GENETIC TRANSFORMATION OF *Amorphophallus paeoniifolius* (Dennst.) Nicolson

by

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THESIS

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2014

DECLARATION

I, hereby declare that this thesis entitled **"GENETIC TRANSFORMATION OF *Amorphophallus paeoniifolius* (Dennst.) Nicolson"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A	Adenine
A_{600}	Absorbance at 600 nm wavelength
bp	Base pair
C	Cytosine
CTAB	Cetyl trimethyl ammonium bromide
CIM	Callus Induction Medium
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
F	Forward primer
G	Guanine
g	gram
h	Hour
kg	Kilogram
M	Molar
mg	milligram
min	Minute
ml	Millilitre

m*M*	Millimolar
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
°C	Degree Celsius
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
R	Reverse primer
rpm	Revolution per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
T	Thymine
TE	Tris-EDTA buffer
Tris HCl	Tris (Hydroxy Methyl) aminomethane hydrochloride
U	Enzyme unit
v/v	volume/volume
w/v	weight/volume

Introduction

1. INTRODUCTION

Amorphophallus paeoniifolius (Dennst.) Nicolson, popularly known as elephant foot yam, is an important edible tropical tuber crop, belonging to the family Araceae. It is a crop of South-east Asian origin, grown widely in Philippines, Malaysia, Indonesia and South eastern Asian countries. The tubers of elephant foot yam are popularly used as vegetables in various delicious cuisines and in preparation of indigenous ayurvedic medicines (Misra *et al.,* 2002a).

It is widely cultivated as a cash crop owing to its high production potential, medicinal and nutritional value (Ravi *et al.*, 2009). Andhra Pradesh, Gujarat (286 ha), West Bengal (12,142 ha) , Kerala (7,662 ha) , Bihar (695 ha), Uttar Pradesh, Azadpur in New Delhi were identified from surveys as the potential elephant foot yam production and marketing centers in India (Srinivas *et al.*, 2012). Among the southern states, besides Andhra Pradesh, Tamil Nadu has a major area of the cultivation of the high yielding varieties of elephant foot yam, *Gajendra and Sree Padma* (Saraswathi *et al.*, 2008), followed by Kerala, where the tuber crop is cultivated in almost every part (Ravindran and George, 2008). Even though it is cultivated throughout Kerala in the homestead gardens, commercial cultivation of this crop is seen in Wayanad, Malappuram, Eranakulam and Thrissur districts(Srinivas *et al.*, 2012).

Occurrence of mosaic disease of elephant foot yam in India has been reported during later parts of 1960s (Capoor and Rao, 1969). Incidence of mosaic disease reported in several states of India like Kerala, Andhra Pradesh and Orissa, has resulted in considerable loss of yield (Babu *et al.*, 2011). A major concern of the viral infection is the reduction in the tuber yield, due to perpetuation of the virus through infected planting materials. In India, 24-88 percent mosaic incidence with a maximum yield loss up to 38 percent was reported from elephant foot yam growing areas in Uttar Pradesh (Nehalkhan *et al.*, 2006). Viral disease incidence of 5-10 percent was observed in other major *A. paeoniifolius* growing states of India, *viz.* Kerala, Andhra Pradesh and Orissa (Babu *et al.*, 2011). In

addition to this, the tubers of the wild plants are highly acrid and cause irritation in throat and mouth due to high content of calcium oxalate present in the tubers.

Considering the wide commercial importance of this crop and the mosaic disease causing yield loss, efforts should be directed at developing disease and pest resistance, enhancing the nutritional quality and improving tuber palatability by developing non-acrid varieties. Though resistance genes can be transferred from wild varieties to conventional cultivars, the low germplasm variability of elephant foot yam is the major limitation. The need to use distantly related varieties for resistance as well as other desirable genes necessitates the use of transgenic technology.

Of all methods of gene transfer, *Agrobacterium*-mediated gene transfer is a good choice on account of the ease of gene transfer, precise integration of DNA sequences along with the transformation marker, higher frequency of stable transformation with many single copy insertions.

To achieve high transformation efficiency using this method, it is necessary to standardise and devise a suitable *Agrobacterium*-mediated transformation protocol because there could be several factors that influence the transformation efficiency such as *Agrobacterium* strain, acetosyringone concentration, co-cultivation time, incubation temperature etc. Hence the present study is focused on *Agrobacterium*-mediated transformation in elephant foot yam with the following objectives

- Mass multiplication of callus cultures of *A. paeoniifolius* and use as explant source for transformation.
- Standardisation of *Agrobacterium*-mediated transformation in *A. paeoniifolius*.
- Molecular analysis and GUS assay for confirmation of transformation.

The study may have future applications in improving the ability of this tuberous crop to withstand different adverse environmental factors, in increasing yield, and enhancing nutritional quality.

Review of literature

2. REVIEW OF LITERATURE

2.1. Amorphophallus paeoniifolius

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is a highly potential tropical tuber crop of Araceae family and subfamily Lasiodeae of the monocotyledons. It is an important tuber crop of tropical and sub-tropical countries because of its yield potential and culinary properties. It is widely grown and consumed in south eastern countries like India, Philippines, Malaysia, and Indonesia.

In India, it is commonly known as *Suran* or *Jimmikand* and is traditionally cultivated on commercial scale in the states of Andhra Pradesh, Tamil Nadu, West Bengal and Kerala. The cultivation of elephant foot yam is slowly spreading to other states like Bihar and Uttar Pradesh also. The most popular variety for commercial cultivation in India is '*Gajendra*', which is a local selection from *Kovur* area of Andhra Pradesh (Srinivas and Ramanathan, 2005). The crop also offers excellent export potential from India, since it is not generally cultivated commercially in other countries. In India, it has gained the status of a cash crop due to its high production potential, market acceptability and lucrative economic returns with a production potential (50-80 t ha^{-1}) (Misra *et al.*, 2002b).

Its tubers are used as vegetable and in preparation of indigenous ayurvedic medicines because it is a good source of protein as well as starch. The crop plays a very important role in the socio-economic development of the country because of its nutritional and pharmaceutical values and high production potential, thereby forming an important source of income for the farmers. Moreover the crop is used in various food preparations and possesses a large number of medicinal values. The tubers are believed to have blood purifying characteristics and are used in medicines for the treatment of piles, asthma, dysentery and other abdominal disorders (Kirtikar and Basu, 1989 ; Misra and Sriram, 2001).

Pharmacological studies showed that the chloroform, acetone and ethanol extracts of the tubers of the wild form of elephant foot yam exhibited significant antibacterial and anti inflammatory activity (Nambisan *et al.,* 2005). Elephant foot yam starch is easily extractable and pure white in colour with good viscosity, stability and suitability for many applications in food industry (Moorthy *et al.*, 1994).

2.1.1 Morphoplogy

Elephant foot yam is a perennial, terrestrial, underground hemispherical depressed dark brown corm of approximately 20-25 cm in diameter which bears flowers and fruits in the month of April – May (Cooke, 1967; Yoganarsimshan, 1996). The genus *Amorphophallus* has an estimated number of 90-170 species occurs in the tropical Asia and Africa (Bogner *et al.,* 1985).

The crop is vegetatively propagated by corms, which is an underground stem commonly used as vegetable. From the central bud of the corm, a solitary dissected tripartite leaf is formed on a long petiole (pseudostem). The large leaves constitute the luxuriant outspreading crown like foliage.

It flowers rarely and the floral primordium arises from the central bud. The solitary inflorescence developed from the bud is comprised of a short peduncle of 5-7 cm long and 15-20 cm broad. It culminates in an enormous spathe on full development. The spathe measures about 8- 10 cm height and about 20-30 cm breadth with a wide open upper part and the base covering the inflorescence. The spathe on the outside is light green at the base and brown towards the top region and the inside is brown. The spadix extends and reaches up to 15-20 cm, holding the female flowers at the proximal portion and male flowers at the distal end and terminates in a dark brown spongy sterile appendage (Misra *et al.*, 2002a).

2.1.2 Propagation and cultivation

It is an underground stem tuber growing well in hot and humid climates. Humid climate promotes the initial stages of plant growth whereas dry climate favours tuber bulking. The ideal temperature for growth ranges between 25°C and 30°C. Being a water loving crop, well distributed rainfall of 1000-1500 mm in addition to well drained; fertile, sandy loam soil rich in organic matter with adequate amount of available plant nutrients is ideal for elephant foot yam cultivation.

Elephant foot yam is a long duration crop and generally matures in 6-7 months. Crop can be harvested at different stages of development starting from 6-7 months of plantation up to 4 years as per requirement. The crop is cultivated as a mixed crop in the fields of banana, ginger and groundnut (Anonymous, 1985).

Plants can be vegetatively propagated through corms which can be planted in the prepared pits (40 cm x 40 cm x 40 cm) filled with decomposed cowdung compost and sandy loam soil. Tubers start sprouting after 2-3 months of storage (Misra *et al.*, 2002b).

Some of the superior varieties released for general cultivation in India are the following:

1. Gajendra: A local variety from Kovur of Andhra Pradesh released by A.N.G Ranga Agricultural University and is recommended for all India cultivation. It has a yield potential of 50-60 t/ha. The tubers are non acrid, well shaped and generally devoid of cormels, with a light orange coloured flesh (Nedunchezhiyan *et al.*, 2006).

2. Sree Padma: The variety was found to be the most suitable for cultivation in southern states, released by Central Tuber Crop Research Institute, Trivandrum. It has a yield potential of 80 t/ha. The tubers are non acrid and generally have one mother corm and a few cormels or propagules.

3. Kusum: This variety is another selection from Kovur (Andhra Pradesh) released by Bidhan Chandra Krishi Vishwavidyalaya (West Bengal), with a yield potential 50-60 t/ha and characters similar to Gajendra variety (Misra *et al.*, 2002a).

Some local cultivars of high yielding, vigorous growth and good cooking qualities are also seen to be cultivated in different regions of Kerala such as:

1) **Kuzhimundan**: high yielding (>3.0 kg/plant) local variety having dwarf plant stature with deep green pseudostem and leaves, light yellow flesh with excellent cooking quality.

2) **Neychena:** the most popular local cultivar in homestead having excellent cooking quality.

3) **Anachena**: gigantic shoot as well as corm which are mainly cultivated for commercial purposes, with a potential yield of up to 80.0 t/ha.

4) **Malanchena**: higher yield and good cooking quality popularly cultivated in the hilly regions.

5) **Perunkalanchena**: vigorous growth with edible petiole and high yield.

6) **Venkuttychena**: white pseudostem with excellent cooking quality (Babu, 2011).

2.1.3 Major nutrient and chemical constituents of elephant foot yam corm

Elephant foot yam is a good source of energy, sugar, starch, proteins as well as minerals. Average nutritional profile contains starch (11-28 percent), sugar (0.7-1.7 percent), protein (0.8-2.60 percent), fat(0.07-0.40 percent) mean energy value (236-566.70 KJ/100g). The most abundant macro mineral is potassium (327.83 mg/100 g), phosphorus (166.91 mg/100 g), calcium (161.08 mg/100 g) and iron (3.43 mg/100 g). Macro mineral and soluble oxalate varies between different varieties: K (230-417mg/100g), P (120- 247 mg/100 g), Ca (131-247

mg/100 g), Fe (1.97-5.56 mg/100 g), Mn (0.19- 0.65 mg/100 g), Zn (0.12-1.92 mg/100 g) and soluble oxalate (6.65-18.50 mg/100 g). The mean soluble oxalate content (13.53 mg/100 g) was safe from the viewpoint of accumulation of urinary oxalate leading to kidney stones (Chattopadhyay *et al.*, 2009).

2.2 *IN VITRO* PROPAGATION

Hartman (1974) was first reported the use of micro propagation for the purpose of producing disease free aroids (*Caladium bicolor*, *Xanthosoma sagittifolium*, and *Colocasia esculenta*) which are free from dasheen mosaic virus by culturing shoot tips, which consist of an apical meristem and a leaf primordium.

In elephant foot yam, plant regeneration has been achieved in some economically important species using corm segments (Asokan *et al.*, 1984; Irawati *et al.*, 1986), leaf (Kohlenbach and Becht, 1998), inflorescence, and bulbil (Zhuang and Zhou, 1987). Scientists have investigated the effect of growth regulators on corm explant in *A. albus* and reported successful induction of adventitious bud from corm-derived callus of *A. albus* and establishment of regenerated plants in soil (Liu *et al.*, 2001; Wu and Xie, 2001; Yan *et al.*, 2005).

Jianbin *et al.* (2008) reported plant regeneration of *A. albus* from petiole-derived callus. Calli were induced at a high frequency of 76.4±3.2 percent from petiole explants excised from two month old plants on Murashige and Skoog (MS) medium supplemented with 5.37 μM α-naphthaleneacetic acid (NAA) and 4.44 μM 6-benzyladenine (BA).

A study reported on the Tissue culture and micropropagation on *A. Albus* compared the induction of different explants on different seasons, and the observed that the leaf and petiole when adopted in May have higher start up rates while the bud has high start up rate in March. Start up rate of buds in March was up to 90 percent; the stem tuber has the lowest contamination when adopted it in July

Ban *et al.* (2009) produced *in vitro* plantlets from tubers of *A. konjac.* For surface sterilization of explants about 1 cm long tubers were excised and washed with tap water, surface sterilized twice with mercuric chloride (0.2 percent) for 1 min, followed by rinsing with sterile distilled water for two to three times; then treated with 75 percent ethanol for 30 s, and followed by rinsing with sterile distilled water for four to five times, each for 5 min. Epidermis of surface sterilized tuber was shaved in sterile distilled water and tubers were cut into small pieces (about 0.5 cm^3), then placed on callus initiation (CIM) medium (MS basal salts (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.3% Phytagel, 0.5 mg/L NAA and 0.5 mg/L 6-BA, pH 6.0) in the dark with a temperature of 26°C for 30 days. The calli were then cut into small pieces, cultured again on CIM medium.

Hu *et al.* (2006) optimised the procedure for *in vitro* corm production and multiplication, the effects of phytohormones, sucrose concentrations and incubation conditions with desirable phytohormone combinations for callus induction, corm formation and corm growth of *A. albus*. The results showed that calli were induced at high frequency from petiole segments on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1.0 mgl^{-1} α-naphthaleneacetic acid (NAA) and 1.0 mgl^{-1} 6-benzyladenine (BA). Compact nodular calli were desirable for corm formation, and optimum corm formation was obtained in the presence of 0.5 mgl^{-1} NAA and 2.0 mgl^{-1} BA. With this auxin and cytokinin combination, an increase in sucrose concentration from 2 percent to 6 percent significantly increased the corm formation rate and favoured corm growth, but negative effects occurred at higher sucrose concentrations. By incubating over a range of temperatures from 19°C - 28°C, 22°C produced the largest numbers of corms and highest mean fresh weight of each corm. Short-day (8 h) or long-day (16 h) photoperiods did not affect corm formation and growth significantly, except that corm weight fell under long-day conditions.

Kamala and Makeshkumar (2013) reported initiation of whitish, yellowish white or pink friable callus from lateral bud and petiole of *A. paeoniifolius*

(Dennst.) Nicolson) within 15-30 days of inoculation on modified MS medium supplemented with 0.5 mgl^{-1} each of BA, 2,4-D and NAA and the days to callusing and the callusing percent differed significantly depending on the explant type.

2.3 GENETIC TRANSFORMATION IN MONOCOTYLEDON

Agrobacterium-mediated transformation has been widely used for research in plant molecular biology and for genetic improvement of crops since 1983. *Agrobacterium* for genetic transformation mostly facilitates stable integration of a single copy of transgenes in plant genome with little or no rearrangement. Hence this method is considered to be associated with far fewer problems like transgene instability, gene silencing and/or co-suppression (Koncz *et al.*, 1994; Hansen *et al.*, 1997).

The suitability of *A. tumefaciens* as a vector for monocotyledon transformation was a highly debated topic until Hooykaas *et al.* (1984) reported transgene expression in *Asparagus*. Better understanding of the crucial steps governing *Agrobacterium* infection of plant tissues and the difficulties encountered in *Agrobacterium*-mediated transformation of monocots made it now possible to transform even difficult monocots.

2.3.1 Choice of explant

Explants are initiated from sterile pieces of a whole plant and may consist of pieces of organs such as leaves or may be specific cell types such as pollen. Many explants features are known to affect the efficiency of culture initiation and transformation. Younger, more rapidly growing tissue or tissue at an early stage of development is most effective.

An *Agrobacterium*-mediated study reported by Ban *et al.*, (2009) in *A. konjac* used calli initiated from tubers for transformation and they obtained a transformation efficiency of 5.7 percent. Several explant types have been used in

regeneration and transformation studies of cassava like friable embryogenic calls (FEC), callus, somatic embryos, cotyledons, axillary buds and chloroplasts. Rossin (2008) and Sahoo *et al.* (2011) reported regeneration frequency (~90 percent) and transformation frequency of 45 percent in indica rice varieties by using calli initiated from rice as explants for *Agrobacterium*-mediated transformation.

2.3.2 Methods of gene transfer

The gene transfer methods and the vectors to be used must be compatible with the plant genotype and the tissues to be treated. Genetic transformation requires penetration of the transgenes through the plant cell wall, facilitated by biological or physical methods. Currently different technologies for gene transfer are available; however, low transformation efficiency and the randomness of integration sites are still limitations. The transformed plant cells are regenerated into whole plants using tissue culture and the transgenes are stably inherited through generations. Common methods for genetic transformation are usually divided into indirect or direct transformation (Qayyum *et al.*, 2009).

Biological methods using bacteria are referred to as indirect, while direct methods are physical; that is, based on the penetration of the cellular wall. Indirect transformation methods introduce plasmids, that is, independent circular molecules of DNA that are found in bacteria, separate from the bacterial chromosome, into the target cell by means of bacteria capable or transferring genes to higher plant species (Broothaerts *et al.*, 2005). The most popular used microorganisms are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, two soil native bacteria capable of transferring a tumor-inducing plasmid to its host, promoting tumor formation (Meyers *et al.*, 2010).

There are a few reports on genetic transformation using direct and indirect methods of gene transfer for different traits such as insect resistance, pest resistance, improving nutritional quality etc.

The biolistic and *Agrobacterium* methods have been successfully used to produce several transgenic monocots by adjusting the parameters that govern efficient delivery and integration of transgene(s) into plant genome. By the late eighties, the first transgenic maize was produced by electroporation and PEG treatment (Rhodes *et al.,* 1988) and the strategies continued to remain the principle methods of monocot transformation until 1990. Although other methods of direct DNA transfer gained momentum with time, nearly all genetically engineered monocots were produced through the use of the particle gun technology only. But the simplicity and stable integration of a single copy of transgene in plant genome with little or no rearrangement boost up the use of *Agrobacterium*-mediated transformation. But initial developments in *Agrobacterium*- mediated genetic transformation of monocots were rather slow and the first breakthrough came only when some monocot species were shown to be susceptible to *Agrobacterium* infection under natural conditions (De Cleene and De Ley, 1976).

Sahoo *et al.* (2011) developed an easy, rapid and highly efficient transformation and regeneration protocol for indica rice varieties using mature seeds as explants and they achieved 46 percent of transformation efficiency.

In the first case of genetically modified *A. konjac* by *Agrobacterium*-mediated transformation procedure reported the transformation efficiency was 5.7 percent (Ban *et al.*, 2009). In other tuber crops also *Agrobacterium*-mediated transformation is found to be successful, cassava (Bull *et al.*, 2009), Potato (Biswas *et al.*, 2010), Taro (He *et al.*, 2008).

2.3.3 *Agrobacterium*-mediated gene transfer

2.3.3.1 *Molecular Mechanism of Agrobacterium-mediated DNA Transfer*

Agrobacterium tumefaciens is a gram-negative bacterium and soil phytopathogen that genetically transforms host plants and causes crown gall tumours at wound sites (Smith and Townsend, 1907). The interaction of

Agrobacterium and eukaryotic cells is the only known mechanism for DNA transport between the different kingdoms in nature. The genetic mechanism of host range determination is still obscure, but it was reported that several virulence (*vir*) genes on the Ti plasmid, *vir*C (Yanofsky and Nester, 1986), *vir*F (Regensburg-Tuink and Hooykaas, 1993), and *vir*H (Jarchow *et al.,* 1991) were involved in determination of the range of plant species.

Agrobacterium is an effective tool for plant genetic engineering, since a portion of the plasmid DNA from *Agrobacterium* is incorporated into higher plant cells and results in crown gall in the host plant (Chilton *et al.,* 1977). Tumour induction is initiated by bacterial recognition of monosaccharide and phenolic compounds secreted by the plant wound site.

Activated *Agrobacterium* transfers a particular gene segment, called transfer DNA (T-DNA), from the Ti plasmid, and T-DNA is stably integrated into the chromosomal DNA in nucleus of the host plant; the genes for opine synthesis and tumour inducing factors in T-DNA are transcribed in the infected cells. This expression of the foreign gene in the host plant results in neoplastic growth of the tumours, providing increased synthesis and secretion of opines for bacterial consumption (Nester *et al.,* 1984).

Agrobacterium is classified based on the type of opine. Different *Agrobacterium tumefaciens* strains produce different opine phenotypes of crown gall tumours, because a particular opine expressed in the tumour is used for particular bacterial growth. Most common *Agrobacterium* strains produce an octopine or nopaline form of opines (Hooykaas and Beijersbergen, 1994).

Tumour inducing (Ti) Plasmid

Several components of *Agrobacterium* are necessary for transferring the piece of bacterial DNA into the plant cell. One component is the chromosomal virulence A (*chv*A) gene, which is on the *Agrobacterium* chromosome and activated by sugars. ChvA protein triggers bacteria to bind to the wounded plant

tissue and to respond to a specific chemical (chemotaxis). The Ti plasmid in bacteria contains the other main components, which are generated or activated efficiently for causing crown gall in host plants after bacteria attach to the plant wound site. The first is T-DNA, which is actually integrated into the plant cell chromosome. The second is the 35 kb virulence (*vir*) region, which is composed of seven loci (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G, and *vir*H). Expression of *vir* genes is triggered by a phenolic compound, which is secreted from the wound site of the host plant. The main functions of Vir proteins are to mediate the T-DNA excision from the Ti plasmid, export of the T-DNA piece from the bacteria, and insert it into the host plant chromosome (Gelvin, 2003). These two components are essential for a successful gene transfer. The Ti plasmid also has other components, an opine catabolism region, a conjugal transfer region, and a vegetative origin of replication of the Ti plasmid (*ori*V). Engler *et al.* (1981) found that these *vir* regions have sequence conservation between the octopine and nopaline Ti plasmids.

Transfer DNA (T-DNA) of Ti Plasmid

T-DNA is the DNA segment transferred into the plant cell. The T-DNA is present on the Ti-plasmid of the wild type *Agrobacterium*, and its size is an average of 25 kb, ranging from 10 to 30 kb. The T-DNA region is flanked and delineated by two 25 bp direct repeats, known as the right border and left border (Sheng and Citovsky, 1996). These border sequences are highly homologous and are targets of the border-specific endonuclease (VirD1/VirD2). The excised single strand of T-DNA from the Ti plasmid is exported from the bacterial cell to the plant cell by the activity of the other *Agrobacterium* Vir proteins. Right border is essential for *Agrobacterium* pathogenecity than left border. Deletion of the right border leads to a reduction of virulence, whereas the left border does not. Additional evidence, that the right border is more important than left border, is that the VirD2 protein can alone bind to the single stranded right border sequence and cleaves a single-stranded T-DNA. The VirD2 protein remained on the 5' end

(right border) of the resulting single stranded T-DNA molecule, termed the T-strand (Jasper *et al.*, 1994).

After T-DNA is integrated into the host plant, opine is synthesised, then secreted out of the cell and imported into *Agrobacterium*. The absorbed opine molecule is catabolised by a specific enzyme in *Agrobacterium*. Opine is degraded into amino acid and the sugar moieties, which can be used as carbon and energy sources for bacterial growth.

2.3.3.2 Structure and Function of Virulence Genes in Ti Plasmid

Induction of vir Gene Expression

Matthysse (1986) reported that gene transfer is started from tight binding between *Agrobacterium* and the host cell. This process is caused by bacterial chromosomal proteins ChvA, ChvB and PscA. Expressions of these proteins are triggered by substances secreted from wound site, such as acetosyringone (Winans, 1992), lignin or flavonoid precursors. Addition of artificial phenolic compounds during bacterial infection of the plant increased gene transfer efficiency. Sugars also assist activation of the major phenolic-mediated, wound-signalling pathway when small amounts of phenolic compounds are secreted from wounded cells.

After bacteria and plant cell binding, the protein in bacteria, which is activated by signal molecules are secreted from the plant wound, is VirA (Sheng and Citovsky, 1996). VirA can amplify the transformation system by detection of monosaccharide in the presence of low concentrations of phenolic compounds. Klee *et al.* (1983) identified the functions of five *vir* regions, from *vir*A through *vir*E, by transposon mutagenesis and genetic complementation. Vir proteins are involved in signal recognition, transcriptional activation, conjugal DNA metabolism, intercellular transport, nuclear import and probably T-DNA integration into the plant nucleus. The major roles of the VirA and VirG proteins are activation of other *vir* genes. VirA activates VirG, which is a cytoplasmic

DNA binding protein and works as a transcriptional activation factor to induce the expression of other *vir* genes. The autophosphorylated histidine residue of VirA by a signal molecule phosphorylates an aspartate residue of VirG. These two proteins initiate the process of T-DNA transport (Winans, 1992). The phosphorylated VirG protein recognises the *vir* genes containing a *vir* box, a conserved 12 bp sequence, and induces the expression of *vir* genes. This conserved region is located at a promoter region of the *vir* genes (Citovsky *et al.*, 1992). VirD1 and VirD2, a heterologous system, act like endonucleases that cut between the third and fourth base pairs of 24 bp right and left border repeats of the T-DNA bottom strand (Wang *et al.*, 1987). A linear single-stranded copy of the T-DNA region, named T-strand, is generated in *vir*-induced *Agrobacterium* cells (Stachel *et al.*, 1985). The T-strand is produced from the 5' to 3' direction, initiating at the right T-DNA border and terminating at the left border, by the endonuclease activity of the VirD protein (Citovsky *et al.*, 1992). VirD2 covalently binds to the right border of the T-strand, and to the 5' end of the remaining bottom strand of the Ti plasmid after the cleavage. The resulting single-stranded gap is repaired after the T-DNA strand is removed. VirD2 in the remaining strand may participate in ligating the left border nick (Sheng and Citovsky, 1996). Howard and Citovsky (1990) described a structural model of the T-strand when it is transferred out of the bacterium and into the plant cells, which is a protein-nucleic acid complex, called a T-complex. This T-DNA transport intermediate has at least three components: a T-strand, a VirD2 and VirE2 single strand DNA-binding protein. Citovsky *et al.* (1989) showed that VirE coats the single stranded DNA and forms a strong, stable, unfolded VirE2- ssDNA complex that is protected from external nucleolyic activity.

The T-complex is 3600 nm long and 2 nm wide, and it seems to contain about 600 molecules of VirE2 and one molecule of VirD2. The predicted molecular mass is 50,000 kD. The structural model suggested that VirD2 and VirE proteins are transported to the plant cell with the T-strand (Citovsky *et al.*,

1989). Another protein from the octopine Ti plasmid, VirC1, helps to generate a T-strand when VirD1 and VirD2 are limiting.

Several putative mechanisms of gene transfer from bacteria to plants have been proposed. One mechanism involves the conjugation machinery to transfer T-DNA into the plant cells. The T-DNA transfer system is similar to an inter-bacterial conjugative transfer system of broad-host range plasmids (Lessl and Lanka, 1994). Eleven *vir*B genes in the Ti-plasmid make proteins that seem to be involved in T-DNA transfer (Lai *et al.,* 2000).

Together with the VirD4 protein, the 11 VirB proteins make up a type IV secretion system necessary for transfer of the T-DNA and several other Vir proteins, including VirE2 and VirF (Christie, 1997). VirD4 may serve as a "linker" to promote the interaction of the processed T-DNA/VirD2 complex with the VirB-encoded secretion apparatus (Hamilton *et al.*, 2000).

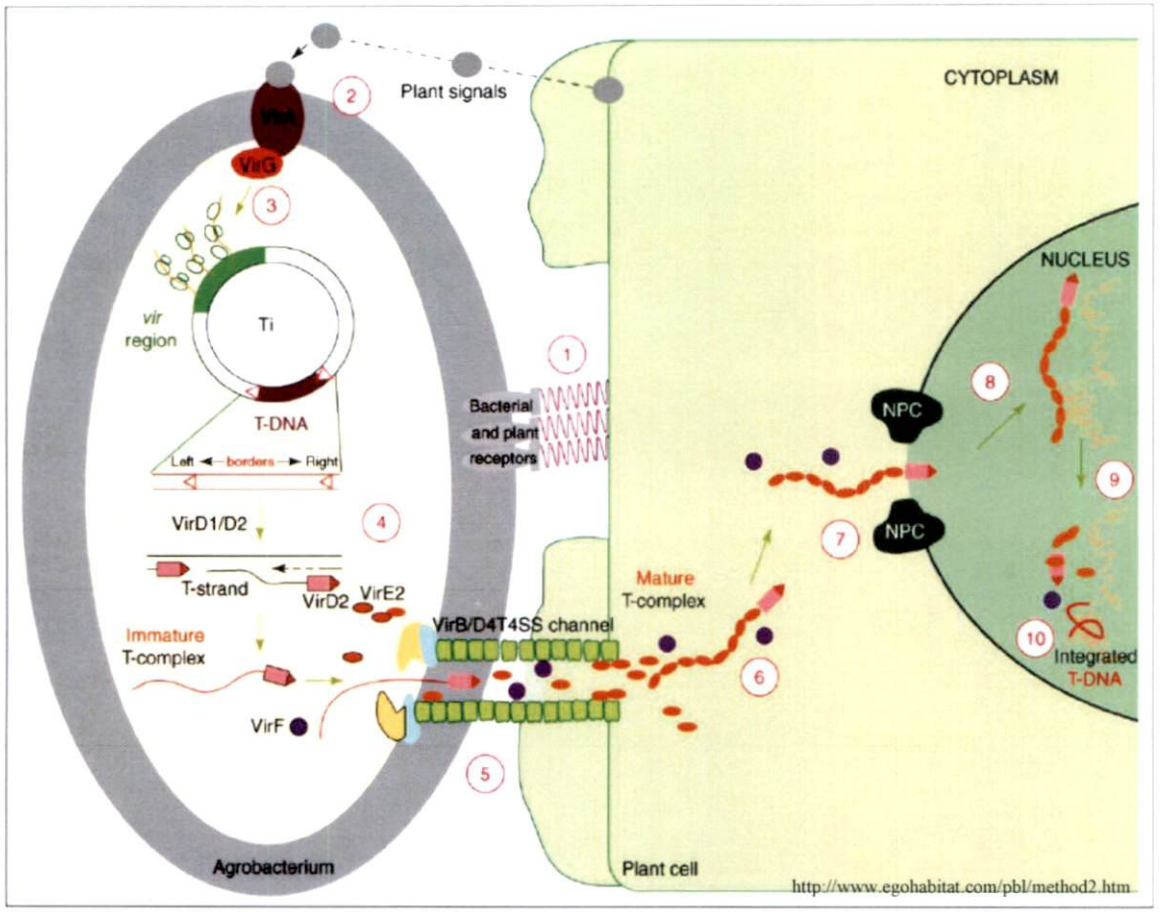
Most VirB proteins either form the membrane channel or serve as ATPases to provide energy for channel assembly or export processes. Several proteins, including VirB2, VirB5, and possibly VirB7, make up the T-pilus (Eisenbrandt *et al.,* 1999; Jones,1996; Schmidt-Eisenlohr *et al.*, 1999). VirB2, which is processed and cyclised, is the major pilin protein. The function of the pilus in T-DNA transfer remains unclear; it may serve as the conduit for T-DNA and Vir protein transfer, or it may merely function as a "hook" to seize the recipient plant cell and bring the bacterium and plant into close proximity to affect molecular transfer.

T-complex, an Intermediate of T-DNA Transfer

The T-complex (protein-nucleic acid complex), which is ssDNA bound with VirD2 and VirE2 proteins, is transported through the *Agrobacterium* channel, composed of the VirB protein family, into the cytoplasm of the host plant cell. The evidence for VirD2 and VirE2 involvement in T-strand transfer was reported by Howard *et al.* (1992) and Tinland *et al.* (1992). VirD2 and VirE2

proteins have specific nuclear localization signals (NLS) to move into the Nucleus. Howard *et al.* (1992) found the functional NLS in the VirD2 protein C terminal region, which has the bipartite consensus motif. According to the analysis of the TDNA integration junction, VirE2 is also required for integration at the 3' end, but not the 5' end of the T-strand (Rossi *et al.,* 1996). These results support a T-DNA complex model, in which VirD2 and VirE2 bind to the 5'and 3'ends specifically (Sheng and Citovsky, 1996).

The successful expression of the transgene depends on the position within the chromosome where the T-DNA integrates. T-DNA can be inserted near or far from transcriptional activating elements or enhancers, resulting in success or failure of activation of T-DNA carried transgenes. The failure of transgene expression (gene silencing) can also be caused by methylation or post-transcriptional gene silencing of multiple copies of transgenes. RNAs from these transgene copies may interfere with each other and then be degraded. In this aspect, the *Agrobacterium*-mediated method is favoured because fewer gene copies are integrated, compared to the direct gene transfer methods (e.g. polyethylene glycol-liposome-mediated transformation, electroporation, or particle bombardment (De la Riva *et al.,* 1998).



A model for the *Agrobacterium*-mediated genetic transformation. The transformation process comprises 10 major steps and begins with recognition and attachment of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system (2). Following activation of the *vir* gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome

Figure 1. Schematic representation of *Agrobacterium*-mediated transformation

2.4 AGROBACTERIUM-MEDIATED PLANT TRANSFORMATION PROTOCOL DEVELOPEMENT

2.4.1 Factors to increase gene expression and transformation efficiency

Transformation efficiency can be increased by manipulation of either the plant or bacteria for enhancing competency of plant tissue and *vir* gene expression, respectively (Chakrabarty *et al.*, 2002). Seedling age and pre-culturing of explants have been tested to increase the transformation efficiency. These trials were conducted to determine the best conditions for plant cell infection or

increasing the number of dividing plant cells before bacterial infection (Amoah *et al.*, 2001; Chakrabarty *et al.*, 2002). To increase the virulence of bacteria by inducing the *vir* gene expression, temperature (Dillen *et al.*, 1997; Chakrabarty *et al.*, 2002; De Clercq *et al.*, 2002), medium pH (Godwin *et al.*, 1991; De Clercq *et al.*, 2002) and chemical inducers, such as acetosyringone (Chakrabarty *et al.*, 2002; De Clercq *et al.*, 2002; Stachel *et al.*, 1985), have been tested. These factors likely enhance bacterial pili formation required for gene transfer between bacteria, as well as between the bacteria and plants. Manipulation of other factors, such as bacterial density, co-cultivation duration, surfactant, and vacuum infiltration, have also increased transformation efficiency in many experiments (Amoah *et al.*, 2001). According to previous experiments, inducing *vir* gene expression seems most important and effective for increasing plant transformation efficiency, regardless of the type of plant being studied.

2.4.1.1 Addition of acetosyringone

The transfer of T-DNA is mediated by the virulence genes in *Agrobacterium*. Transcription of these genes is induced by various phenolic compounds released by wounded plant cells. Induction of *vir* genes can also be achieved *in vitro* by co-cultivation of *Agrobacterium* with wounded plant cells or tissues in media containing signal molecules such as AS. AS has been commonly utilized in most transformation research for both dicotyledon and monocotyledon plants to enhance transformation efficiency (Frame *et al.*, 2002; Kant *et al.*, 2007).

However, the effect of AS on *Agrobacterium*-mediated transformation is known to vary according to the plant species. Msikita, (2007) reported that wounded explants of cassava with 200 µM acetosyringone was found to be suitable for successful transformation in Cassava (*Manihot esculenta* Crantz).

Fullner *et al.* (1996a) reported that *Agrobacterium* did not produce pili without 200 µM acetosyringone at both 19°C and 25°C. Tripathi *et al.* (2010) reported that 50µM Acetosyringone was suitable to obtain the high transformation frequency to introduce agronomically desired gene in *Oryza sativa* variety Pusa

Basmati- 1 (IET- 10364). Results from a wheat inflorescence transformation experiment showed that T-DNA cannot be transformed to the plant tissue without acetosyringone (Amoah *et al.*, 2001).

Xin and Feng (1995) observed several tendencies of effect of acetosyringone concentration in *Populus* hybrid NC-5331 such as 1) exogenous acetosyringone did not always enhance the gene transformation frequency, which was dependent on its concentration and the other factors 2)acetosyringone preferred higher pH for higher transformation efficiency 3) the most beneficial range of acetosyringone was between 25 and 75 μM.

Sheikholeslam and Weeks (1987) reported that the use of acetosyringone during co cultivation has been shown to increase *Agrobacterium*-mediated transformation frequencies.

2.4.1.2 Co-cultivation duration

The explant chosen for transformation in its most receptive stage is exposed to the *Agrobacterium* culture in the induction medium at an optimum bacterial density. The transformation efficiency is determined by the co-cultivation media and the co-cultivation duration.

Co-cultivation for 2 to 7 days has been normally used in *Agrobacterium*-mediated transformation under various co-cultivation temperatures (Cervera *et al.*, 1998; Han *et al.*, 2000; Mondal *et al.*, 2001;; Somleva *et al.*, 2002). Co-cultivation for 3 days resulted in high transformation efficiency, and transformation efficiency reached a maximum at day 5 in citrange (*Citrus sinensis* L. Osbeck x *Poncirus trifoliate* L. Raf.) (Cervera *et al.*, 1998). They reported that more than 5 days caused bacterial overgrowth and decreased the transformation efficiency. Many transformation experiments in different plant species, such as tea (*Camellia sinensis* L.), cauliflower, white spruce (*Picea gluca*) and citrange, showed that 2 to 3 days of co-cultivation resulted in high transformation efficiency under room temperature co-cultivation conditions (Lopez *et al.*, 2004; Chakrabarty *et al.*,

2002; Le *et al.*, 2001; Cervera *et al.*, 1998). Therefore 2 to 3 days co-cultivation has been routinely used in most transformation protocols, since longer co-cultivation causes bacterial overgrowth that covers the leaf tissue and causes toxicity under room temperature co-cultivation conditions.

Cervera *et al.* (1998) reported that 5 days co-cultivation prevented callus formation and resulted in poor plant regeneration. The co-cultivation duration recommended from published protocols for tobacco leaf disk transformation varies from one research group to another. They are 2, 3 and 4 days at 26°C, 24°C, and room temperature co-cultivation conditions (Svab *et al.*, 1995). Most of the previous reports indicated that 2 to 3 days were optimal at 25°C co-cultivation conditions, regardless of plant species. No experiments have been conducted yet to find an optimal co-cultivation duration at lower than 25°C.

2.4.1.3 Temperature effect on transformation

One aspect of pilus biology that may be important for transformation is its temperature lability. Although *vir* gene induction is maximal at approximately 25°C to 27°C (Alt-Moerbe *et al.*, 1988) the pilus of some, but not all, *Agrobacterium* strains is most stable at lower temperatures (approximately 18 to 20°C) (Lai *et al.*, 2000).

Temperature effects on plant transformation have been studied by a few groups (Dillen *et al.*, 1997; Chakrabarty *et al.*, 2002; De Clercq *et al.*, 2002), after conjugal transfer of Ti plasmid was shown to be sensitive to temperature (Fullner *et al.*, 1996b). Dillen *et al.* (1997) tested the effect of temperature on transgene expression in two plant systems, *Phaseolus acutifolius* and *Nicotiana tabacum*. The optimal temperature for *Phaseolus* callus transformation was 22°C and for tobacco was 19°C to 22°C. *Phaseolus* callus was incubated with bacteria at different temperatures from 15°C to 29°C for 3 days. Expression of GUS at 25°C was lower than at 22°C. Very low levels of GUS expression were detected at 27°C and no expression at 29°C and 15°C. They showed highest GUS expression at 22°C co-cultivation. They reported similar results with the tobacco experiment.

In both studies, GUS expression was dramatically reduced when the temperature was increased from 22°C to 25°C. GUS expression was low at 27°C and undetectable at 29°C.

Chakrabarty *et al.* (2002) evaluated a number of factors that increased the *Agrobacterium*-mediated transformation efficiency of hypocotyl explants of cauliflower. They showed that co-cultivation at 22°C resulted in higher GUS expression than at 28°C. De Clercq *et al.* (2002) also reported that co-cultivation at 22°C resulted in higher GUS expression than temperatures above 22°C in *Phaseolus acutifolius*.

The optimal co-cultivation temperatures for most monocots ranged between 23°C to 25 °C (Rashid *et al.*, 1996; Arencibia *et al.*, 1998; Enriquez-Obregon *et al.*, 1998; Hashizume *et al.*, 1999; Salas *et al.*, 2001).

The temperature at which the *Agrobacterium* is grown prior to transformation is also important for *vir* gene induction. *Vir* gene induction occurs at 28°C while increasing temperatures during bacterial growth starts to decrease the induction of these genes. This was shown by Jin *et al.* (1993) when they monitored the expression of *vir* genes at 28°C, 32°C, and 37°C by measuring the β-galactosidase activity in *Agrobacterium tumefaciens* A243MX which contains a virB::lacZ fusion. *Vir* gene expression was dramatically decreased as the temperature increased. An explanation for the decline in *vir* gene expression is that VirA and VirG which are required for *vir* gene induction. Signal transduction is sensitive to temperature of 32°C and above. VirA autophosphorylation and phosphate transfer to VirG was inhibited at elevated temperatures and *vir* gene expression is inactivated.

2.4.1.4 Agrobacterium strains and vectors

The strain of *Agrobacterium* and its ability to produce opines was another important factor governing monocot transformation (Hooykaas *et al.*, 1984). The *Agrobacterium*-mediated transformation method was improved by developing

modern binary Ti vectors after the removal of all the genes for tumour induction and opines synthesis. Ti plasmids without the tumour inducing function are called disarmed plasmids (nononcogenic Ti plasmid). Ti plasmids have been engineered to separate T-DNA and *vir* regions into two distinct plasmids, resulting in a binary vector and a *vir* helper plasmid, respectively (Hoekema *et al.*, 1983). Since disarmed binary plasmids, containing the T-DNA region, do not have the ability to move a T-DNA into the plant, they need the help of another separate plasmid containing the *vir* genes. Many *Agrobacterium* strains containing non-oncogenic *vir* helper plasmids LBA 4404, GV3101 MP90, AGL0, EHA101, and its derivative strain EHA 105 have been developed (Gelvin, 2003).

Binary vector plasmids are small and easy to handle in *E. coli* and *Agrobacterium,* when the wild type Ti plasmid is around 200 kb. The sizes of the processed binary vectors from wild type Ti plasmids have been reduced to less than 10 kb. The binary vector has a replication origin for both *E. coli* and *Agrobacterium*, an antibiotic selectable marker for bacteria and plants, are reporter gene and a T-DNA region containing a multiple cloning site for insertion of genes of interest (Klee *et al.*, 1983).

Transformation efficiency of five *Agrobacterium tumefaciens* strains GV2260, LBA4404, AGL1, EHA105, and C58C1 was evaluated in *Nicotiana tabacum* L. cultivar Samsun and the highest transformation rate (20 percent) was obtained with the *Agrobacterium* strain LBA4404, followed by EHA105, GV2260, C58C1 and AGL1, and the transformation efficiencies of all *Agrobacterium* strains were significantly different (P\0.05), except between LBA4404 and GV2260 (Bakhsh *et al.*, 2014).

A comparison of different *Agrobacterium* strains demonstrated that AGL0, a hyper virulent strain containing a disarmed pTiBo542 plasmid was better at generating wheat transformants than other strains tested (Lazo *et al.*, 1991).

pCAMBIA vectors

The pCAMBIA vector backbone is derived from the pPZP vectors (Hajdukiewicz *et al.,* 1994). The pUC18 polylinker was used in some vectors, but pUC8 and pUC9 polylinkers were also used to simplify the choice of cloning enzyme. Plant selection genes in the pCAMBIA vectors are driven by a double enhancer version of the CaMV35S promoter and terminated by the CaMV35S polyA signal. Reporter genes feature a hexa-Histidine tag at the C-terminus to enable simple purification on immobilised metal affinity chromatography resins. The sequence for this tag occurs between the first *Nhe*I site and the unique *Pml*I site. Gene of interest may be inserted in place of the reporter gene. Insertion without a stop codon and in frame at the (first) *Nhe*I site will append a hexa-Histidine tag to your protein of interest. Insertion without a stop codon and in frame at the *Pml*I site will append a stop codon. Insertion at the *Bst*EII site will add neither a tag nor a stop codon.

Nomenclature of pCAMBIA vectors:

The four digit numbering system works as follows:

First digit - indicates plant selection: 0 for absence; 1 for hygromycin resistance; 2 for kanamycin;and 3 for phosphinothricin.

Second digit - indicates bacterial selection: 1 for spectinomycin/streptomycin resistance; 2 for chloramphenicol; 3 for kanamycin; 4 for spec/strep and kanamycin.

Third digit - indicates polylinker used: 0 for pUC18 polylinker; 8 for pUC8 polylinker; 9 for pUC9 polylinker.

Fourth digit - indicates reporter gene(s) present: 0 for no reporter gene; 1 for *E.coli gusA*; 2 for *mgfp5*; 3 for *gusA:mgfp5* fusion; 4 for *mgfp5:gusA* fusion; 5 for *Staphylococcus* sp. *gusA* (GUSPlus).

Fifth digit - notes some other special feature. So far this has been used only with: pCAMBIA1305.1 and plasmids derived from it, where the .1 denotes the absence of a signal peptide from the GUSPlus™ protein; and pCAMBIA1305.2 where the .2 denotes the presence of the GRP signal peptide for *in planta* secretion of the GUSPlus™ protein.

Lagging letter - X indicates that the reporter gene lacks its own start codon and the vector is for creating fusions to the reporter; Z indicates presence of a functional *lacZa* for blue-white screening; a/b/c indicates the reading frame for fusions with the Fuse and Use vectors.

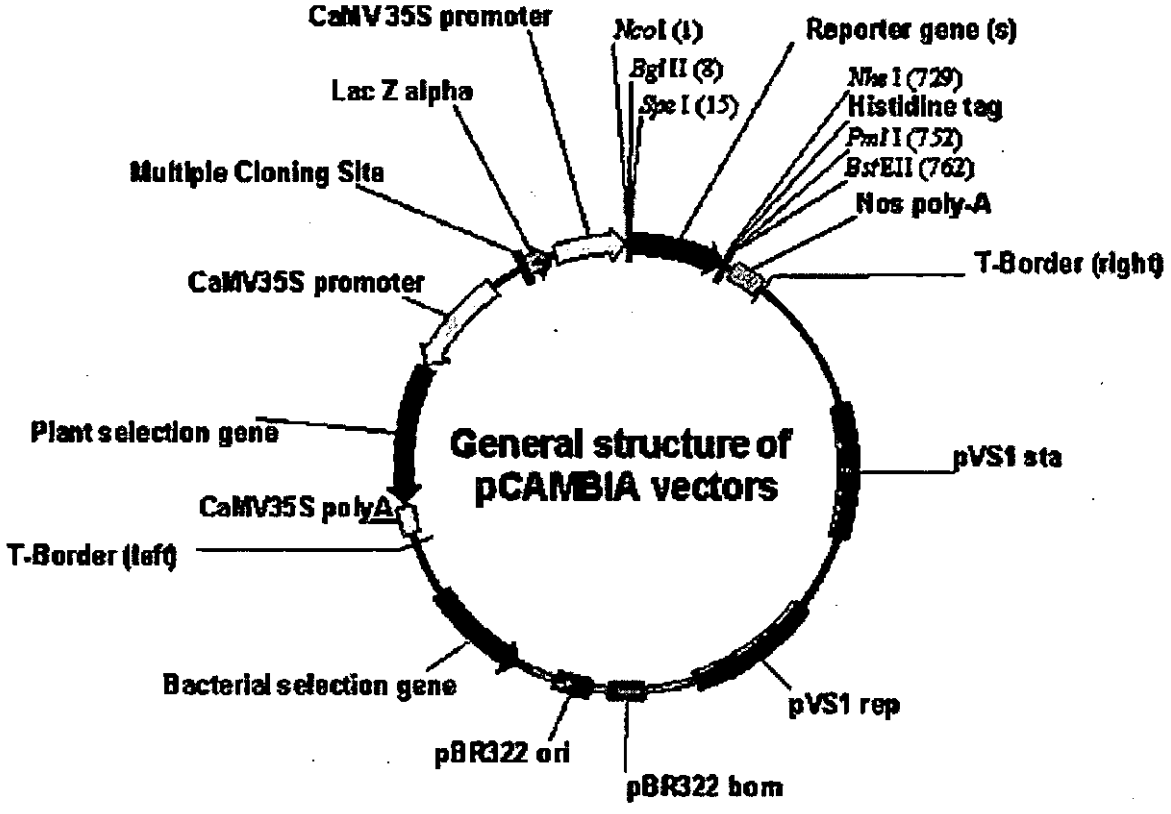


Figure 2. General structure of pCAMBIA vectors

2.4.2 Elimination of bacteria after co-cultivation

The successful elimination of *Agrobacterium* (following sufficiently long co-cultivation periods to transform cells) from regeneration media in transformation protocols is important for higher transformants recovery and increased transformation efficiency (Zhao *et al.*, 2001; Zhang *et al., 2003*).

Either a gentle rinsing of explants with fresh inoculation medium (Zhao *et al.*, 2001) and/or use of antibiotics such as cefotaxime, carbenicillin, tricarcillin, timentin etc. for suppressing or eliminating the residual *Agrobacterium* enhanced the transformation efficiency of several monocots (Naureby *et al.*, 1997; Bottinger *et al.*, 2001). However, the detrimental effects of high concentrations of antibiotics like cefotaxime on explants and reduction in the transformation frequency by several folds was investigated by Ishida *et al.* (1996).

The antibiotic chosen should be such that, it efficiently kills the bacteria; at the same time it does not affect the growth and organogenesis of the explants. Different antibiotics effectively eliminate *Agrobacterium*, the most common being cefotaxime (CF), carbenicillin (CA), vancomycin (VA) and timentin (Nauerby *et al.*, 1997).

Ticarcillin is a β-lactam antibiotic, very active against Gram-positive bacteria and certain species of Gram-negative bacteria such as *A. tumefaciens*. However, it is β-lactamase sensitive. Clavulanic acid is a specific inhibitor of β-lactamase and protects ticarcillin against inactivation by β-lactamase. So, ticarcillin/potassium clavulanate is an effective combination against *Agrobacterium* species.

A study reported by Ling *et al.* (1998) showed that concentration of 150 mgl^{-1} was high enough to eliminate *A. tumefaciens* strain LBA4404 on agar plates with inoculated tomato explants. No difference in effectivity on the elimination of *Agrobacterium* was observed between ticarcillin/potassium clavulanate (150 mgl^{-1}) and cefotaxime (500 mgl^{-1}). Callus formation and shoot regeneration were

significantly stimulated on medium containing ticarcillin/potassium clavulanate. Enhancement of callus formation and shoot regeneration might be caused by release of auxin-like compounds as has been reported for carbenicillin and penicillin G.

Where as cefotaxime in culture medium shows no obvious inhibition effect on callus growth in tomato, but it significantly reduced shoot regeneration. Additionally, a strong decrease of callus growth, shoot regeneration, and transformation efficiency appeared in combination with transformation and kanamycin. It is likely that transformed cells of tomato are more sensitive to cefotaxime and kanamycin.

2.4.3 Selection of transformed tissues

The choice of selective agent is vital for the successful selection of transformed cells from nontransformed cells. Selectable markers are a convenient method to distinguish between transformed and nontransformed tissues. Plants do not have an antibiotic resistance gene naturally. Transformed tissues contain selectable markers and survive on selective media containing antibiotics, while non-transformed cells, which do not have an antibiotic resistance gene, are killed on the antibiotic media.

Antibiotic resistance genes, usually the kanamycin (neomycin phosphotransferase II) resistance gene or hygromycin (hygromycin phosphotransferase) resistance gene, have been used as selectable markers. Reporter genes, such as β-glucuronidase (GUS), luciferase or green fluorescent protein (GFP), are important components of the T-DNA region. Expression of reporter genes is visualized in the transformed tissue through staining or auto-fluorescence. Transformed tissue can be distinguished from non-transformed tissue by reporter gene expression.

2.4.3.1 Neomycin phosphotransferase II (NPT II)

The *nptII* (or neo) gene was isolated from the transposon Tn5 of *Escherichia coli* and it encodes *nptII* (E.C. 2.7.1.95), also known as neomycin phosphotransferase II which inactivates different antibiotics of the amino-glycosylated group, such as kanamycin A, B and C, geneticin, neomycin, among others. In the plant cells these antibiotics, when applied in appropriate concentrations, act in the synthesis of proteins in mitochondria and chloroplast, reducing the chlorophyll synthesis and inhibiting the growth of untransformed cells (Brasileiro and Aragon, 2001). In this way, the transgenic plants which synthesize the enzyme NPTII can tolerate concentrations of amino-glycosylated antibiotics that are lethal in untransformed plants (Benveniste and Davies, 1973).

Regulation of *nptII* expression may be changed in various ways to alter the selection conditions. Elevation of transcription levels with strong promoters, like the cauliflower mosaic virus 35S promoter or the enhanced 35S promoter, raised the level of *nptII* enzyme activity and tolerance to kanamycin without creating instability in the expression of the *nptII* gene (Sanders *et al.*, 1987).

Fraley *et al.* (1983) were successful in raising independent transgenic of both petunia and tobacco with the *nptII* and *nptI* genes as selection marker genes using 50 mgl^{-1} kanamycin and other aminoglycosides as selection agents, *nptII* gene is preferentially used over *nptI* gene for plant transformation; however *nptI* is preferred for bacterial selection.

The *nptII* gene is the most frequently used selectable marker gene for generating transgenic plants for research purposes. The gene is very efficient in model research species such as *Arabidopsis* and tobacco, which represent 15–73 percent of the dicot species or rice and maize, which are the most common monocots used in published studies (4–33 percent).There have been no reports of adverse effects of *nptII* gene on humans, animals or the environment.

Yu *et al.* (2003) compared the effect of kanamycin and geneticin on regeneration of papaya from root tissue, and they found that somatic embryogenesis of calli derived from papaya root explants is strongly inhibited by kanamycin. The studies recommend lower concentration of geneticin (12.5- 25 mgl^{-1}) to avoid these adverse effects.

2.4.3.2 Hygromycin phosphotransferase (hpt)

The *hpt* (or aph IV) gene of *Escherichia coli* codes for the enzyme HPT (E.C. 2.7.1.119) confers resistance to the antibiotic hygromycin B (Waldron *et al.*, 1985). When hygromycin occupies their ribosomal binding site of the elongation factor 2 (EF-2) in prokaryotic cells, consequently the elongation of polypeptide chain is inhibited and protein synthesis interrupted, causing the same symptoms described for the other aminoglycoside antibiotics. In plant cells, these antibiotics exert its effect on mitochondria and chloroplast, acting in the same manner by impairing protein synthesis. These organelles have ribosomes that are similar to those found in bacteria and are also susceptible to aminoglycoside antibiotics. Therefore, in the presence of antibiotic, the plant tissue will show a chlorosis, caused by the lack of chlorophyll synthesis and inhibition of growth (Benveniste and Davies, 1973).

The *nptII* gene is the most frequently used selectable marker gene for generating transgenic plants. Because plant species display differential sensitivity to antibiotics, the *hpt*-hygromycin selection system tends to be used when plant cells display high tolerance to kanamycin (Twyman *et al.*, 2002). Even though production of transgenic cassava with the *nptII*-paromomycin selection system has been established and demonstrated (Abhary *et al.*, 2011) the hpt-hygromycin system has been reported to be more efficient.

A study reported by Ban *et al.* (2009) demonstrated 22.5 mgl^{-1} hygromycin for selection of transformed elephant foot yam. In the presence of 22.5 mgl^{-1} hygromycin, the shooting ability of the calli were restricted. Higher

concentrations of hygromycin in the medium were toxic to calli, causing tissue softening, blackening and consequently resulting in disc death.

Bull *et al.* (2009) used M S medium supplemented with 15 mgl^{-1} hygromycin, and 250 mgl^{-1} Carbenicillin for efficient production of transgenic plants by *Agrobacterium*-mediated transformation of cassava (*Manihot esculenta* Crantz).

2.4.4 Histochemical gus expression

Reporter genes serve as indicators to study transgenic events by facilitating visual identification of successfully transformed tissues from a large background of non-transformed tissues. The most popular of these systems is the GUS reporter gene (GUS A) is derived from the *E.coli* GUS operon (also known as *uidA* operon). The GUS A reading frame of operon encodes for a 68 kD β - glucuronidase protein which, in its active form, assembles in to a homo- tetramer which can catalyze the hydrolysis of a wide variety β-glucuronides and β-galactouronides. Jefferson (1987) reported that activity of GUS protein in plant tissues can be detected with a histochemical chromogenic assay in which 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) is used as substrate.

Stable plant transformation requires a considerable investment in time before the expressed proteins can be analyzed. In contrast, transient gene expression systems are rapid, flexible and straight forward. The transient expression assays described ensure that most errors and technical problems with gene expression can be identified and resolved before making stable transformants (Kapila *et al.*, 1996).

Efforts to increase GUS gene expression have been conducted by adding an intron or optimizing codon efficiency. The pCAMBIA Company developed a GUS gene that has a catalase intron for preventing GUS expression in bacteria (Ohta *et al.*, 1990), and they found that a GUS gene isolated from *Staphylococcus*

resulted in 10 times higher expression in rice tissues than GUS from *E. coli* (Jefferson *et al.*, 1987).

2.4.5 Molecular analysis of transgenes

PCR amplification of the marker gene or transgene is often taken as an indication of transgenic status of regenerates. However, Southern analysis is essential to prove the integration of the foreign gene into host genome (Potrykus, 1991). Also, Southern hybridization is useful to assess the number of independent insertions of transgene. Since single, unaltered transgene insertions are ideal for analysis, it is also necessary to check the presence of multicopy tandem insertions and other rearrangements at the given locus.

Routinely a first PCR screen of the transformants is based on the detection of stable integration of the selection marker gene. To verify the integration of constructs containing an *nptII* selection marker cassette, the transformants DNA was analysed by PCR amplification using *nptII* specific primer (Schween *et al.*, 2002).

Ban *et al.* (2009) used PCR analysis using primers specially amplifying an internal 753 bp fragment of the *aiiA* gene for molecular detection of transgenic plants of *Amorphophallus konjac.* The expected 753 bp *aiiA* band was found in 21 lines (about 61.7 percent efficiency) and was absent in untransformed controls, and the size of the amplified fragment was in coincidence with that of the positive control (*aiiA* gene was also amplified from the pU1301-aiiA plasmid). In order to determine whether T-DNA integration had taken place and how many copies of T-DNA were present in plant genome. Those PCR-positive plants were further used for southern blot hybridization.

DIG-labelled probes and chemiluminescent substrates have proved to be a popular method for nucleic acid detection because of susceptibility, quicker results, and dependability (Shu *et al.*, 1999). DIG polymerase chain reaction (PCR) labelled probes were used to detect transgenes in barley, rice,

and *Nicotiana benthamiana* (McCabe *et al.*, 1997). DIG-labelled probes have also been used successfully in detecting transgenes in other transgenic plants, such as rice, potato, sugar beet, maize, and wheat. DIG-based dot blot hybridization has been reported to provide a dependable, easy, and cost-effective tool for plant molecular breeders, in which a large number of single nucleotide polymorphisms can be analyzed for breeding purposes (Dietzgen *et al.*, 1999).

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Genetic transformation of *Amorphophallus paeoniifolius* (Dennst.) Nicolson was conducted at the Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2013- 2014. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 SOURCE OF EXPLANT

Amorphophallus paeoniifolius (Dennst.) Nicolson cultivar *Gajendra* was used for the study. Callus was initiated from the petiole and leaves of *in vitro* plantlets in callus induction medium (CIM). The initiated calli were sub cultured periodically in CIM (Appendix I) to obtain sufficient calli for transformation study.

3.1.1 Composition of media

Murashige and Skoog modified 3B- ½ concentration of NH_4NO_3 and KNO_3 (Duchefa Biochemie) supplemented with various plant growth hormones were used for different plant tissue culture experiments. YEB were used for bacterial culture during the study (Appendix II).

3.1.2 Preparation of medium

Standard procedures (Murashige and Skoog, 1962) were followed for the preparation of plant tissue culture media. Murashige and Skoog modified 3B- ½ concentration of NH_4NO_3 and KNO_3 (2.6 g l^{-1}) and Sucrose (20 gl^{-1}) and appropriate concentration of plant hormones was dissolved in 980 m l sterile distilled water and pH of the medium was adjusted to 5.5- 5.6 using 0.1 M NaOH/HCl, and made up the volume to one litre. Agar was then added at the rate 7.25 g l^{-1} and the medium was heated with intermittent stirring to mix agar. Then the medium was dispensed to the Schott Duran reagent bottles. Autoclaving was

done at 121°C and 1.06 kg/cm^2 pressure for 20 minutes to sterilise the medium. The medium was allowed to cool and stored in culture room until use.

For preparation of medium for bacterial culture, the media components were dissolved in the order stated in Appendix II and adjusted the pH to 7.5 with 0.1 M NaOH/HCl. For solidified medium 7.25 g l^{-1} agar was added to the medium. Autoclaving was done at 121°C and 1.06 kg cm^{-2} pressure for 20 minutes to sterilize the medium. The medium was allowed to cool and stored at culture room until use.

3.1.3 Maintenance of aseptic environment.

All the culture vessels, media and instruments used for handling tissues as well as the explants were sterilized by autoclaving or using sterilizing agents. All the aseptic measures were carried out in a clean laminar air flow cabinet fitted with UV lamp.

3.1.4 Culture conditions

The cultures were incubated under light intensity of 2500 lux and temperature of 25 ± 2°C with 16 h light/8 h dark cycle. Humidity in the culture room varied between 60 to 80 percent.

3.2 EVALUATION OF ELEPHANT FOOT YAM CALLI FOR SENSITIVITY TO ANTIBIOTICS

Sensitivity of elephant foot yam calli to antibiotics (Geneticin, Hygromycin and Ticarcillin) was evaluated to utilize it as a marker system for selection purpose.

3.2.1 Geneticin

The sensitivity of the elephant foot yam calli for geneticin was studied by culturing the calli in CIM containing different concentration of antibiotics ranging from 0 mg l^{-1} to 25mg l^{-1}.

Murashige and Skoog modified 3B- ½ concentration of NH_4NO_3 & KNO_3 with BA (0.5 mg l^{-1}), NAA (0.5 mg l^{-1}), 2, 4-D (0.5 mg l^{-1}) and Agar (7.25 mg l^{-1}) was used for the selection. The medium was prepared in conical flasks and stored at room temperature.

On the day of experiment the medium was melted and cooled to a temperature 40°C. The melted and cooled medium and the geneticin stock (100mg ml^{-1}) was taken to the laminar air flow cabinets. The required antibiotic concentration as per the treatment was added to the medium and mixed thoroughly by gentle swirling (with out any air bubble formation) and it was dispensed 15 ml to sterilised empty Petri dishes (90 x 15 mm) and allowed to cool and solidify. When the medium was cooled to room temperature and solidified, the calli were inoculated. The petri dishes were sealed with parafilm and kept in culture room.

The response of the calli at different antibiotic concentration was evaluated at periodic intervals (weekly) based on the scoring as follows:

Score	Culture Response
+	Turning black and dead
++	Partially discolored
+++	Healthy tissue

Similarly the procedure was repeated for hygromycin (0 mg l^{-1} to 50 mg l^{-1}) and ticarcillin (0 mg l^{-1} to 750 mg l^{-1}) (APPENDIX VIII).

3.3 AGROBACTERIUM MEDIATED TRANSFORMATION

3.3.1 Bacterial strains and binary vectors

Three strains of *Agrobacterium tumefaciens* viz, AGL0, LBA4404, GV3103 with 2 different binary vectors (viz, pOYE153, pCAMBIA 1305.2) were used for the study.

1. AGL0/pOYE 153

The T-DNA of pOYE153 vector contains the GUSA reporter gene under the control of CaMV35S promoter and the selectable marker gene *nptII.* AGL0 strain is cultured in YEB medium (Appendix II) with 2mM $MgSO_4$ and 80mg ml^{-1} kanamycin.

2. LBA4404 /pOYE 153

The T-DNA of pOYE153 vector contains the GUSA reporter gene under the control of CaMV35S promoter and the selectable marker gene *nptII.* LBA4404 strain is cultured in YEB medium with 2mM $MgSO_4$ and 80mg ml^{-1} kanamycin and 20 mg l^{-1} rifampicin.

3. GV3103/ pCAMBIA 1305.2

The T-DNA of pCAMBIA 1305.2 (APPENDIX IX) vector contains the GUSPlus reporter gene under the control of CaMV35S promoter and the selectable marker gene *hpt.* GV3103 strain is cultured in YEB medium with 2mM $MgSO_4$ and 80 mg ml^{-1} hygromycin and 20 mg l^{-1} rifampicin.

3.3.2 Preparation of *Agrobacterium* suspension for co-cultivation

Three *Agrobacterium* strains with two binary vectors were grown on petri plates with Luria Agar medium (LA) (Appendix II). *Agrobacterium* suspension for co-cultivation was prepared by picking a single colony from LA plate. This was inoculated in 5 ml YEB medium containing the required antibiotics and 2

mM $MgSO_4$. The inoculated medium was kept in shaking incubator 28°C with 200 rpm and incubated for 72 h.

The 3 days old YEB culture was inoculated in 25 ml YEB with appropriate antibiotics, 2 mM $MgSO_4$ and 200 µM acetosyringone. The inoculated medium was kept in shaker overnight. The overnight culture was spun in a centrifuge at 5000 rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet obtained was resuspended in 25 ml liquid MS medium with 200 µM acetosyringone. The centrifugation was repeated and the washed pellet was resuspended in 25 ml liquid MS with 200 µM acetosyringone.

3.3.3 Preparation of plant material for co-cultivation

Callus was used as the explants for co-cultivation experiments. The callus was precultured in CIM, one day before co-cultivation to be in active cell division. Three 1.0 cm^2 callus was subcultured in a 60 x 15 mm petri plate. The plate was incubated overnight in culture room.

3.3.4 Co-cultivation

The pre cultured callus was used for the co-cultivation experiments. Prepared *Agrobacterium* suspension was slowly pipetted (500 µl) on the calli and kept it for 3 minutes. After 3 minutes, excess *Agrobacterium* suspension was pipetted out from the plate. The plates were sealed with parafilm and incubated at 22°C in dark for 2 days.

3.3.5 Elimination of *Agrobacterium* after transformation

After co-cultivation, the callus was washed 5-6 times with Liquid MS containing 500 mg l^{-1} ticarcillin and blotted dry with sterile filter paper, but not allowed the callus to over dry. Then the callus were transferred to sterile petri plate containing regeneration media with 500 mg l^{-1} ticarcillin for elimination of *Agrobacterium* and incubated for one week.

3.3.6 Optimization of different parameters for transformation

Dfferent parameters affecting transformation efficiency ie., acetosyringone concentration, incubation temperature, number of days of co- cultivation and efficient Agrobacterium strain for transformation was studied.

Experimental design

For the optimisation of acetosyringone concentration, co- cultivation medium with 5 different concentration of acetosyringone ie., 0 µM, 100 µM, 200 µM, 300 µM and 400 µM was used for transformation.

The co cultivated calli were incubated at 4 different incubation temperatures ie., 20 °C, 22 °C, 24 °C, 26 °C and 28 °C for the optimisation of suitable incubation temperature of transformation.

For the optimisation of number of days of co cultivation, the calli were co cultivated for 0 days (The calli Washed just after transformation), 1 day, 2 days, 3 days and 4 days.3 replications was maintained for each treatment.

3.3.7 Selection of transformed tissue

After one week, the transformed tissues were selected on regeneration medium (Appendix I) containing ticarcillin 500 mg l^{-1}, geneticin 20 mg l^{-1} (for calli co-cultivated with AGL0/pOYE153 and LBA4404/pOYE153) and hygromycin 10 mg l^{-1} (for calli co-cultivated with GV3103/pCAMBIA1305.2). The tissues were maintained by sub culturing once in a month. The transformed tissues were grown in medium containing antibiotic for 8 months.

3.4 ANALYSIS OF TRANSFORMANTS

3.4.1 Gus histochemical assay

A histochemical assay was used to detect the expression of the GUS gene (β-glucuronidase). Transient expression of the GUS gene was visualised 24-30 h

after GUS staining. Transformed tissues were incubated in GUS Assay buffer (Appendix VI) containing 10 mg ml^{-1} X-gluc for 24- 30 h at 37°C in dark. Explants were rinsed several times with 70 percent ethanol to remove chlorophyll in order to bleach the explants and enhance the blue stain. GUS expressing cells were observed as blue spots on the explants under a microscope. Each spot was scored as one transformation event.

3.4.2 PCR Analysis

Total genomic DNA and RNA was isolated from each putative transformed and non-transformed plants for PCR analysis.

3.4.2.1 DNA Isolation

CTAB method of DNA extraction (Doyle and Doyle, 1990) with slight modifications was used for genomic DNA isolation.

β-mercaptoethanol was added fresh to the CTAB extraction buffer (Appendix IV) to give a final concentration of 0.2 percent (v/v). The solution was heated to 60°C in water bath (ROTEK, India). The samples (100 mg) were chilled and pulverized to a fine powder in liquid nitrogen using a sterile mortar and pestle and transferred in to a sterile 2 ml centrifuge tubes containing 1 ml of freshly prepared warm extraction buffer. The content was homogenized by gentle inversion. The samples were incubated at 60 °C in water bath for 30 min with intermittent shaking. Then it was centrifuged at 10,000 rpm for 10 min at RT. The supernatant was transferred to another sterile eppendorf tubes with a sterile pipette tip. To this 10 µl RNase was added and incubated at 37°C for 1 h. The homogenate was then extracted with an equal volume of 24: 1 (v/v) chloroform/isoamyl alcohol and mixed well by inversion for 5-10 min. The homogenate was centrifuged (Hermle, Table top refrigerated centrifuge) at 15000 rpm for 10 min at RT. To the aqueous phase, 0.8 volume of chilled isopropanol was added and mixed by inversion. The mixture was then incubated at -20°C for at least 1 h or overnight to precipitate the nucleic acid. After

incubation, the precipitated DNA was pelletized by centrifugation at 15000rpm for 10 min at 4 °C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (70 percent) twice, each time centrifuging at 12000 x *g* for 5 min at RT and discarding the supernatant. The pellet was air dried for 30-40 min and dissolved in 50 µl of sterile distilled water. The extracted DNA samples were then stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.4.2.2 Agarose gel electrophoresis

The most common method to assess the integrity of genomic DNA is to run an aliquot of the DNA sample on agarose gel. The gel was run using horizontal gel electrophoresis unit. Aliquot of PCR mix (10 µl) was loaded on agarose gel (0.8 percent) made of 0.5 X TAE buffer (Appendix III). The gel was run at 5 Vcm^{-1} until the dyes migrated $3/4^{th}$ of the distance through the gel. The gel was visualized and documented under the gel documentation system (Alpha Innotech) using 'Alpha Imager Software'.

3.4.2.2 PCR analysis

DNA samples isolated from putative transformants using the CTAB method were used for PCR analysis.

For the analysis of transformants of AGL0/pOYE153 and LBA4404/pOYE153, *nptII* gene was amplified using primers NPTII-loc (Forward) 5'- GCA CGT ACT CGG ATG GAA GCC-3' and NPTII-ups(Reverse) 5'- TCG CCG CCA AGC TCT TCA GC - 3' and GUS gene was amplified using GUSI (Forward) 5'- GGG CAT TCA GTC TGG ATC - 3' and GUSII (Reverse) 5'- GTG CGG ATT CAC CAC TTG – 3' primers.

For the analysis of transformants of GV3103/pCAMBIA, isolated DNA was amplified with *hpt* specific primer HygF (Forward) 5'- CGT CTG CTG CTC CAT ACA AG -3'and HygR (Reverse) 5'- ATT TCA TAT GCG CGA TTG CT-3'. GUS gene was amplified using GusplusF(Forward) 5'- TTA ACG AAG CGA

GCA ATG TG -3' primer and GusplusR (Reverse) 5'- GAC GCT TGG ATG GTT CTT GT -3'.

PCR Analysis with *nptII* specific primers

The components of the mixture were optimized as listed below:

Water	:	18.2 µl
10X PCR buffer	:	2.5 µl
dNTP (10 mM each)	:	1 µl
Forward primer (10 pmol μl^{-1})	:	0.5 µl
Reverse primer (10 pmol μl^{-1})	:	0.5 µl
Template DNA	:	2 µl
Taq DNA polymerase(0.05 U μl^{-1}):		0.3 µl
Total volume	:	25 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. Final extension was done at 72 °C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system.

PCR Analysis with GUS specific primers

The components of the mixture were optimized as listed below:

Water	:	17.2 µl
10X PCR buffer	:	2.5 µl
dNTP (10 mM each)	:	1 µl

Forward primer (10 pmol μl^{-1})	:	1 µl
Reverse primer (10 pmol μl^{-1})	:	1 µl
Template DNA	:	2 µl
Taq DNA polymerase (0.05 U μl^{-1})	:	0.3 µl
Total volume	:	25 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min. Final extension was done at 72 °C for 15 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system.

PCR Analysis with *hpt* specific primer

The components of the mixture were optimized as listed below:

Water	:	17.2 µl
10X Taq buffer A	:	2.5 µl
dNTP (10 mM each)	:	1 µl
Forward primer (10 pmol μl^{-1})	:	1 µl
Reverse primer (10 pmol μl^{-1})	:	1 µl
Template DNA	:	2 µl
Taq DNA polymerase (0.05 U μl^{-1})	:	0.3 µl
Total volume	:	25 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and

extension at 72 °C for 1 min. Final extension was done at 72 °C for 15 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system.

PCR Analysis with GusPlus specific primer

The components of the mixture were optimized as listed below:

Water	:	18.8 µl
10X PCR buffer	:	2.5 µl
dNTP (10 mM each)	:	1 µl
Forward primer (10 pmol μl^{-1})	:	1.2 µl
Reverse primer (10 pmol μl^{-1})	:	1.2 µl
Template DNA	:	2 µl
Taq DNA polymerase (0.05 U μl^{-1})	:	0.3 µl
Total volume	:	25 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1min and extension at 72 °C for 1 min. Final extension was done at 72 °C for 15 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system.

3.4.3 ANALYSIS OF TRANSFORMANTS BY BLOTTING

3.4.3.1 Probe preparation

Probe was prepared with Thermo Scientific™ North2South™Biotin Random Prime Labelling Kit.

The random prime labelling method is based on the procedure of (Feinberg and Vogelstein, 1983) wherein random hepta nucleotides containing all possible sequences anneal to a denatured DNA template and act as primers for complementary strand synthesis by DNA Polymerase (Klenow fragment, 3′-5′ exo). Inclusion of biotinylated nucleotides in the reaction mixture ensures that the newly synthesized DNA strands are labelled with biotin.

3.4.3.2 Labelling Reaction

In a micro centrifuge tube, ~100ng of linear DNA (PCR elute of *nptII* or *hpt* gene) was diluted for labelling to a final volume of 24µl in nuclease-free water. For the positive control, 0.5 µl of control DNA (125 ng) was diluted to 24 µl. 10 µl of hepta nucleotide mix was added and the DNA template was denatured by boiling the tube for 5 minutes. Quickly froze the denatured DNA solution by placing the tube on ice for 5 minutes. Briefly centrifuged the tube to collect liquid at the bottom of the tube and the tube was placed on ice.

In the order stated, following components were added to the prepared sample solution on ice:

- 10 µl of 5 X dNTP mix
- 5 µl of 10 X Reaction buffer
- 1 µl Klenow fragment

(Final volume = 50 µl)

Contents were mixed by flicking or brief vortexing of the tube. Centrifuged briefly to collect liquid at the bottom of the tube. The labelling reaction was incubated for 60 minutes at 37°C and the enzyme was inactivated by adding 2 µl of 500 mM EDTA, pH 8.0. The reaction was adjusted to contain 0.5M ammonium acetate by adding 5 µl of 5 M NH_4OAc to 50 µl labelling reaction and mixed well. 2 volumes of 100 percent ethanol was added to the above reaction mixture and mixed well. Chilled the tube at -20°C for 15 minutes followed by centrifugation at 4°C for 30-60 minutes at maximum speed (> 10,000 X *g*). The supernatant was discarded carefully and washed the DNA pellet once by adding ice-cold 70 percent ethanol and centrifuging for 30-60 minutes at maximum speed. Carefully discarded the supernatant and the pellet was dissolved in 100 µl of 1 X TE (APPENDIX V) and store at -20°C.

3.4.3.3 Estimation of probe labelling efficiency

Probe labelling efficiency was estimated using 'Thermo Scientific Chemiluminescent Nucleic Acid Detection Module'. It is a complete system for the detection of biotin-labelled nucleic acids for various blotting applications including Northern/Southern blots.

3.4.3.4 Dot blot by hand spotting

A positively-charged nylon membrane was equilibrated in TE Buffer for at least 10 minutes. Dilutions of the labeled DNA were prepared from both the control DNA and the experimental probe with TE from 10^{-1} to 10^{-7}. The equilibrated membrane was placed on to a clean, dry paper towel and allowed excess buffer to absorb into the membrane, but did not allow the membrane dry out. 2 µl of samples were spotted onto the hydrated membrane and allowed the samples to absorb into the membrane. Immediately, UV cross linked the membrane by placing the nucleic acid side facing the UV radiation for 66 s.

For the detection and analysis of UV cross linked DNA, the membrane was blocked by adding blocking buffer and incubated for 15 minutes with gentle shaking.

Conjugate/blocking buffer solution was prepared by adding 50 µl of the stabilized Streptavidin-Horseradish Peroxidase conjugate to 16 ml blocking buffer (1:300 dilution) and it was a added to the container after decanting blocking buffer from the membrane and incubated for 15 minutes with gentle shaking. Membrane was transferred to a new container and rinsed briefly with 20 ml of 1 X wash solution and it was washed four times for 5 minutes each in 20 ml of 1 X wash solution with gentle shaking. Then the membrane was incubated for 5 minutes with gentle shaking in 30 ml of substrate equilibration buffer in a new container. Chemiluminescent substrate working solution was prepared by adding 6 ml Luminol/Enhancer Solution to 6 ml stable peroxide solution. The membrane was removed from substrate equilibration buffer and carefully blotted an edge of the membrane on a paper towel to remove excess buffer. The membrane was placed in a clean container or onto a clean sheet of plastic wrap placed on a flat surface. Then the membrane was placed nucleic acid side down onto a puddle of the working solution and incubated for 5 minutes without shaking. The membrane was removed from the working solution and blotted on a paper towel for 2-5 seconds to remove excess buffer without allowing the membrane to become dry. Moist membrane was wrapped in plastic wrap and placed in film cassette. A X-ray film was exposed to membrane for 4 minutes and then the film was developed by immersing in developer solution for 3 minutes followed by 1 minute rinsing in distilled water and 3 minutes in Fixer solution. Finally, the developed X- ray film was washed in running tap water and air dried.

3.4.3.5 Nucleic acid spot hybridization

Nucleic acid spot hybridization was performed with North2South® Chemiluminescent Hybridization and Detection Kit. This system combines an enhanced luminol substrate for horseradish peroxidase (HRP) with optimized

hybridization and blocking conditions that ensure consistent results with sensitivity equal to or exceeding ^{32}P.

Positively charged nylon membrane of appropriate size was used for hybridization and it was equilibrated in TE Buffer for at least 10 minutes. 4 µl sample was spotted on the membrane and UV cross linked by the nucleic acid side facing the UV radiation for 66 sec. The blot was placed in hybridisation bottles and sufficient hybridisation buffer was added to completely cover the blot. The blot was prehybridised for at least 30 minutes at 55°C for DNA hybrids and 65°C for RNA: RNA hybrids. At the end of prehybridisation reaction, the biotinylated DNA probe (*nptII* probe for LBA4404/pOYE153 and AGL0/pOYE153 transformants, *hpt* probe for GV3103/pCAMBIA1305.2 transformants) was denatured by heating at 100°C for 10 minutes and quickly placed in ice for 5 minutes. After prehybridisation, the denatured probe (10 µl) was added to the hybridisation buffer and incubated overnight with shaking at 55°C.

On the next day, the North2South® Hybridization stringency wash buffer (2 X) was equilibrated to RT and prepared 1 X concentration of the same in sterile ultrapure water. Stringency washes were performed by washing the blot three times with 1 X stringency wash buffer for 15-20 minutes per wash with agitation at 55°C.

The stringency wash buffer was decanted and sufficient blocking buffer was added generously to cover the membrane and incubated for 15 minutes with shaking or rotating at RT. Next, streptavidin-HRP conjugate (1:300) was added to the blocking buffer and incubated for 15 minutes at RT with agitation. After the addition of streptavidin-HRP conjugate, the membrane was washed four times for 5 minutes with 1 X wash buffer at RT with agitation. Substrate equilibration buffer was added to new container and placed the blot and incubated for 5 minutes at RT with agitation.

The moist membrane was placed on a tray or a piece of plastic wrap and covered with the substrate working solution and incubated for 5 minutes at RT

and ensured that the membrane was fully covered with substrate. Substrate solution was drained from the membrane surface and the moist membrane was transferred to a cassette and covered with plastic wrap and then exposed to X-ray film and developed as described for estimation of probe quality.

3.4.3.6 Southern Hybridisation

The DNA samples and the isolated plasmids (GenElute Plasmid Miniprep Plasmid isolation Kit, SIGMA-ALDRICH) of AGL0/pOYE153, LBA4404/pOYE153 and GV3103/pCAMBIA (positive control) were restriction digested with *Eco*RI restriction enzyme.

Restriction digestion

Reaction mix contains:

Tango Buffer - 6µl

DNA - 10µl

*Eco*RI - 2µl

Nuclease free water - 12µl

Incubated the reaction mixture at 37°C for 18 h

Southern blotting

Agarose gel (0.8 percent) was prepared with 1X TAE and added 1µl of ethidium bromide. Restricted samples were loaded on the prepared gel and run at 80V till full separation of bands.

To fragment long DNA, the gel was depurinated in 0.5N HCl for 45 minutes at 37°C with gentle shaking. After depurination (APPENDIX VII), the gel was rinsed with sterile distilled water. Then the DNA was denatured by placing the gel in 250 ml denaturation solution (APPENDIX VII) for 45 minutes at RT with gentle shaking. The denaturation solution was washed out from the gel with sterile distilled water. Again the gel was soaked in 250 ml neutralization solution (APPENDIX VII) for 45 minutes at RT.

DNA Transfer

Kept the gel tray (Used for casting the gel) upside down in a tray containing 200 ml of 20X SSC (APPENDIX VII). A Whatman No.3 filter paper wick was placed on the gel tray platform in such a way that the ends are dipped well in 20X SSC and wet it with 20X SSC. The air bubbles were removed by rolling a sterile glass rod on it. The gel was laid on the wick such that the bottom even side of the gel faces up; again a glass rod was rolled on it to remove the air bubbles.

Nylon membrane was cut to the size of the gel and wetted in sterile distilled water followed by 20X SSC. The membrane was laid over the gel without any air bubbles. Similarly, two Whatman No.3 filter paper was cut to the size of the gel and wetted with 20X SSC and placed it over the membrane. Then a dry whatman No.3 filter paper to the size of the gel was placed over the wet filter papers. Above this crude filter papers to the gel size were stacked on top to a height of 8-10 cm with weight over it and allowed for 18 h to transfer takes place. The Nylon membrane was removed from the blot and rinsed briefly with 20X SSC. Air dried membrane was UV cross linked by placing the nucleic acid side down on UV transilluminator for 66 seconds. Hybridisation was performed with North2South® Chemiluminescent Hybridization and Detection Kit and developed on an X-ray film.

Results

4. RESULTS

The results of the study entitled "Genetic transformation of *Amorphophallus paeoniifolius* (Dennst.) Nicolson" conducted at the Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014 are presented in this chapter.

4.1 ESTABLISHMENT OF *IN VITRO* CULTURE

4.1.1 Callus Induction

Callus cultures were initiated using petiole and leaves of *in vitro* plantlets of elephant foot yam cv. Gajendra in callus induction medium. The calli developed from the explants after four weeks were globular, hard and creamy white in color. These calli exhibited proliferation without differentiation. The uppermost and peripheral cells of the callus were actively dividing.

When maximum growth was observed, subculture was done by excising the matured calli into 4-5 clumps of 1cm^2 size and placed on fresh callus induction medium. Subculture was repeated periodically with 20 days interval, when the callus biomass increased two to four times. Fifteen days old calli were found to be suitable for transformation study. Calli sufficient for the transformation study were obtained after 3 months of subculture (Plate 1.).

4.2 SENSITIVITY OF ELEPHANT FOOT YAM CALLI TO ANTIBIOTICS

Experiments were carried out to evaluate the sensitivity of the elephant foot yam calli to Geneticin, Hygromycin and Ticarcillin.

4.2.1 Geneticin

Calli were tested for their sensitivity for geneticin (Plate 2.). The response of the calli at different antibiotic concentration was weekly evaluated.

The growth and proliferation of elephant foot yam calli was partially inhibited by geneticin at lower concentrations (10 to 15 mg l^{-1}), and almost completely inhibited at concentrations of 20 mg l^{-1} or higher (Table 1). On medium supplemented with geneticin, a partial discoloration was observed from

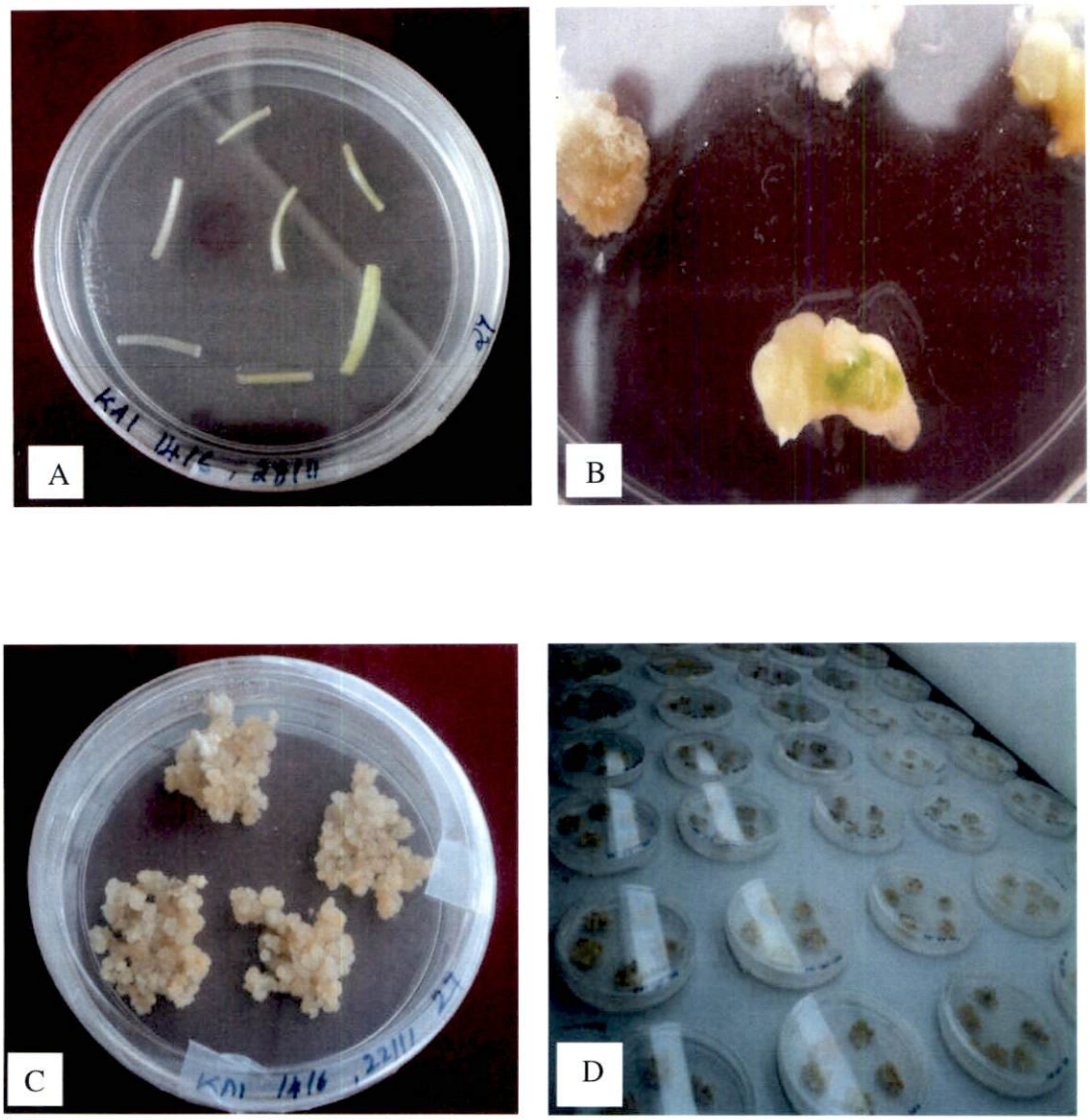


Plate 1. Establishment of *in vitro* culture of elephant foot yam

(A) Petiole as explants for callus multiplication (B) Initiated calli from petiole (C) Proliferated Callus (20 days after subculture) (D) Mass multiplication of calli

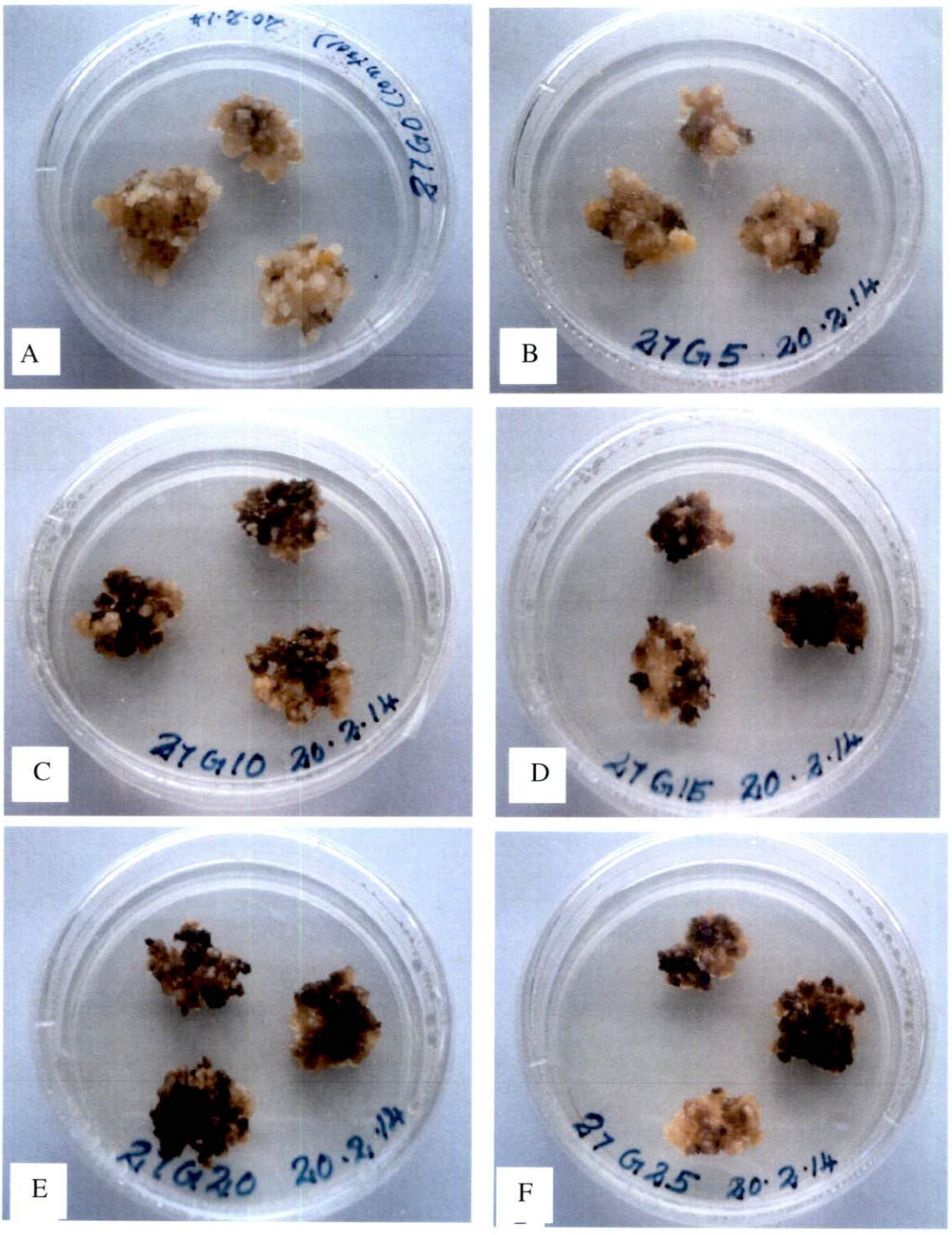


Plate 2. Sensitivity of elephant foot yam tissues to geneticin

(A) Control (Without geneticin) (B) Geneticin 5 mg l^{-1} (C) Geneticin 10 mg l^{-1}

(D) Geneticin 15 mg l^{-1} (E) Geneticin 20 mg l^{-1} (F) Geneticin 25 mg l^{-1}

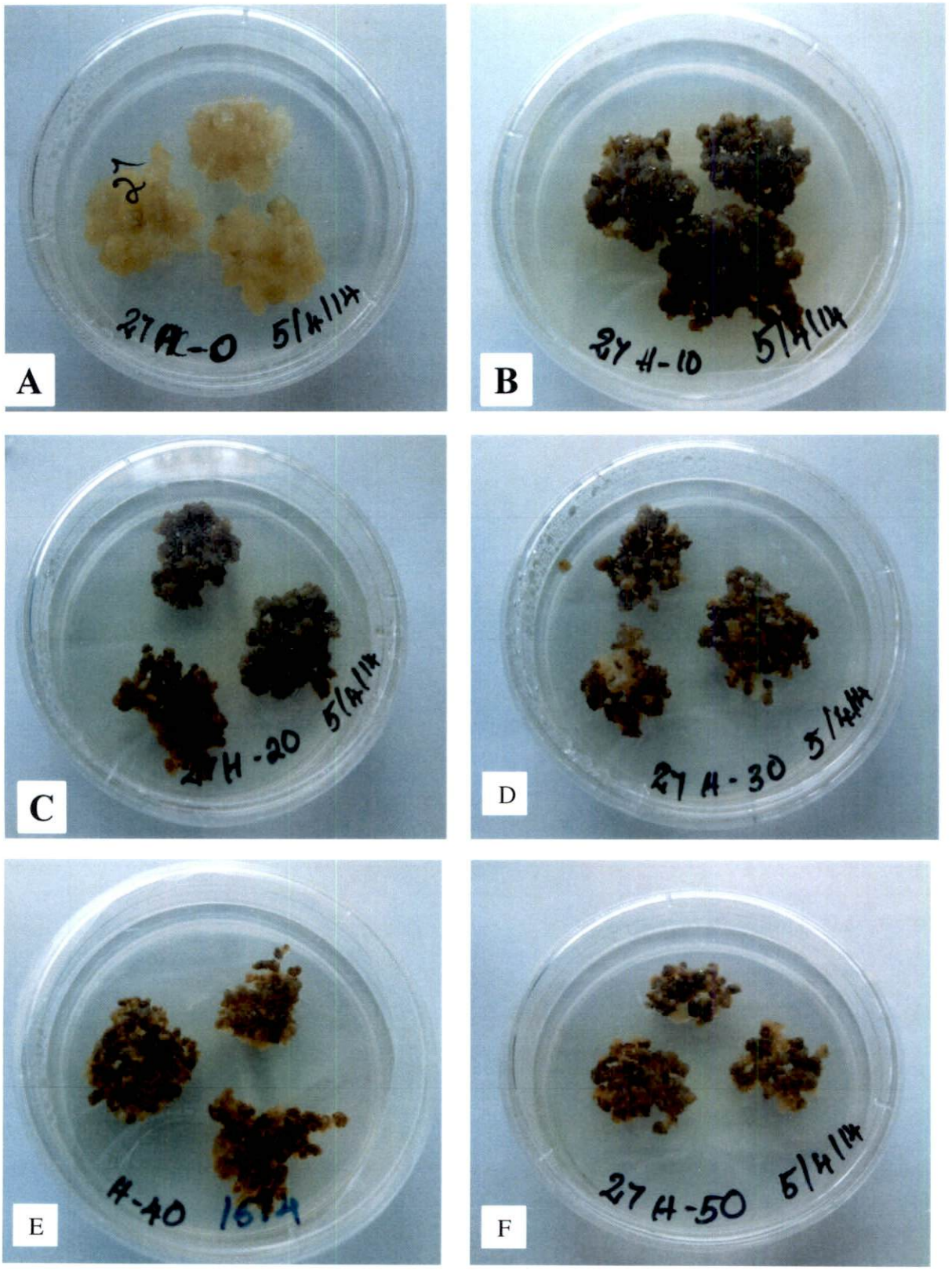


Plate 3. Sensitivity of elephant foot yam tissues to hygromycin

(A) Control (Without hygromycin) (B) Hygromycin 10 mgl^{-1} (C) Hygromycin 20 mgl^{-1} (D) Hygromycin 30 mgl^{-1} (E) Hygromycin 40 mgl^{-1} (F) Hygromycin (50 mgl^{-1})

Table 1. Sensitivity of calli to different doses of geneticin

Sl. No	Geneticin (mgl^{-1})	Sensitivity (Weeks)					
		I Week	II Week	III Week	IV Week	V Week	VI Week
1	0	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)
2	5	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	++ (2/9)
3	10	+++ (9/9)	++ (3/9)	++ (6/9)	++ (6/9)	++ (8/9)	++ (8/9)
4	15	+++ (9/9)	++ (6/9)	+ (8/9)	+ (8/9)	+ (8/9)	+ (8/9)
5	20	+++ (9/9)	++ (7/9)	+ (8/9)	+ (9/9)	+ (9/9)	+ (9/9)
6	25	++ (8/9)	+ (7/9)	+ (8/9)	+ (9/9)	+ (9/9)	+ (9/9)

Mean of 9 replications:

+++: Healthy tissue

++ : Partially discolored

+ : Turning black and dead

the first week itself and it leads to complete dead and black tissues in the second week. From the second week onwards partially discolored calli was observed for lower concentrations of geneticin (10 mg l^{-1}) (Figure 3).

The sensitivity calli to geneticin was measured in terms of concentrations of geneticin that caused 50 percent (half-lethal dose, LD_{50}), 90 percent (LD_{90}), and 100 percent (lethal dose, LD_{100}) death of explants. The LD_{50}, LD_{90} and LD_{100} of geneticin were ~7, ~12 and 20 mg l^{-1} respectively on after 6 weeks of culture.

4.2.2 Hygromycin

Calli were tested for their sensitivity for hygromycin (Plate 3.). The response of the calli at different antibiotic concentration was weekly evaluated.

The calli showed complete death and discoloration at lower concentration of hygromycin (10 mg l^{-1}) (Table 2). On the second week onwards, callus in 30 mg l^{-1} hygromycin showed partial discoloration. On the third week, completely dead calli was observed in 50 mg l^{-1} hygromycin (Figure 4).

The sensitivity of calli to hygromycin was measured in terms of concentrations of hygromycin that caused 50 percent (half-lethal dose, LD_{50}), 90 percent (LD_{90}), and 100 percent (lethal dose, LD_{100}) death of explants. The LD_{50}, LD_{90} and LD_{100} of hygromycin were ~5 mg l^{-1} on 6 weeks of culture.

4.2.3 Ticarcillin

To observe the effect of ticarcillin on callus growth, calli were cultured on medium containing different concentration of ticarcillin (0 mg l^{-1} -750 mg l^{-1}). The response of the calli at different antibiotic concentration was weekly evaluated (Plate. 4).

The calli were fully healthy and multiplied in all the treatments for the first 3 weeks (Table 3.) In the fourth week the calli in ticarcillin 750 mg l^{-1} showed

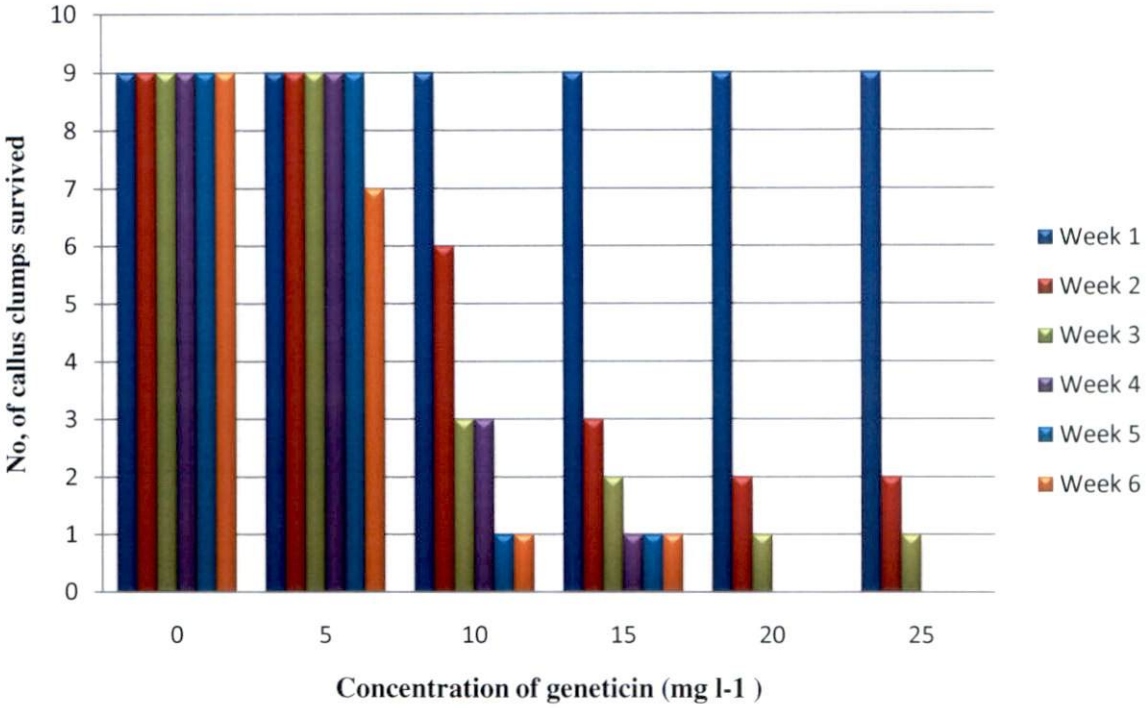


Figure 3. Sensitivity of calli to different doses of geneticin

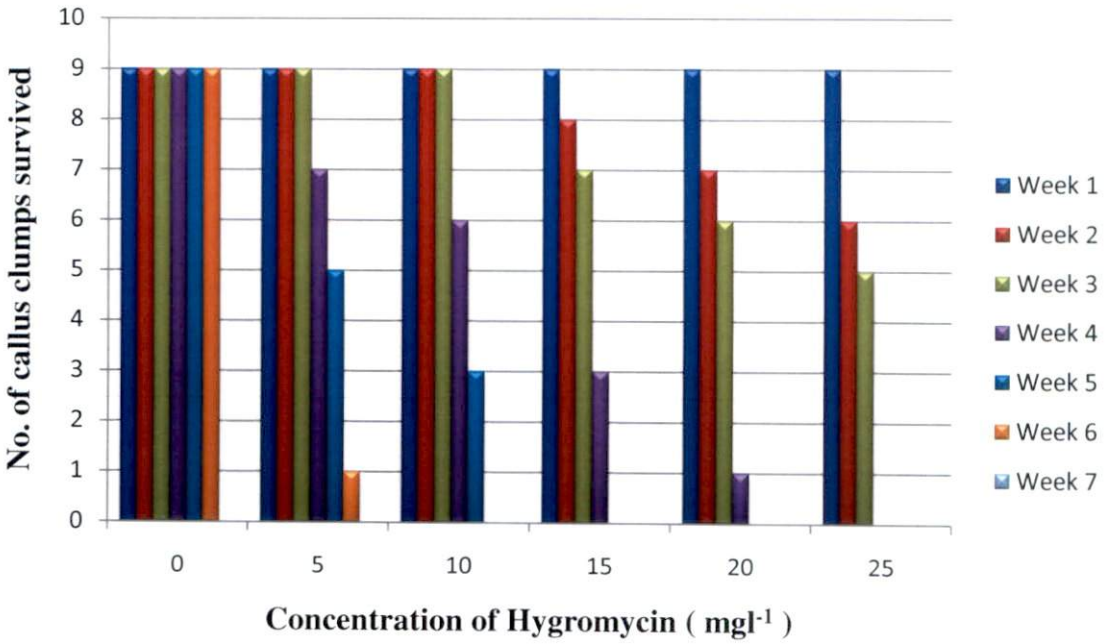


Figure 4. Sensitivity of calli to different doses of hygromycin

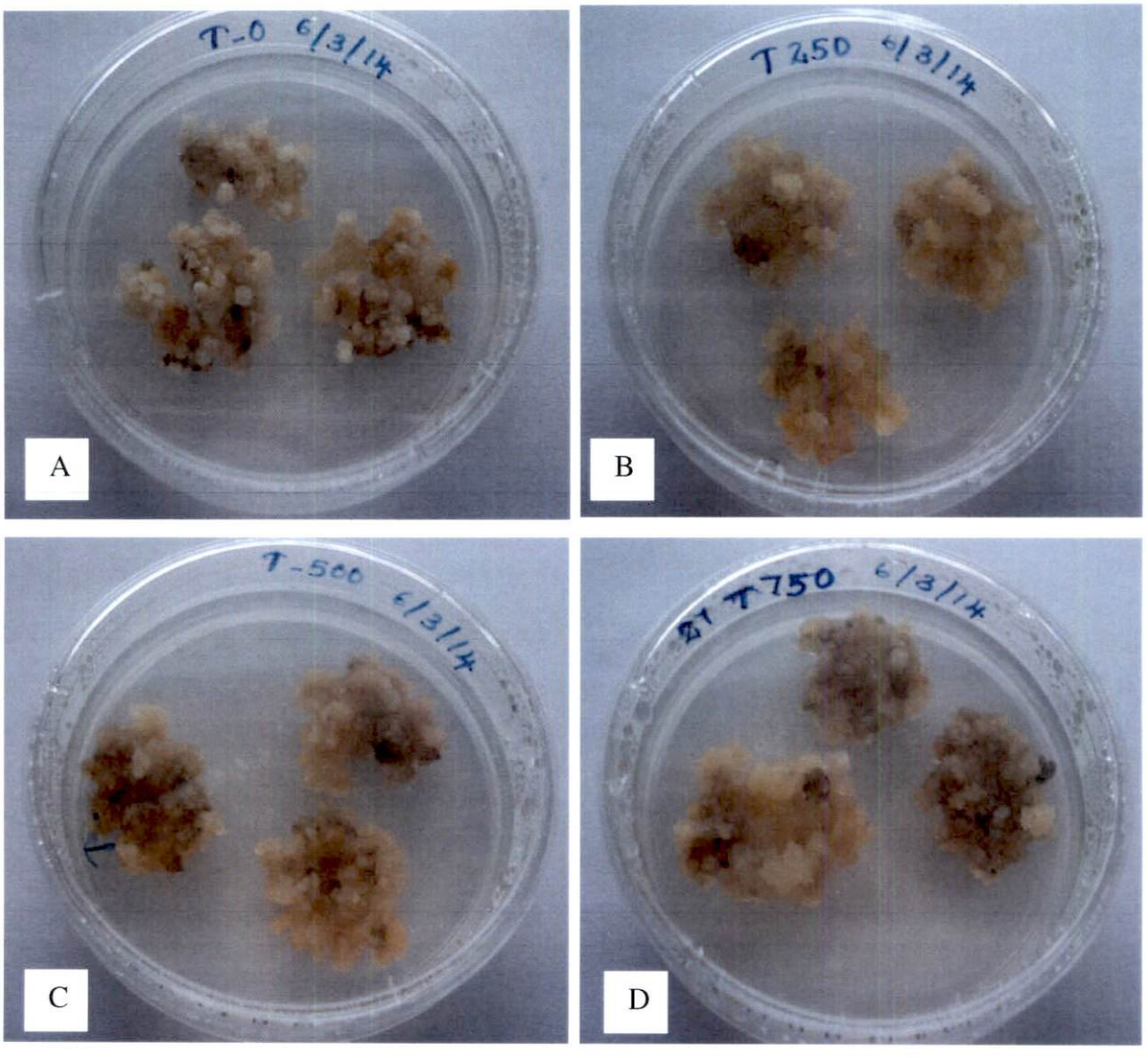


Plate 4. Sensitivity of elephant foot yam calli to ticarcillin

(A) Control (Without ticarcillin) (B) Ticarcillin 250 mg l^{-1} (C) Ticarcillin 500 mg l^{-1} (D) Ticarcillin 750 mg l^{-1}

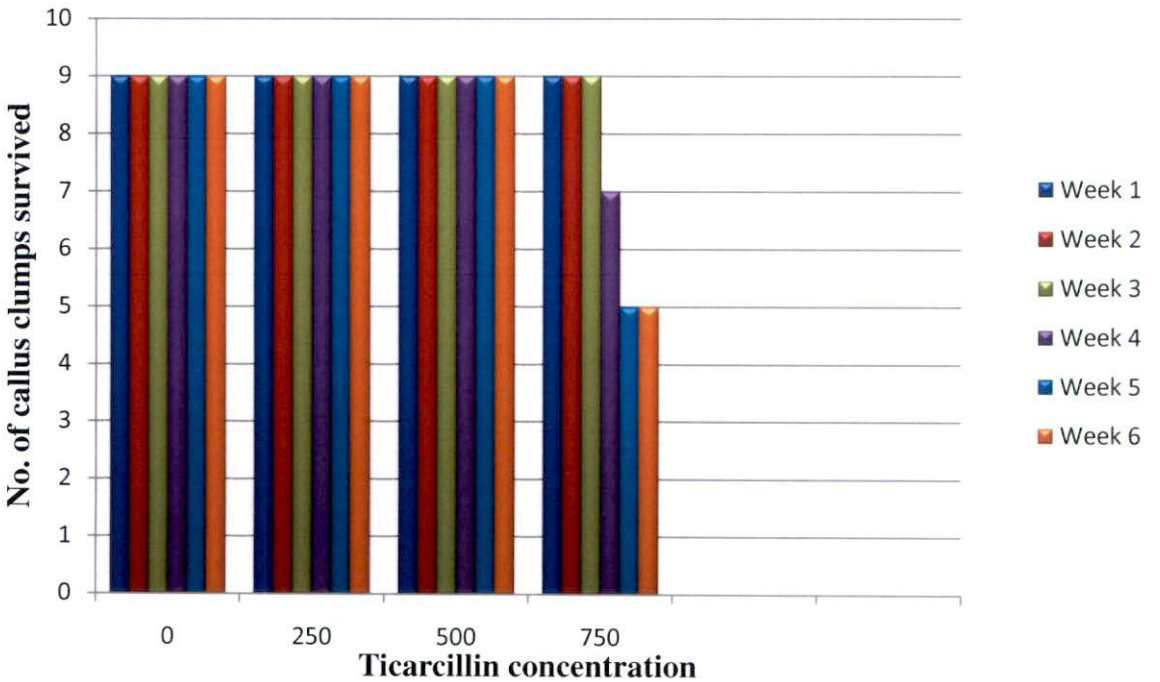


Figure 5. Sensitivity of Calli to different doses of ticarcillin

Table 2. Sensitivity of calli to different doses of hygromycin

Sl. No	Hygromycin (mgl^{-1})	Sensitivity (weeks)						
		I Week	II Week	III Week	IV Week	V Week	VI Week	VII Week
1	0	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)
2	10	+++ (9/9)	+++ (9/9)	+++ (9/9)	++ (3/9)	++ (6/9)	+ (8/9)	+ (9/9)
3	20	+++ (9/9)	+++ (9/9)	+++ (9/9)	++ (4/9)	+ (6/9)	+ (9/9)	+ (9/9)
4	30	+++ (9/9)	++ (8/9)	++ (9/9)	+ (9/9)	+ (9/9)	+ (9/9)	+ (9/9)
5	40	+++ (9/9)	++ (9/9)	++ (9/9)	++ (9/9)	+ (9/9)	+ (9/9)	+ (9/9)
6	50	+++ (9/9)	++ (9/9)	+ (9/9)	+ (9/9)	+ (9/9)	+ (9/9)	+ (9/9)

Mean of 9 replications:

+++: Healthy tissue

++ : Partially discolored

+ : Turning black and dead

Table 3. Sensitivitv of calli to different doses of ticarcillin

Sl. No	Ticarcillin (mgl^{-1})	Sensitivity (weeks)					
		I Week	II Week	III Week	IV Week	V Week	VI Week
1	0	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)
2	250	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)
3	500	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)	+++ (9/9)
4	750	+++ (9/9)	+++ (9/9)	+++ (9/9)	++ (2/9)	+ (4/9)	+ (4/9)

Mean of 9 replications:

+++: Healthy tissue

++ : Partially discolored

+ : Turning black and dead

partial discoloration and the extent of discoloration was increased on subsequent weeks (Figure 5). The calli on ticarcillin 0 mgl^{-1} to 500 mg l^{-1} was observed healthy in all the treatments. With the statistical analysis using ANOVA the lowest concentration of ticarcillin lethal to calli is ~ 650 mg l^{-1}(LD0 ~ 650 mg l^{-1}).

4.3 *AGROBACTERIUM*-MEDIATED TRANSFORMATION

4.3.1 Preparation of *Agrobacterium* suspension for co- cultivation

The overnight grown culture (18 h) of *Agrobacterium* with OD_{600} 0.6-0.8 was found to be suitable for *Agrobacterium* mediated transformation in elephant foot yam calli. It was also observed that overgrown cultures of *Agrobacterium* suspension significantly reduced the number of tissues transformed and resulted in the death of explants.

4.3.3 Effect of acetosyringone concentration on transformation efficiency

Agrobacterium strains were cultured in YEB medium containing different concentration of acetosyringone (0 µM, 100 µM, 200 µM, 300 µM, 400 µM). These cultured strains were used for transformation of elephant foot yam calli.

Among the different treatments, highest average calli (34.2860) showing GUS positive was obtained from 100µM acetosyringone with the LBA4404/pOYE153 strain. But 100µM acetosyringone is not sufficient to produce transformants (0.000) with GV3103/pCAMBIA. AGL0/pOYE153 gave maximum number of GUS stained calli (28.2391) with 400 µM acetosyringone. GV3103/pCAMBIA also required 400 µM acetosyringone to give maximum number of transformants (21.5896) (Table 4).

Table 4. Effect of acetosyringone concentration on transformation efficiency of elephant foot yam calli.

Agrobacterium strains	Average number of GUS positive calli at acetosyringone concentration					
	0µM	100µM	200µM	300µM	400µM	Mean of different *Agrobacterium* strains.
AGL0/pOYE153	0.9249 (1.3874)	4.9799 (2.4453)	16.0715 (4.1317)	19.1713 (4.4902)	28.2391 (5.4073)	11.7622 ^{b}(3.5724)
LBA4404/pOYE153	17.0931 (4.2536)	34.2860 (5.9402)	26.1681 (5.2123)	17.0931 (4.2536)	8.9932 (3.1612)	19.8318 ^{a}(4.5641)
GV3103/pCAMBIA1305.2	0 (1.0000)	0 (1.0000)	6.9838 (2.8255)	10.9159 (3.4519)	21.5896 (4.7528)	5.7916 ^{c}(2.6060)
Mean of each treatment	3.9004 ^{d}(2.2137)	8.7875 ^{c}(3.1285)	15.456 ^{b}(4.0566)	15.5266 ^{b}(4.0653)	18.7180 ^{a}(4.4405)	

* Figure in the parenthesis shows the square root of transformed values

* Mean value with same alphabet in the superscript does not differ significantly.

* The data is average of three replicates

Comparison of different treatments showed that treatment with 200 μM and 300 μM concentration of acetosyringone gave significantly similar mean of transformants, whereas 100 μM and 400 μM significantly gave a different set of results. The lowest number of transformants was obtained with 0 μM acetosyringone. The total number of transformants with 400 μM of acetosyringone was maximum when compared to that of other treatments (Figure 6 and 7).

4.3.2 Effect of number of days of co-cultivation on transformation efficiency

Calli were co cultivated with *Agrobacterium* for further infection process in dark condition for zero, one, two, three and four days at 26°C in culture room. The effect of number of days of co cultivation on transformation was compared on the GUS expression of 14 day old selected calli.

All GUS activity values were pooled from three separate replications and averaged with a standard deviation at each day. The highest average of GUS activity (23.9577) was obtained from 2 days co cultivated calli. All the treatments are statistically significant except Day1 (2.1729) and Day 4 (1.9181). Statistically lowest average of GUS activity was observed in Day 0 (1.5017) (Table 5).

Least square means of different treatments of strains, showed that maximum number of transformants were obtained from the explants co cultivated with LBA4404/pOYE153 for 2 days, followed by AGL0/pOYE153 strain for 2 days (Figure 8 and Figure 9).

4.3.4 Effect of incubation temperature of co-cultivation on transformation efficiency

Temperature has been considered as a factor affecting the efficiency of Agrobacterium to transfer the T-DNA to plant cells. The present investigation of the effect of temperature during co cultivation in elephant foot yam calli revealed that it plays an important role in transformation efficiency. Temperature of 28°C was found to be optimal to support the highest transient transformation frequency in elephant foot yam and there was no significant difference between 22°C, 24°C

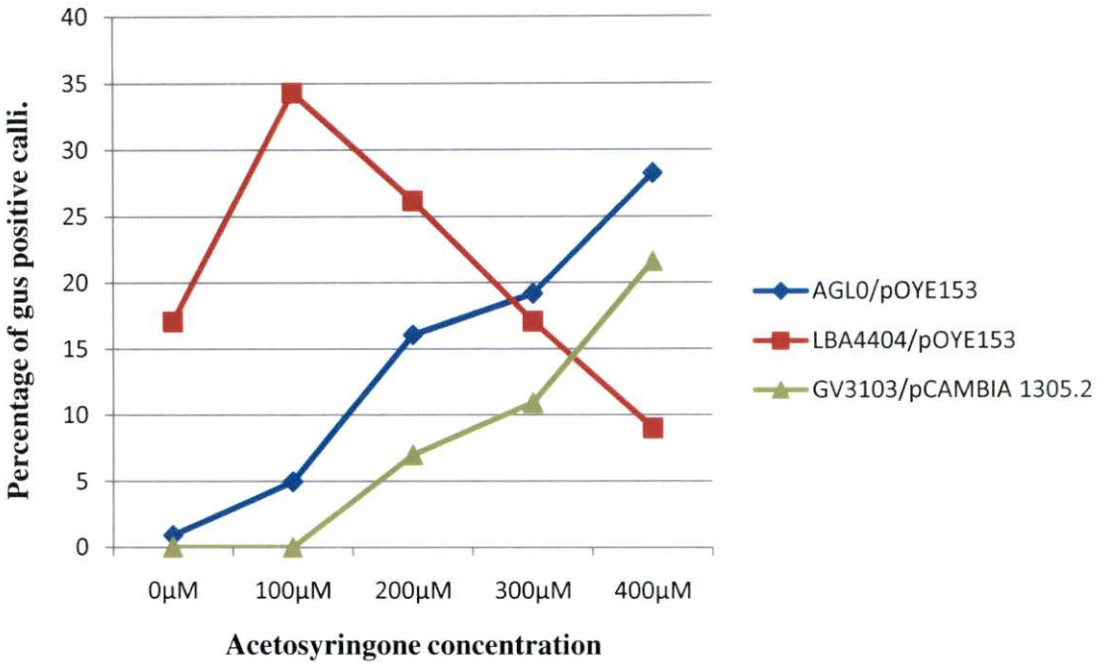


Figure 6. Transformation efficiency of 3 different *Agrobacterium* strains at different acetosyringone concentration

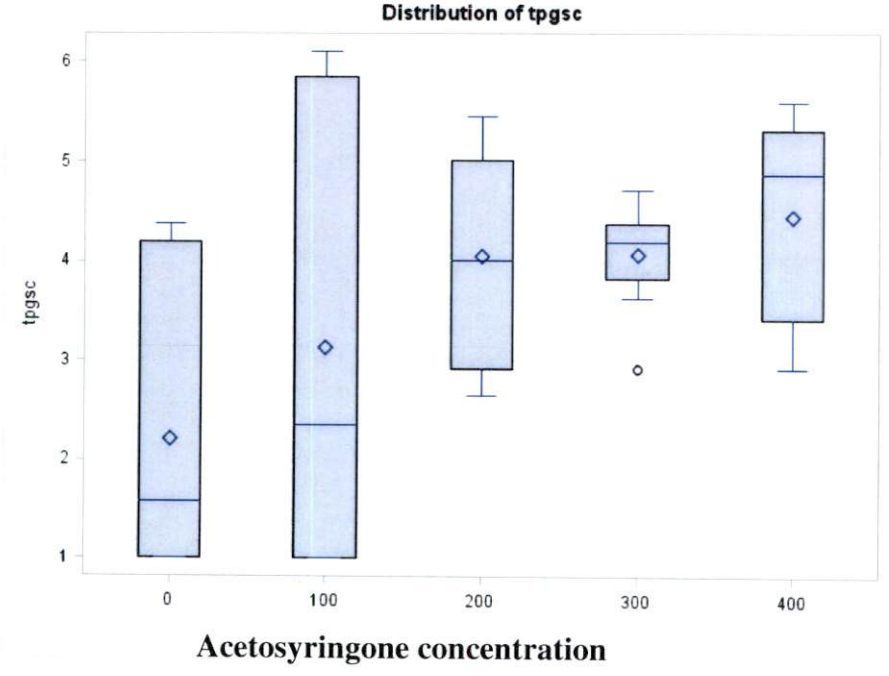


Figure 7. Distribution of transformed calli at different acetosyringone concentration

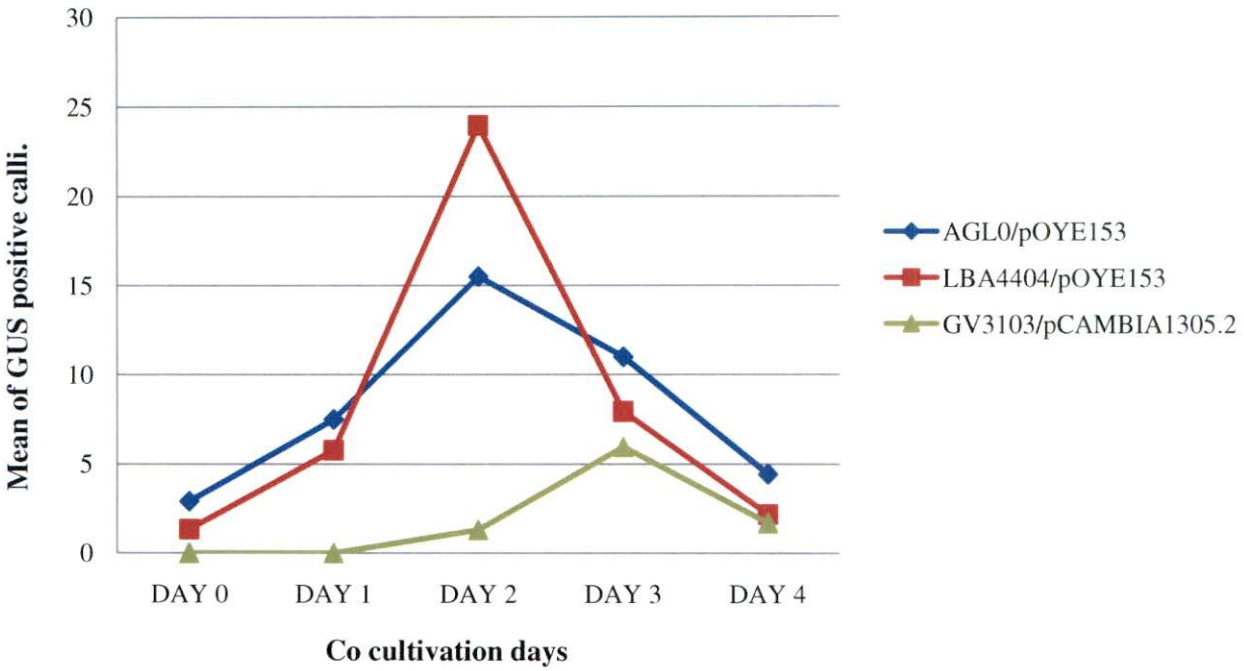


Figure 8. Transformation efficiency of 3 different *Agrobacterium* strains at different days of co cultivation

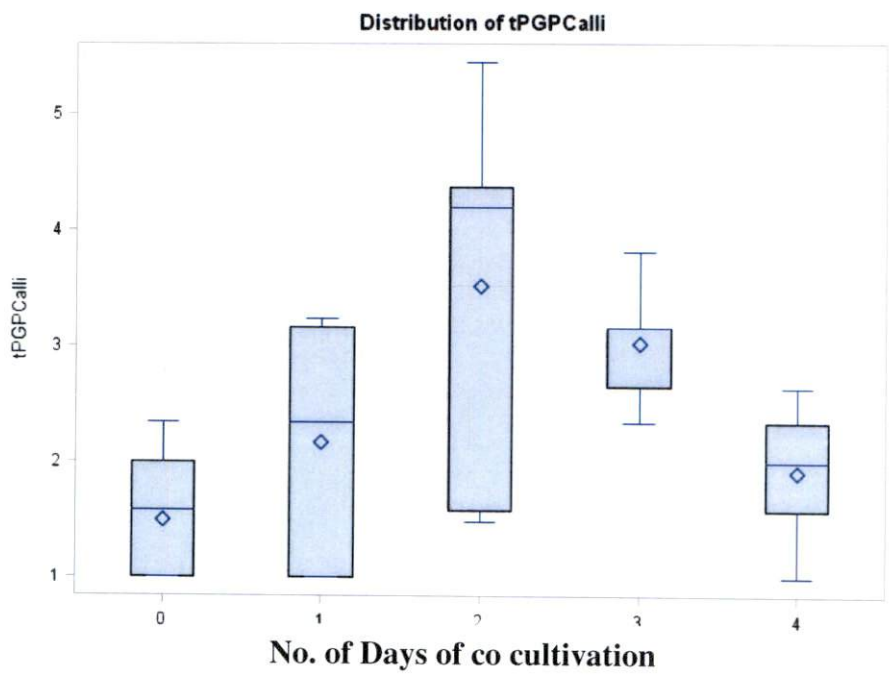


Figure 9. Distribution of transformed calli at each day of co-cultivation

and 26°C treatments (Table 6). Statistical analysis of the number of transformants obtained from different strains showed that higher temperature is favourable for AGL0 with pOYE153 (31.2847) and GV3103 with pCAMBIA 1305.2 (27.0496) whereas LBA4404/pOYE153 gave maximum efficiency at 22°C (24.6083) and the efficiency decreased with increase in temperature (Figure 10 and Figure 11).

4.4 SELECTION OF TRANSFORMANTS

Transformants were selected on regeneration medium containing ticarcillin 500 mg l^{-1}, geneticin 20 mg l^{-1} (for calli co-cultivated with AGL0/pOYE153 and LBA4404/pOYE153) and hygromycin 10 mg l^{-1} (for calli co-cultivated with GV3103/pCAMBIA1305.2). The tissues were maintained by sub culturing once in a month. In eight weeks, the transformed tissue developed green sprouts. The transformed tissues were grown in medium containing antibiotic for 8 months (Plate 5 –Plate 8).

4.5 DNA ISOLATION

Two month old GUS positive transformed calli were used for genomic DNA extraction by CTAB method. The agarose gel electrophoresis (0.8 perecent) of the extracted genomic DNA showed the presence of good quality and unsheared bands on the gel (Figure 12).

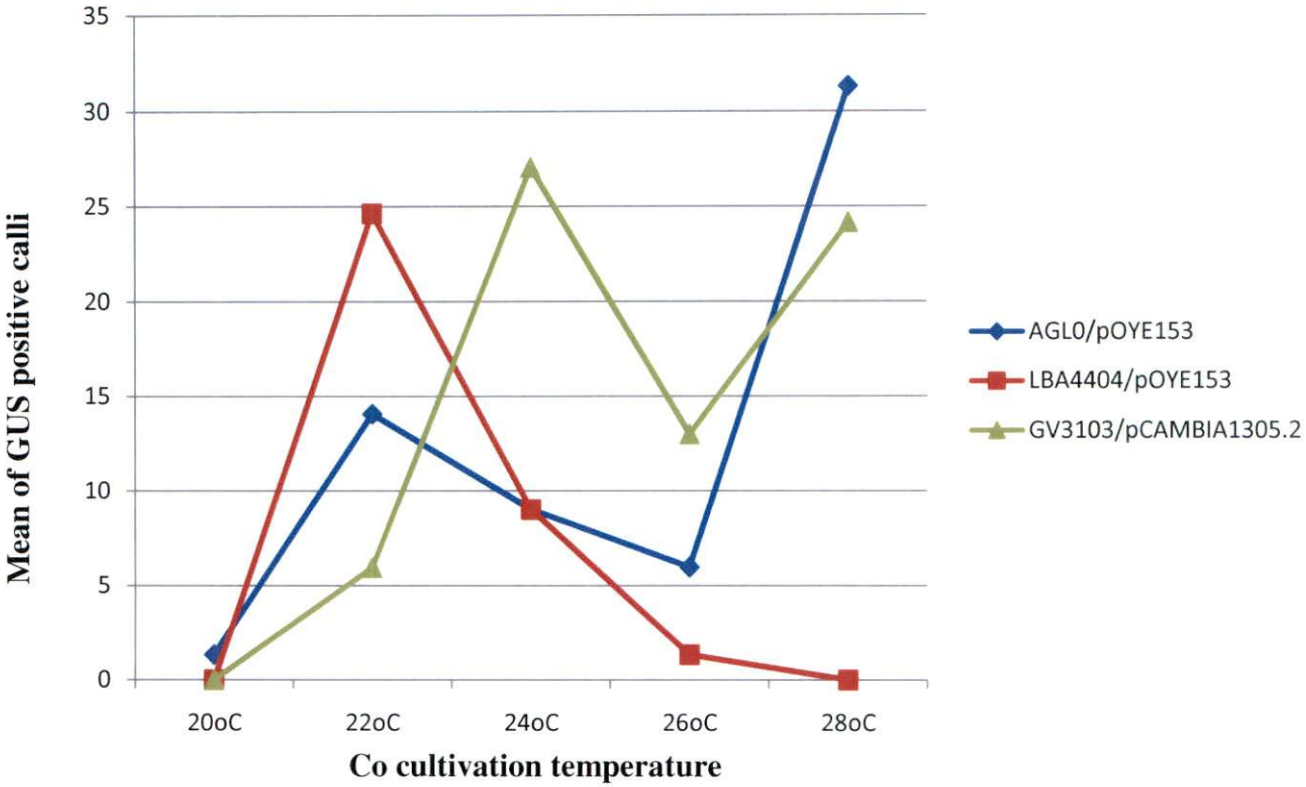


Figure 10. Transformation efficiency of 3 different *Agrobacterium* strains at different co-cultivation temperature

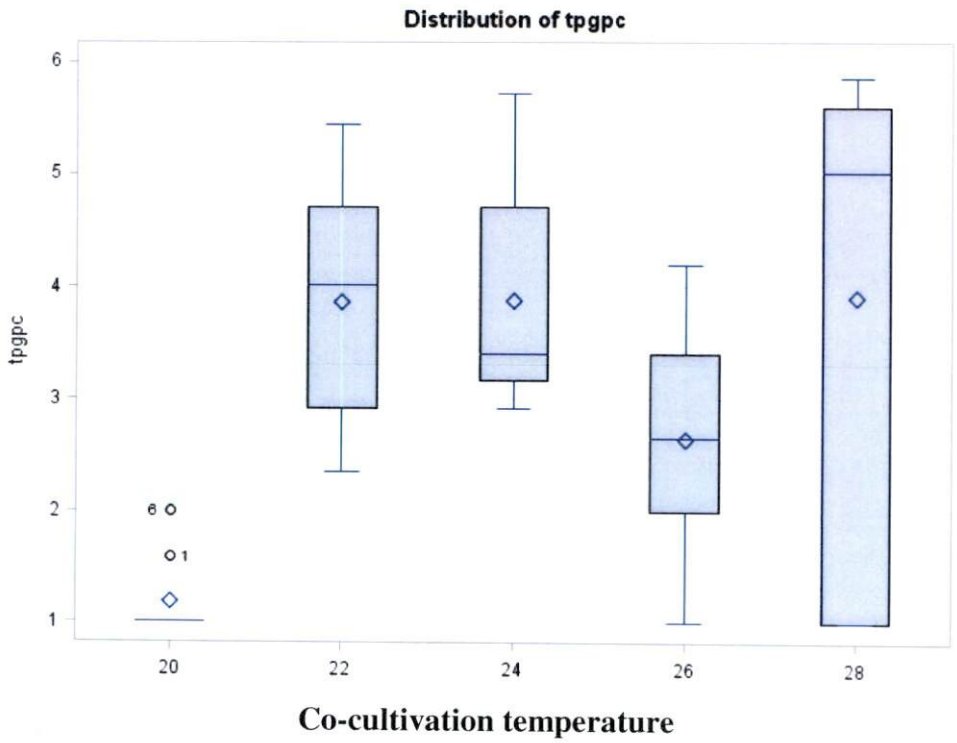


Figure 11. Distribution of transformed calli at different incubation temperature

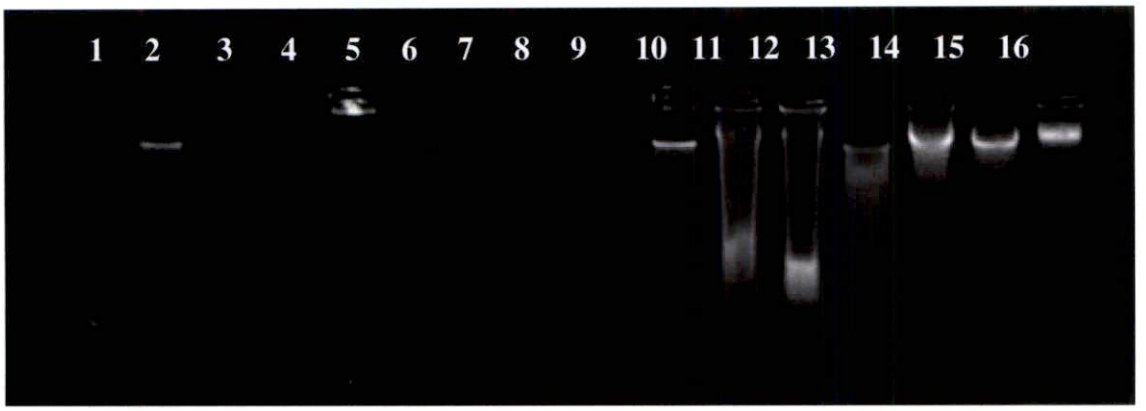


Figure 12. DNA isolation from transformed callus by CTAB method

1-5 AGL0/pOYE153

6-10 LBA4404/pOYE153

10-16 GV 3103/pCAMBIA1305.2

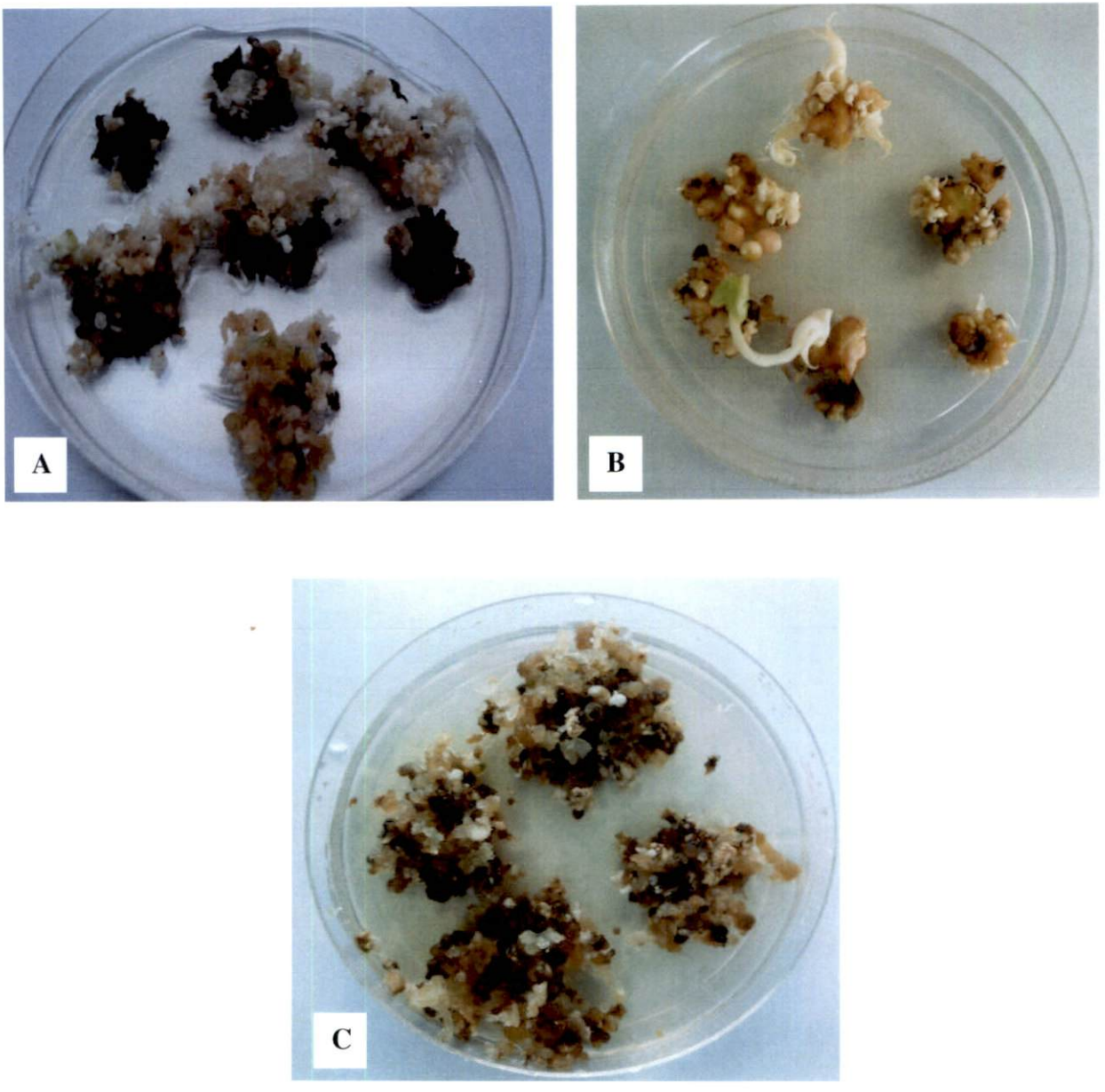


Plate 5. AGL0/pOYE153 transformed calli in selection medium

(A) After 2 months (B) After 6 months (C) Untransformed control calli in selection medium

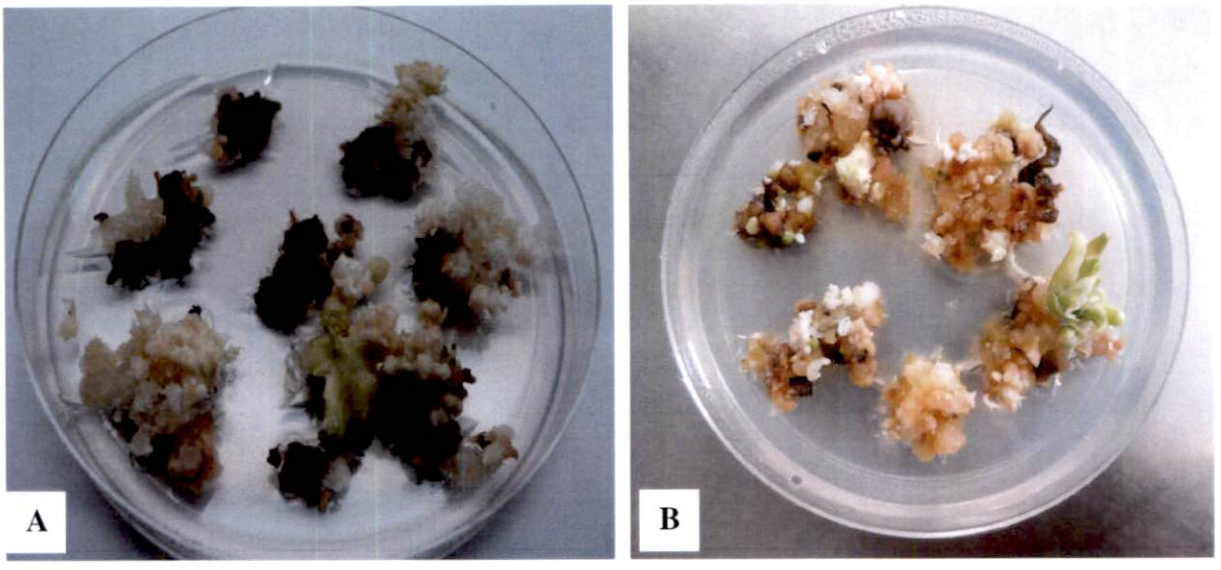


Plate 6. LBA4404/pOYE153 Calli in selection medium

(A) After 2 months (B) After 6 months

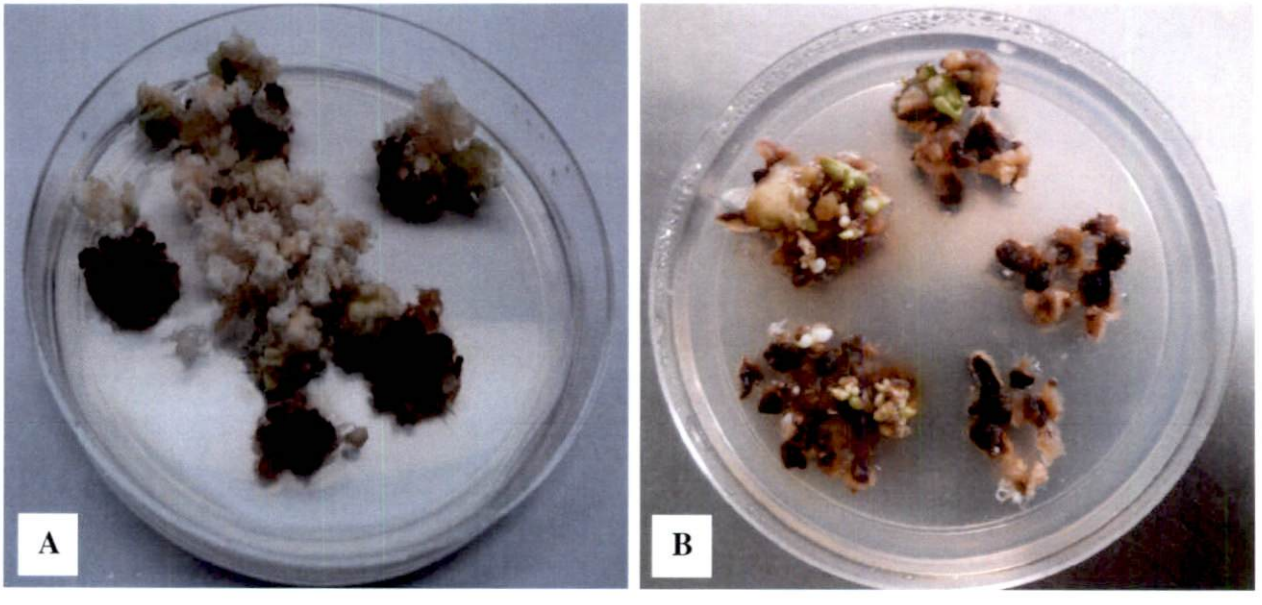


Plate 7. GV3103/pCAMBIA1305.2 Calli in selection medium

(A) After 2 months (B) After 6 months

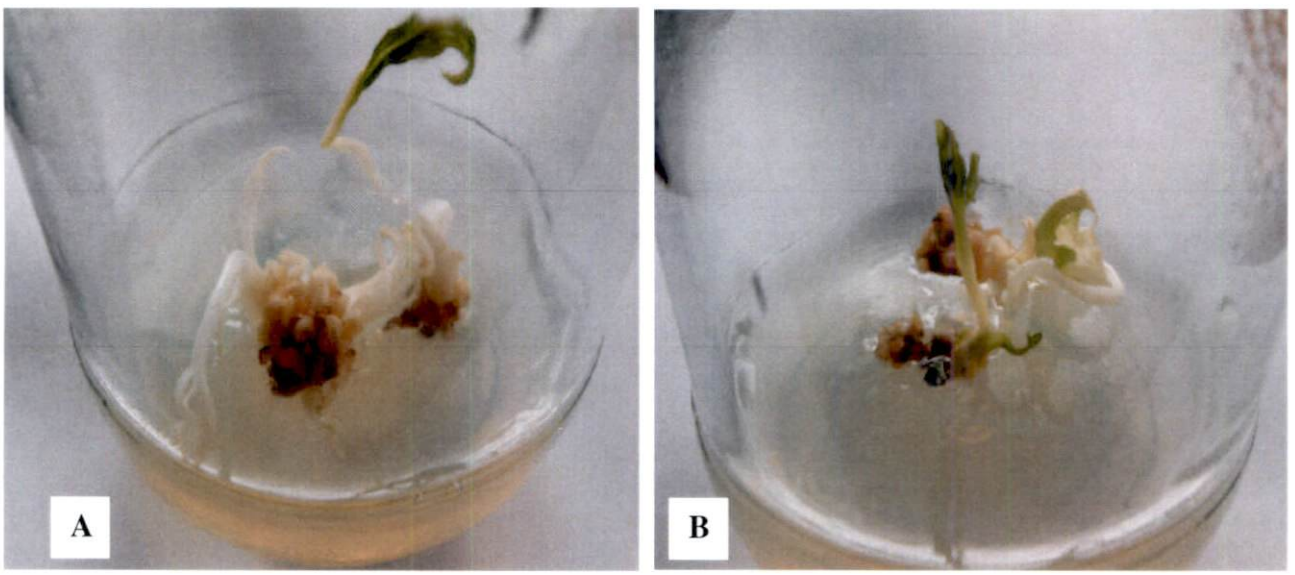


Plate 8. Regenerated plantlet of elephant foot yam (A) AGL0/pOYE153 Regenerated plantlet (B) LBA4404/pOYE153 Regenerated plantlet

Table 5. Effect of co-cultivation duration on transformation efficiency of elephant foot yam calli.

Agrobacterium strains	Average number of GUS positive calli cocultivated for					
	Day 0	Day 1	Day2	Day3	Day 4	Mean different *Agrobacterium* strains.
AGL0/pOYE153	2.9122 (1.9779)	7.5027 (2.9159)	15.4970 (4.0616)	10.9927 (3.4630)	4.4303 (2.3303)	7.70132 (2.9498)
LBA4404/pOYE153	1.3318 (1.5270)	5.7739 (2.6026)	23.9577 (4.9957)	7.9407 (2.9901)	2.1745 (1.7817)	6.7256 (2.7795)
GV3103/pCAMBIA1305.2	0 (1.0000)	0 (1.0000)	1.2978 (1.5158)	5.9656 (2.6392)	1.6966 (1.6421)	1.4317 (1.5594)
Mean of each treatment	1.25510 ^{d}(1.5017)	3.7214 ^{c}(2.1729)	11.4213 ^{a}(3.5244)	8.1857 ^{a}(3.0308)	2.6791 ^{c}(1.9181)	

* Figure in the parenthesis shows the square root of transformed values
* Mean value with same alphabet in the superscript does not differ significantly.
* The data is average of three replicates

Table 6. Effect of co cultivation temperature on transformation efficiency of elephant foot yam calli.

Agrobacterium strains	Average number of GUS positive calli co cultivated at					
	20°C	22°C	24°C	26°C	28°C	Mean of different *Agrobacterium* strains.
AGL0/pOYE153	1.3318 (1.5270)	14.0656 (3.8814)	8.9932 (3.1612)	5.9656 (2.6392)	31.2847 (5.6819)	11.5117 (3.5372)
LBA4404/pOYE153	0 (1.000)	24.6083 (5.0604)	8.9932 (3.1612)	1.3318 (1.5270)	0 (1.0000)	10.4122 (3.3782)
GV3103/pCAMBIA 1305.2	0 (1.0000)	5.9457 (2.6354)	27.0496 (5.2961)	12.9888 (3.7401)	24.1402 (5.0140)	4.5210 (2.3497)
Mean of each treatment	0.3822 (1.1757)	13.8926 (3.8591)	13.9993 (3.8729)	5.94586 (2.6355)	14.1998 (3.8987)	

4.6 PCR ANALYSIS OF PUTATIVE TRANSFORMANTS

4.6.1 Amplification using *nptII* & *GUSA* specific primer

Analysis of putative transformants of LBA4404/pOYE153 and AGL0/pOYE153 were done using *nptII* and GUSA primers. For the negative control, the genomic DNA of untransformed calli was isolated and used in PCR analysis whereas, for the positive control, single colony of LBA4404/pOYE153 and AGL0/pOYE153 was used. Also a non template control was used during PCR which contains all the elements of a typical PCR but not any DNA sample.

PCR was performed with gene specific primers, which amplified a single band at 280 bp for *nptII* and double band of 800bp and 780bp size for GUSA primer. This confirmed the presence of two transgenes within the *in vitro* grown transformed plant genome regenerated in presence of geneticin selection. No amplification was observed from untransformed plant DNA and the non template control, which indicate that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *nptII* and GUSA specific primers are shown in Figure 13- 16.

4.6.2 Amplification using *hpt* specific primer & GUS plus primer

hpt and GUS plus primers were used for the analysis of transformants of GV3103/pCAMBIA1305.2 putative transformants. For the negative control, the genomic DNA of untransformed calli was isolated and used in PCR analysis. Colony PCR of GV3103/pCAMBIA1305.2 was used as positive control. A non template control is also used during PCR which contains all the elements of a typical PCR but not any DNA sample. The PCR results with *hpt* and GUSA specific primers are shown in Figure 17 and Figure 18.

PCR performed with *hpt* and GUSA gene specific primers amplified the expected 300 bp long DNA fragment, for both gene. This confirmed the presence of these two transgenes within the *in vitro* grown transformed plant genome regenerated in presence of hygromycin selection. No amplification was observed from untransformed DNA and the non template control.

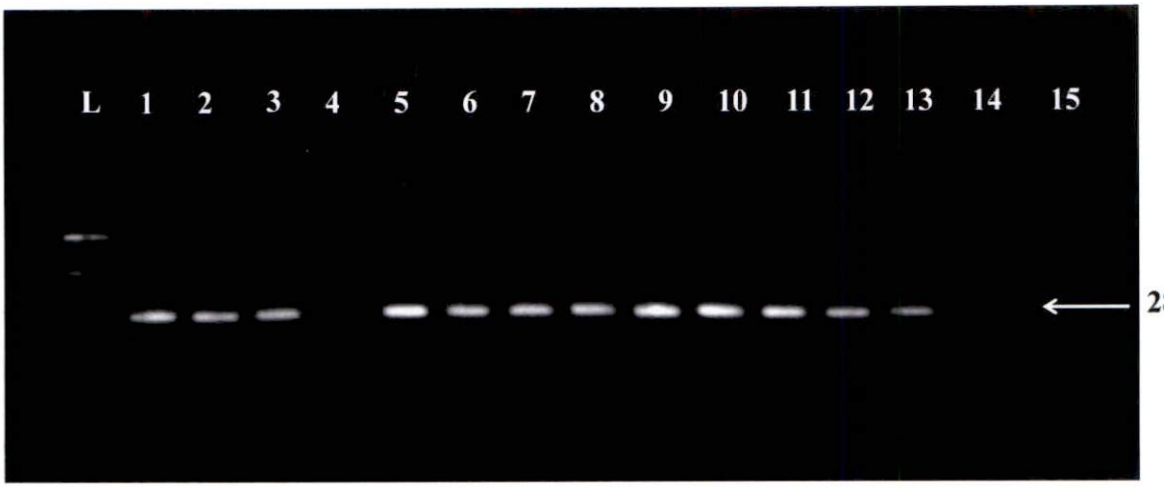


Figure 13. PCR analysis of AGL0/ pOYE153 transformed elephant foot yam calli DNA using *nptII* (loc/ups) primer

L-100bp ladder

1-13 AGL0/pOYE153 transformed callus

14- Untransformed callus

15- Non Template Control

Figure 14. PCR analysis of LBA4404/ pOYE153 transformed elephant foot yam calli DNA using nptII (loc/ups) primer

L-100bp ladder

1-Untransformed callus

2-15 LBA4404/pOYE153 transformed callus

Figure 15. PCR analysis of AGL0/ pOYE153 transformed elephant foot yam Calli DNA using *GUS* specific primer.

L-100bp ladder

1-7 AGL0/pOYE153 transformed callus

8 - Untransformed callus

9 - Non template Control

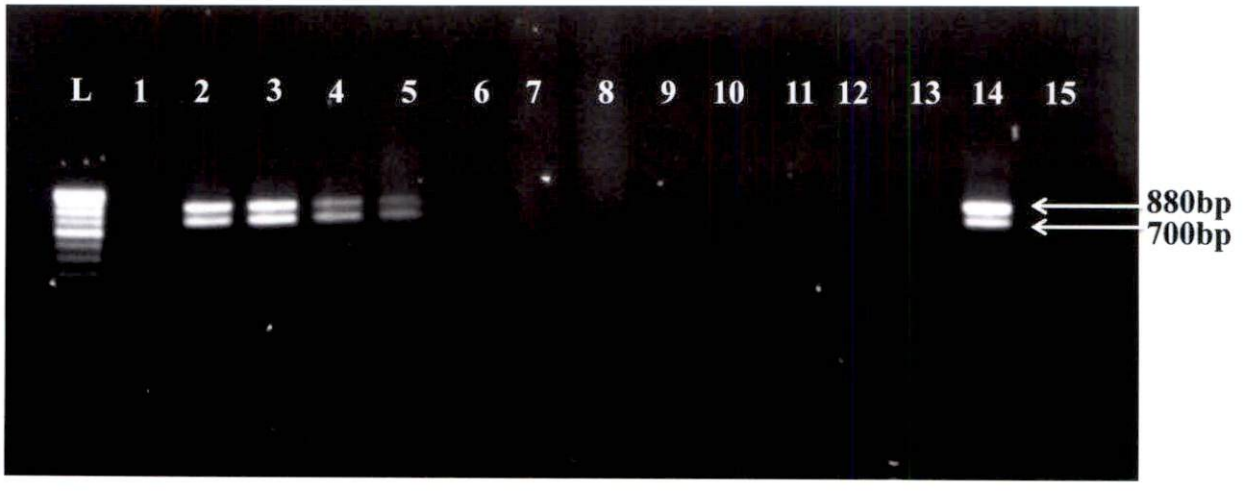


Figure 16. PCR analysis of LBA4404/ pOYE153 transformed elephant foot yam calli DNA using *GUS* primer

L-100bp ladder

1-12 LBA4404/pOYE153 transformed callus

13- Untransformed callus

14- LBA4404/pOYE153 colony

15- Non template Control

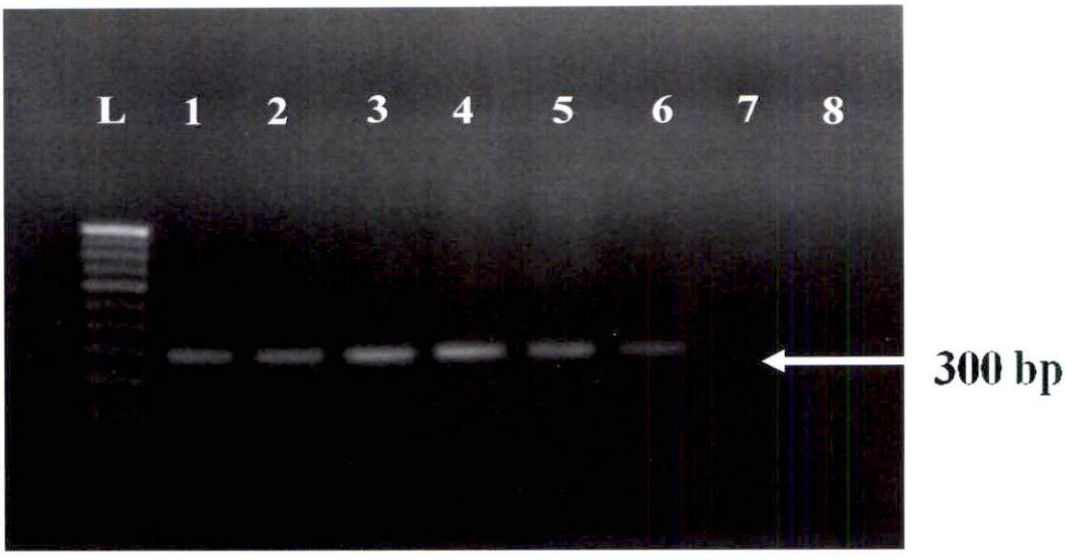


Figure 17. PCR analysis of GV3103/pCAMBIA1305.2 transformed elephant foot yam calli using *hpt* primer

L- 100bp ladder

1-6 GV3103/pCAMBIA1305.2 transformed callus

7 - Untransformed callus

8- Nontemplate control

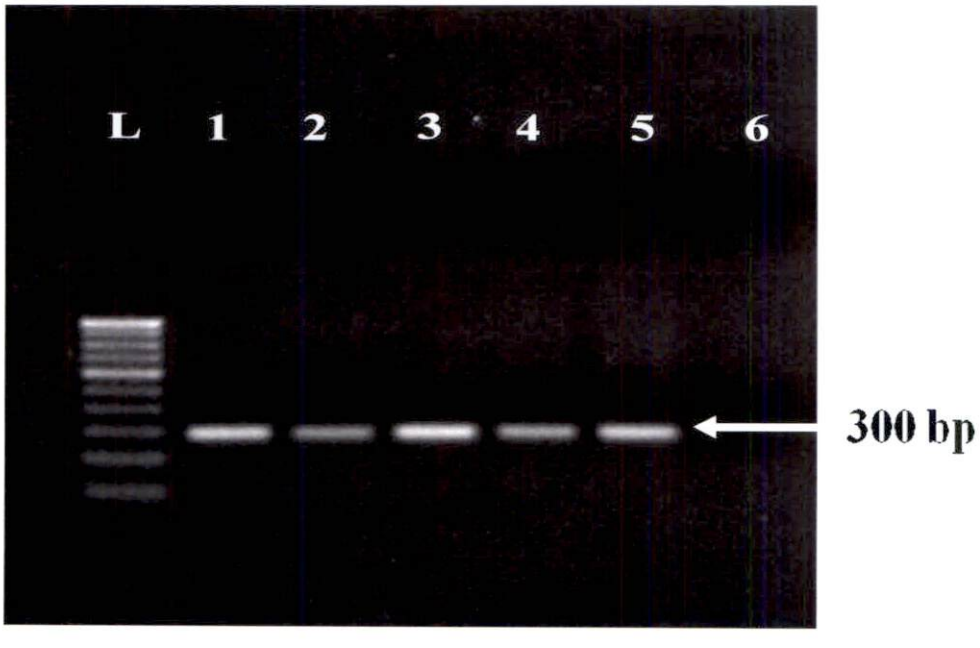


Figure 18. PCR analysis of GV3103/pCAMBIA1305.2 transformed elephant foot yam calli using *GUS* primer

L-100bp ladder

1-5 GV3103/pCAMBIA1305.2 transformed callus

6 - Untransformed callus

4.7 GUS HISTOCHEMICAL ASSAY

T-DNA region of pOYE153 and pCAMBIA1305.2 contain GUSA gene which enables the transgenic tissues to develop blue colour following their incubation in X-gluc chromogenic substrate. In order to monitor the gene expression of transformed calli, GUS staining was performed. For the negative control, the untransformed calli was used for GUS assay. Transformed tissues gave intense blue colour upon GUS staining. The blue colour indicated the integration of GUSA gene into regenerated calli as compared to non-transformed calli where no blue colour was observed. The staining pattern varied among the calli from fully stained to partially stained (Plate 9).

4.8 ANALYSIS OF TRANSFORMANTS BY BLOTTING

The integration pattern of the T- DNA region in the genome of the putative transgenic lines was confirmed by Nucleic acid spot hybridization (NASH) and Southern hybridization analysis.

4.8.1 Probe preparation

Biotin labelled probe was prepared and the probe quality was checked with "Chemiluminescent Nucleic acid Detection Module".

Two different DIG-labelled probes targeting *nptII* gene and *hpt* gene were prepared by random prime labelling. Dilutions of each were spotted on a nylon membrane and analyzed by the direct detection procedure. For reference, dilutions of a DIG-labelled control DNA were included on the same membrane. Chemiluminescent luminol substrate was used to visualize the DIG signal in the spots with an exposure time of 3 minutes. The amount of DIG-labelled DNA in each spot (estimated from the amount of starting template) is shown in the (Figure 19).

The spots corresponding to 0.041 pg of *nptII* DNA (10^{-6}) and 0.0041 pg of *hpt* DNA (10^{-7}) was visible (Table 7). Control probe gave spot only up to 0.41 pg. The quality of the sample DNA probes (*nptII* and *hpt*) was found to be higher than

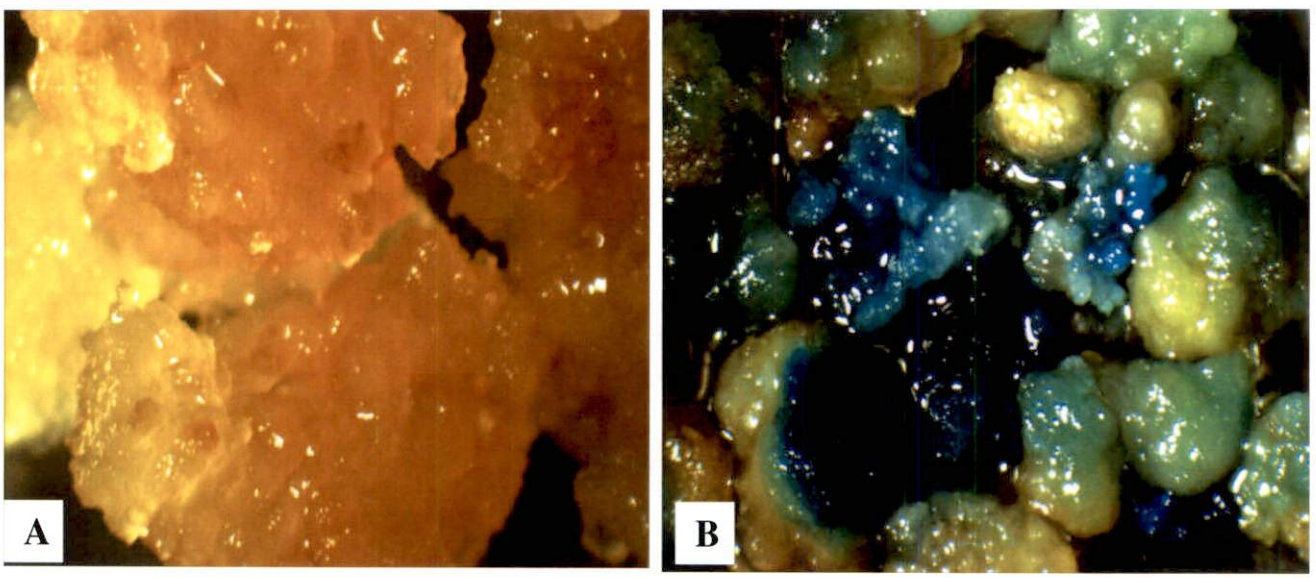


Plate 9. GUS assay of elephant foot yam calli

(A) Control calli (Untransformed) (B) Transformed elephant foot yam calli positive for GUS assay

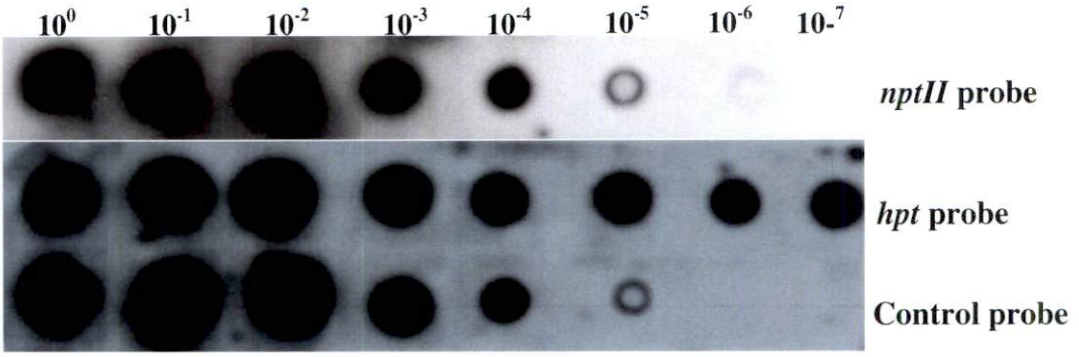


Figure 19. Probe quality check

Table. 7. Concentration of spotted probes

Sl. No.	Dilutions	Concentration of control probe (pg/µl)	Concentration of *nptII* probe (pg/µl)	Concentration of *hpt* probe(pg/µl)
1	10^0	5208.3	4160	4160
2	10^{-1}	520	416	416
3	10^{-2}	52	41	41
4	10^{-3}	5.2	4.1	4.1
5	10^{-4}	0.52	0.41	0.41
6	10^{-5}	0.052	0.041	0.041
7	10^{-6}	0.0052	0.0041	0.0041
8	10^{-7}	0.00052	0.00041	0.00041

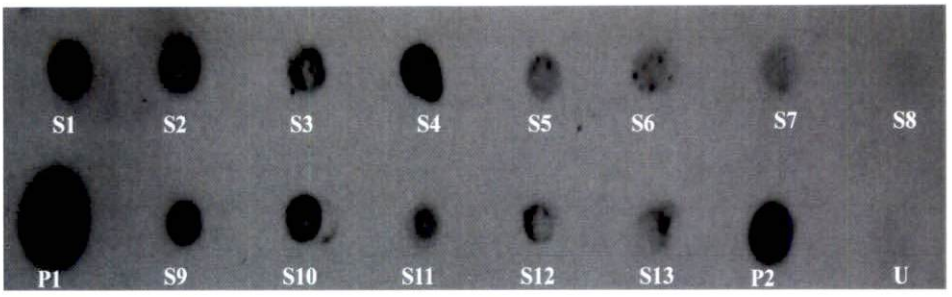


Figure 20. Nucleic acid spot hybridisation of AGL0/pOYE153 and LBA4404/pOYE153 transformants using *nptII* probe

S1- S8 : AGL0/pOYE 153 Transformants

S9- S13 : LBA4404/pOYE 153 transformants

P1 : *nptII* Probe

P2 : Positive Control (pOYE153 plasmid)

U : Untransformed Calli

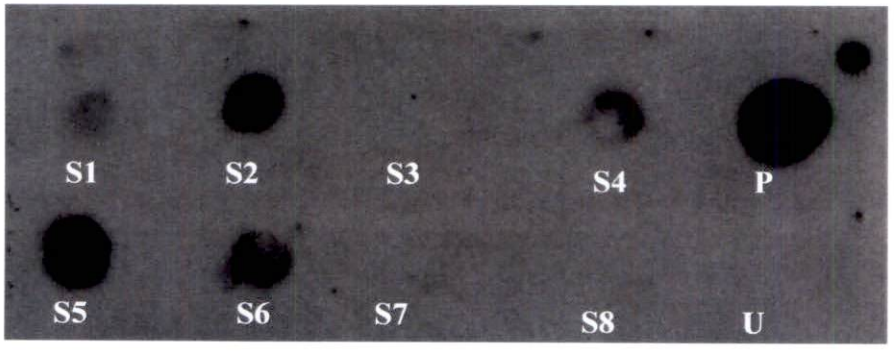
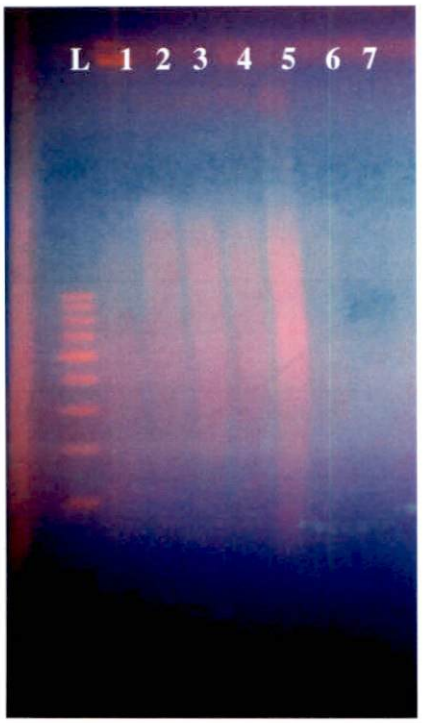


Figure 21. Nucleic acid spot hybridisation of GV3103/pCAMBIA 1305.2 transformants using *hpt* probe

S1-S8: GV3103/pCAMBIA 1305.2 transformants

P : pCAMBIA 1305.2 plasmid (positive control)

U : Untransformed calli



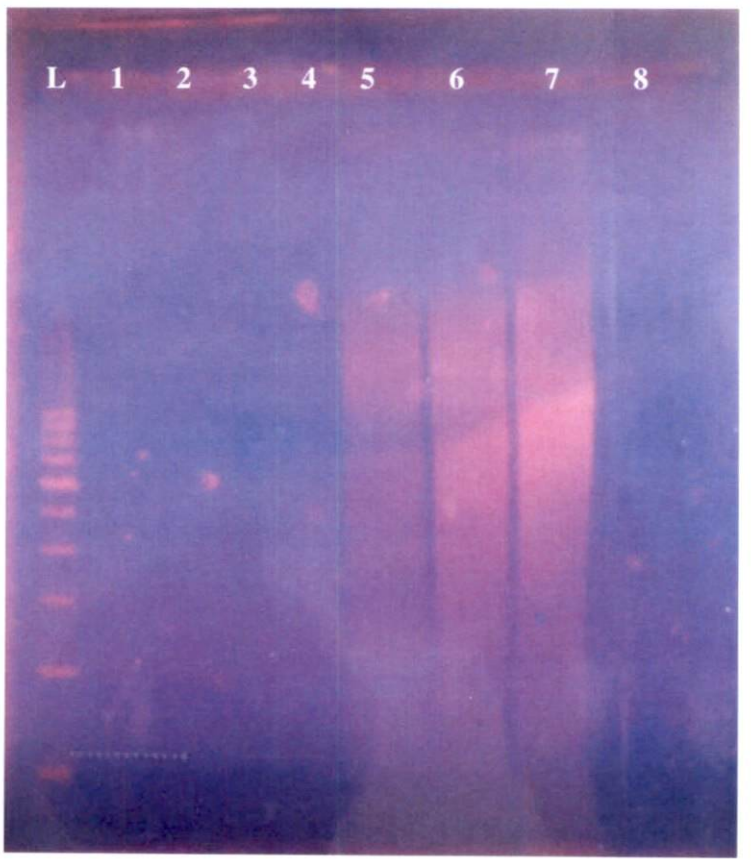
L : 500bp ladder

1-3: AGL0/pOYE153 transformants DNA

4-6: LBA4404/pOYE153 transformants DNA

7 : AGL0/pOYE153 plasmid

Figure 22. Restriction digestion of DNA isolated from AGL0/pOYE153 and LBA4404/pOYE15 transformants using *EcoR*I



L : 500bp ladder

1-7: GV3103/pCAMBIA1305.2 transformants

8 : pCAMBIA1305.2 plasmid

Figure 23. Restriction digestion of DNA isolated from GV3103/pCAMBIA 1305.2 transformants using *EcoR*I

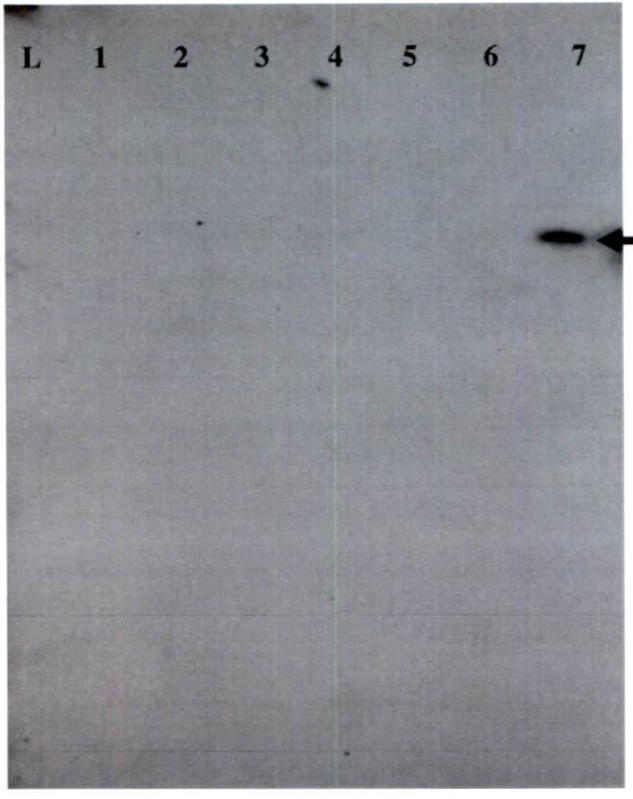


Figure 24. Southern blot analysis of AGL0/pOYE153 and LBA4404/pOYE153 transformants using *nptII* probe

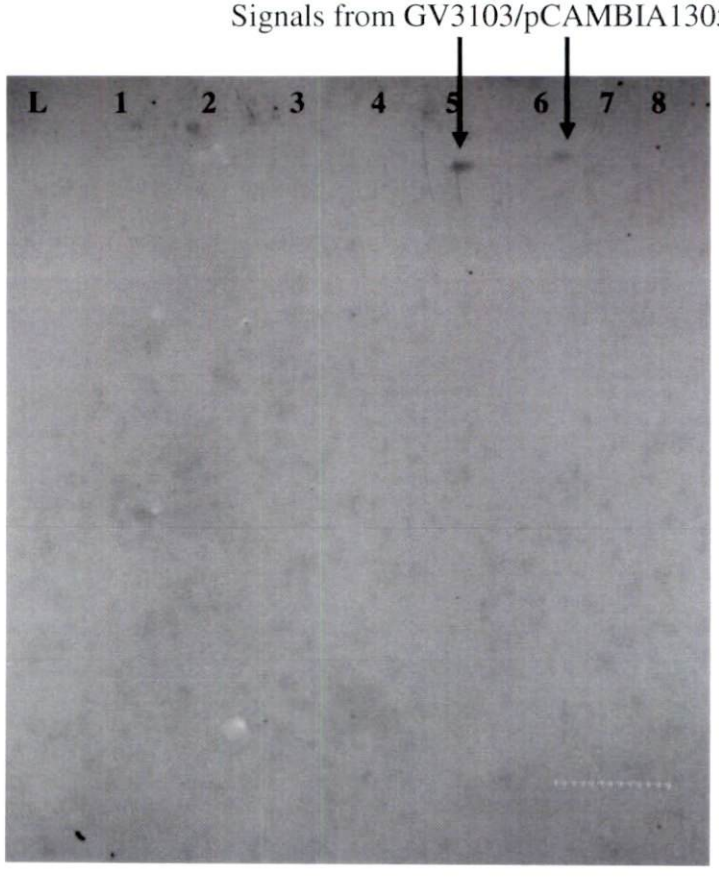


Figure 25. Southern blot analysis of GV3103/pCAMBIA 1305.2 transformants using *hpt* probe

control probe. Hence these probes (10^{-6}) were used to detect the transgene incorporation in the transformed plants through NASH or Southern blot.

4.8.2 Nucleic acid spot hybridization

nptII probe

Isolated DNA of putative transformants of AGL0/pOYE153 and LBA4404/pOYE153 was spotted (4 µl) on positively charged nylon membrane after denaturation and hybridised with biotin labelled *nptII* probe (4.16 pg/µl). All the DNA samples gave signals with varying intensity, which indicated the presence of transgene in transformed plant from where DNA was isolated. pOYE153 plasmid was used as positive control which gave an intense signal compared to other samples. Isolated DNA of untransformed calli was used as negative control which did not give any signal on exposure to X- ray film (Figure 20).

Hpt probe

Denatured DNA samples (8 nos.) of GV3103/pCAMBIA1305.2 were spotted on positively charged nylon membrane and hybridised with biotin labelled *hpt* probe (4.16 pg/µl). Five samples gave signals on exposure to X- ray film with varying intensity. The intensity of the spots was very low compared to the signals from AGL0/pOYE153 and LBA4404/pOYE153 DNA samples. pCAMBIA 1305.2 plasmid was used as positive control which gave intense signal whereas the untransformed DNA did not give any signal (Figure 21).

4.8.3 Southern analysis using *nptII* probe

Southern blot analysis of the six geneticin resistant putative transformants were performed using the DIG labelled *npII* probe under high stringency conditions.

When the genomic DNA of the putative transformants was digested with the restriction enzyme *EcoR*I. The genomic DNA of untransformed calli was used as a negative control and pOYE153 plasmid was used as positive control. Completely sheared DNA of transgenic calli was observed in the gel when stained

with ethidium bromide where as the plasmid lane doesn't show any staining (Figure 22 and 23).

When the genomic DNA of the putative transformants of GV3103/pCAMBIA1305.2 was digested with the restriction enzyme *EcoR*I. The genomic DNA of untransformed calli was used as a negative control and pOYE153 plasmid was used as positive control. Completely sheared DNA of transgenic calli was observed in the gel when stained with ethidium bromide where as the plasmid lane doesn't show any staining.

When the digested DNA of AGL0/pOYE153 and LBA4404/pOYE153 transformants were developed on X- ray film with *nptII* probe the sample DNA did not give any banding pattern, whereas positive control gave a single band of 5kb.

Two DNA samples of GV3103/pCAMBIA1305.2 developed on X ray film with *hpt* probe gave a single faint band of high molecular weight near to the well , whereas the plasmid lane did not give any banding pattern (Figure 24 and 25).

Discussion

5. DISCUSSION

Tuber crops are the third most important food crop after cereals and legumes. They constitute either staple or important subsidiary food for about a fifth of the people in the world.

The genus *Amorphophallus* comprises of about 90 species (Ahlawat, 2003). Among different species, *A. paeoniifolius* is the most important one which is used as vegetable and possess medicinal values. *Gajendra* is the most popular variety released for commercial cultivation in India by A.N.G Ranga Agricultural University. The variety received wide acceptance among the farmers because of its economic potential, medicinal and nutritional value.

When compared to other tuber crops, the production and area under cultivation of elephant foot yam is considerably low. However, in the recent years, considerable increase in cultivation area taken place due to high economic return and large scale commercial cultivation is there in many parts of Bihar, Jharkhand and Uttarpradesh. This crop was attacked by many pest and diseases. In addition, acridity of the tubers and lack of knowledge about the medicinal and nutritional value, limits its widespread cultivation. Therefore, efforts should be directed at developing disease and pest resistance, enhancing the nutritional quality and improving tuber palatability by developing non-acrid varieties.

Developing new varieties through traditional breeding is a time consuming process. Moreover, elephant foot yam presents a lot of barriers for breeding because flowering is a rare phenomenon in many aroids and the exact condition that triggers flowering in elephant foot yam has not yet been worked out. Female sterility, usually encountered in members of Araceae, is another reason for failure of natural seed set in elephant foot yam. These factors restrict the opportunities of traditional breeding methods in this crop, necessitating the application of transgenic technology. However, only few reports on the genetic transformation of elephant foot yam are available in the literature. The present study was undertaken at the Central Tuber Crop Research Institute, Thiruvananthapuram to

obtain a successful transformation event in *Amorphophallus paeoniifolius* (Dennst.) Nicolson.

Advances in transgenic technologies provide new opportunities for manipulation of the plant genome. This will have significant impact on expanding and diversifying the gene pool of crop plants. It can also facilitate the introduction of specific genes and shorten the time required for the production of new varieties. Resilience of this crop to abiotic and biotic stress factors and changing climatic conditions may also be improved. By allowing the direct transfer of genes responsible for disease resistance and better shelf life, new varieties suiting consumer preferences can be developed.

Among the different gene delivery system available, Agrobacterium mediated one is the most widely used to introduce foreign gene in to dicots (Weising *et al.*, 1988) and in some monocots (Eady *et al.,* 2000).

Earlier, Agrobacterium mediated transformation was considered difficult in monocotyledons. Recent advances in understanding the biology of the infection process and availability of gene promoters and selectable markers has improved the progress of genetic transformation in monocotyledons (Smith and Hood, 1995). High frequency of transformation, broad host range, high rate of expression and stable integration of inserted gene have made *Agrobacterium*-mediated gene transfer system the most popular one among the different gene transfer methods.

The first requirement of transgenic breeding is the ability of the genetically manipulated tissue to give rise to whole plants. In elephant foot yam, calli derived from petiole or leaf tissues are reliable for plant regeneration and transformation.

In order to establish sufficient calli for transformation, the petiole and leaves of the *in vitro* plants of elephant foot yam cv. Gajendra were cultured in callus induction medium and mass multiplied by sub culturing in 20 days interval. The actively dividing cells are those seen on the peripheral side of the calli. The

established calli consists of different types of callus such as translucent and friable, slimy and yellow coloured; nodular compact and yellowish.

Compact and yellow-coloured calli could be multiplied easily. They gave rise to globular calli on multiplication. These types of calli were found to be efficient for transformation studies due to ease of handling during the washing step after co cultivation.

Jianbin *et al.* (2008) reported that BA and NAA at approximately equal concentration favour callus induction. Anil *et al.* (2012) reported that petiole slices of *A. paeoniifolius* (Dennst.) Nicolson cultured on MS medium containing BA and NAA produced callus within four weeks of culture. The induced calli were soft, pinkish white, powdery and fragile in nature. Primary calli on subculture to respective media turned in to a compact, nodular, white mass of cells which later turned in to a blackish or greenish hard structure.

Similar findings were reported by Hu *et al.* (2006) that nodular, compact callus was a desirable tissue type and have the capacity to multiply and easily differentiated into shoots and other organs. In some other species, a similar type of callus was also considered an alternative tissue type that could multiply and easily differentiate into shoots or other organs (Teng, 1997; Te-Chato and Lim, 2000).

Similar morphogenetic events have been reported by Irawati *et al.* (1986) in tissue culture of *A. paeoniifolius* (Dennst.) Nicolson where the globular structures were developed from the long-time conserved calli and could produce shoots and roots if transferred to fresh medium. Kamala and Makeshkumar (2013) also reported that the callus was found to be more suitable for transformation due to its ease of regeneration as well as high transformation efficiency. Ban *et al.* (2009) also used callus cultures of *A. konjac* for Agrobacterium mediated transformation for soft rot disease resistance development.

Normally the plant cells are sensitive to antibiotics so the selectable marker genes are introduced into plant genome to express a protein generally with an enzymatic activity, which allows distinguishing transformed from non-transformed cells. These selectable marker genes enable the transformed cells to survive on medium containing the selective agent, while non-transformed cells and tissues die.

Several factors influence the efficiency of an antibiotic as a selection agent. The antibiotic used must be toxic to the normal plant cells but the products from dead tissue should not affect the growth of the transformed cells. Thus the most effective selection agent is those which either inhibits growth or slowly kills the untransformed tissue. Optimal selection pressure is the lowest level of antibiotic needed to kill the untransformed cells completely. All these factors necessitate the evaluation of sensitivity of different antibiotics to select the optimum concentration which is required to inhibit the growth. Hence the sensitivity of elephant foot yam calli to different doses of geneticin and hygromycin were evaluated.

It was observed that, the elephant foot yam calli were sensitive to geneticin at lower concentrations. Complete death of the calli was observed with 20 mg l^{-1} from the sixth week treatment. Kamala and Makeshkumar (2013) reported successful selection of elephant foot yam transformants with geneticin at a concentration 15 mg l^{-1}. Gnasekaran (2014) also reported that geneticin was found to be the most suitable selection agent for transformation in orchid, and has effectively killed PLBs at 30 mg l^{-1}. Niklaus *et al.* (2011) also reported that the most effective amino glycoside concentration suitable for the improved cassava transformation protocol is 20 mg $l^{-1}$and 25 mg l^{-1}geneticin using *nptII* as resistance gene.

The calli of elephant foot yam showed total inhibition of growth at hygromycin 5 mg l^{-1} from sixth week treatment. Elephant foot yam calli was found to be highly sensitive to hygromycin. The results were contrary to the observations recorded by Ban *et al.*, (2009) that the presence of 22.5 mg l^{-1}

hygromycin, inhibit the shooting ability of the elephant foot yam calli higher concentrations of hygromycin in the medium were toxic to calli, causing tissue softening, blackening and consequently resulting in disc death and survival rate of 5.7 percent was obtained with 22.5 mg l^{-1} hygromycin in selection medium. Raja *et al.* (2010) used 50 mg l^{-1} hygromycin for successful selection of transformants of wheat (*Triticum aestivum* L.) .This indicates that there is variation in the sensitivity to antibiotic depending on the genotype, physiological condition and size of the explants.

A successful Agrobacterium mediated plant transformation requires efficient procedures for suppressing bacteria following co-cultivation and subsequently for selecting transformed cells. As the plant tissue is affected by various components in culture media during plant transformation, antibiotics used for suppressing *Agrobacterium* might have negative effects on plant tissues and regeneration. Hence there is need to standardise the optimum concentration of an antibiotic. Ticarcillin 650 mg l^{-1} is the lowest lethal concentration of elephant foot yam calli. Concentration below 650 mg l^{-1} can be used for the successful elimination of Agrobacterium without affecting the regeneration potential of explant. Kamala and Makeshkumar (2013) also reported successful elimination of Agrobacterium in elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) transformation using 500 mg l^{-1} ticarcillin. Ling *et al.* (1998) reported that Ticarcillin/potassium clavulanate was an effective antibiotic against *A. tumefaciens*. A concentration of 150 mg l^{-1} was high enough to eliminate *A. tumefaciens* strain LBA4404 in Agrobacterium mediated transformation of tomato (*Lycopersicon esculentum* Mill.).

Once an efficient *in vitro* regeneration protocol and the sensitivity levels of the antibiotics have been developed, the next requirement for a successful genetic transformation is the selection of an efficient gene delivery system. Agrobacterium mediated transformation, as it has several advantages as compared to direct gene transfer, was used with three strains of *Agrobacterium tumefaciens* viz, AGL0, LBA4404, GV3103 with two different binary vectors were used for

the study. There are differences in susceptibility between species and even between cultivars

Hence transformation with different strains harbouring different selectable marker was used. As elephant foot yam tissues were sensitive to geneticin and hygromycin, the vectors with geneticin and hygromycin were selected. pOYE153 vector contains geneticin resistance gene and pCAMBIA 1305.2 harbour the hygromycin resistant gene as selectable markers.

Acetosyringone is a type of phenolic compound which is well known to increase *Agrobacterium vir* gene activity. In monocots acetosyringone, the key factor for transformation was not present naturally. Elephant foot yam, being a monocot it was assumed that acetosyringone not secreted by nature. The effect of different concentration of acetosyringone was studied by the growing the Agrobacterium in YEB with different concentrations (0µM, 100µM, 200µM, 300µM, 400µM). Significantly higher GUS staining was achieved at the addition of 400µM acetosyringone in the co cultivation media. It is hypothesized in this study that acetosyringone increases transformation efficiency when included in co-culture media during transformation by *Agrobacterium*. Very few GUS positive calli were recorded when acetosyringone was omitted from the media. Raja *et al.* (2010) reported similar results in wheat (*Triticum aestivum* L.) application of 400 µM acetosyringone promoted the production of resistant calli and higher the number of GUS expressing calli. Goredema (2013) reported that all the explants treated with 400 µM acetosyringone concentration were transiently transformed in *Nicotiana benthamiana.* The results are similar to Rashid *et al.* (2001) who reported that in their study the resistant calli only emerged by the application of higher concentration of acetosyringone i.e., 400µM.

Terada and Shimamoto (2004) reported that maximum number of calli showing blue color with maximum frequency of blue patches were found on those calli co-cultivated with with acetosyringone at 400µM. They also reported that increase in the concentration of acetosyringone enhance the GUS activity.

Time of co-cultivation is one of the main factors affecting *Agrobacterium* mediated gene transformation. Very few transformants were recorded when duration of co-cultivation was increased to four days. Two day co-cultivation duration was better over zero, one, three and four day co-cultivation. When co-cultivated for three days, elimination of *Agrobacterium* from the explants was difficult whereas in four days co-cultivation there was complete necrosis and death of explants. In one day co-cultivation period, percent mortality was less but the percent GUS expression was reduced considerably. It was found that, percentage of transformation could be increased just by increasing the co-cultivation period while keeping the bacterial density and incubation period constant. But, prolonged co-cultivation period (more than three days) was found to promote overgrowth of bacteria on the infected explants and also explants showed browning on selection media. Finally, these explants failed to regenerate. Correspondingly the transformation percentage was found to decrease with the decrease (less than two days) of co-cultivation period. Therefore, two-three days of co-cultivation was determined to be the best for elephant foot yam. Ban *et al.* (2009) also reported successful transformation in *A. konjac* for resistance to soft rot disease with 2 days of co cultivation.

Temperature has been considered as a factor affecting the capacity of *Agrobacterium* to transfer the T-DNA to plant cells (Karami, 2008; Sreeramanan and Xavier, 2010). The present investigation of the effect of temperature during co cultivation in elephant foot yam calli revealed that temperature plays an important role in transformation efficiency. Higher temperature, 28°C was found to be optimal to support the highest transient transformation frequency in elephant foot yam and there was no difference between 22°C and 24°C. Dramatic transient expression reduction occurred when temperature decreased from 22 to 20°C. The results are contradictory to various studies in which optimal temperatures for transformation ranged from 19 to 25°C. However, due to high inconsistency across plant species and tissue used, it is possible to affirm that the optimal temperatures varied depending on these factors. Movahedi *et al.* (2014) reported

co cultivation at 28°C produced the highest transformation efficiency in poplar, with significant differences in efficiency between the temperatures tested.

There were differences in transformation efficiency with respect to the different strain of *Agrobacterium.* Maximum percent of GUS stained tissue (24.5%) of transformants was obtained with the strain LBA4404 with pOYE153 vector. AGL0/pOYE153 and GV3103/pCAMBIA 1305.2 strains give 14 percent and 6 percent GUS positive tissue. Thus difference in transformation efficiency according to the bacterial strain was evident. The *Agrobacterium* infectivity is because of the interaction between the plant cell and the bacterial cell. The host range is a much more complex process, which is under the genetic control of multiple factors within both the bacterium and the plant host. The infectivity is improved by the use of right strain of bacteria, varying host genotype, manipulating explants physiology etc.

Comparison of transformation efficiency of five *Agrobacterium tumefaciens* strains in *Nicotiana tabacum* L. by Bakhsh *et al.* (2014) revealed that the highest transformation rate (20%) was obtained with the *Agrobacterium* strain LBA4404, followed by EHA105, GV2260, C58C1 and AGL1. Bull *et al.* (2009) reported successful transformation in friable embryogenic calli of cassava using LBA4404 strain. Transformation efficiency of 36.37% was observed for callus when compared to swollen petiole explants in elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) expressing glucuronidase reporter gene (Kamala and Makeshkumar, 2013).

GUS histochemical assay and PCR was done to confirm transformation. GUS assay proved to be effective in confirming the transformation event within a short period after transformation (Kamala and Makeshkumar, 2013).The GUS assay of transformed callus showed blue colour and further confirmation was done by PCR analysis with specific primers which is the usual method generally followed in several cases for confirmation of transgenic events. PCR amplification of transgenic callus of (*Amorphophallus paeoniifolius* (Dennst.) Nicolson with GUS specific primers, which survived in the selection medium,

gave a band at 400 bp, while the non transformed control callus failed to give amplification (Kamala and Makeshkumar, 2013).

Geneticin and hygromycin select the rare integration events from the stable integration event. Transformed calli survived the antibiotic screening whereas untransformed calli were effectively killed. Among the calli selected by antibiotics, few GUS negatives were observed. It is possible that the GUS negatives among the selected calli may be escapes. DNA was isolated from transformed and untransformed calli (Control). The DNA samples were amplified by PCR with the *nptII, hpt* and GUS and GUS plus primers designed for *nptII*, *hpt* and *GUSA* genes in the vector. The amplified products were separated by agarose gel electrophoresis. *nptII* primer yielded a 280 bp single band and GUS primer yielded two bands of size 880bp and 700bp. *hpt* primer gave a band of size 300bp. The amplification achieved with these primers further confirms the presence of transgene in the genomic DNA.

Nucleic acid spot hybridisation of putative transformants of AGL0/pOYE153, LBA4404/pOYE153 and GV3103/pCAMBIA1305.2 revealed the specificity of the probes and the number of transformants obtained for each strain. All the transformants of AGL0/pOYE153 and LBA4404/pOYE153 gave spots of varying intensity when hybridised with *nptII* probe whereas only 5 out of the 8 transformants of GV3103/pCAMBIA1305.2 gave positive for *hpt* probe and the intensity of spot was low when compared to the spots obtained with *nptII* probe. The positive control (pOYE153 and pCAMBIA1305.2 plasmid) showed intense signal, which indicated the quality of the probe used. Untransformed DNA doesn't gives any spots, indicates the specificity of the probe and the stringency of the washing conditions.

The DIG-labelled *nptII* probe constitutes geneticin resistance of *nptII* gene cassette in plasmid pOYE153. The probe is thus designed to confirm successful insertion of the T-DNA into the genome of geneticin resistant putative transformants. It is expected that a band would be present in the genomic DNA of transformants, while being absent in the wild type, which lacks the *nptII* gene.

Hybridization with this probe would allow further conclusions to be drawn as to the nature of the T-DNA insertions. When digesting the genomic DNA with specific restriction enzymes, the number of bands obtained reveals how many insertion events have occurred in the genomic DNA of each transformants. It is most favourable that a transformation system produce single insertion events at a single locus, as this will minimize any difficulties with later recovery of the tagged sequences. The band sizes indicate randomness of integration and may also give some idea as to whether tandem repeats have occurred (depending upon which restriction enzymes are used). (Covert *et al.*, 2001; de Groot *et al.*, 1998; Dobinson *et al.*, 2004; Bundock *et al.*, 1999 ; Shi *et al.*, 1995; Kahmann and Basse, 1999; Meyer *et al.*, 2003).

A single band corresponding to the positive control pOYE153, visible after 30 min exposure, indicated that successful hybridisation of the DIG-labelled *nptII* probe had taken place with complementary sequences having high homology. But the absence of bands for six geneticin resistant putative transformants was not expected. It is possible that the concentration of DNA (10µl) used in the blot was too low for detection of T-DNA inserts. Genomic DNA was present, as evident in gel analysis prior to the blot. However, insufficient transfer to the membrane could be the reason (although sufficient for the controls). Another possibility is the nature of probe labelling used for detection. A more suitable probe label might give positive results. Radioactive labelled probes (e.g. ^{32}P- dNTPs) are commonly used for detecting T-DNA inserts in the genomic DNA.

The DIG-labelled *hpt* probe constitutes hygromycin resistant *hpt* gene cassette in the plasmid pCAMBIA1305.2. A single band corresponding to the putative transformants lane, which are visible after 30 min exposure, indicated that successful hybridisation of the DIG-labelled *hpt* probe has taken place with complementary sequences having high homology. But the absence of band for positive control was not expected. However, due to limited time, higher concentrations of pure DNA and radioactive probe labelling could not be tested.

Summary

6. SUMMARY

A study on genetic transformation of *Amorphophallus paeoniifolius* (Dennst.) Nicolson was conducted at the Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2013- 2014. The important findings of the above studies are summarised in this chapter.

Callus of elephant foot yam can be initiated from petiole and leaves within four weeks of culture in CIM. The established calli consists of different types of callus; compact and yellow-coloured calli could be multiplied easily. This type of calli in 15 days growth stage was found to be suitable for transformation studies.

Elephant foot yam calli were sensitive to geneticin and hygromycin at lower concentrations. Complete death of the calli was observed with 20 mgl^{-1} geneticin and 5 mgl^{-1} from sixth week treatment. So, these concentrations of antibiotics can be used for the successful selection of elephant foot yam transformants.

Ticarcillin 650 mgl^{-1} is the lowest lethal concentration of elephant foot yam calli. Concentration below 650 mgl^{-1} can be used for the successful elimination of *Agrobacterium* without affecting the regeneration potential of explants. 500 mgl^{-1} used in this study found to be suitable for successful elimination of *Agrobacterium* without affecting the regeneration potential of explants

There are multiple factors involved in *Agrobacterium*-mediated transformation that influence the success or failure of the transfer of gene of interest into plants and their subsequent stable integration and expression. In this study, acetosyringone increases transformation efficiency when included in co-culture media during transformation by *Agrobacterium*. Addition of 400 µM acetosyringone in *Agrobacterium* culture medium gave significantly higher number of GUS expressing calli.

Two to three days of co-cultivation was determined to be suitable for elephant foot yam and increase in co cultivation days reduced the transformation efficiency and increased mortality percent.

Temperature plays an important role in transformation efficiency. Higher temperature, 28°C was found to be optimal for highest transient transformation in elephant foot yam calli.

Difference in transformation efficiency according to the bacterial strain was evident in the study and maximum percent of GUS stained tissue of transformants was obtained with the strain LBA4404 with pOYE153 vector (24.5 percent) followed by AGL0/pOYE153 (14 percent) and GV3103/pCAMBIA 1305.2 (6 percent).

GUS histochemical assay and PCR was done to confirm transformation. PCR amplification of the DNA of the calli survived in selection medium yielded an expected band size of 280 bp for *nptII* primer, two bands of size 880bp and 700bp for GUS primer, 300bp single band for *hpt* primer and GUSPlus primer. No amplification was obtained for untransformed calli DNA. The amplification achieved with these primers further confirms the presence of transgene in the genomic DNA.

Nucleic acid spot hybridisation of putative transformants revealed the specificity of the probes and the number of transformants obtained for each strain. All the transformants of AGL0/pOYE153 and LBA4404/pOYE153 gave spots of varying intensity when hybridised with *nptII* probe whereas only 5 out of the 8 transformants of GV3103/pCAMBIA1305.2 gave positive for *hpt* probe and the intensity of spot was low when compared to the spots obtained with *nptII* probe. Whereas untransformed DNA doesn't give any spots, indicates the specificity of the probe and the stringency of the washing conditions.

Southern hybridisation with DIG-labelled *nptII* probe and *hpt* probe was done to draw conclusions on nature of the T-DNA insertions. *nptII* probe give single band corresponding to the positive control pOYE153 on 30 minutes exposure. No band was visible for geneticin resistant putative transformants may be due to too low for detection of T-DNA inserts in DNA samples. DIG-labelled *hpt* probe give a single band corresponding to the putative transformants lane

(GV3103/pCAMBIA1305.2) on 30 minutes exposure. Which indicate the successful hybridisation of the DIG-labelled *hpt* probe on blot.

The investigation of various factors that influence T-DNA delivery is an important step in the *Agrobacterium*-mediated transformation of elephant foot yam calli. Therefore, an efficient *Agrobacterium*-mediated transformation protocol for elephant foot yam was successfully established.

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Appendices

APPENDIX I

MEDIUM FOR PLANT TISSUE CULTURE

Callus Induction medium (CIM)	Dissolved 2.6 g Murashige and Skoog modified 3B- ½ concentration of NH_4NO_3 and KNO_3, BA (0.5 mg l^{-1}, NAA (0.5 m gl^{-1}) , 2,4-D (0.5 mg l^{-1}) and 2 percent sucrose in 950 ml sterile distilled water. The pH was adjusted to 5.5-5.6 using 1N NaOH/HCl. Made up the volume to 1 L and 7.25 g l^{-1} Agar was added. Autoclaved at 121°C and 1.06 Kg cm^{-2} pressure for 20 minutes.
Plant regeneration medium	Dissolved 2.6 g Murashige and Skoog modified 3B- ½ concentration of NH_4NO_3 & KNO_3, BA (5 mg l^{-1}), NAA (1 mg l^{-1}) and 2 percent sucrose in 950 ml sterile distilled water. The pH was adjusted to 5.5-5.6 using 1N NaOH/HCl. Made up the volume to 1 L and 7.25 g l^{-1} Agar was added. Autoclaved at 121°C and 1.06 Kg cm^{-2} pressure for 20 minutes.

APPENDIX II

MEDIUM FOR BACTERIAL CULTURE

1. **Yeast extract broth (YEB)**

Beef extract (Himedia) - 3 gl^{-1}

Yeast extract (Himedia) - 1 gl^{-1}

Casein enzyme

hydrosylate((Himedia) - 5 gl^{-1}

Sucrose(Duchefa Biochemie) - 5 gl^{-1}

pH was adjusted to 7.5 with 1N NaOH/HCl . Sterilized by autoclaving.

2. **Luria Agar (LA)**

Suspended 35.0 grams of LA (HiMedia) in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

APPENDIX III

TAE Buffer

Tris base - 242g

Glacial acetic acid - 57.1 mL

100 mL of 0.5 M EDTA (pH 8.0)

APPENDIX IV

CTAB DNA Extraction Buffer

CTAB - 2%

PVP - 2%

Tris- HCl (pH 8.0) - 100 mM

EDTA - 25 mM

NaCl - 2 M

β-mercaptoethanol - 0.2 % (v/v) freshly added prior to DNA extraction

APPENDIX V

TE Buffer

Tris- HCl (pH 8.0) - 10 mM

EDTA - 1 mM

APPENDIX VI

GUS Assay Buffer

0.5ml of 0.1M $K_3Fe(CN)_6$

0.5ml of 0.1M $K_4Fe(CN)_6.3H_2O$

0.94ml of 0.2M $NaH_2PO_4.H_2O$

3.04ml of 0.2M $NaHPO_4.2H_2O$

200µl of 10% Triton X-100

5-10 mg of x-Gluc (dissolved in 200µl of DMSO)

Make up to 10 ml with sterile distilled water.

APPENDIX VII

REAGENTS FOR SOUTHERN HYBRIDISATION

Denaturation Solution

NaCl (1M) - 29.22 g

NaOH (0.5M) - 10.00g

Dissolved in 400 ml of Sterile distilled water and made up to 500 ml. Autoclaved and stored at RT.

Neutralization Solution (pH -7.0)

NaCl (1.5 M) - 43.88 g

Tris Cl (0.5 M) - 30.28 g

Dissolved in 350 ml of sterile distilled water and adjusted the pH to 7 with con. HCl and made up to 500 ml. Autoclaved and stored at RT.

20X SSC

NaCl - 175.3 g

Sodium citrate - 88.2 g

Dissolved in 350 ml of sterile distilled water and adjusted the pH to 7 with con. HCl and made up to 500 ml. Autoclaved and stored at RT.

APPENDIX VIII

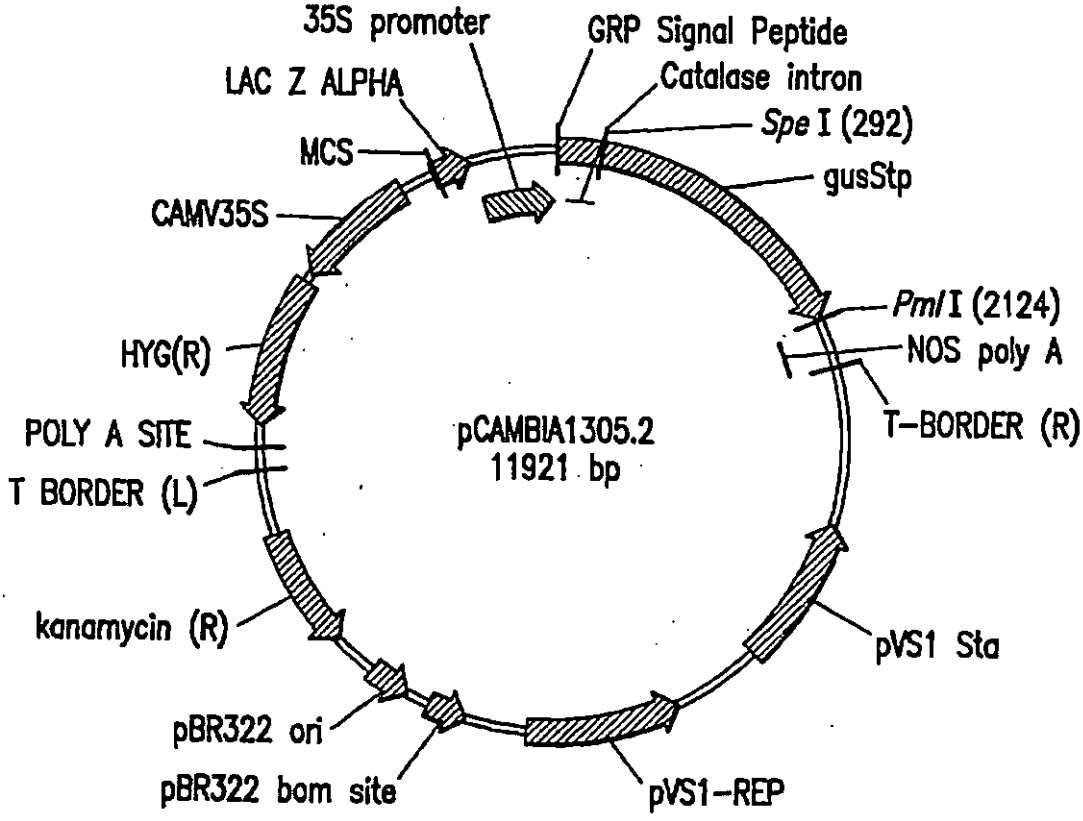
STOCK PREPARATION

Antibiotic	Stock solution concentration	Solvent	Method of sterilization	Storage temperature
Rifampicin*	20 mg/ml	Methanol	No need to sterilize	-20°C
Kanamycin	80mg/ml	Sterile distilled water	Filter sterilization	-20°C
Geneticin	100mg/ml	Sterile distilled water	Filter sterilization	-20°C
Hygromycin	500mg/l	Sterile distilled water	Filter sterilization	-20°C
Ticarcillin	500mg/ml	Sterile distilled water	Filter sterilization	-20°C
Magnesium Sulphate	1M	Sterile distilled water	Filter sterilization	-20°C
Acetosyringone*	100mM	DMSO	No need to sterilize	-20°C

* Light sensitive: container should be covered with foil.

APPENDIX IX

Vector map of pCAMBIA1305.2



Abstract

GENETIC TRANSFORMATION OF *Amorphophallus paeoniifolius* (Dennst.) Nicolson

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Abstract of the

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9. ABSTRACT

A study on *Agrobacterium*-mediated genetic transformation of *Amorphophallus paeoniifolius* (Dennst.) Nicolson was conducted at the Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014.

Calli were initiated using petiole and leaves of *in vitro* plantlets of elephant foot yam cv. *Gajendra* in callus induction media. After four weeks of incubation, actively dividing globular, hard and creamy white calli were developed. Subculture of developed calli was repeated periodically (20 days) in CIM with an approximate size of one cm^2. 15 days old calli was found to be suitable for transformation study. Calli sufficient for the transformation study was obtained after 3 months of subculture.

Experiments were conducted to evaluate the sensitivity of elephant foot yam calli to different doses of antibiotics *viz.* geneticin, hygromycin, ticarcillin. It was observed that complete death and discoloration of the calli obtained with 20 mgl^{-1} geneticin and 10 mgl^{-1} hygromycin from sixth week treatment. Statistical analysis of sensitivity response of calli indicated that LD_{100} was 20 mgl^{-1} and 5 mgl^{-1} with geneticin and hygromycin respectively.

Sensitivity of the calli to ticarcillin was studied and the responses are analysed with ANOVA. The lowest lethal concentration of ticarcillin was found to be 650 mgl^{-1}. So, concentration below 650 mgl^{-1} can be used for the successful elimination of *Agrobacterium* without affecting the regeneration potential of explant. 500 mgl^{-1} ticarcillin used in this study was observed sufficient for the successful elimination of *Agrobacterium* without affecting the regeneration potential of calli.

For the optimization of parameters affecting transformation, experiments were conducted for the standardisation of optimum concentration of acetosyringone, time of co cultivation, temperature of co cultivation, and suitable *Agrobacterium* strain.

In a study conducted for standardisation of optimum concentration of acetosyringone, increasing number of transformants was obtained with increase in acetosyringone. Significantly higher GUS staining of calli (21.5896) was achieved with the addition of 400μM acetosyringone in the co cultivation media.

The effect of number of days of co cultivation on transformation was compared on the GUS expression of 14-day old selected calli. Two-three days of co-cultivation was determined to be the suitable for elephant foot yam because prolonged co-cultivation period (more than three days) was found to promote overgrowth of bacteria and subsequent death of the calli. Correspondingly the transformation percentage was found to decrease with the decrease (less than two days) of co-cultivation period.

Investigation of the effect of temperature during co cultivation in elephant foot yam calli revealed that temperature plays an important role in transformation efficiency. Higher temperature, 28°C was found to be optimal to support the highest transient transformation frequency in elephant foot yam and dramatic transient expression reduction occurred when temperature decreased from 22 °C to 20°C.

Transformation efficiency with respect to the different strain of *Agrobacterium* was investigated and the results showed that maximum percent of GUS stained tissue (24.5 percent) of transformants was obtained with the strain LBA4404 with pOYE153 vector followed by AGL0/pOYE153 (14 percent) and GV3103/pCAMBIA 1305.2 (6 percent).

GUS assay of transformed callus showed blue colour and confirmation was done by PCR analysis with specific primers and southern blotting. PCR amplification of the DNA of the calli survived in selection medium yielded an expected band size of 280 bp for *nptII* primer, two bands of size 880bp and 700bp for GUS primer, 300 bp single band for *hpt* primer and GUSPlus primer. No amplification was obtained for untransformed calli DNA.

Nucleic acid spot hybridisation of putative transformants of elephant foot yam further confirmation of the presence of transgene in the DNA. Hybridisation with *nptII* probe yield spots of varying intensity for all the transformants of AGL0/pOYE153 and LBA4404/pOYE153. Whereas only 5 out of the 8 transformants of GV3103/pCAMBIA1305.2 gave positive for *hpt* probe and the intensity of spot was low when compared to the spots obtained with *nptII* probe.

Southern hybridisation with DIG labelled *nptII* probe yield a band for positive control (pOYE153 plasmid) whereas the bands in sample lane was not observed. It is possible that the concentration of DNA (10µl) used in the blot was too low for detection of T-DNA inserts. Hybridisation with *hpt* probe gave a single band corresponding to the putative transformants lane, which are visible after 30 min exposure indicated that successful hybridisation of the DIG-labelled *hpt* probe. But the absence of band for positive control was not expected.