shoot proliferation was poor and was accompanied by bacterial overgrowth at all stages of selection and failed to be controlled even on the third cycle of selection. In general, a 2 to 7-day co-cultivation period is considered ideal for *Agrobacterium*-mediated transformation in many plant species (Sujatha and Sailaja, 2005; Subramanyam *et al.*, 2015). Hence, in the present investigation two days co-cultivation period was preferred (Table 5.4 - 48 hours – 2 days). After two days of co-cultivation, leaching of explants was observed and the explants were washed with sterile half strength mMS liquid medium containing 250 mg/l cefotaxime (data not shown) to prevent the over growth of *Agrobacterium* in the infected explants. Then these infected explants were subsequently placed in the selection medium. Cefotaxime totally arrested the overgrowth of *Agrobacterium* which leads to the death of the explants. In contrast to our results, Cefotaxime, which was used for suppressing *Agrobacterium*, was found to severely inhibit shoot regeneration from root explants of *Arabidopsis* (Valvekens *et al.*, 1988). Coherent to our results, in the case of *J. curcas*, cefotaxime (250 mg/l) did not inhibit both of the callus induction and shoot regeneration capacity of its cotyledon explants and therefore, cefotaxime was used to inhibit *Agrobacterium* after co-cultivation with cotyledon explants (Li *et al.*, 2006; 2008).

Most of the studies on determining factors for enhancing transformation efficiency through *A. tumefaciens* - mediated gene transfer employ a transient *gus* assay as an index of T-DNA transfer. However, in the present study inferences were drawn based on the frequency of shoot survival after three cycles of selection. This

methodology is promising as the data are based on stable integration of the introduced gene.

5.3.6. Shoot development and plantlet establishment

Leaf explant produced multiple shoots with no base callusing on medium with 0.3 mg/l TDZ. On medium with 0.1 mg/l BA for 1 week prior to co-cultivation, the CN elongated and showed characteristic swelling at the meristematic region. Subculture followed by co-cultivation and culture of CN on medium with 0.3 mg/l TDZ and 0.6 mg/l PF - 68 resulted in enormous expansion of the meristematic region with a large number of tiny green coloured protuberances. Transfer of these cultures to selection medium with 0.3 mg/l TDZ with 0.6 mg/l PF - 68 and kanamycin (50 mg/l) led to the differentiation of these protuberances into shoot-like structures (Table 5.2). During the second and third cycles of selection, non-transformed cultures became necrotic while putative transformants continued shoot proliferation. Transfer of cultures subjected to three cycles of selection to medium with 0.6 mg/l of PF - 68 with GA_3 (0.3 mg/l) facilitated shoot elongation. All the elongated shoots with two or three distinct nodes developed roots on medium supplemented with 1.5 mg/l IBA and 0.6 mg/l $AgNO_3$. The rooted shoots were successfully acclimatized (Plate - 9).

5.3.7. Histochemical *gus* analysis

The plant obtained after transformation was undergone the gus assay. The leaves and shoots were taken for the experiment. These samples were transferred to X – Glu solution and incubated overnight at 37 ^{o}C. Then the samples were treated with acetic acid: ethanol in the ratio of 2:1. The *gus* expressed in the meristematic region was significantly influenced by the pre - culture of explants, co – cultivation period, acetosyringone concentration and the bacterial cell density. The *gus* expression of transgenic plants were studied for nearly four months. The histochemical *gus* analysis of the shoots recovered after three cycles of selection were positive for *gus* expression (Plate - 9). The intensity of *gus* staining was higher in young leaves and shoot primordia. The *Agrobacterium* strain is another important factor potentially influencing the efficiency of genetic transformation. Generally, the *Agrobacterium* strains available for different plant species may vary according to the susceptibility of plant species to the strains. Based on the *gus* expression LBA4404 was superior and more effective than

the other strains used in *T. cucumerina* L (Subramanyam *et al.*, 2015), castor (Sujatha and Sailaja, 2005; McKeon *et al.*, 2003), in Jatropha (Li *et al.*, 2008) in cotton (Sunil Kumar and Rathore, 2001).

5.3.8. Molecular confirmation

5.3.8.1. PCR analysis

In the present study, molecular analysis was carried out through PCR. The amplification was confirmed by the presence of *npt* II and *gus* gene in kanamycin resistant putative transformed plants that were obtained after three cycles of selection co-cultivated with LBA4404 *Agrobacterium* strain harbouring the binary plasmid pBAL2. The gene specific primers for *npt*II and *gus* were used for PCR amplification it's given 680 bp and 1.9 kb amplified fragment in respectively. There was no amplified product in untransformed shoots (control) (Plate 10). Transformation frequency of *Agrobacterium tumefaciens* was estimated on the basis of PCR positive plant and in relation to the total number of cocultivated explants. The frequency of recovery putative transformants after three cycles of selection worked out at 16 in 1,366 (1.17%) CN cultured (Table 5.5, 5.6; Plate 10).

5.3.8.2. Southern Hybridization

The fidelity of the amplified gene fragment was verified by subjecting the PCR gels to Southern blot hybridization. These genomic DNAs were digested with *Eco*RI and *Hind*III. Southern blot analysis of genomic DNA showed one copy of the *npt*II gene (Plate 9). Hybridization signals were not detected in the digested DNA of the control plant. Hybridization of PCR- amplified products with the probe showed a signal at the 690bp position while in the case of genomic DNA digested with *Hind*III the DNA fragments hybridized at the 1.9bp position. Southern hybridization results showed that the stable integration of foreign DNA in the transgenic plants.

5.4. CONCLUSION

This study is the first successful attempt to develop a stable transformation system for *T. cucumerina* via *A. tumefaciens* (strain LBA4404) mediated transfer using CN from 10^{th} day *in vivo* seedlings. With this protocol, a primary transformants can be developed within 4 months from culture initiation. To date, there are no protocols

described for the genetic transformation of *T. cucumerina* using vegetative explants. By this study, a reliable system of plant regeneration is developed for *T. cucumerina*, the meristem-based transformation system described in this report serves as a useful tool for the introduction of desirable genes into this highly industrialized crop.

Table 5.1. The effect of pre culture on growth response of leaf explants after co-cultivation

Pre – culture (in days)	Total number of explants cultured	Number of explants responded	Percentage of response
0	150	69	46.0
2	150	64	42.6
4	150	76	50.6
6	148	58	39.1
8	150	37	24.6
10	147	26	17.6
12	146	23	15.7
14	150	20	13.3

Table 5.2. The effect of selection marker sensitivity with growth regulators TDZ (0.3 mg/l) and PF – 68 (0.6 mg/l)

Antibiotics (mg/l)	Percentage of response	Mean number of shoots / explants
Kanamycin		
00	43.2	6.3
25	17.6	3.6
50	9.6	2.0
75	3.5	1.2
100	-	-
125	-	-
150	-	-
175	-	-
Hygromycin		
00	37.6	5.2
01	18.5	3.3
03	4.6	1.6
05	-	-
07	-	-
09	-	-
11	-	-
13	-	-

Table 5.3 Effect of acetosyringone concentration on transformation efficiency using GUS

Acetosyringone Concentration (µl)	No. of leaf explants	No. of Shoots responded for GUS	Percentage of response
00	50	10	20.0
25	50	17	34.0
50	50	23	46.0
75	50	31	62.0
100	50	36	72.0
125	50	32	64.0
150	50	29	58.0
175	50	21	42.0
200	50	16	32.0

Table 5.4 Effect of co-culture duration on transformation efficiency of leaf explants

Co-cultivation (in hours)	No. of explants cultured	Number of shoots responded GUS	Percentage of response
00	50	3	6.0
06	50	6	12.0
12	50	10	20.0
24	50	14	28.0
36	50	20	40.0
48	50	26	52.0
72	50	9	18.0
96	50	4	8.0

Table 5.5. Preliminary gene transfer with *Agrobacterium* strain pBAL 2 and Kanamycin 50 mg/l (Selection Marker) using 2 days preculture explants with 100 mg/l acetosyringone on their response on leaf explants

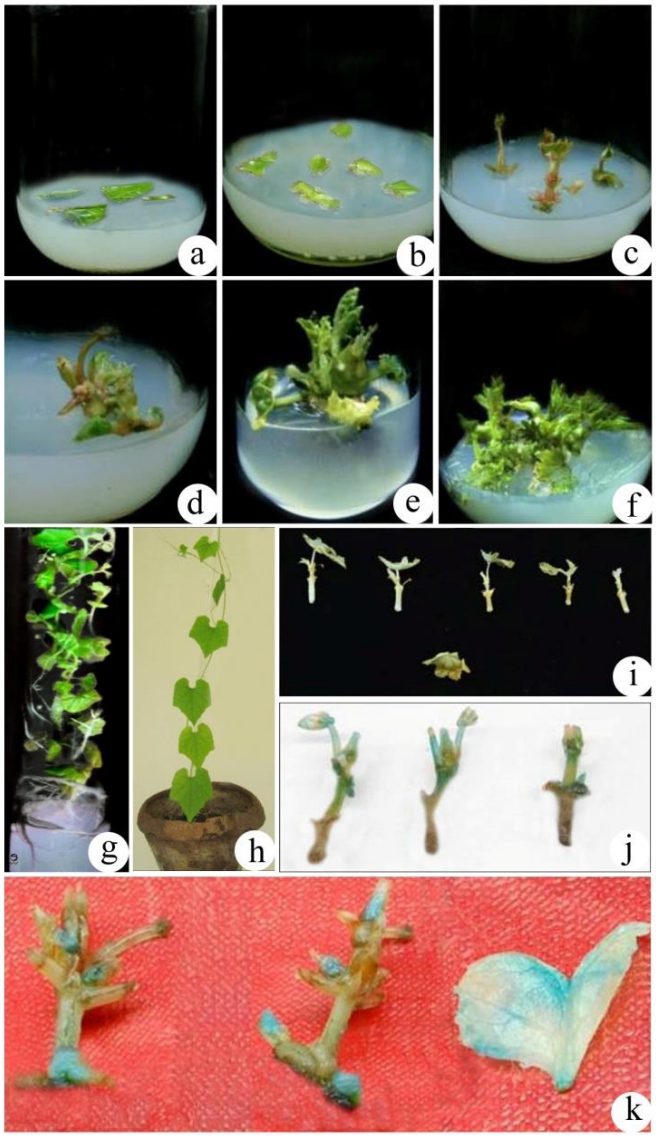
Experiments	No. of explants Infected (A)	No. of KanR shoots (B)	Percentage of response (B/A)	No. of shoots PCR positive (C)	Transformation efficiency (C/A)
1	131	41	31.2	3	2.2
2	123	44	35.7	2	1.6
3	142	34	23.9	1	0.7
4	144	51	35.4	2	1.3
5	136	42	30.8	1	0.7
6	137	39	28.4	1	0.7
7	146	49	33.5	2	0.6
8	135	30	22.2	0	0.0
9	129	35	27.1	2	1.5
10	143	36	25.1	2	0.6

Table 5.6. Summary of transformation using *Agrobacterium* strain with pBAL2 and Kanamycin (selection Marker) on their response on leaf explants

No. of explants infected (A)	No. of KanR shoots (B)	Percentage of response (B/A)	No. of shoots PCR positive (C)	Transformation efficeiency (%) (C/A)
1366	401	29.3	16	1.17

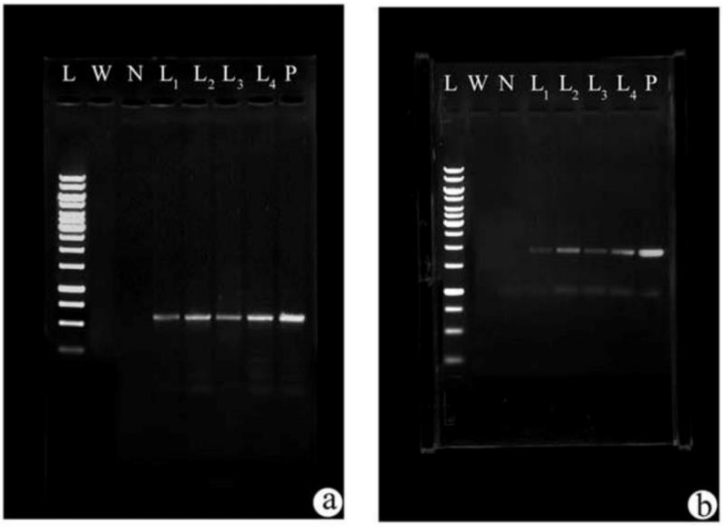
PLATE 9

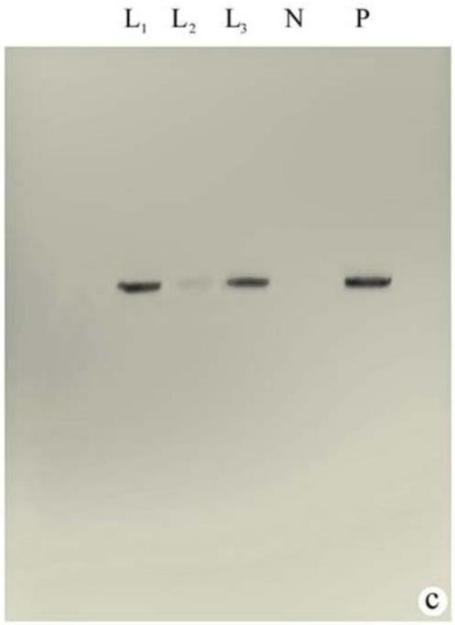
Agrobacterium mediated genetic transformation of *Trichosanthes cucumerina* L. using pBAL2 Vector



a. Preculture of explants
b, c & d. Shoot bud initiation from leaf explants in selection medium
e & f. Multiple shoot initiation & proliferation
g. Rooting
h. Hardening
i. Control shoot & leaf
j & k. Histochemical localization of Gus activity in node, shoots and leaves

PLATE 10





a. PCR analysis of putatively transformed plants using *npt* II
L – Ladder W - Water Control
N – Untransformed plant DNA (negative control)
L1 to L4 – Transformed plant DNA P – Plasmid DNA (positive control)

b.* PCR analysis of putatively transformed plants using *Gus
L – Ladder W - Water Control
N – Untransformed plant DNA (negative control)
L1 to L4 – Transformed plant DNA P – Plasmid DNA (positive control)

c. Southern hybridization of putatively transformed plants
P – Plasmid DNA (positive control)
L1 to L3 – Transformed plant DNA
N – Untransformed plant DNA (negative control)