DEVELOPMENT OF *IN VITRO* REGENERATION AND GENETIC TRANSFORMATION SYSTEMS IN PUMPKIN (*Cucurbita moschata* Poir.)

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "Development of *in vitro* regeneration and genetic transformation systems in Pumpkin (*Cucurbita moschata* Poir.)" 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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BA	Benzyl Adenine
2,4 – D	2,4 – Dichlorophenoxy acetic acid
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
Kin	Kinetin
h	Hour
min	Minutes
MS	Murashige and Skoog's medium
%	Per cent
µl	Micro litre
T-DNA	Transfer DNA
^{0}c	Degree celcius
g l^{-1}	Grams per litre
mg l-1	Milligrams per litre
µM	Micro molar
µg	Micro grams
ml	Milli litre
NAA	Naphthalene Acetic Acid
GUS	Glucuronidase
npt	Neomycin phospho transferase
YEM	Yeast Extract Mannitol medium
nm	Nano metre
HCl	Hydrochloric acid
psi	Pounds per square inch
rpm	Revolutions per minute
M	Molar
uv	Ultra violet
NaOH	Sodium hydroxide
cm	Centi metre
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
TDZ	Thidiazuron
C. moschata	*Cucurbita moschata*
A. tumefaciens	*Agrobacterium tumefaciens*
OD	Optical density

Introduction

1. INTRODUCTION

Pumpkin (*Cucurbita moschata* Poir.) occupies a prominent place among vegetables owing to its high productivity, nutritive value, good storability, long period of availability, better transport qualities and extensive cultivation in subtropical and tropical parts of the world. The pumpkin is very high in fibre and ß-carotene which has recently received credit for cancer preventing qualities.

A major limitation to successful production of pumpkin worldwide is infection by diseases that severely limit yield and for which adequate levels of native resistance are not available (Vengadesan *et al.,* 2005). To date, genetic improvement of pumpkin has been achieved mainly by conventional breeding techniques. However, their introduction via these techniques is very low, expensive and limited because of the interspecific and intergeneric reproductive barriers (Guis *et al.,* 2000). The potential benefits of virus resistant transgenic pumpkin include reduction in the use of pesticides for vector control, improved crop quality and possibility of developing varieties with multiple virus resistance. Hence, it is imperative to develop other avenues of disease management. Plant transformation technology has become a versatile platform for cultivar improvement as well as for studying gene function in plants. The development of an *Agrobacterium* based transformation system for pumpkin would greatly expand the germplasm base of this species by allowing the introduction of specific cloned genes of interest.

Genetic transformation facilitates the introduction of only specifically desirable genes without co-transfer of any undesirable genes from donor species, which normally occurs by conventional breeding methods. The two most important pre-requisites for the success of this method are the availability of a plant regeneration system from the explants and a suitable method for transformation.

Plant transformation is performed using a wide range of tools such as *Agrobacterium* Ti plasmid vectors, microprojectile bombardment, microinjection,

chemical (PEG) treatment and electroporation of protoplasts. All these methods have advantages unique to each of them. Transformation using *Agrobacterium tumefaciens* and microprojectile bombardment are currently the most extensively used methods.

Among the available genetic transformation systems, *Agrobacterium* – mediated gene transfer is considered as more efficient. The efficiency of this technique can be influenced by many factors including the variety to be transformed, the bacteria used, the explant and the presence of inductive virulence genes.

For successful integration of *Agrobacterium* – mediated transformation into pumpkin improvement programmes, procedures and protocols must be standardized for efficient, large scale selection of transformed cells and rapid regeneration of transgenic plants. Since cucurbits are infected with *Agrobacterium* (Smarrelli *et al.,* 1986), many researchers introduced foreign genes by *Agrobacterium* mediated method. It is quite essential to standardize transformation system in pumpkin using the *npt* II gene.

Hence, the present study was undertaken with the following objectives:

1) To standardize a viable in *vitro regeneration* system in Pumpkin
2) To develop a protocol for genetic transformation in Pumpkin.

Review of literature

2. REVIEW OF LITERATURE

Pumpkin (*Cucurbita moschata* Poir.) is one of the most important summer vegetable grown all over India on a commercial scale. Because of its high carotene and good keeping quality, it is considered as a vegetable of immense value. It is an important cucurbitaceous vegetable grown in different parts of the country for its immature and mature fruits. They are used as fresh vegetable, processed food and stock feed. Pumpkin seeds are a good source of iron, zinc, essential fatty acids, potassium and magnesium. Pumpkins are orange coloured because they contain massive amounts of lutein, alpha and beta carotene which turn to vitamin A in the body. The crop is specially known for its low cost of production and long keeping quality (Thamburaj and Singh, 2004)

The improvement work in pumpkin has been undertaken with the objective of conferring resistance to pests and diseases. Viruses that affect pumpkin growth includes cucumber, squash, watermelon and zucchini yellow mosaic. Disease symptoms will vary but generally the plants are stunted. The leaves show mottling mosaic, crinkling and twisting and shortened internodes and flowering is adversely affected. The complete control of virus diseases has not been possible. In the cucurbits grown in rainy season, insect vectors like white fly spread the viruses rapidly. The use of insecticides on pumpkin to suppress aphid populations has not resulted in effective control of the spread of the viruses. The only solution to control viruses is the development of virus resistant cultivars (Chandra and Mishra, 2003).

The regeneration systems from tissue and cell culture have contributed to the development of producing transgenic pumpkin. Transformation technology could be used to protect pumpkin against biotic stresses. The incorporation of virus resistance genes into pumpkin has been the goal of many breeding programmes. However, resistant genes are derived from wild species; they are not simply inherited and or are recessive, so introgression of these genes into

horticulturally acceptable species is not an easy task. All of these improvements depend upon the existence of a genetic transformation system. The production of transgenic plants depend on three major factors viz, a) efficient introduction of foreign gene b) establishment of efficient regeneration system for the crop under consideration and c) reliable and efficient selection of transformed callus or regenerated plants (Hansen and Wright., 1999). Since cucurbits are infected with *Agrobacterium* (Smarrelli *et al.,* 1986), many researchers introduced foreign genes by *Agrobacterium* mediated method. Moreover, in this transformation system, an intact DNA segment can be integrated into the plant genome at low copy numbers (Hansen *et al*., 1994). On the other hand, microprojectile mediated transformation method was also been employed in cucurbits (Chee and Slightom, 1992; Schulze *et al*., 1995).

2.1 APPLICATION OF *IN VITRO* CULTURE TECHNIQUES IN TRANSFORMATION

Development of an efficient and reproducible regeneration system is a pre-requisite for genetic transformation of plants to ultimately recover the plants. Indeed, it is the totipotency of plant cells that underlies most plant transformation systems. Direct organogenesis, indirect organogenesis and somatic embryogenesis have been effectively utilized in production of genetically transformed plants.

2.2 *IN VITRO* CULTURE STUDIES IN CUCURBITS

The *in vitro* culture technique holds promise for large scale production of propagating materials. Micropropagation has been considered as an alternative method for production of extensive hybrids for example, triploid seedless watermelons are now commercially produced through micropropagation. As the cucurbit family contains many dioecious species, the technique is extremely useful. For example, the propagation of pointed gourd and ivy gourd is not possible through seeds due to germination problem (Hoque *et al*., 1998). The cutting method is labour intensive and also requires lot of vines; micro propagation is now being used to multiply the quality materials.

2.2.1 Regeneration via organogenesis

Organogenesis is a process of differentiation by which plant organs namely roots, shoots, stem etc., are formed. Plant production through organogenesis can be achieved by two modes:

1) Organogenesis through callus formation with *de novo* origin,
2) Emergence of adventitious organs directly from the explant.

The ratio of auxins to cytokinins determines the route of morphogenesis *in vitro.* Medium containing high auxin levels will induce callus formation. Lowering the auxin and increasing the cytokinin concentration favoured shoot formation and the converse situation favoured root formation (Warren, 1991).

2.2.1.1 EXPLANT SOURCE

For any given species or variety, a particular explant may be necessary for successful plant regeneration. The season of the year, donor conditions of the plant, age and physiological state of donor plant contribute to the success of organogenesis in cell cultures. The different explants used for organogenesis in cucurbits include cotyledonary explants, protoplast, epicotyl and hypocotyl segments, petiole, shoot tip and nodal segments.

Cotyledonary explants were used for regeneration in Cucumber, *Cucumis figarei*, melon, bottle gourd, winter squash, summer squash, ash gourd and watermelon.

Cade *et al.* (1987) reported shoot regeneration from cotyledons of *Cucumis sativus* L. after subculturing and or transferring to another medium and keeping the explants in the dark. The calli appeared as smooth yellow to orange colour. The growth and differentiation of callus tissues derived from cotyledons was obtained (Kim *et al*., 1988). Msikita *et al* (1988) attempted *in vitro* regeneration from excised cucumber seed tissues. They obtained only male flowers *in* vitro. Gambley and Dodd (1989) described a technique for production

de novo of shoots arising from adventitious buds and are confined to a specific region at the base of the cotyledon. Addition of silver nitrate did not improve either bud initiation or shoot regeneration (Niedz *et al*., 1989) but induced plant regeneration only in distal cotyledons (Mohiuddin *et al., 1997).*

Colijn Hooymans *et al* (1988) isolated protoplasts from cotyledons, hypocotyls and leaves of cucumber cultured on MS medium with added sucrose, mannitol, NAA and 2iP. Amorphous callus from this medium formed embryoids when transferred to hormone free MS medium. Embryoid growth was stimulated by reduction of sucrose from 3% to 2% and shoots formed in 2% of embryoids. An efficient method was developed for obtaining callus from cotyledon, hypocotyl and leaf protoplasts. Of the factors studied that affected protoplast isolation and culture, the key step for reproducibility of results and high planting efficiency was preculture of the material from which the protoplasts were to be isolated. The frequency of callus giving rise to plantlets was on an average 1.5% with a maximum of 12.5% (Garcia Sogo *et al*., 1991).

Tabei *et al* (1995) reported shoot organogenesis in wild *Cucumis sp.* viz. *Cucumis figarei* and *Cucumis metuliferus* from 3 day old cotyledons after forced sprouting.

In melon, explants from 7 day old seedlings provided better results compared to 2 day old and 4 day old seedlings (Souza *et al.,* 2006). Well developed shoots were formed from the calli as reported by Moreno *et al* (1985) and Ortz *et al* (1987). Mackay *et al* (1988) reported that cultures placed in the dark formed a creamy white friable callus which when transferred to light developed small green zones throughout the callus clumps but no shoot formation occurred.

Efficient plant regeneration was reported from bottle gourd by Han *et al* (2004). The shoot regeneration frequency in 4-day old seedlings was higher than those in 2-day old seedlings and 6-day old seedlings. Light irradiation provided during both the seed germination and adventitious shoot induction periods significantly increased the shoot regeneration for proximal cotyledons. This result agrees with the report indicating that the distal cotyledons of cucumber are less responsive than proximal cotyledons (Mohiuddin *et al*., 1997).

Proximal parts of cotyledons from 4 day old seedlings was used for efficient plant regeneration in winter squash (Lee *et al*., 2003). Similar results were obtained by Ananthakrishnan *et al* (2003).

Thomas and Sreejesh (2004) obtained callus induction and plant regeneration in ash gourd. The calli remained friable with whitish or pale yellowish colour.

Adventitious buds were induced in cotyledon segments of watermelon by Krug *et al* (2005). Histological studies showed that organogenesis occurs directly, without callus formation on epidermal and subepidermal layers of the explant. Seedling age was reported to be an important factor for obtaining high frequency adventitious shoot regeneration in watermelon (Choi *et al*., 1994). Explants were collected from seedlings younger than 5 day old as young cotyledons are physiologically very active and respond efficiently to exogenous hormones (Dong and Jia, 1991).

Epicotyl explants from 9 day old seedlings were also proved to be highly effective in shoot bud induction in *Cucumis melo* cv. Pusa Madhuras (Jain and More, 1992).

Hypocotyl explants showed successful regeneration via direct organogenesis in summer squash (Pal *et al*., 2007) and indirect organogenesis in cucumber (Rajasekeran *et al*., 1983) and muskmelon (Abak and de Vaulx, 1980).

2.2.1.2. GROWTH REGULATORS

The growth, differentiation and organogenesis of tissues become feasible only on the addition of growth regulators to a medium. The ratio of hormones required for root or shoot induction varies considerably with the tissue, which seems directly correlated to the quantum of hormones synthesized at endogenous levels within the cells of the explant.

Auxins and cytokinins are the widely used growth regulators for shoot induction and root induction in cucurbits. Apart from these, gibberellins and abscisic acid were also employed in some species.

The first successful report of organogenesis via callus phase was obtained from musk melon hypocotyls and peduncle explants cultured on MS medium containing 0.2 mg l^{-1} NAA and 0.2 mg l^{-1} Kinetin (Abak and de Vaulx, 1980).

Rajasekeran *et al* (1983) obtained male flowers *in vitro* by culturing cucumber cotyledons on MS medium supplemented with 0.5 mg l^{-1} BA and 1.5 mg l^{-1} 2, 4-D.

MS medium supplemented with 1.5 mg l^{-1} IAA and 6 mg l^{-1} Kinetin was found to produce well developed shoots from melon cv. Amarillo Oro cotyledons via callus phase (Moreno et al., 1985).

In melon, cotyledons cultured on MS medium supplemented with 6 mg l^{-1} Kinetin and 1.5 mg l^{-1} IAA placed in light formed green nodular callus and visible shoots while in dark formed creamy white friable callus and only one shoot primordia (Mackay *et al*., 1988).

Shoots developed from cucumber cotyledons cultured on MS medium containing 0.5 µM 2,4-D and 5 µM BA (Kim *et al.*, 1988). Zeatin favoured root formation in both epicotyl and cotyledonary explants (Jain and More, 1992). They also obtained direct shoot and root in MS medium supplemented with 1 mg l^{-1} IAA and 5 mg l^{-1} Kinetin. Msikita *et al* (1988) reported that embryonic axes developed shoots faster than cotyledons within 3 weeks of culturing in MS medium containing 2 mg l^{-1} BA and 0.1 mg l^{-1} NAA. Cotyledon explants cut into small pieces cultured on MS medium containing 4 mg l^{-1} or less cytokinin resulted in *de novo* production of multiple shoots (Gambley and Dodd, 1989).

The micropropagation in squash has been reported by Chee (1991) through shoot apices. Seedling derived shoot apices were cultured on MS medium with 0.8 mg l^{-1} BA and 0.1 mg l^{-1} kinetin and the regenerated plantlets were successfully transferred to soil.

In African horned cucumber, petiole explants cultured in MS medium supplemented with 1 mg l^{-1} 2,4-D and 1 mg l^{-1} BA produced friable yellow callus and further shoot development was obtained on medium containing 4 µl zeatin (Raharjo and Punja, 1993).

Ficcadenti and Rotino (1995) attempted indirect organogenesis in melon var reticulatus and inodorus. They obtained two types of calli namely compact green organogenic tissue and white friable callus from cotyledons cultured on MS medium supplemented with 2.8 µM BA and 1 µM ABA for 4 weeks.

Silver nitrate treatment induced shoot regeneration only in distal cotyledons of cucumber (Mohiuddin *et al.*, 1997). Flowers initated from seeds cultured on MS medium containing 2 mg l^{-1} BA with or without gibberellic acid by Ameha *et al* (1998).

In pointed gourd, shoots initiated and proliferated from nodal cuttings on MS medium containing 1µM IAA and 0.2 µM IBA (Mythili and Thomas, 1999). The ideal time for subculture was 4 weeks and if delayed to 8 weeks, it resulted in a decline in transferable nodes.

Shoots produced within 2 weeks of culture of hypocotyls explants of melon on MS medium containing 4.4 µM BA (Curuk *et al.,* 2002).

Lee *et al* (2002) obtained shoot regeneration and elongation from proximal parts of cotyledons of Korean and Japanese winter squash cultured on MS medium containing 1 mg l^{-1} BA.

Direct organogenesis was obtained from bottle gourd on MS medium containing 3 mg l^{-1} BA (Han *et al.,* 2003). BA was considered as a crucial factor for the adventitious shoot regeneration. This agrees with the results reported by Sarowar *et al* (2003) using a *Cucurbita* interspecific hybrid. They also observed that the enhancement of *in vitro* adventitious shoot induction as well as the increase in the number of shoots per explant can be achieved using silver nitrate.

Shoots recovered by culturing cotyledons in MS medium supplemented with 1 mg l^{-1} BA in melon (Dirks and Buggenum, 1989), summer squash (Ananthakrishnan *et al*., 2003) and winter squash (Lee *et al.,* 2003).

Thidiazuron has been exploited for the first time to produce shoots from leaf callus of cucumber (Selvarai *et al.,* 2003).

Shoots regenerated from callus after 5 weeks of culture on MS medium supplemented with 4 mg l^{-1} BA and 0.2 mg l^{-1} NAA in ash gourd (Thomas and Sreejesh, 2004).Similar results were reported in *Cucumis melo* (Niedz *et al.,* 1989) and *Citrullus lanatus* (Dong and Jia, 1991).

Culturing the cotyledon explants of watermelon in MS medium containing 1 mg l^{-1} BA and 10% coconut water induced direct organogenesis (Krug et al., 2005).

Pal *et al* (2007) reported indirect organogenesis in summer squash by culturing hypocotyl explants on MS medium containing 2.5 mg l^{-1} 2,4 D and 0.5 mg l^{-1} TDZ.

2.2.2 Regeneration via somatic embryogenesis

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to reproducible regeneration of non zygotic embryos capable of germinating to form complete plants (Razdan, 2003). These embryos pass through globular, heart-shaped, torpedo-shaped and cotyledonary embryo stages to give rise to complete plantlets. It can occur in either of two distinct ways: 1) from the differentiated cells of a newly transferred piece of whole plant tissue without the proliferation of undifferentiated tissue; and 2) from the unspecialized, unorganized and dedifferentiated cells of callus tissues or suspension cultures.

For initiation of embryogenic tissues, differentiated explants often require extensive proliferation through unorganized callus cycles, death or disruption of surrounding explant cells, and or high levels of synthetic auxin such as 2, 4 D or picloram. Cell isolation is manifested early in somatic embryogenesis by the formation of a cuticle. Each pro embryonic group of cells becomes separated from surrounding cells by thickened, cutinized walls on the outer surface. These cells divide in an assymetrical way and their daughter cells give rise to typical cell clumps which have been called proembryogenic masses or embryogenic clusters. For the production of vigorous plantlets, a period of embryonic growth and maturation is needed before germination. Once a sufficient quantity of embryogenic tissue is obtained, the next phase is to stop proliferation and allow the tissue to form somatic embryos. Clumps of embryogenic tissue are transferred

to a maturation medium containing suitable supplements, which promotes maturation of the somatic embryos. High auxin levels can inhibit development and growth of the shoot meristem if young proembryos are not transferred to a low auxin or zero auxin medium after induction. Individual mature somatic embryos are picked from the clumps and placed on germination medium containing a mixture of nutrients needed for early plant development. The somatic embryos germinate to form roots and shoots, similar to plants germinating from seeds. The plants propagated from somatic embryos are called emblings or somatic seedlings or somatic embryo derived plantlets (Chandra and Mishra, 2003).

2.2.2.1. EXPLANT SOURCE

Age, physiological state, genotype and orientation of the explant, while in contact with the medium influence the induction of somatic embryogenesis. These aspects govern the disruption of explant tissue integrity, callus friability and isolation of cells in order to enhance somatic embryogenesis in various species (Merkle *et al.,* 1995)

Diverse types of cucurbit explants are able to undergo somatic embryogenesis, but seedling material such as cotyledon and hypocotyls tissues, is especially successful. The developmental stage of plants has also shown to be important: ovules from flower buds of *Cucurbita moschata* Poir. at anthesis resulted in better embryogenic response than those from flower buds one day before anthesis (Kwack and Fujieda, 1988). The embryo sacs degenerated and nucellar cells gave rise to pro embryos which developed further to give embryos.

Cotyledons were used as explants for inducing somatic embryogenesis in squash (Chee, 1971), Cucumber (Rebecca *et al*., 1988; Kim and Janick, 1989; Cade *et al*., 1990; Lou and Kako, 1994; Yang *et al*., 2003) and melon (Gray *et al.,* 1993; Whan and Suh , 1998; Stipp *et al*., 2001).

Tabei *et al* (1991) studied various factors and conditions involved in plant tissue and cell culture techniques that influenced regeneration from plant tissue in melon and found that cotyledon explants of mature seeds and young seedlings possess high ability for embryogenesis. Moreover, embryogenesis was controlled by the concentration of auxin in the regeneration media.

Seeds and seed cuttings were used in melon (Homma *et al.,* 1991; Trigiano and Gray, 1996), cucumber (Kageyama *et al.,* 1991; Hassanein, 2003).

Hypocotyls were used in pumpkin (Jelaska, 1972), melon (Hanana *et al*., 2002) and cucumber (Rodaree *et al.,* 2003).

Leaf tissue used in cucumber (Kuijpers *et al.,* 1996), squash and melon (Kintzios *et al*., 2002). The ability of leaf explants to regenerate plants had high heritability.

Roots rarely gave good results, although their competence was demonstrated with cucumber by Trulson and Shahin (1986).

Oridate *et al* (1992) also noted a significant genotype effect in somatic embryogenesis from melon seeds. Transfer of the frequency of somatic embryogenesis from superior responding cultivars to inferior cultivars was demonstrated.

Firmly crushed zygotic embryos were used to induce somatic embryogenesis in pumpkin (Leljak and Jelaska, 1995).

Kintzios *et al* (1997) studied the effect of light on the induction, development and maturation of *Cucurbita pepo* somatic embryos after prolonged incubation of cultures for *in vitro* conservation purposes. Further embryoid development to the torpedo shape stage and embryoid maturation was

significantly affected by exposure of the cultures to light during the induction phase.

2.2.2.2. GROWTH REGULATORS

Somatic embryogenesis in cucurbits requires two media: an induction medium for embryogenic cell determination and a maturation medium allowing embryogenic development (Debeaujon and Branchard, 1993). The presence of auxin in the medium is generally essential for embryo initiation. The callus is initiated and multiplied on a medium rich in auxin which induces differentiation of localized group of meristematic cells called embryogenic clumps. Somatic embryo development follows the transfer of cells or callus to media lacking auxin or with reduced levels of the same auxin or with similar or reduced levels of a weaker auxin. The addition of reduced nitrogen in the medium helps in both embryo initiation and maturation.

Kwack and Fujieda (1988) obtained direct somatic embryogenesis in pumpkin nucellus without any growth regulators. In most studies on cucurbits, somatic embryogenesis proceeded from induced embryogenic determined cells, through a callus stage.

Orczyk and Malepszy (1987) noted the efficiency of 2, 4 D and 2, 4, 5 T on cucumber tissue. The mode of regeneration depended upon 2,4 D concentration such that a high concentration led to somatic embryogenesis while a lower concentration induced caulogenesis as was observed for cucumber (Rajasekeran *et al*., 1983 and melon (Tabei *et al.,* 1991).

Media with NAA or IBA produced embryos that were able to undergo complete development whereas most embryos were restricted to the globular stage on media with 2,4 D in cucumber (Garcia Sogo, 1988; Cade *et al.,* 1990; Kageyama *et al.,* 1991 and Yang *et al.,* 2003) and melons (Whan and Suh, 1998). According to Bergervoet *et al* (1989), the use of NAA in cucumber suspension

+media instead of continuous 2,4 D changed the pattern of morphogenesis into caulogenesis rather than embryogenesis.

Chee (1991) cultured somatic embryos of *Cucurbita pepo* originated from shoot apex derived callus on MS medium supplemented with 1.2 mg l^{-1} 2,4,5-T, 0.8 mg l^{-1} BA and 0.1 mg l^{-1} kinetin. Immature somatic embryos developed into plantlets after transfer to MS medium with 0.05 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin. Regenerated plants appeared morphologically normal and set fruits which germinated normally. Chee (1992) initiated embryogenic callus tissues from cotyledons of mature seeds cultured in MS medium supplemented with either 22.7 µM 2,4 D or a combination of 4.7 µM 2,4,5 T, 4 µM BA and 0.5 µM kinetin. Clusters of somatic embryos were found in callus tissue. Maturation of these somatic embryos was achieved by transfer of embryogenic callus tissues to MS medium supplemented with 0.5 µM NAA and 0.25 µM kinetin. Regenerated mature plants were morphologically normal and set fruits containing seeds that germinated normally.

BAP was the most frequently used cytokinin for both melon (Trulson and Shahin, 1986., Rhimi *et al*., 1992 and Hanana *et al.,* 2002) and cucumber (Custers *et al.,* 1988).

Thidiazuron was found to be superior to BAP for the induction phase in cultures of melon (Gray *et al*., 1993) and watermelon (Compton and Gray, 1991).

The combination of 1.0 mg l^{-1} 2, 4 D and 0.1 mg l^{-1} BAP was efficient for induction of somatic embryogenesis in melon calli (Oridate and Oosawa, 1986) and cucumber calli (Punja *et al.,* 1990a).

Somatic embryo maturation was commonly accomplished with growth regulator free media, although cytokinins (Chee 1991; Trulson and Shahin, 1986) or rarely, Abscisic acid (Ladyman and Girard, 1983, 1992) or gibberellins

(Orczyk *et al.,* 1988; Tabei *et al.,* 1991) were also used. An exception to this was found for squash tissue where exogenous auxin was permanently required for induction and continuous development of embryos (Jelaska, 1986).

Cytohistological analysis of somatic embryos in cucumber was carried out by Tarkowska *et al* (1994). They observed that early globular embryos occur on 9th day, heart shaped embryos on 14th day and morphologically mature embryos on 19th day after pollination.

Explants and protoplasts were reported to have different growth regulator requirements. Orczyk and Malepszy (1985) observed that the auxins that were effective for obtaining cucumber plantlets from leaf explants and suspension cultures were ineffective for initial steps of protoplast culture. Cucumber (Colijn-Hooymans *et al*., 1988; Malepszy, 1988) and melon (Debeaujon and Branchard, 1991) protoplasts were successfully cultivated in an initial solid medium and the isolated microcalli were then transferred to solid induction medium.

Ziv and Gadasi (1986) suggested the use of double-layer culture with activated charcoal in the lower agar layer and ABA in the top liquid layer. This technique increased the frequency of regenerated plantlets and improved their morphological development.

2.3 *AGROBACTERIUM TUMEFACIENS* AS A VECTOR FOR PLANT GENETIC ENGINEERING

The Agrobacterium system was historically the first successful plant transformation system, marking the breakthrough in plant genetic engineering in 1983. The *Agrobacterium tumefaciens* mediated transformation system is the most widely used method for introducing foreign genes into dicotyledonous plant species (Weising *et* al., 1988). Both the efficiency of transformation and the broad host range of *Agrobacterium tumefaciens* among dicot species contribute to its widespread use. The breakthrough in gene manipulation in plants came by

characterizing and exploiting plasmids carried by the bacterial plant pathogens *Agrobacterium tumefaciens* (Smith and Townsend, 1907) and *Agrobacterium rhizogenes* (Riker, 1930) which are the causative agents of the wide spread plant diseases crown gall and hairy root respectively, These provide natural gene transfer, gene expression and selection systems.

Agrobacterium are gram negative rods that belong to the bacterial family Rhizobiaceae. These are found near soil level at the junction of plant stem and root and invade wounded plants (Primrose and Twyman, 2006). The exudates produced upon wounding induce the *vir* genes of *Agrobacterium tumefaciens* which in turn stimulates the transfer of a portion of the bacterial DNA into the plant cell where it ultimately becomes integrated into the plant chromosome (Chilton *et al.*, 1977; Thomashow *et al*., 1980, 1987).

Opine synthesis is a property conferred upon the plant cell when it is colonized by *Agrobacterium tumefaciens.* The type of opine produced is determined by the bacterial stain (Bomhoff *et al.,* 1976; Montaya *et al.,* 1977). Zaenen *et al* (1974) first noted that virulent strains of *Agrobacterium tumefaciens* harbor large plasmids (200-250 Kb) known as Ti plasmid. These bacteria induce neoplastic growth on most dicots and gymnosperms (De Cleene and De Leney, 1976) but also on a few monocots (Bytebier *et al.,* 1987; Eady *et al.,* 2000) where it results in so called crown gall tumours. The crown gall tissue represents oncogenic transformation, since the undifferentiated callus can be cultured *in vivo* even if the bacteria are killed with antibiotics, and yet retains its tumorous properties. *In vivo*, the infection requires wounding of the plant tissue (Kahl, 1982). After attachment to the cell walls of wound activated plant cells, *Agrobacterium* transfer part of their Ti plasmid (the T-region) into the nucleus of the host plant, where it becomes stably integrated (T-DNA). This carries genes that confer both unregulated growth and the ability to synthesize opines upon the transformed plant tissue.

The transferred bacterial DNA, the T-DNA, is bordered by 25 base pair repeats which are conserved sequences involved in the processing, transfer and possibly the integration of the T-DNA into the plant nuclear DNA (Wang *et al.,* 1984). Between the borders are genes that code for hormone production which cause the formation of tumors characteristic of the crown gall disease (Thomashow *et* al., 1984). For genetic transformation, the hormone genes and often the opine genes, are deleted and replaced with a selectable marker gene and genes of interest.

The vir gene complex is the main cluster of genes controlling infection by *Agrobacterium tumefaciens* (Stachel and Nester, 1986). Two of these genes, vir A and vir G, are constitutively expressed at a low level and control the plant induced activation of the other vir genes. VirA gene product produced constitutively, acts as an antenna by receiving the wound stimulus and then transmitting an induction signal to the virG genes (Stachel *et al.,* 1986). DNA transfer itself is initiated by a site specific endonuclease formed by the products of the vir D1 and vir D2 genes (Yanofsky *et al.,* 1986). This introduces either single strand nicks or a double strand break at the 25-bp borders of the T-DNA (Veluthambi *et al.,* 1987). After cutting, a single stranded T-strand is generated unidirectionally from the right border and is coated with vir E2, a single stranded DNA binding protein. The whole complex is then transferred through the pilus and into the plant cell (Konkolikova-Nicola *et al.,* 1985). The *Agrobacterium* gene transfer system represents a highly adapted form of bacterial conjugation applied to plant cells (Lichtenstein, 1987).

Once transferred to the nucleus, the T-DNA is covalently integrated into the plant genome through a process of illegitimate recombination in one to several copies (Lemmers *et al.,* 1980). The integration sites are randomly distributed throughout the plant genome. Multiple insertions as well as aberrant integration patterns may occur (De Frammod *et al*., 1986) including tandemerization in direct (Zambryski *et el*., 1980) and inverted repeats (Jones *et al*., 1987), rearrangements

and truncation of T-DNA and of plant target sequences (Weising *et al*., 1988). After its integration, T-DNA adopts eukaryotic features of chromatin organization and DNase I /(hyper) sensitivity (Coates *et al*., 1987).

As early as 1980, it was possible to transfer foreign DNA sequences to plant cells either by transposon mutagenesis or by site specific insertion of genes into T-DNA, followed by recombination with a wild type Ti plasmid (Matzke and Chilton, 1981). However, these early attempts were time consuming and cumbersome and resulted in low transformation frequencies. More efficient vector systems have subsequently been developed to facilitate bacterial genetic manipulations and to allow selection and regeneration of transformants.

Initially the problem was addressed by the construction of cointegrate vectors in which new genes are introduced via homologous recombination into an artificial T-DNA already present on the Ti plasmid produced a so-called intermediate vector (Zambryski *et al*., 1983). Binary vector systems in which the vir genes and the disarmed T-DNA containing the transgene are supplied on separate plasmids (Hoekma *et al.,* 1983; Bevan, 1984).

2.3.1 Chimeric gene vectors

The nuclear plant gene consists of different regions, each involved in different functions of transcription and translation of mRNA. Starting with 5' end, there is a promoter region that is involved in the initiation of transcription, together with enhancer/silencer regions that confer regulation of expression, a transcriptional start or cap site, and the so called CAAT and TATA boxes, which help in binding RNA polymerase. One or more untranslated or intron regions are present within the transcribed region. The end of the translation region is determined by a stop codon and followed by a terminator at the 3' end polyadenylation signal (Chawla, 2002).

Plants are usually transformed with relatively simple constructs in which the gene of interest is coupled with an appropriate promoter, 5' leader and 3' terminator sequences to ensure efficient transcription, stability and translation of mRNA. The promoter can be of plant, viral or bacterial origin. Some promoters confer constitutive expression, whereas others may be selected to permit tissue specific expression or environmentally inducible expression.

Plant viruses which are dependent on plant transcription and translation factors have been used as sources of regulatory elements. Most commonly used are the promoters of the 35S RNA of the cauliflower mosaic virus (*CaMV*). It directs high levels of expression in most tissues. Others such as maize ubiquitin 1 promoter, *rbcs* (ribulose biphosphate carboxylase small sub unit), *Adh* 1 (alcohol dehydrogenase), *nos* (nopaline synthase) and the rice actin promoter / intron sequences are often preferred for expression in monocots.

The control of gene expression plays an important role in plant development and it is obvious that consideration has to be given to the regulation of gene introduced artificially. As the DNA constructs become more sophisticated, they will contain complex chimeric nucleotide sequences that are a combination of enhancer and silencer sequences, transcription promoters and terminators, protein-coding open reading frames possibly with organelle targeting or signal sequences, selectable markers, reporter genes and vector sequences. Thus, it is the chimeric gene constructs that are used for expression of transferred genes.

2.3.2 Gene fusion markers in transgenic plants (Reporter genes)

A reporter gene is a test gene whose expression results in a quantifiable phenotype. A reporter system is useful in the analysis of plant gene expression and standardization of parameters for successful gene transfer in a particular technique. The principle of using reporter genes in studying molecular processes in a living cell means that in the natural gene, a synthetic modification is

introduced in order to either simplify the detection of the gene product or to distinguish it from similar or identical genes in the genome.

A reporter gene produces a protein that is easily detectable in transformed organisms. Often, the protein possesses an enzymatic activity that can turn a colourless substrate into a coloured product. Thus, it is able to see the location and amount of gene expression in a transformed organism by looking at the location and intensity of the coloured product (Hooykaas and Shilperoort, 1992).

NPT II or Neomycin phosphotransferase II is the most widely used marker. It imparts kanamycin resistance so that the transformed tissue can be selected on kanamycin. The npt II gene derived from the Tn5 transposon, inactivates kanamycin, neomycin and G418 by phosphorylation. This gene was used as a successful marker in melon (Dong *et al*., 1991; Valles and Lasa, 1994; Guis *et al.,* 2000; Awatef *et al.,* 2007), cucumber (Ganapathi and Perl Treves, 2000; Soniya and Das, 2002; Kose and Koc, 2003; Sarmento *et al*., 1992; Raharjo *et al*., 1996) and bottle gourd (Sul *et al*., 2005).

Hygromycin phosphotransferase (hpt) gene was originally derived from *Escherichia coli*. This inactivates the antibiotic hygromycin. This gene was successfully tried in cucumber (Tabei *et al*., 1994).

The firefly luciferase gene (lux) has been used as a marker in transgenic plants (Ow *et al*., 1986) but the enzyme is labile and difficult to assay with accuracy. Disarmed strain of *Agrobacterium tumefaciens* was used to mediate the transfer and expression of a reporter gene encoding firefly luciferase in the genome of the cucumber hybrid Bambina (Sapountzakis and Tsaftaris, 1996).

New gene fusion systems were developed that are easy to quantitate, highly sensitive and moreover allowing analysis of genes whose products are of

moderate and low abundance in contingent on a complete absence of any intrinsic reporter activity in plants.

Jefferson *et al* (1987) developed the *Escherichia coli* β glucuronidase gene as a reporter gene system for transformation of plants. It can be assayed histochemically to localize GUS activity in cells and tissues. β glucuronidase, which is encoded by the uid A locus is a hydrolase that catalyzes the cleavage of β glucuronide bond in a variety of useful substrates, many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. Uid A encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions.

Shetty *et al* (1997) used GUS reporter gene for transforming melon plants. Transgenic melon plants carrying GUS gene was obtained by Kennedy *et al* (2004).

Genes conferring resistance to herbicide are also used as selectable marker. The bar gene isolated from *Streptomyces hygroscopicus* confers resistance to the herbicide phosphinothricin. The bar gene codes for the enzyme phosphinothricin acetyl transferase (PAT), which converts PPT into non herbicidal acetylated form. This gene has been inserted and expressed in cucumber (Sapountzakis and Tsaftaris, 2002; Soon *et al.,* 2003; Vengadesan *et al*., 2005) and bottle gourd (Han *et al*., 2005).

2.3.3 *Agrobacterium* mediated plant genetic transformation

The *Agrobacterium* system was historically the first successful plant transformation system, making the breakthrough in plant genetic engineering in 1983 (Fraley *et al*., 1983; Herrera-Esterella *et al*., 1983). Since then this gene delivery system has been used widely in a number of crops and transgenic plants of commercial importance were produced (Lindsey, 1992). The ability of *Agrobacterium* to transfer a fragment of its DNA to the plant cell provides a

powerful tool for plant biotechnology, and therefore, *Agrobacterium* – mediated DNA transfer is one of the most commonly used techniques of plant transformation (Ziemienowicz, A., 2001). The *Agrobacterium* mediated transformation has the following advantages-

i) It is a natural means of transfer

ii) *Agrobacterium* is capable of infecting intact plant cells, tissues and organs

iii) It is capable of transferring large fragments of DNA very efficiently without substantial rearrangements

iv) Stability of gene transferred is excellent

v) Integration of T DNA is a relatively precise process

Efficient transformation methods require good control of the regeneration step through either organogenesis or somatic embryogenesis. The efficiency of regeneration has been found to be highly dependent on the stage of development and the growth conditions (Yadav *et al.,* 1996) and the genotype (Ficcadenti and Rotino, 1995).

2.3.3.1 Preculture

One of the critical factors in achieving high frequencies of transformation is the preculture of explants on the inoculation medium prior to co-cultivation. The preculturing is required to make the cells more amenable to transformation. It makes the explant tissue competent enough to withstand the bacterial infection and the related stress that follows it.

Vasudevan *et al* (2007) precultured the cotyledon explants of cucumber for 5 days in shoot bud induction medium to induce shoot bud at the proximal region and more GUS expression. Beyond 5th day, cotyledon explants produced shoots but most of them were escapes due to pre emergence of shoot primordia from the explants before agro infection.

2.3.3.2 Inoculation time and media

The explant, in their most receptive stage is exposed to the *Agrobacterium* culture in the inoculation medium at an optimum bacterial density. Both the composition of the inoculation media and time of inoculation may have a role in the efficiency of transformation.

For inoculation, regeneration medium of the explant that can support bacterial growth like Murashige and Skoog medium is used. It can also be the bacterial culture medium. The pH of the medium usually maintained range from 5.5 to 5.7.For infection the explants are mostly immersed in the inoculation medium for a time varying 5 min to 30 min.

Sarmento *et al* (1992) tried various inoculation times ranging from 5 min to 60 min during their transformation studies in pickling cucumber. Using MS medium for inoculation they found that more than 45 min prolonged exposure, the explants become necrotic and died.

Nora *et al* (2001) reported that young, non expanded leaves of melon showed successful transformation when immersed in an *Agrobacterium* suspension culture (OD_{600} = 0.4 to 0.6) for 20 min under gentle agitation.

Transformation works in cantaloupe melon revealed that the inoculation time of 30 min was ideal on MS medium supplemented with 1 µM BAP and 0.2 µM GA_3 (Guis *et al.,* 2000).

The most effective bacterial infection was observed when cotyledon explants of 4 day old seedlings of bottle gourd was immersed in the bacterial inoculum for 20 min and then washed in the same medium consisting of MS medium supplemented with BA and silver nitrate for 10 sec (Han *et al.,* 2005).

Soniya and Das (2003) reported that infection was most effective when cucumber cotyledon explants were infected with *Agrobacterium* strain LBA4404 for 15 min on shoot induction medium consisting of MS medium supplemented with 4 mg l^{-1} BA and 1 mg l^{-1} Indole propionic acid.

Awatef *et al* (2007) obtained successful plant transformation from Tunisian *Cucumis melo* when the cotyledon explants from 10 day old seedlings were inoculated with an overnight culture of *Agrobacterium* OD_{600} = 0.8 for 20 min.

In cucumber, Vasudevan *et al* (2007) reported that an inoculum density of 1.0 OD_{600} was optimum for the infection of cotyledon explants for 10 min in induction medium consisting of MS medium supplemented with BA and ABA.

2.3.3.4 Co-cultivation

Co-cultivation period plays an important role in the success of transformation. It is during this period that the *vir* genes are activated and T-DNA transferred into plant cell. The length of the co-cultivation period should always be the shortest interval necessary to obtain the maximum frequency of transformation in terms of the number of transgenic plants recovered following co-cultivation.

Raharjo *et al* (1996) during their transformation experiments in pickling cucumber tried different co-cultivation periods ranging from 48 to 96 hrs to get the best transformation efficiency. The frequency of embryogenic callus formation ranged from 0 to 12%.

Ezura *et al* (2000) reported that ethylene affects the *Agrobacterium* mediated gene transfer to the explants excised from melon cotyledons. Application of 10µM AVG inhibited ethylene production from explants.

Cotyledon explants of cucumber were co-cultivated with *Agrobacterium* for two days. The survival rates after co-cultivation on PPT and kanamycin were 5% and 8% respectively (Ganapathi and Perl Treves, 2000).

Kennedy *et al* (2004) obtained melon calli and embryos in embryo induction medium consisting of liquid MS supplemented with 2 mg l^{-1} 2,4 D and 0.1 mg l^{-1} BA after 9 weeks of bacterial inoculation. Over 80% of these were transgenic.

In bottle gourd, the optimum length of co-cultivation period was 6-8 days resulting in an infection frequency of 96.8-100%. The blue area indicating the transient expression of the GUS gene was initially observed after 2 days of co-cultivation (Han *et al.,* 2005).

A two day co-cultivation period was found optimal for cucumber cv. Poinsett 76 which led to the production of significantly higher rate of GUS expression. The optical density greater thn 1.0 OD_{600} and co-cultivation period beyond 3 days led to bacterial overgrowth and leaching of bacteria from explants (Vasudevan *et al*., 2007).

Awatef *et al* (2007) reported that in Tunisian *Cucumis melo, Agrobacterium* infected explants developed green organogenic calli. Cotyledon explants formed transgenic shoots, but transformation frequency was low 6.66%.

2.3.3.5 Elimination of bacteria after co-cultivation

After co-cultivation, the explants are transferred to media containing bacteriostatic agent to kill the bacteria. Complete elimination of the bacteria from the explant after co-cultivation is very essential otherwise it will interfere with the growth and organogenesis of the explant. Elimination of the bacteria from the explant is done by use of antibiotics. The most commonly used antibiotics for this purpose are carbenicillin and cefotaxime.

Carbenicillin 50 mg l-1 was used for inhibiting bacterial growth after co-cultivating cucumber explants (Sarmento *et al*., 1992).

Cotyledon explants co-cultivated with bacteria were washed with liquid selection medium containing 500 mg l^{-1} Cefotaxime in bottle gourd (Han *et al*., 2005) and sterile distilled water containing 300 mg l^{-1} Cefotaxime in cucumber (Vasudevan *et al*., 2007).

In Tunisian *Cucumis melo*, the explants were freed from bacteria by washing with liquid MS medium containing 300 mg l^{-1} Claventin (Awatef *et al.,* 2007).

2.3.3.6 Selection of transformed cells

The explants are transferred to a selection medium containing appropriate selection agent depending on the plant selectable marker employed for transformation. Resistant or tolerant tissues are continuously grown and selected on selection agent followed by regeneration (Kumaresan, V. 2001).

The frequency of calli developed from cucumber explants on Kanamycin containing medium following *Agrobacterium* transformation was influenced by explant size, bacterial concentration and length of exposure to the bacteria (Sarmento *et al.,* 1992). The selection medium comprised of the shoot regeneration medium supplemented with 100 mg l^{-1} Kanamycin and 300 mg l^{-1} Cefotaxime (Ganapathi and Perl Treves, 2000). Nishibayashi et al (1996) compared the effects of kanamycin and hygromycin on the selection of transgenic cucumber. Hygromycin resistant calli were selected under the presence of 20-80 mg l^{-1} hygromycin (Tabei *et al*., 1994). Although kanamycin allows to grow callus of explants uninfected *Agrobacterium,* hygromycin is more effective to suppress callus formation from uninfected *Agrobacterium* (Nishibayashi *et al*., 1996). Phosphinothricin was employed for selection of transformants by Vasudevan *et al* (2007). The advantage of using phosphinothricin for the

screening procedure is that it has a localized effect i.e., only those plant cells that uses the active bar gene have the ability to detoxify the herbicide.

In melon, Kanamycin (Valles and Lasa, 1994; Guis *et al.,* 2000) and Carbenicillin (Nora *et al*., 2001) were used as the selection agents for transformation.

Phosphinothricin at 2 mg l-1 was used for selection of herbicide resistance (bar) genes in bottle gourd (Han *et al.,* 2005).

2.3.3.7 Analyses of transformed plant cells

Selection and growth of plant cells on selective media provide initial phenotypic evidence for transformation. However, spontaneous variants with increased resistance to many chemicals can be readily selected in plant tissue culture. This includes resistance to kanamycin (Owens, 1981), the most commonly used selection agent for plant transformation. Therefore biochemical and molecular evidence is essential to confirm expression and integration of transferred genes.

The presence of a selectable marker allows selection of transgenic cells but does not guarantee 100% co-transmission and/or expression of the other genes on the same section of T-DNA. Independently selected transgenic plants often show varying degrees of gene expression. Several factors other than the absolute effects of the regulatory sequences can influence the magnitude of gene expression. These include copy number, position effects resulting from the site of insertion and methylation of the transferred genes.

The integration of the foreign genes into the plant nuclear genome can be determined by via Southern analyses and the use of the Polymerase Chain Reaction (PCR). PCR is a new and powerful technique for confirming DNA insertion in transgenic plants (Lassner *et al*., 1989). Primers can be designed

which simultaneously amplify specific genes or T-DNA regions on the T-DNA that are expected to be integtrated into the genome of plants. Advantages include the rapid manner in which large collection of transgenic plants can be analysed and the very small amount of plant tissue required.

The presence of latent *Agrobacterium* that multiplies in the plant tissue in spite of antibiotic application confounds the results obtained by PCR analysis of putative transgenic plants. The contaminating plasmid vector becomes double stranded after renaturation and is cut by a restriction enzyme having site(s) within the PCR amplicon. As a result amplification by PCR is not possible whereas the genomic DNA with a few copies of the transgene remains single stranded leading to amplification by PCR (Nain *et al*., 2005). The integration of the transgene into the melon genome was confirmed by Awatef *et al* (2007). In cucumber (Vasudevan *et al.,* 2007) PCR amplification confirmed the presence of bar gene in the putative transformants. Similar results were obtained in bottle gourd by Han *et al* (2005).

Southern Analyses allows the number of copies and nature of the integration of the specific genes or DNA regions to be determined. In melon, genomic DNA when digested with Hind III, which cuts only once within the T-DNA but not in the transgene, released only one fragment from transformed plants but not from untransformed, plants (Guis *et al.,* 2000). Vasudevan *et al* (2007) carried out Southern hybridization on genomic DNA from GUS positive shoots developed on cucumber cotyledon explants.

2.4 PARTICLE BOMBARDMENT METHOD FOR EFFICIENT PLANT TRANSFORMATION

Particle bombardment method has evolved into a useful tool allowing direct gene transfer to a broad range of cells and tissues. The technique involves accelerating DNA coated particles (microprojectiles) directly into intact tissues or cells. It is conveniently used for evaluating transient expression of different gene

constructs in intact tissues. The likelihood of recovering a transformed plant through DNA bombardment is dependent on the frequency of hitting the target tissue, the frequency with which bombarded tissue can be propagated or regenerated and the frequency of stable gene expression (Fillatti, 1987). A plasmid containing the *cat* reporter gene driven by the CaMV35S promoter was tested to determine whether DNA could be delivered three days after bombardment. Analysis of the epidermal tissue revealed high levels of transient CAT activity (Klein *et al.*, 1987).

The microprojectile method was used to transfer DNA into embryogenic callus of cucumber and stably transformed lines were obtained. Genomic blot hybridization analyses showed that a high percentage (16%) of the cucumber explants were transformed with Nos-NPTII however, only about 25% of the transgenic plants expressed the gene (Chee and Slightom, 1992).Schulze *et al* (1995) reported that biolistic transformation of cucumber using embryogenic suspension cultures showed activity of the uidA gene about one year after bombardment, indicating a high stability of the non selectable gene. All selected plants were proved to be nptII positive and no escapes could be detected.

Materials and Methods

3. MATERIALS AND METHODS

The investigations on *Agrobacterium* mediated genetic transformation of pumpkin, *Cucurbita moschata* Poir. were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara from October 2005 to July 2008. The study was aimed at standardizing *in vitro* regeneration and transformation systems in pumpkin variety Saras. Details regarding the experimental materials used and the methodologies adopted for various experiments are described below.

3.1 SOURCE OF EXPLANT

Seedlings of Cucurbita moschata Poir. var. Saras raised under in vitro conditions on Murashige and Skoog (MS) medium served as the source of explant for the study.

3.2 CULTURE MEDIUM

3.2.1 Chemicals

The major and minor elements required for the preparation of media were of analytical grade and procured from M/s Sisco Research Laboratories (SRL), British Drug House (BDH) and M/s Merck India Ltd. The amino acids, vitamins and chemicals used for molecular biology work were obtained from M/s Merck India Ltd., SRL and Sigma Chemicals, USA. The antibiotics were obtained from Himedia.

3.2.2 Glass wares and plastic wares

Borosilicate glasswares of Corning/Borosil were used for the experiment. The glasswares were cleaned initially by soaking in potassium dichromate solution for 12h followed by thorough washing with jets of tap water in order to remove completely all traces of potassium dichromate solution. They were further cleaned with 0.1 per cent teepol detergent solution and were washed thoroughly with water and rinsed twice with double distilled water. These were then air dried in hot air oven at 105^{0}c for 24h and later stored in cupboards free of dust till further use.

The plastic wares used for the experiment were procured from Tarsons India Ltd. and Axygen. The plastic wares were autoclaved at 121°C by applying 15 psi pressure for 20 min and stored in cupboards free of dust till use.

3.2.3 Composition of media

Basal MS medium (Murashige and Skoog, 1962) supplemented with different plant growth regulators were used for plant tissue culture in the present study. Yeast Extract Mannitol (YEM) medium was used for culturing Agrobacterium tumefaciens strain EHA 105 during the study. The basal composition of these media are given in Appendix-I.

3.2.4 Preparation of the tissue culture medium

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of plant tissue culture media. Stock solutions of major and minor elements were prepared and stored in pre-cleaned amber coloured bottles in refrigerated conditions.

For preparation of the medium, a clean steel vessel, rinsed with distilled water was used. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. A little amount of distilled water was added to it and later on, required quantities of sucrose and inositol were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of the medium was adjusted between 5.6 and 5.8 using 0.1N NaOH or HCl.

For solid medium, after adjusting the pH, agar was added at 0.75 per cent (w/v) concentration. The medium was stirred and heated to melt the agar, and was poured when hot into culture vessels and were plugged with non-absorbent cotton. The medium was then dispensed to test tubes (15 cm x 2.5 cm) at the rate of 15 ml each or to conical flasks of required size at the rate of 50 ml in 100 ml conical flask, 100 ml in 250 ml conical flask and 250 ml in 500 ml conical flask. Vessels containing media were sterilized in an autoclave at 121°C by applying 15 psi pressure for 20 min

(Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in culture room until used.

3.2.5 Preparation of YEM medium for Agrobacterium

For preparation of the media, clean steel vessels, rinsed with distilled water were used. The ingredients were weighed on electronic balance and were added into the vessels. The ingredients were dissolved in little amount of distilled water. The desired volume was made up by adding distilled water. The pH of the media was adjusted to 7.0 using a standard pH meter by adding either 0.1N NaOH or HCl.

Agar was added at the rate of 20 g l^{-1} for solid media. The media were stirred and heated to melt the agar and were poured when hot into conical flasks (100 ml). Fifty ml medium was poured in each conical flask and the conical flasks were plugged with non-absorbent cotton. Autoclaving was done at 121°C at 15 psi for 20 min to sterilize the medium. The sterilized flasks were kept in the culture room until used.

3.3 TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic manipulations were carried out under the hood of a laminar air flow cabinet fitted with UV lamp for extra protection.

3.4 CULTURE CONDITIONS

The cultures were incubated at 26 ± 2^0c in an air conditioned culture room with 16 h photoperiod (1000 lux) supplied by fluorescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing.

3.5 STANDARDIZATION OF SURFACE STERILIZATION PROCEDURE

In order to formulate an effective surface sterilization technique, mercuric chloride (0.1%) at two different time periods was used.

3.5.1 Effect of surface sterilant on culture establishment

The mature seeds were treated with 0.1 per cent solution of mercuric chloride at two time intervals namely, 6 min and 10 min.

The sterilization treatments were carried out under aseptic conditions in a laminar flow cabinet and the sterilized seeds were inoculated in MS basal and half MS basal for comparing the survival percentage.

3.6 EXPLANTS

Various explants viz. cotyledons, cotyledonary node, hypocotyls and leaf segments were used for the study.

3.6.1 Establishment of aseptic plants

Seeds of *Cucurbita moschata* Poir. var Saras obtained from the Department of Olericulture, College of Horticulture, Vellanikkara were used for the study. The seeds were washed in water containing few drops of teepol and rinsed with running tap water to remove teepol. The seeds were taken to the laminar flow and surface sterilized with $HgCl_2$ (0.1 per cent) for one minute. It was then washed free of $HgCl_2$ by rinsing with three changes of sterile distilled water. The seeds were drained on sterile blotting paper. The surface sterilized seeds were incubated in basal MS medium containing 2 per cent sucrose. The cultures were incubated in the culture room.

3.6.2 Preparation of explants

The *in vitro* raised seedlings were taken out of the culture tubes in a laminar flow cabinet on pre-sterilized steel plates. The seedlings were cut with sterile blade to separate cotyledons and hypocotyls. The hypocotyls were cut approximately 2-2.5 cm below the cotyledonary node, separated from the roots. The leaf segments were cut into 1 cm^2 (approximately) with a sterile blade.

3.7 STANDARDIZATION OF CALLUS MEDIATED REGENERATION

3.7.1 Standardization of explants

Cotyledonary node, cotyledonary leaf segments, hypocotyl segments and taken from *in vitro* raised seedlings were cultured in MS media supplemented with different combinations of auxins and cytokinins. The leaf segment cut into 1 cm^2 was inoculated with proximal end touching the medium. The hypocotyl segments (2-2.5 cm long) were placed horizontally in the culture medium. Cotyledons were taken from intact seeds and were dissected into well defined explant types, namely, basal cotyledon explants, distal cotyledon explants and cotyledonary axis explants. The cultures were incubated in the culture room. Observations with respect to callus induction and proliferation of different explants were noted.

3.7.2 Standardization of growth regulators

The explants were cultured in MS medium supplemented with different concentrations of auxins and cytokinins. The cultures were incubated in the culture room. Observations regarding the response of explants were recorded.

Table.1. Different media combinations used for callus induction from different explants of *Cucurbita moschata* Poir. var. Saras

Sl. No.	Media combinations
1	MS + 0.5 mg l^{-1} 2,4 – D
2	MS + 0.7 mg l^{-1} 2,4 – D
3	MS + 1.0 mg l^{-1} 2, 4 – D
4	MS + 2.5 mg l^{-1} 2, 4 – D
5	MS + 0.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BA
6	MS + 5 mg l^{-1} 2, 4 – D + 0.1 mg l^{-1} TDZ
7	MS + 1.0 mg l^{-1} Picloram
8	MS + 5.0 mg l^{-1} Picloram

9	MS + 0.1 mg l^{-1} TDZ
10	MS + 0.5 mg l^{-1} TDZ
11	MS + 0.1 mg l^{-1} TDZ + 5 mg l^{-1} Picloram
12	MS + 0.5 mg l^{-1} NAA
13	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA
14	MS + 2.0 mg l^{-1} NAA + 0.5 mg l^{-1} BA
15	MS + 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA
16	MS + 3.0 mg l^{-1} NAA + 0.5 mg l^{-1} BA
17	MS + 3.0 mg l^{-1}NAA + 1.0 mg l^{-1}BA
18	MS + 0.2 mg l^{-1} NAA + 4.0 mg l^{-1} BA
19	MS + 0.2 mg l^{-1}NAA + 0.2 mg l^{-1}Kin
20	MS + 0.3 g l^{-1} Casein hydrolysate + 0.2 mg l^{-1} NAA + 2.0 mg l^{-1}Kin
21	MS + 1.0 mg l^{-1} BA
22	MS + 3.0 mg l^{-1} BA
23	MS + 5.0 mg l^{-1} BA
24	MS + 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA
25	MS + 1.0 mg l^{-1} IAA + 5.0 mg l^{-1} Kin
26	MS + 1.5 mg l^{-1} IAA + 6.0 mg l^{-1} Kin

3.7.3 Establishment of callus cultures

Media composition as well as the suitable explant were standardized and utilized for the establishment of callus cultures. Cotyledonary leaves, hypocotyls, cotyledonary node and cotyledons were cultured in test tubes in media containing different growth regulators. Callus cultures were maintained by subculturing every 21st day on fresh medium containing the same growth regulator combination. The cultures were kept in light for callus induction.

The observations on time taken for callusing, number of explants callused and callus growth was recorded. Callus Index (CI) was calculated based on the percentage of explant callused and callus growth score as given below:

$$CI = P \times G$$

where P is the percentage of explant callusing and G is the average growth score of the callusing explants. The maximum growth score of four was given when the surface of the media in the tube was fully covered by callus.

3.8. STANDARDIZATION OF *IN VITRO REGENERATION*

3.8.1 Explant source

The cotyledonary nodal segments taken from *in vitro* seedlings served as the source of explants for inducing callus mediated organogenesis in *Cucurbita moschata* Poir.

3.8.2 Production of multiple shoots

The cotyledonary nodal segments produced white friable callus in MS medium containing 1 mg l^{-1} BA. The multiple shoots were produced when the callus was cultured in different combinations of growth regulators. The cultures were maintained in the culture room. Observations regarding the response of explants to different media combinations were recorded.

Table.2. Different media combinations used for *in vitro* regeneration from cotyledonary node explants in *Cucurbita moschata* Poir.

Sl. No.	Media combinations
1	MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kin
2	MS + 1.5 mg l^{-1}IAA + 1.0 mg l^{-1} BA
3	MS + 1.5 mg l^{-1} IAA + 6.0 mg l^{-1} Kin

3.8.3 Rooting

Multiple shoots were excised using a sterile blade and inoculated in MS medium containing 1 mg l^{-1} IBA. The cultures were maintained in the culture room. Observations regarding the percentage of rooting were documented.

3.8.4 Hardening

Rooted plantlets were taken out of the culture tubes and washed in running tap water to remove the adhering media. These plants were planted in pots containing sterile potting mixture. These tissue culture regenerants were kept in net house for 2 weeks.

3.8.5 Planting out

The hardened plants were transferred to pots (capacity-2kg) containing sterile potting mixture in net house. Regular watering of the plants was done. Observations regarding the percentage of plants established were documented.

3.9. AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION

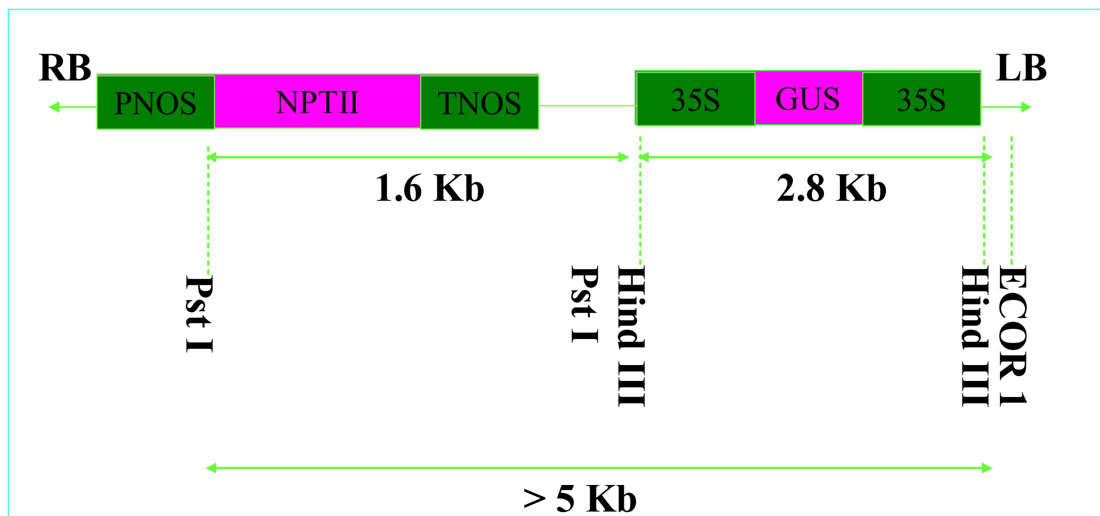
3.9.1 Evaluation of the sensitivity of explants to various antibiotics

The cotyledons were tested for their sensitivity to various antibiotics, in order to select a suitable marker for *Agrobacterium* mediated genetic transformation. The antibiotics used for testing sensitivity were kanamycin and cefotaxime.

The callus induction medium namely, MS + 5 mg l^{-1} 2,4 –D + 0.1 mg l^{-1} TDZ was supplemented with 50 mg l^{-1}, 100 mg l^{-1}, 150 mg l^{-1} and 200 mg l^{-1} of each antibiotic, separately. The medium containing the antibiotic was dispensed to sterile disposable petriplates and allowed to cool and solidify. When the medium cooled down to room temperature, the explants were inoculated. A control having no antibiotic was also maintained. Observations regarding the response of explants to each antibiotic were recorded.

3.9.2 *Agrobacterium tumefaciens* strain

Agrobacterium tumefaciens strain EHA105 was used for the study. The EHA 105 contains the plasmid p35SGUSINT. This plasmid contains *gusA* gene fused to the *CAMV 35S* promoter and the *npt II* gene controlled by the nos promoter (Fig.1). The intron inserted in the protein coding region of the GUS INT gene prevents the translation of the corresponding mRNA in *Agrobacterium tumefaciens*.



NPT II - Neomycin phosphotransferase
GUS - β glucuronidase
NOS - Nopaline synthase promoter

RB - Right border
LB - Left border
35S - 35S Promoter

Fig.1. p35SGUSINT

Table.3. Details of constructs used for transformation studies in pumpkin

Name of construct	Strain of *Agrobacterium*	Plant selection marker	Gene	Source of construct
p35S GUSINT	EHA105	Kanamycin	GUS	NRCPB, IARI, New Delhi

3.9.3 Culturing of *Agrobacterium tumefaciens* strain EHA 105

The bacterial culture was maintained in YEM medium containing 50 mg l^{-1} kanamycin and 20 mg l^{-1} rifampicin.

The YEM medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use, the media was melted and then cooled to 40 to 50°C. Inside a laminar flow cabinet, the antibiotics were added to the medium and mixed thoroughly by vigorous shaking. The medium was then distributed in sterile petriplates and allowed to solidify and attain room temperature. A single colony was scooped from the previous bacterial culture plate using sterile bacterial loop and streaked on to the freshly prepared solidified medium. The newly streaked bacterial plate was sealed with parafilm and incubated in inverted position at room temperature (28-30°C). The bacterial strain was subcultured once in a month.

3.9.4 Maintenance of strains

The strain was maintained as bacterial stabs and glycerol stocks.

3.9.4.1 Preparation of stabs

The YEM medium containing Kanamycin 50 mg l^{-1} and Rifampicin 20 mg l^{-1} was poured in sterilized culture tubes and allowed to solidify. The transfer loop was flamed, cooled and plunged in a single cell bacterial colony. The loop loaded with bacteria was used to stab the solid medium in the culture tube. The stabs were incubated at 27ºC. The stabs showing good growth of bacteria were further stored in refrigerator at 4-6°C till further use.

3.9.4.2 Preparation of glycerol stock

Liquid YEM medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use the required antibiotics were added to the medium and shaken well in a laminar flow. Using a sterile bacterial loop, a single colony of the bacteria from the culture was taken and inoculated into the YEM liquid medium and shaken well to form a uniform bacterial suspension. The conical flask was plugged and kept in shaker (120 rpm) at 28°C for 18 to 24 hours according to the required density of the bacteria.

Inside the laminar air flow, 800 µl of the bacterial suspension was added to a sterile eppendorf tube. To this 200 µl autoclaved glycerol was added and stored at -20^{0}C.

3.9.5 Plasmid isolation from *Agrobacterium tumefaciens*

Binary vector constructs were isolated from the bacterial strains to confirm their presence using alkali lysis method (Birnhoim and Doly, 1979). Composition of various reagents used for plasmid isolation is given in Appendix II.

3.9.5.1 Procedure

YEM media (5 ml) containing 50 mg l^{-1}Kanamycin and 20 mg l^{-1} Rifampicin was taken in test tubes. To these, about 10 bacterial colonies were inoculated and incubated in a shaker (120 rpm) for 36-48 hours at 28°C. 1.5 ml aliquot of the broth was transferred into an eppendorf tube and centrifuged at 5000 rpm for 3 min at 4°C. The pellet was saved. To this 500 µl of ice cold solution II was added and incubated on ice for 30 min. 200 µl of freshly prepared solution I was added and mixed gently by inverting the tube 5 times. 150 µl of ice-cold high salt solution (solution III) was added, mixed gently and incubated on ice for 1 hour. This was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected promptly, added 1 ml 100 per cent ethanol, incubated on dry ice (-20°C) for 30 minutes and centrifuged at 14,000 rpm for 10 min at 4°C. The pellet was air dried and resuspended in 200 µl solution IV and incubated on ice for 30 min. 100 µl of ice cold 100 per cent ethanol was added, incubated on dry ice for 15 min, centrifuged at 14,000 rpm for 10 min at 4°C. The pellet was saved and dissolved in 200 µl TE

buffer. The resuspended pellet was extracted once with 200 µl phenol: chloroform:isoamyl alcohol (25:24:1), centrifuged at 14,000 rpm for 10 min at 4°C. The aqueous phase was collected and pelleted the DNA by addition of 0.5 volume of solution V and 2.5 volume of 100 per cent ethanol for 1 hr at 20°C. The pellet was washed with 70 per cent alcohol and dissolved in TE buffer (pH - 8.0) and checked on 0.7 per cent agarose gel.

3.9.6 Standardization of *Agrobacterium* mediated transformation

Agrobacterium mediated genetic transformation of *Cucurbita moschata* Poir var. Saras was standardized with construct having *gus* reporter gene. Cotyledonary leaf segments and cotyledons were used as the explants for transformation. Callus induction medium for cotyledon explants namely MS medium supplemented with 5 mg l^{-1} 2,4 - D and 0.1 mg l^{-1} TDZ was used for the study.

3.9.6.1 Preparation of explants

Cotyledon explants were extracted from mature seeds inside the laminar flow cabinet. The cotyledons were disinfested by agitation for 6 min in 100 ml of an aqueous solution of Teepol and was rinsed twice with sterile water and placed for immediate use in sterile distilled water. The cotyledons were dissected using sterile scalpel blade into well defined explant types. Each cotyledon yield one cotyledonary axis, four basal cotyledon explants and four distal cotyledon explants. The segments were placed in callus induction medium in sterile petriplates. The petriplates were sealed with parafilm and incubated in culture room for 2 weeks. The callused explants were cut into small bits and placed in callus induction medium and incubated in culture room for 2 days.

3.9.6.2 Preparation of Agrobacterium culture

The required volume of liquid YEM medium was prepared in conical flask and kept in culture room until use. Kanamycin (50 mg l^{-1}) and rifampicin (20 mg l^{-1}) were added to the medium on the day of inoculation and shaken well in a laminar flow. A single colony of the bacteria from the culture was taken using a sterile bacterial loop, and inoculated into the YEM liquid medium and shaken well to form a uniform bacterial suspension. The conical flask was plugged and kept in shaker (120 rpm) at 28°C for 18 to 24 hours according to the required density of the bacteria. The

bacterial density was measured in spectronic 20 spectrophotometer at a wavelength of 600 nm.

3.9.6.3 Preparation of inoculation media

For the preparation of inoculation media, liquid MS medium was used. The *Agrobacterium* suspension after overnight incubation was centrifuged at 10000 rpm for 15 minutes at 4°C. The supernatant was discarded and the bacterial pellet was washed thrice with liquid MS. The bacterial pellet was re-suspended in MS liquid medium to allow the required bacterial density.

3.9.6.4 Standardization of inoculum density

Transformation was tried with cotyledonary leaf segments and cotyledons at different inoculum density (bacterial concentration) ranging from OD_{600} = 0.5 to 0.8.

3.9.6.5 Standardization of infection time

The explants were immersed in *Agrobacterium* suspension for different time periods namely 5, 10, 15 and 20 minutes. The explants were then blotted dry with sterile blotting paper.

3.9.6.6 Standardization of co-cultivation period

The blot dried explants were transferred to regeneration medium (without antibiotics) in petriplates. The cultures were maintained at 26 ± 2°C.

The regeneration medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use, the media was melted and then cooled to 40 to 50°C. Inside a laminar flow cabinet the medium was distributed in sterile petriplates and allowed to solidify and attain room temperature. The blot dried hypocotyl explants were inoculated into the media in an upside down position. The cultures were maintained at 26 ± 2°C.

The period of co-cultivation tried ranged from one to four days.

3.9.6.7 Pre-selection

The explants after co-cultivation were washed with liquid MS containing 250 mg l^{-1} cefotaxime. The explants were then blot dried and inoculated in pre-selection medium containing bacteriostatic agent to kill the bacteria (regeneration medium + 250 mg l^{-1} cefotaxime).

The callus induction medium was prepared in required volume in conical flask and kept in culture room. On the day of use, the media was melted and cooled to 40-50°C. The antibiotics were added to the medium under aseptic conditions and mixed thoroughly by vigorous shaking. The medium was then distributed in sterile petriplates and allowed to solidify and attain room temperature. The co-cultivated explants were then transferred to the solidified media and cultured under 26 ± 2°C for a week.

3.9.6.8 Screening of transformants

The explants were transferred to screening media in culture tubes after pre-selection. The screening media consisted of callus induction medium containing antibiotics was used as selecting agent and the bacteriocidal agent. Control explants (without infection) were also maintained in the screening medium.

The cultures in the screening media were sub cultured every 2 to 3 weeks. All the cultures were maintained in the culture room under light.

3.9.6.9 Transient expression of GUS reporter gene (histochemical assay)

The histochemical GUS assay was carried out as described by Jefferson *et al*. (1987). The transient expression of GUS can be monitored after 3 days of transfer to the pre-selection medium. The composition and preparation of X-Gluc stain is as follows:

Reagents

X-gluc	: 10 mg
Dimethyl sulfoxide (DMSO)	: 100 µl
1M sodium phosphate pH 7	: 1 ml
Triton	: 1 ml
Sterile Distilled water	: 18 ml

Ten mg X-Gluc was dissolved in 100 µl dimethyl sulfoxide (DMSO). Sodium phosphate and triton were added and finally the volume made upto 20 ml with sterile distilled water. Aliquots of 1 ml were taken in storage vial, wrapped with aluminium foil and stored at -20°C.

The explants were incubated in X-Gluc stain for 24-48 hrs at 37°C in dark. The transformed and control explants were subjected to X-Gluc assay. Following overnight incubation, the explants were cleaned and kept in 100 per cent ethanol for preservation. Assayed tissues were observed under a microscope. The number of GUS expressing calli that developed on Kanamycin selection medium after 3 days was determined by counting the number of calli with blue zones after X-Gluc incubation.

3.10. MOLECULAR CHARACTERIZATION OF TRANSGENIC PLANTS

The transgenic plants were tested for stable integration and expression of genes by PCR using *npt* II primers.

3.10.1. Plant DNA isolation

DNA was isolated from callus tissues of transformed plantlets and control plants following Doyle and Doyle method (Doyle and Doyle, 1987). The composition of various reagents used for plant DNA isolation is given in Appendix II.

3.10.1.1 Procedure

Plant sample weighing 0.5 g was ground with liquid nitrogen, 6 ml of 1X extraction buffer, 50 µl β-mercapto ethanol and a pinch of sodium metabisulphate using an autoclaved mortar and pestle. To this ground material, 6 ml pre warmed lysis buffer and 1 ml sarcosine were added and transferred into a 50 ml centrifuge tube. The tubes were kept in a water bath at 65° C for 10 minutes. The tubes were removed from the water bath and equal volume of chloroform:Isoamyl alcohol mixture (24:1 v/v) was added and mixed by gentle inversion. The tubes were centrifuged at 10,000 rpm for 10 min at 4°C. Transferred the clear aqueous phase to a new tube. 0.6 volume of ice-cold isopropanol was added and mixed gently and then kept in – 20°C deep freezer for 30 minutes for the complete precipitation of DNA. The DNA pellet was

collected by centrifugation of tubes at 10,000 rpm for 10 min at 4ºC. The supernatant was discarded and the pellet was washed with 70 per cent ethanol by centrifuging at 10,000 rpm for 10 min at 4ºC. The supernatant was discarded and the pellet was air-dried. The fresh pellet was dissolved in 100 µl of TE and stored at – 20ºC.

3.10.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis is the standard method for the separation, identification and purification of DNA and also RNA fragments ranging in size from a few hundred to 20 Kb. The method described by Sambrook *et al.* (1989) was used to carry out electrophoresis.

3.10.2.1. Materials

a) Agarose

Agarose : 1.0 per cent (for genomic DNA)

Agarose : 0.7 per cent (for PCR samples)

b) 50x TAE buffer (pH 8.0)

Tris base	- 242.0 g
Glacial acetic acid	- 57.1 ml
0.5 M EDTA (pH 8.0)	- 100 ml
Distilled water	- 1000 ml

The solution was prepared and stored at room temperature.

c) Electrophoresis unit, power pack, casting tray, comb.

d) 6x Loading/Tracking dye

Bromophenol blue	- 0.25 %
Xylene cyanol	- 0.25 %
Glycerol	- 30 %

The dye was prepared and kept in fridge at 4ºC

e) Ethidium bromide solution

The dye was prepared as a stock solution of 10 mg ml^{-1} in water and was stored at room temperature in a dark bottle.

f) UV transilluminator

g) Alpha imager TM 1200 documentation and analysis system.

3.10.2.2. Procedure for casting, loading and running the gel

Four hundred ml of electrophoresis buffer (1x TAE) was prepared to fill the electrophoresis tank and to prepare the gel. The open ends of the gel-casting tray were sealed with a cellophane tape and placed on a perfectly horizontal leveled platform. Agarose (1.0 per cent for genomic DNA and 0.8 per cent for PCR) was added to 1x TAE, boiled till the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was added to a final concentration of 0.5 $\mu g\ ml^{-1}$ as an intercalating agent of DNA, which will help in its visualization in UV rays. It was then poured into the gel mould and the comb was placed properly and allowed to solidify. After the gel was completely set (30-45 minutes at room temperature), the comb and cellophane tape were removed carefully. The casted gel was placed in the electrophoresis tank with the wells near the cathode and submerged with 1x TAE to a depth of 1 cm. A piece of cellophane tape was pressed on a solid surface and 1 µl 6x loading buffer was dispensed in small quantity on the tape. A quantity of 3-5 µl of DNA was added to each dot (In the case of PCR products, 10.0-15.0 µl) mixed well by pipetting in and out for 2 to 3 times. Then the mixture was loaded into the wells, with the help of the micropipette. Lambda DNA (*Hind* III/*Eco*R I double digest) was also added in one of the wells as a molecular weight marker. The cathode and anode were connected to power pack and the gel was run at a constant current of 60 volts. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

3.7.8.4 PCR analysis of npt II gene

Polymerase chain reaction was carried out using the primers designed for *npt* II gene. Two primers of *npt* II gene were used. The expected size of the fragment was 600bp. Primers used for PCR amplification were: 5’ CAA TCG GCT GCT CTG ATG CCG 3’ and 5’ AGG CGA TAG AAG GCA ATG CGC 3’.

Composition of the reaction mixture for PCR (25.0 µl)

a) Genomic DNA - 1µl (1:9 dilution)
b) 10X Taq assay buffer - 2.5µl
c) dNTP mix (1mM) - 1µl
d) Forward primer (2.5 pM) - 1µl

e) Reverse primer (2.5 pM) - 1µl

f) Taq polymerase (0.6U) - 2µl

g) Autoclaved distilled water - 16.5µl

The reaction mixture was given a momentary spin for thorough mixing of the cocktail components and15 µl of mineral oil was added to each tube to prevent the evaporation during the thermal cycling. Then the PCR tubes were loaded in a thermal cycler (PTC-200^{TM} Programmable Thermocycler MJ Research).

Thermal cycler program

Profile 1: 94 ºC for 2 min - Initial denaturation

Profile 2 : 94 ºC for 45 sec - Denaturation

Profile 3 : 54 ºC for 1 min - Annealing

Profile 4 : 72 ºC for 2 min – Extension

(Profiles 2–4: 30 cycles)

Profile 5 : 72 ºC for 10 min - Final extension

Profile 6 : 4 ºC for 15 min to hold the sample.

Amplified DNA fragments were electrophoresed on 0.7% agarose ethedium bromide gel, observed under ultra-violet and documented using the gel documentation.

Results

4. RESULTS

The results of the investigations on the 'Development of *in vitro* regeneration and genetic transformation systems in Pumpkin (*Cucurbita moschata* Poir),' carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, from October 2005 to July 2008 are presented in this chapter.

4.1 PRODUCTION OF AXENIC PLANTS AS EXPLANT SOURCE

The results of the trial for standardization of basal media and surface sterilant are presented in Table 4. Among the different basal media tried, full MS medium gave higher sprouting percentage when compared to half strength MS medium. Mercuric chloride (0.1%) treatment for 6 min treatment was found to be effective in sterilizing the mature seeds. The youngest seedlings emerged with unexpanded cotyledons, green at the proximal end with a short hypocotyl and one or more roots (Plate.1.a, b, c and d). The seeds germinated within 1 week and the germination recorded was 90 per cent.

4.2 STANDARDIZATION OF CALLUS MEDIATED REGENERATION PROTOCOL

4.2.1 Effect of growth regulators on hypocotyl explants

4.2.1.1 Response to auxins

The data on the response to various concentrations of auxins on induction of callus from hypocotyl explants are presented in Table 5. It was observed that percentage of callusing increased with increasing concentration of 2,4-D. Maximum callusing percentage and callus index were recorded with 2.5 mg l^{-1} 2,4-D. The callus was initiated by swelling of basal part of the hypocotyl, followed by the appearance of dark green ring like lump of tissue around the circumference of the cut ends. Roots were produced from the middle and basal regions at higher concentrations of 1 mg l^{-1} and 2.5 mg l^{-1}. (Plate.2).

Table.4. Effect of basal media and surface sterilants on culture establishment

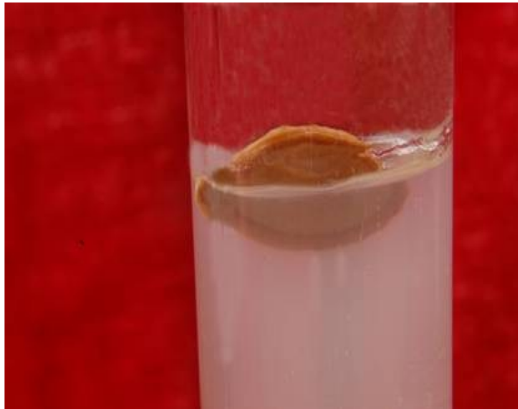
Sl. No.	Basal Media	Sterilant	Concentration (%)	Duration	Survival (%)
1	MS Basal	Mercuric chloride	0.1	6 min	90
2	MS Basal	Mercuric chloride	0.1	10 min	72
3	Half MS	Mercuric chloride	0.1	6 min	75
4	Half MS	Mercuric chloride	0.1	10 min	66

Average of eighteen observations
Culture duration – 3 weeks

Table.5. Effect of auxins on hypocotyl explants of pumpkin on callus induction

Sl. No.	Media combination	Response	Growth score	Callus Index
1	MS + 2,4-D (4 µM)	White callus	1	83.3
2	MS + 2,4-D (0.5 mg l^{-1})	Creamy yellow callus	1	83.3
3	MS + 2,4-D (1 mg l^{-1})	White callus, rooting	3	250
4	MS + 2,4-D (2.5 mg l^{-1})	White callus, rooting	3	300
5	MS + Picloram (5 mg l^{-1})	Creamy yellow friable callus	4	300

Average of eighteen observations
Culture duration – 3 weeks



MS basal – after 2 days

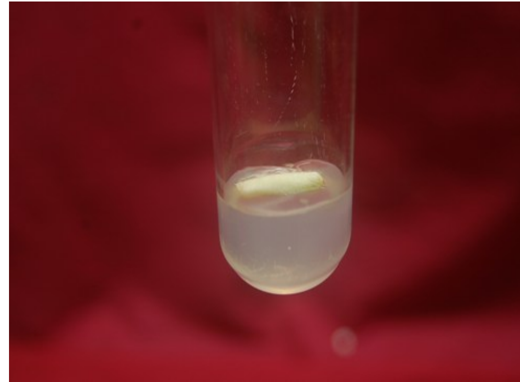
Half MS basal – after 2 days

Mercuric chloride – 10 min (1 week)
Medium – MS basal

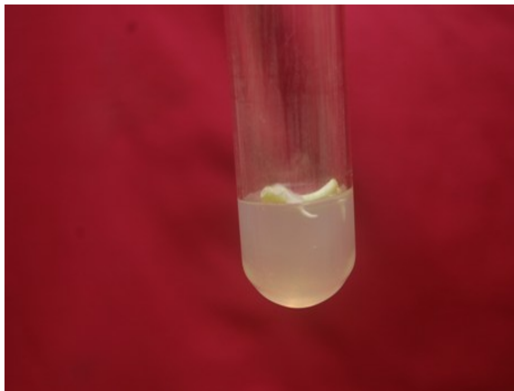
Mercuric chloride – 6 min (1 week)
Medium – MS basal

Plate 1. Production of axenic seedlings as explant source

MS + 2,4 – D (4µM)

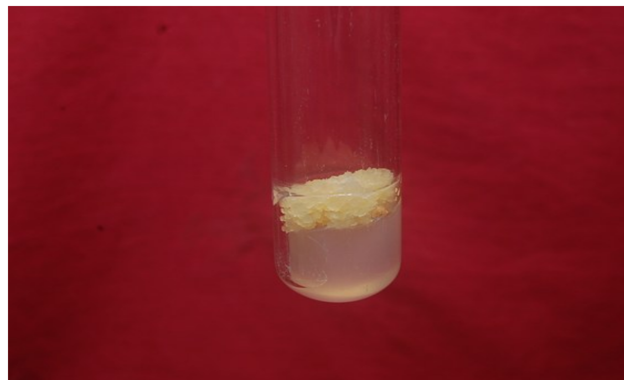


MS + 2,4-D (0.5 mg l^{-1})



MS + 2,4 –D (1 mg l^{-1})

MS + 2,4-D (2.5 mg l^{-1})



MS + Picloram (5 mg l^{-1})

Plate 2. Effect of Auxins on hypocotyl explants

With picloram at concentration of 5 mg l^{-1}, friable calli were produced which covered all over the surface of the explant within 2 weeks of culture (Plate.2).

4.2.1.2 Response to Thidiazuron

Hypocotyl explants taken from one week old aseptic cultures were cultured in MS medium supplemented with thidiazuron. It was observed that the callusing was very negligible at concentration below 0.5 mg l^{-1}. At 0.5, white friable callus was produced at the surface of the explants (Plate.3). The data on the percentage of callusing and callus index are given in Table 6.

4.2.1.3 Combined effect of auxins with cytokinins

The effect of different growth regulator combinations for callus induction from hypocotyl explants is presented in Table 7. Maximum response to callusing and callus index were observed with combinations of BAP (4 mg l^{-1}) and NAA (0.2 mg l^{-1}) and also with NAA (0.2 mg l^{-1}) and Kinetin (0.2 mg l^{-1}). The callus produced was white coloured and friable with roots produced from the middle and basal regions (Plate.4).

The addition of casein hydrolysate to the medium containing NAA (0.2 mg l^{-1}) and Kinetin (2 mg l^{-1}) produced white coloured friable calli. Thick roots were produced from the middle and bottom regions of the horizontally placed explants (Plate.4).

4.2.2 Effect of growth regulators on cotyledonary explants

4.2.2.1 Response to auxins

Cotyledonary leaf explants taken from one week old axenic seedlings were cultured in MS medium containing different combinations of auxins. The data on the percentage of callusing are given in Table 8.

The results clearly show that BA concentrations namely 1 mg l^{-1}, 3 mg l^{-1} and 5 mg l^{-1} produced morphogenetically different callus. It was observed that at lower

Table.6. Effect of thidiazuron on callus induction of hypocotyl explants

Sl. No.	Media combination	Response	Growth score	Callus Index
1	MS + TDZ (0.1 mg l^{-1})	Nil	0	0
2	MS + TDZ (0.5 mg l^{-1})	Swelling and callusing	1	55.5

Average of eighteen observations
Culture duration – 3 weeks

Table.7. Combined effect of auxins and cytokinins on callus induction and proliferation of hypocotyl explants

Sl. No.	Basal Medium	Auxin (mg l^{-1})	Cytokinin (mg l^{-1})	Response	Growth score	Callus Index
1	MS	NAA(0.2)	BA (4.0)	White friable calli and thick roots	3	300
2	MS	NAA (0.2)	Kin (0.2)	White friable calli with thin hairy roots	3	300
3	MS + Casein hydrolysate (0.3 g l^{-1})	NAA (0.2)	Kin (2)	White callus, thick roots	3	233.33

Average of eighteen observations
Culture duration – 3 weeks

Plate 3. Hypocotyl swelling and callusing – MS + TDZ (0.5 mg l^{-1})

MS + BA (4 mg l^{-1}) +
NAA (0.2 mg l^{-1}

MS + NAA (0.2 mg l^{-1}) +
Kin (0.2 mg l^{-1})

MS + Casein Hydrolysate (0.3 g l^{-1}) + NAA (0.2 mg l^{-1}) + Kin (2 mg l^{-1})

Plate 4. Effect of combination of auxin and cytokinin on hypocotyl explants

concentration, the callus produced was white in colour and concentrating at the proximal end of the explant. At higher concentration, the amount of callus produced was less and the callus becomes brown in colour (Plate.5).

NAA at concentration of 0.5 mg l^{-1} was effective in inducing callusing at the ends of the leaf segments and the callus produced was white in colour (Plate.5).

With picloram at concentration of 5 mg l^{-1}, creamy yellow friable callus was obtained all over the surface of the explants (Plate.5).

4.2.2.2 Response to Thidiazuron

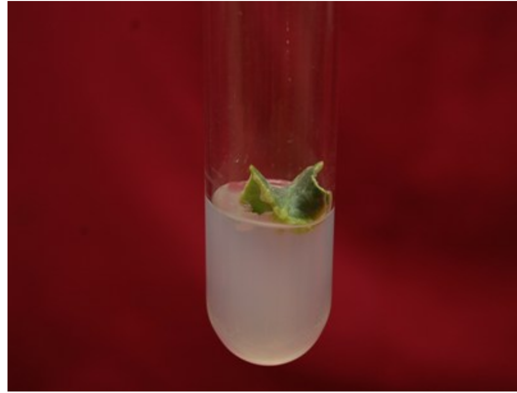
The percentage of callusing and the callus index of explants in medium containing thidiazuron are presented in Table 9. Callusing was found to be negligible on culturing the explants in medium containing thidiazuron at concentration of 0.5 mg l^{-1}. Multiple roots were arising from the ends of few leaf bits (Plate.6).

4.2.2.3 Effect of different combinations of auxins and cytokinins

The effect of different combinations of auxins and cytokinins for callus induction is presented in Table 10. Callusing was obtained in all the different combinations with highest callusing percentage with BA (4 mg l^{-1}) and NAA (0.2 mg l^{-1}). White compact callus and thick roots were obtained after 4 weeks of culture period. 2,4-D was found to be almost similar to NAA in inducing callus in combination with BA. NAA in combination with kinetin resulted in callus induction and the callus produced were white and friable. Further roots were initiated with the same combination supplemented with casein hydrolysate (Plate.7).

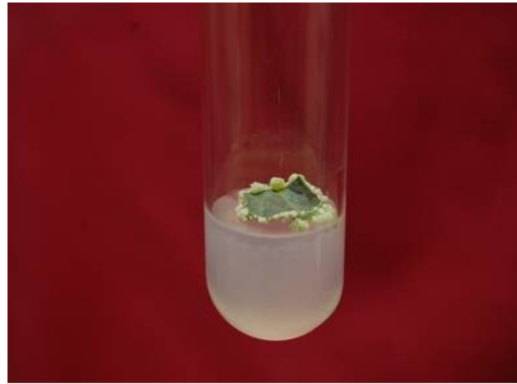
The explants showed callusing and rooting in medium supplemented with different combinations of IAA and kinetin. The rooting percentage was less compared to medium containing BA and NAA. Similar results were obtained with explants cultured in medium containing BA and IAA (Plate.7.A and B).

MS + BA (1 mg l^{-1})

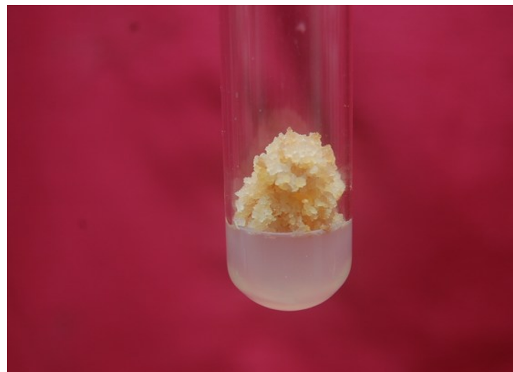


MS + BA (3 mg l^{-1})

MS + BA (5 mg l^{-1})



MS + NAA (0.5 mg l^{-1})



MS + Picloram (5 mg l^{-1})

Plate 5. Effect of auxins on callus induction in cotyledonary leaf explants

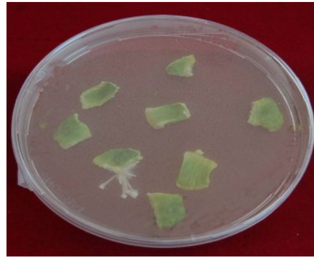
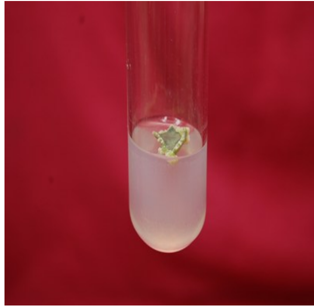
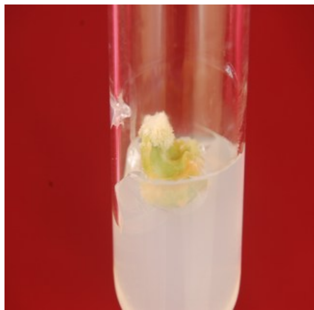


Plate 6. Cotyledonary leaf – MS + TDZ 0.5 mg l^{-1})



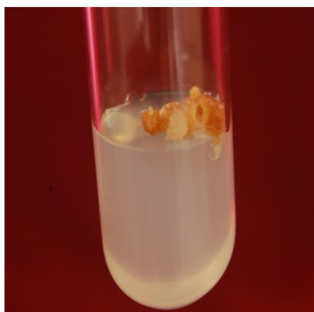
MS + NAA (0.5 mg l^{-1})
+ BA (1.0 mg l^{-1})

MS + NAA (2.0 mg l^{-1})
+ BA (0.5 mg l^{-1})



MS + NAA (2.0 mg l^{-1})
+ BA (1.0 mg l^{-1})

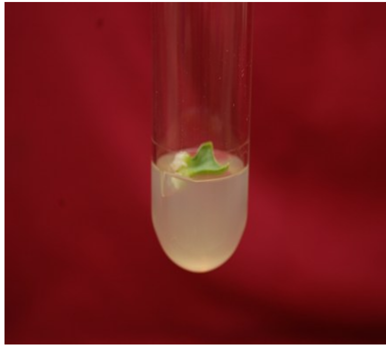
MS + NAA (3.0 mg l^{-1})
+ BA (0.5 mg l^{-1})



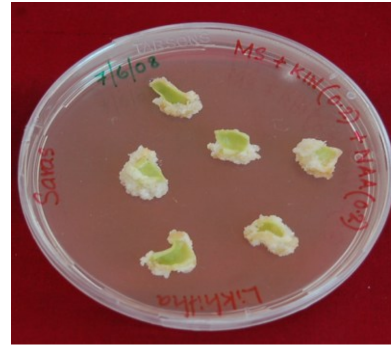
MS + NAA (3.0 mg l^{-1})
+ BA (1.0 mg l^{-1})

MS + NAA (0.2 mg l^{-1})
+ BA (4.0 mg l^{-1})

Plate 7A. Effect of combination of auxin and cytokinin on callus induction in cotyledonary leaf explants

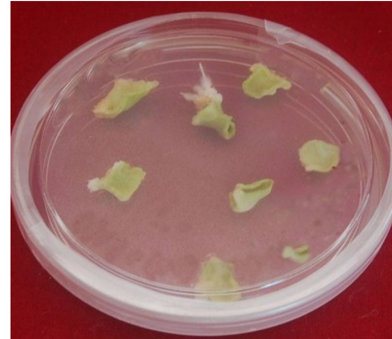


MS + 2,4 – D (0.5 mg l^{-1})
+ BA (1.0 mg l^{-1})



MS + NAA (0.2 mg l^{-1})
+ Kin (0.2 mg l^{-1})

MS + Casein hydrolysate (0.3 g l^{-1})
+ NAA (0.2 mg l^{-1}) + Kin (2.0 mg l^{-1})



MS + IAA (1.5 mg l^{-1})
+ BA (1.0 mg l^{-1})

MS + IAA (1.0 mg l^{-1})
+ Kin (5.0 mg l^{-1})

MS + IAA (1.5 mg l^{-1})
+ Kin (6.0 mg l^{-1})

Plate 7 B. Effect of combination of auxin and cytokinin on callus induction in cotyledonary leaf explants

Table.8. Effect of auxins on callus induction and proliferation on cotyledonary leaf explants

Sl. No.	Auxins (mg l^{-1})	Response	Growth score	Callus Index
1	MS + BA (1 mg l^{-1})	White callus	3	300
2	MS + BA (3 mg l^{-1})	Green callus	2	200
3	MS + BA (5 mg l^{-1})	Brown callus	2	200
4	MS + NAA (0.5 mg l^{-1})	White callus	2	111.11
5	MS + Picloram (5 mg l^{-1})	Creamy yellow friable callus	4	300

Average of eighteen observations
Culture duration – 3 weeks

Table.9. Effect of Thidiazuron on callus induction and proliferation of cotyledonary leaf explants

Sl. No.	Media combination	Response	Growth score	Callus Index
1	MS + TDZ (0.1 mg l^{-1})	Nil	0	0
2	MS + TDZ (0.5 mg l^{-1})	Callusing with rooting	1	55.5

Average of eighteen observations
Culture duration – 3 weeks

Table.10. Combined effect of auxins and cytokinins on callus induction and proliferation of cotyledonary leaf explants

Sl. No.	Basal Medium	Auxin (mg l^{-1})	Cytokinin (mg l^{-1})	Response	Growth score	Callus Index
1	MS	NAA(0.5)	BA (1.0)	White friable calli	2	111.11
2	MS	NAA (2.0)	BA (0.5)	White callus	2	133.33
3	MS	NAA (2.0)	BA (1.0)	White callus	2	109.87
4	MS	NAA (3.0)	BA (0.5)	White callus	2	200
5	MS	NAA (3.0)	BA (1.0)	White callus	2	200
6	MS	NAA (0.2)	BA (4.0)	Callusing, thick roots	3	300
7	MS	2,4-D (0.5)	BA (1.0)	White callus	1	44.44
8	MS	NAA (0.2)	Kin (0.2)	White callus	3	266.67
9	MS + Casein hydrolysate (0.3 g l^{-1})	NAA (0.2)	Kin (2.0)	Callusing, rooting	1	22.2
10	MS	IAA (1.5)	BA (1.0)	Callusing, rooting	2	200
11	MS	IAA (1.0)	Kin (5.0)	Callusing, rooting	2	200
12	MS	IAA (1.5)	Kin (6.0)	Callusing, rooting	2	200

Average of eighteen observations

Culture duration – 3 weeks

4.2.3 Effect of growth regulators on cotyledons

Basal cotyledon explants, distal cotyledon explants and cotyledonary axis explants were cultured in MS medium containing different growth regulators (Plate.8).

4.2.3.1 Effect of picloram

Cotyledon explants produced white compact callus when cultured in medium containing 5 mg l^{-1} picloram (Table 11, Plate.8).

4.2.3.2 Effect of combination of auxin and cytokinin

The cotyledon cultures produced the maximum amount of callus when cultured in MS medium containing 5 mg l^{-1} 2, 4 – D and 0.1 mg l^{-1} TDZ. The effect of different combination of auxins and cytokinins on callus induction of cotyledon explants is presented in Table 12 (Plate.8).

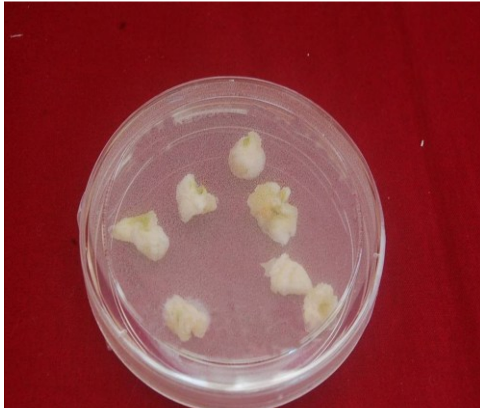
4.3 *IN VITRO* REGENERATION

4.3.1 Standardization of Indirect organogenesis

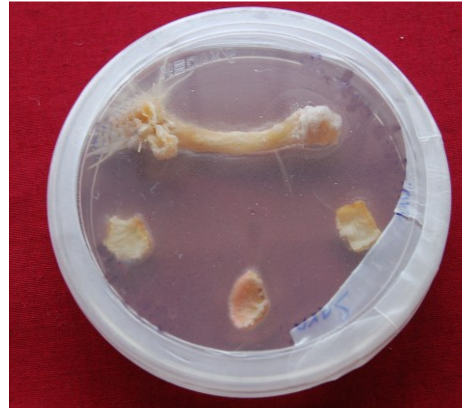
4.3.1.1 Standardization of explants

Among the explants tried, only cotyledonary nodal segments induced multiple shoots from the calli cultured on different combinations of growth regulators (Table 13). The highest number of shoots (4.8) was obtained when the nodal explants were cultured in the presence of BA (1 mg l^{-1}) and Kinetin (1 mg l^{-1}) (Plate.9). The combinations MS + IAA (1.5 mg l^{-1}) + Kinetin (6 mg l^{-1}) and MS + IAA (1.5 mg l^{-1}) + BA (1 mg l^{-1}) produced average number of 3.66 and 2.97 shoot buds respectively along with callusing.

After 21 days of regeneration, the multiple shoots were cut into 4-5 pieces and inoculated in MS medium containing different growth regulators and without any growth regulators for rooting. The data is given in Table 14. The rooting was obtained in all the different combinations. Profuse rooting with more number of multiple shoots was obtained with medium containing IBA (1.0 mg l^{-1}) (Plate.10). The plants turned



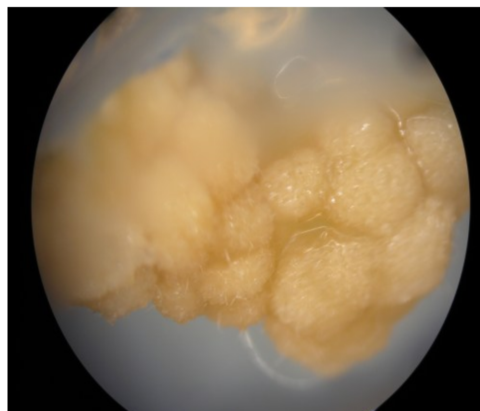
MS + Picloram (5 mg l^{-1})



MS + 2,4 –D (5 mg l^{-1})
+ TDZ (0.1 mg l^{-1})

MS + IAA (1.5 mg l^{-1})
+ Kin (6.0 mg l^{-1})

MS + BA (4 mg l^{-1})
+ NAA (0.1 mg l^{-1})



MS +Picloram (5 mg l^{-1})
+ TDZ (0.1 mg l^{-1})

Plate 8. Callus induction from cotyledon explants

Table.11. Effect of picloram on callus induction and proliferation in cotyledon explants of pumpkin

Sl. No.	Picloram (mg l^{-1})	Response	Growth score	Callus Index
1	1	Nil	0	0
2	5	White callus	4	144

Average of nine observations

Culture duration – 3 weeks

Table.12. Combined effect of auxins and cytokinins on cotyledon explants

Sl. No.	Basal Medium	Auxin (mg l^{-1})	Cytokinin (mg l^{-1})	Response	Growth score	Callus Index
1	MS	2,4 – D (5)	TDZ (0.1)	Creamy white calli, cotyledon axis germinated	4	308
2	MS	IAA (1.5)	Kin (6.0)	Enlargement	1	33.33
3	MS	BA (4.0)	NAA (0.2)	Creamy yellow callus	2	133.4
4	MS	Picloram (5)	TDZ (0.1)	Creamy yellow callus	3	93.7

Average of nine observations

Culture duration – 3 weeks

Table.13. Response of cotyledonary node segments in MS medium containing different growth regulators

Sl. No.	Media combination (mg l^{-1})	Percentage regeneration	Average no. of shoot buds/explant	Response
1	MS + BA (1) + Kin (1)	54.5	4.8	Regeneration + Callusing
2	MS + IAA (1.5) + BA (1)	35.5	2.97	Regeneration + Callusing
3	MS + IAA (1.5) + Kin (6)	64.7	3.66	Regeneration + Callusing

Average of eighteen observations

Culture duration – 3 weeks

Table.14. Effect of different rooting media on cotyledonary node segments of pumpkin

Sl. No.	Media combination (mg l^{-1})	Response	Average no. of roots per shoot
1	MS Basal	Rooted, turned brown	3.8
2	MS + IBA (1)	Profuse rooting	7.6
3	MS + Casein hydrolysate (0.3 g l^{-1}) + NAA (0.2) + Kin (2)	Thick roots	2.7

Average of eighteen observations

Culture duration – 3 weeks

Plate 9. Multiple shoots produced from cotyledonary node explants in MS + BA (1.0 mg l^{-1}) + Kin (1.0 mg l^{-1})

Plate 10. Rooting of *in vitro* derived shoots in MS + IBA (1 mg l^{-1})

brown and dried in basal MS medium after profuse rooting. The roots produced in MS medium supplemented with casein hydrolysate (0.3 g l^{-1}) + NAA (0.2 mg l^{-1}) + Kinetin (2 mg l^{-1}) were thicker and less when compared to the other combinations tried. The number of days taken for root induction was 15 days.

Rooted plantlet were hardened for 15 days in small pots containing sterile sand and then planted in big pots containing potting mixture. The percentage of plants established is given in Table.15.

4.4 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

4.4.1 Sensitivity of explants to antibiotics

Successful transformation using *Agrobacterium* utilizes various antibiotics for the elimination of *Agrobacterium* from the culture. The optimum concentrations of antibiotics are to be standardized and the toxicity of explants towards the lethal dose have to be evaluated.

Various experiments were carried out to find out the optimum level of antibiotics and results are presented hereunder.

4.4.1.1 Sensitivity of cotyledon explants to Kanamycin

The response of cotyledon explants to kanamycin at 50, 100, 150 and 200 mg l^{-1} were tested. The data is given in table 16.

White friable callus was successfully induced on kanamycin free regeneration medium (control). Kanamycin at the concentration of 50 mg l^{-1} induced callusing in 33% of the cotyledon explants tested. Callusing was inhibited when the level of kanamycin was 100 mg $l^{-1,}$ and at a concentration of 200 mg l^{-1}, browning of explant tissue was observed (Plate.11). Hence kanamycin 200 mg l^{-1} was considered as minimal lethal.

Table.15. Plant out success under controlled conditions

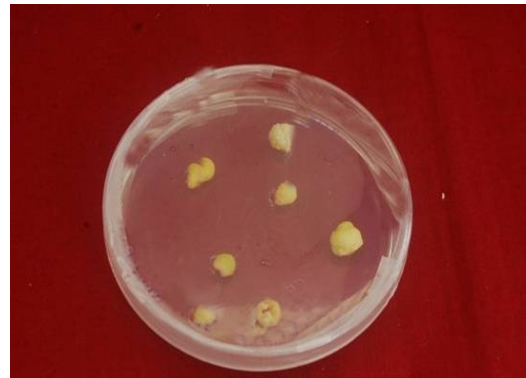
Total no. of hardened plants	No. plants survived	Percentage of plant out success
15	12	80

Table.16. Response of cotyledon explants to different concentrations of kanamycin

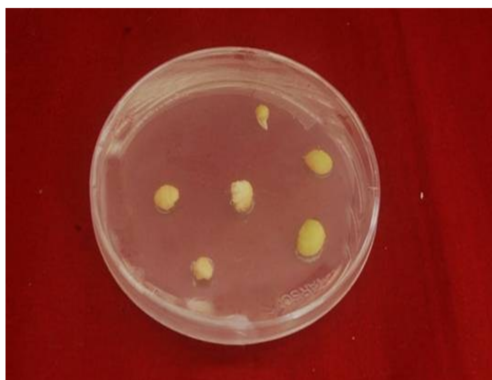
Concentration (mg l^{-1})	Response	Remarks
50	Bleaching	Sensitive
100	Callusing inhibited	Sensitive
150	Callusing inhibited	Sensitive
200	Callusing inhibited + browning of tissue	Sensitive

Medium – MS + 5 mg l^{-1} 2,4-D + 0.1 mg l^{-1} TDZ
Culture duration 4 weeks
Sub culturing 2 weeks interval

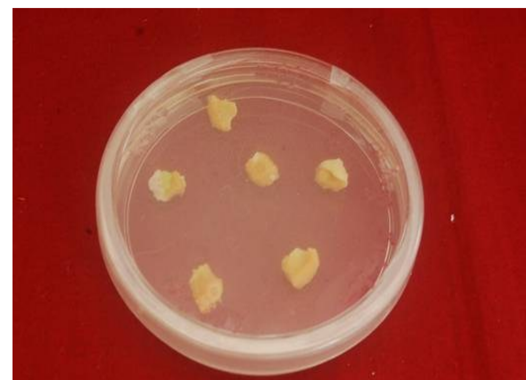
50 mg l^{-1} - Bleaching of tissues



100 mg l^{-1} - Callusing inhibited



150 mg l^{-1} - Callusing inhibited



200 mg – Callusing inhibited
+ Browning

Control plate – Creamy yellow embryogenic callus initiated from cotyledon explants

Plate 11. Sensitivity of cotyledon explants to kanamycin

4.4.1.2 Sensitivity of explants to cefotaxime

Sensitivity of cotyledonary explants to varying concentrations of cefotaxime was tested. The data is given in table 17. Callus was induced from the explants at all concentrations tested.

4.4.2 Cultural characteristics of *Agrobacterium*

The *Agrobacterium* strains EHA 105 was streaked on Yeast Extract Mannitol (YEM) medium. Bacterial colonies were appeared two days after streaking. Colonies were round with smooth margin, convex, colourless and mucoid in nature (Plate.12).

4.4.3 Plasmid isolation from *Agrobacterium*

Plasmid DNA was isolated from the *Agrobacterium* strain EHA 105 following the alkali lysis method. Electrophoresis of plasmid DNA on 0.7% agarose gel showed a single DNA band having a relative molecular wt. of approximately 14 kb. There was no RNA contamination (Plate.13).

4.4.4 Standardization of *Agrobacterium* mediated genetic transformation

Agrobacterium mediated genetic transformation of *Cucurbita moschat*a Poir var. Saras was standardized with EHA 105 having *gus* reporter gene. The coding region of the gus reporter gene contains an intron that prevents translation by *Agrobacterium tumefaciens*. The time of infection, density of bacterial population and co-cultivation period were standardized. Transformants were screened in selection medium containing 200 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime antibiotics. Transformants were subjected to X-gluc assay for confirmation of transformation.

4.4.4.1 Standardization of inoculum density

Transformation was tried at different inoculum densities (0.5, 0.6, 0.7 and 0.8 OD_{600}nm) (Plate.14). The cotyledon explants were used for the experiment. A 2 day period for co-cultivation and 10 minutes infection time were fixed for the study. The results of the experiment are given in Table 18.

Table.17. Response of explants to different concentrations of cefotaxime

Concentration (mg l^{-1})	Response	Remarks
50	Callusing	Resistant
100	Callusing	Resistant
150	Callusing	Resistant
200	Callusing	Resistant
250	Callusing	Resistant

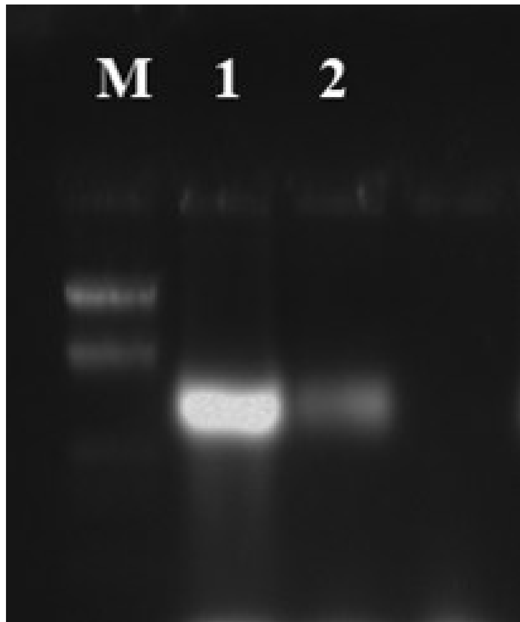
Medium – MS + 5 mg l^{-1} 2,4-D + 0.1 mg l^{-1} TDZ
Culture duration 4 weeks
Sub culturing 2 weeks interval

Table.18. Standardization of inoculum density using *Agrobacterium* strain EHA 105

Pre-culture (days)	Bacterial density (OD_{600}nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Callusing rate (%)
2	0.5	10	48	+	70.0
2	0.6	10	48	+	80.0
2	0.7	10	48	+	50.0
2	0.8	10	48	+	15.0
Control	-	-	-	-	100.0

Callusing rate after 30 days of co-cultivation
Medium – MS + 5 mg l^{-1} 2,4-D + 0.1 mg l^{-1} TDZ

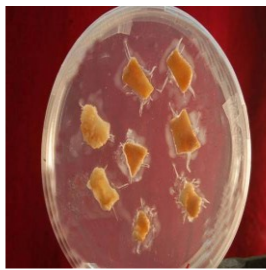
Plate 12. *Agrobacterium tumefaciens* strain EHA 105 on YEM medium



Lane 1 – Molecular Marker
(lambda DNA/Hind III/EcoRI)

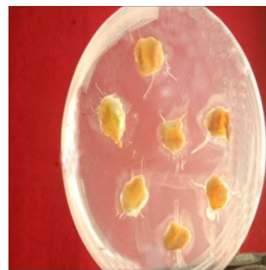
Lane 2 & 3 – p35SGUSINT

Plate 13. Plasmid profile

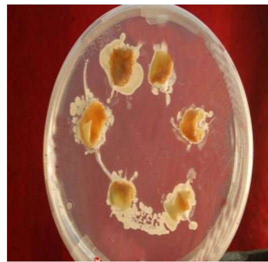


0.5 OD

0.6 OD

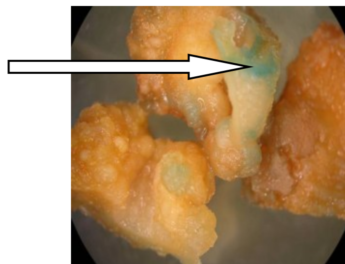


0.7 OD

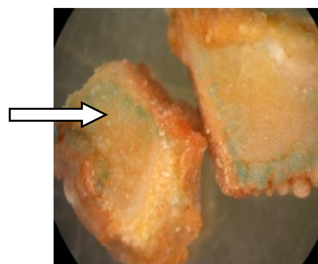


0.8 OD

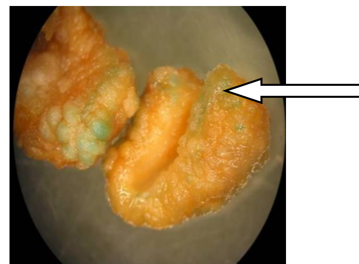
Plate 14. Bacterial growth on the surface of cotyledon explants
(Standardization of inoculum density)



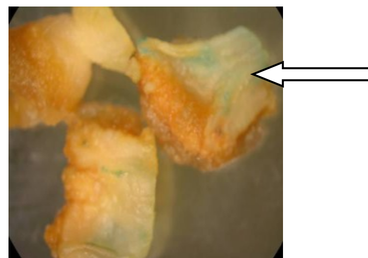
0.5 OD



0.6 OD



0.7 OD



0.8 OD

Plate 15. GUS spots observed under microscope
(Different inoculum densities)

The histochemical GUS assay was performed 3 days after co-cultivation. Both control and transformed plants were assayed. The results showed blue coloured areas on the surface of the transformed explants. When these blue coloured areas were examined under microscope, clear blue specks were observed (Plate 15). The blue coloured areas were more when the bacterial density was 0.6 OD_{600}nm.The control plants did not show any blue coloured areas.

When the bacterial density was 0.6 OD_{600} nm, 80% of the explants showed callusing after 30 days of co-cultivation. When the bacterial density was 0.8 OD_{600} nm, 15% callusing was obtained after 30 days. Control plants did not show any callusing.

4.4.4.2 Standardization of infection time

The infection time's viz. 5, 10, 15 and 20 minutes were tried for the transformation study (Plate.16). A 2-day period for co-cultivation and a bacterial density of 0.6 OD_{600}nm were fixed for the study. The data is given in Table 19.

Transient GUS assay was performed 3 days after co-cultivation. Blue coloured areas were present on the surface of transformed explants. These explants when observed under microscope blue specks were seen indicating transformation. The blue coloured areas were more on the transformed explants that were co-cultivated for 10 minutes (Plate.17).

The callusing rate of transformed plants after 30 days of co-cultivation was 90% in case of 10 minutes infection time. When the infection time was more than 10 min, the explants turned brown. The control plants did not show any callusing in the media containing kanamycin 200 mg l^{-1}.

4.4.4.3 Standardization of co-cultivation period

The duration of co-cultivation with *Agrobacterium* is one of the factors affecting transformation efficiency. The effect of co-cultivation duration on pumpkin (*Cucurbita moschata* Poir var. Saras) was examined using cotyledon explants

Table.19. Standardization of infection time using *Agrobacterium* strain EHA 105

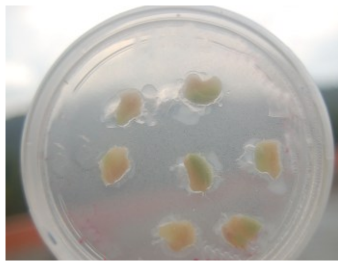
Pre-culture (days)	Bacterial density (OD_{600}nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Callusing rate (%)
2	0.6	5	48	+	75.0
2	0.6	10	48	+	90.0
2	0.6	15	48	+	60.0
2	0.6	20	48	+	-
Control	-	-	-	-	100.0

Callusing rate after 30 days of co-cultivation
Medium – MS + 5 mg l^{-1} 2, 4-D + 0.1 mg l^{-1} TDZ

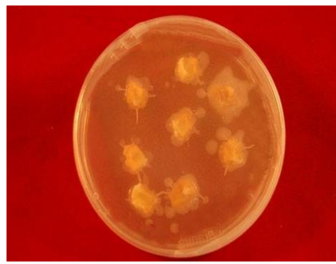
Table.20. Standardization of co-cultivation period using *Agrobacterium* strain EHA 105

Pre-culture (days)	Bacterial density (OD_{600}nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Callusing rate (%)
2	0.6	10	24	+	60.0
2	0.6	10	48	+	80.0
2	0.6	10	72	+	35.0
2	0.6	10	96	+	20.0
2	0.6	10	-	-	75.0
Control	-	-	-	-	100.0

Callusing rate after 30 days of co-cultivation
Medium – MS + 5 mg l^{-1} 2, 4-D + 0.1 mg l^{-1} TDZ



5 min

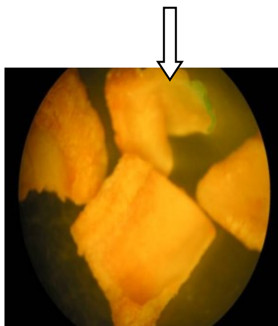


10 min

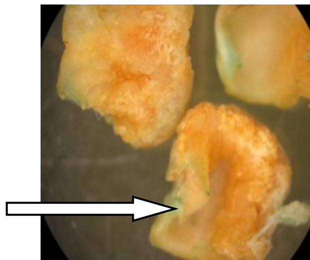
15 min

20 min

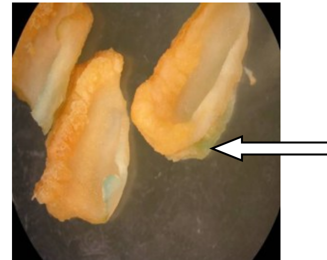
Plate 16. Bacterial growth on the surface of cotyledon explants (Standardization of infection time)



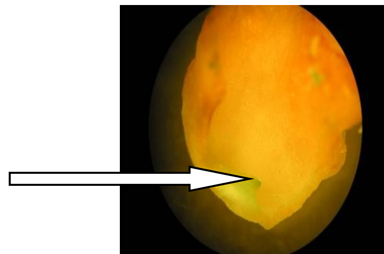
5 min



10 min



15 min



20 min

Plate 17. GUS spots observed under microscope (Different infection time periods)

(Plate.18). A 10 minutes infection time and a bacterial density of 0.6 OD_{600}nm were fixed. The explants were co-cultivated with *Agrobacterium* for 1, 2, 3 and 4 days. The data is given in Table 20.

Transient GUS assay was performed 3 days after co-cultivation. Blue coloured areas were present on the surface of explants that were co-cultivated for 1, 2, 3 and 4 days. When these blue coloured areas were examined under microscope, distinct blue specks were observed (Plate.19). Blue coloured areas were not present on the surface of explants that were immediately transferred to the pre selection medium after co-cultivation (no co-cultivation). Control plants also did not show any blue specks.

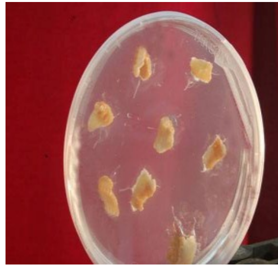
The transformation frequency was very low after one day of co-cultivation but increased rapidly when the co-cultivation was prolonged to two days, reaching a maximum at four days. Bacterial overgrowth was seen when there is a prolonged co-cultivation period of more than 2 days. The bacterial overgrowth was removed by culturing explants in selection medium containing 500 mg l^{-1} cefotaxime.

4.4.4.4 Confirmation of transformation

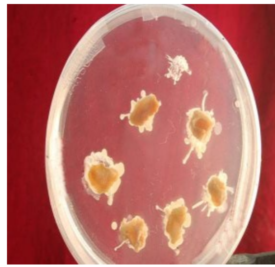
Molecular confirmation of the presence of *npt* II gene in the transformed cotyledon explants was gained by PCR with specific primers. PCR was used to demonstrate the presence of T-DNA in the transgenic plants.

4.4.4.4.1 Isolation of genomic DNA

Genomic DNA from independently obtained transgenic plants and one non-transformed plant was isolated using Doyle and Doyle method. Upon electrophoresis on 0.8% agarose gel, intact DNA was observed in all the wells (Plate.20 and 21).

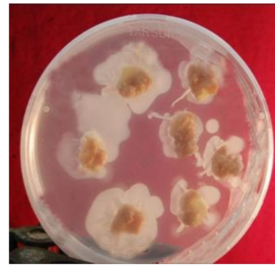


24 hrs



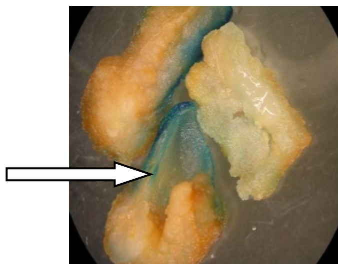
48 hrs

72 hrs

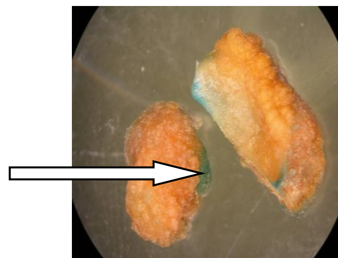


96 hrs

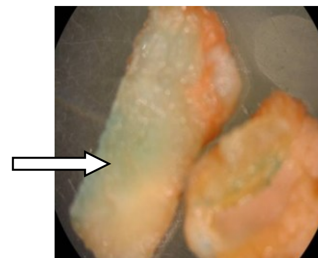
Plate 18. Bacterial growth on the surface of cotyledon explants (Standardization of different co-cultivation periods)



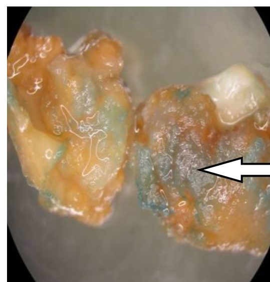
24 hrs



48 hrs

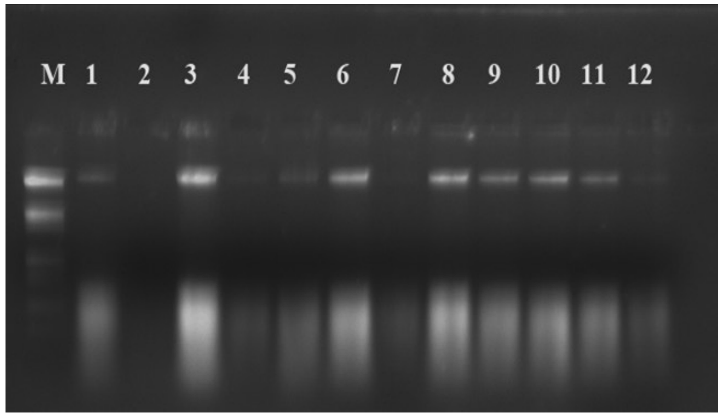


72 hrs



96 hrs

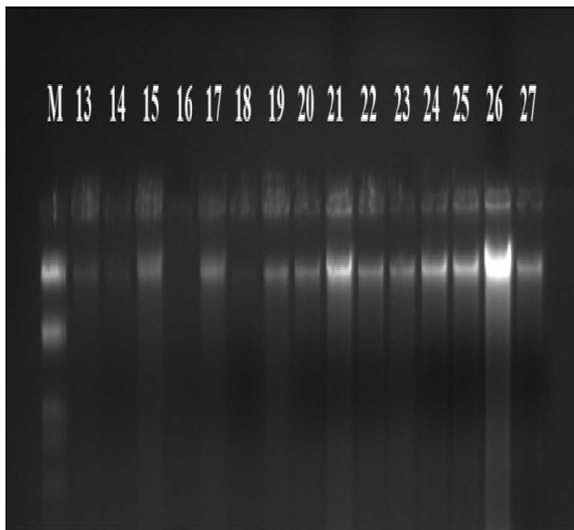
Plate 19. GUS spots observed under microscope (Different co-cultivation periods)



M – Molecular Marker (lambda DNA/Hind III/ Eco RI)

1,2,3,4,5,6,7,8,9,10,11,12– Genomic DNA from non-transformed cotyledons

Plate 20. Gel electrophoresis of genomic DNA from non-transformed tissues



M – Molecular Marker (lambda DNA/Hind III/ Eco RI)

13, 14,15,16,17 – Genomic DNA from transformed cotyledons (0.6 OD_{600} nm)

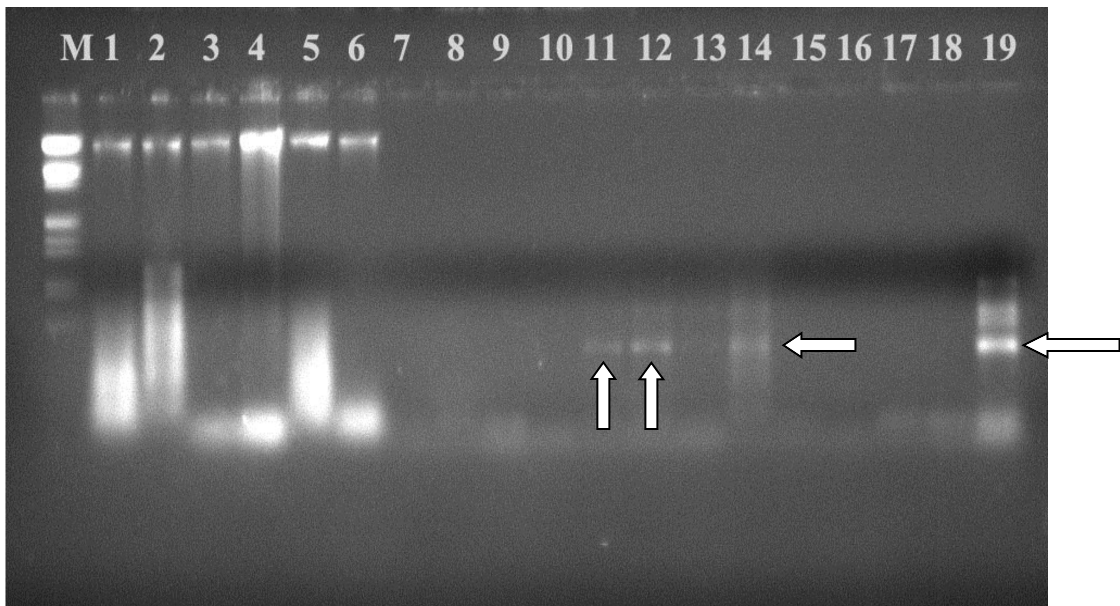
18, 19,20,21,22 – Genomic DNA from transformed cotyledons (10 min infection)

23, 24,25,26,27 – Genomic DNA from transformed cotyledons (48 hrs co-cultivation)

Plate 21. Gel electrophoresis of genomic DNA from transformed tissues

4.4.4.4.1.1 PCR analysis of npt II gene

The kanamycin resistant calli were screened by PCR using genomic DNA from transformed and from non-transformed calli as negative control. The plasmid DNA EHA 105 was used as positive control. Two specific primers derived from *npt II* gene sequences were used to detect a 600 bp fragment. The amplified DNA samples were electrophoresed on 1.0% agarose gel (Plate.22). The results showed that only DNA from the transformed plants and the positive control showed amplification of the 600bp fragment. The control plants did not amplify a 600 bp fragment corresponding to *npt II* gene (Plate.22). Thus, PCR analysis of genomic DNA confirmed the introduction of *npt II* gene in the genome of transgenic pumpkin calli.



M – Marker DNA

1, 2, 3, 4 – Genomic DNA from transgenic tissue

5, 6 – Genomic DNA from normal tissue

7, 8, 9, 10, 11 – Non-transformed control

12, 13 – Transformed control (10 min infection)

14, 15 – Transformed control (48 hrs co-cultivation)

16, 17 – Transformed control (0.6 OD_{600} nm)

18 – Negative control

19 – Positive control

Plate 22. PCR Analysis of *npt* II gene

Discussion

5. DISCUSSION

Pumpkin (*Cucurbita moschata* Poir.) is one of the most important summer vegetable grown all over India on a commercial scale. Conventional breeding methods have led to considerable genetic improvement. Considering its tremendous importance in the world's vegetable basket, both conventional and transgenic approaches have been used in order to confer antibiotic resistance. With the rapid development of genetic engineering techniques based on the knowledge of gene structure and function, plant breeding has been dramatically broadened. Tools of genetic engineering can be exploited as an additional method for the introduction of agronomically useful traits into established cultivars. *In vitro* gene transfer is the laboratory technique of transferring refined desirable genes across taxonomic boundaries into plants and animals from other plants, animals and microbes, or even to introduce artificial, synthetic or chimeric genes into plants. The crop is highly susceptible to pests and diseases. The incorporation of resistance genes is an important tool in genetic engineering. *Agrobacterium tumefaciens* mediated transformation is an effective and widely used approach to introduce desirable genes into plants. Antibiotic resistance genes have the ability to selectively inactivate certain antibiotics and consequently protect cells against these antibiotics. The most widely used antibiotic resistance marker for the selection of transformed cells is the *npt* II gene, which confers resistance to the antibiotics neomycin and kanamycin. In the present study an attempt was made to transform pumpkin (*Cucurbita moschata* Poir.) var. Saras with *gus* gene. The results obtained in the study are discussed in this chapter based on the earlier reports and possible interpretations.

5.1 PRODUCTION OF AXENIC PLANTS AS EXPLANT SOURCE

Axenic plants were raised as a source of explant from mature seeds of pumpkin. Treatment with 0.1 per cent mercuric chloride for six minutes was effective in sterilizing the seeds. Among the different basal media tried for culture establishment, MS basal was found to be better compared to half MS basal. MS medium with desired salt composition enhanced the early germination of seeds compared to half MS medium.

Generally MS media is used for the *in vitro* studies in most of the cucurbitaceous crops. In the present study, the percentage germination was higher in Murashige and Skoog (MS) medium compared to half MS basal. MS medium has been identified for seed germination in winter squash (Lee *et al*., 2003) and summer squash (Pal *et al*., 2007).

5.2 STANDARDIZATION OF CALLUS MEDIATED REGENERATION

For successful plant tissue culture, it is best to start with an explant rich in undetermined cells because such cells are capable of rapid proliferation. Once established, the callus can be propagated definitely by subdivision. Usually callus cultures are maintained in the dark because light can induce differentiation of callus cells. Many reports on callus induction of plants *in vitro* from various explants of cucurbits are available (Ananthakrishnan *et al*., 2003; Sarowar *et al.,* 2003). Seedling age and explant type has shown to be important for morphogenesis induction, since competent cells for adventitious shoot formation in cucurbits seem to be restricted to specific cotyledon regions (Choi *et al*., 1994; Compton and Gray, 1994). For watermelon, it has been shown that organogenic competent cells concentrated at the proximal region of the cotyledon, since most of the adventitious buds develop at the explant basal region (Krug *et al.,* 2005).

In view of these facts, the standardization of callus mediated regeneration technique in *Cucurbita moschata* Poir. var. Saras was attempted.

5.2.1 Standardization of explants

The best explant for callus induction and proliferation in *Cucurbita moschata* Poir. was found to be cotyledons. Cotyledons of mature seed were used for callus induction and regeneration in cucurbits (Dirks and Buggenum, 1989; Dong and Jia, 1991; Shetty *et al*., 1992). The regeneration ability of cotyledons of mature seeds was found to be higher than that of cotyledons from 7-day old seedlings. Based on these observations, it was assumed that the ability of cotyledons for callus induction and regeneration gradually decreased when the duration of the period after germination increased. This is

in confirmation with the studies of Tabei *et al* (1991) in melon and Tabei *et al* (1993) in watermelon. Among explants derived from seedlings at the same age, the proximal part of cotyledon showed a higher frequency of callus induction when compared with the distal part. This result agrees with the report indicating that the distal cotyledons of cucumber are less responsive than proximal cotyledons (Mohiuddin *et al*., 1997).

Cotyledonary leaf segments excised from one week old seedlings responded to callus induction at a much higher percentage than explants collected from two week old seedlings, as young leaf segments are physiologically very active and respond efficiently to exogenous hormones (Dong and Jia, 1991). Studies with cucumber demonstrated that the frequency of polyploid cells was much lower in leaf explants, when compared with cotyledons and hypocotyl (Colijn-Hooymans *et al*., 1988).

Hypocotyl segments were found to be less responsive than the cotyledons. Calli derived from hypocotyls were mostly friable and creamy in colour. This can be used to initiate organogenic calli. Callus morphology was in agreement with Thomas and Sreejesh (2004). Calli induced from hypocotyl explants were larger in size than those from the cotyledons.

5.2.2 Standardization of growth regulators

The use of plant growth regulators is essential for the control of morphogenesis in tissue culture. Combination of phytohormones often determines the course of morphogenesis. The ratio of auxins to cytokinins influences the balance between root and shoot organogenesis from cultured tissues. A high cytokinin/auxin ratio results in the formation of shoots and a low cytokinin/auxin, results in roots. The nature of any callus depends on the explant tissue from which it has arisen and also on the composition of medium used to induce and maintain it.

5.2.2.1 Effect of Auxins

Auxin at a moderate to high concentration is the primary hormones used to produce callus.

MS medium supplemented with 2,4-D is among the most widely used auxins for *in vitro* callus induction in a wide range of cucurbits. Successful induction of callus was achieved from hypocotyls using 2,4-D. Maximum callus was recorded with 2.5 mg l^{-1} 2,4-D and the calli produced at all the levels of 2,4-D used were white coloured and friable except at a concentration of 0.5 mg l^{-1}. This is in agreement with the findings of Pal *et al* (2007) in summer squash. Picloram induced creamy friable callus from hypocotyl and cotyledonary leaf explants and white callus from cotyledon explants since at higher concentration, root formation fails to occur and callus formation takes place. White callus was observed all over the edges of the explant and the amount of callus was less compared to 2,4 –D. This justifies the observation made by Srivastava *et al* (1989) that shoot organogenesis was inhibited when NAA was added to the induction medium.

5.2.2.2 Effect of cytokinins

Cytokinins have an important role in shoot induction. These are often used to stimulate growth and development.

BA at different concentrations induced callusing from cotyledonary leaf explants and the explants turned brown when concentration was more than 3 mg l^{-1}. BA has been reported as highly effective for cucurbit organogenesis (Compton and Gray, 1993, Souza, 1996). Thidiazuron promoted callus induction from both hypocotyl and cotyledonary leaf explants. It is thought to be involved in the regulation of purine cytokinin metabolism and may directly act as a cytokinin or in combination with cytokinins. TDZ was less effective for callus induction and shoot differentiation than BA. This was in accordance with the findings of Pal *et al* (2007) in summer squash and Niedz *et al* (1989) in melon.

5.2.2.3 Effect of combination of auxins and cytokinins

The combination of auxin and cytokinin had tended to stimulate callus proliferation and shoot regeneration was not achieved, because the conditions of shoot induction from de-differentiated callus and proliferation was not favourable.

BA was combined with several kinds of phytohormones to improve the regeneration frequency, but only callus induction was obtained in all the different combinations. BA combined with NAA, 2,4-D and IAA resulted in callus production. Rhizogenesis was observed when the explants were inoculated in MS medium containing BA supplemented IAA and higher concentration of NAA. It seems that the presence of auxins is required for increasing the efficiency of callus induction in pumpkin. BA and IAA have been most often used for shoot organogenesis in melon (Tabei *et al*., 1991) and cucumber (Shetty *et al*., 1992). BA in combination with 2,4 – D produced white callus at the proximal end of the cotyledonary leaf segments which was negligible compared to NAA and IAA. The induction of callus of *Cucumis metuliferus* on MS medium containing 2,4-D and BA followed by transfer to medium containing zeatin gave the highest regeneration frequency (Raharjo and Punja, 1993). Kinetin proved to be more efficient in callus induction compared to BA. This supported the report of Souza *et al* (2006) in melon cotyledon and leaf explants. Organic supplement namely Casein hydrolysate was used in combination with NAA and Kin. Callus induction and prolific root formation was observed. Casein hydrolysate serves as a source of organic nitrogen for the callus cell cultures.

Cotyledon explants cultured in MS medium containing 2,4-D and TDZ was most effective in inducing clumps of callus tissue. The cotyledonary axis germinated *in vitro.* Similar results were obtained by Msikita *et al* (1988) in cucumber using NAA and BA. TDZ in combination with picloram produced creamy yellow callus which when subcultured didn't show any signs of shoot regeneration. The substitution of TDZ for the auxin requirement for organogenesis demonstrates that TDZ may possess an auxin-like property or may impinge upon the endogenic auxins by modifying their biosynthesis or metabolism.

5.3 *IN VITRO* REGENERATION

An essential step towards development of transgenic plants through *Agrobacterium* mediated transformation is the development of an efficient and reproducible regeneration protocol.

Cotyledonary nodal segments alone were capable of inducing multiple shoots *in vitro* out of all the explants tried for regeneration.

5.3.1 Standardization of growth regulators

MS medium supplemented with BA, IAA and Kin were used for standardizing regeneration from cotyledonary nodal segments.

Benzyl adenine (BA) is an important cytokinin, widely used for inducing multiple shoot formation in many species. Media treatments involving cytokinins and IAA with a view to induce multiple shoots gave poor response coupled with callusing and limited differentiation. Combination of BA with Kinetin resulted in more number of shoot buds/explant. This may be due to the fact that cytokinin alone or in combination with other cytokinin induced more number of multiple shoots as reported by Thomas and Sreejesh (2004) in ash gourd.

The multiple shoots were rooted in MS medium containing different growth regulators. Rooting was more profuse in medium containing IBA compared to the other combinations. IBA is widely used plant growth regulator for root induction in cucurbits (Sarowar *et al*., 2003; Thomas and Sreejesh, 2004 and Krug *et al.*, 2005). In some cases, rooting in plant growth regulator free medium during organogenesis has been reported in *Cucurbita pepo* (Ananthakrishnan *et al*., 2003) and *Cucurbita maxima* (Lee *et al*., 2003). In Pumpkin, though rooting was observed to a certain extend, the explants dried at a later stage. The difference in rooting response may be a result of genotype or cultural conditions.

5.4 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

5.4.1 Sensitivity of different explants to antibiotics

Selection of the transformed cells is a key factor in developing successful methods for genetic transformation; otherwise the untransformed cells would overgrow leading to the loss of transformed cells. This is done by the use of certain selectable

marker genes that are present in the vector along with gene of interest. Genes conferring resistance to antibiotics are generally used as selection markers. Once the tissue is transformed with the antibiotic resistance genes, it is grown in the medium containing antibiotic resistance genes. The cells which are transformed with the antibiotic resistance gene would multiply and grow normally, whereas, the untransformed cells are killed.

The usefulness of a particular antibiotic depends on several factors. The best selection agents are those that either inhibit growth or slowly kill the non transformed cells so that the dying cells do not overwhelm the transformed ones. Optimal selection pressure will use the lowest level of antibiotic needed to kill untransformed tissues. This necessitates the evaluation of sensitivity of plant tissue to antibiotics and the minimum concentrations of antibiotics required to inhibit growth. In Cucurbits, the antibiotic kanamycin has been successfully used as a selection agent at concentrations of 25-200 mg l^{-1} (Sarmento *et al*., 1992).

In the present study, sensitivity of cotyledon explants of *Cucurbita moschata* Poir. var. Saras to kanamycin and cefotaxime at different doses were evaluated.

Kanamycin is the most commonly used selection agent for plant transformation (Owens, 1981). Sensitivity of the explants to kanamycin at five different concentrations was tested. Kanamycin at the concentration of 50 mg l^{-1} induced callusing in 33 per cent of explants tested. The application of kanamycin to the regeneration medium above 150 mg l^{-1} concentration strongly inhibited the callus induction and started browning. Hence, kanamycin 200 mg l^{-1} was selected as cut off level for the selection of transformants. The differences in selective kanamycin levels are due to the different genotypes tested.

Complete elimination of the bacteria from the explant after co-cultivation is very essential; otherwise it will interfere with the growth and organogenesis of the explant. Overgrowth of the bacteria causes death of the explant and disrupts the experiment. Elimination of the bacteria from the explant is done by the use of antibiotics. The

antibiotic chosen should be such that it efficiently kills the bacteria, at the same time it does not affect the growth and morphogenesis of the explants. The antibiotic used for this purpose was cefotaxime. This is in agreement with the report of Vasudevan *et al* (2007) in cucumber. Cefotaxime at a concentration of 250 mg l^{-1} was considered as optimum for elimination of bacteria from the explant.

5.4.2 Standardization of *Agrobacterium* mediated transformation

Agrobacterium mediated genetic transformation of *Cucurbita moschata* Poir. var. Saras was standardized with the *Agrobacterium* strain EHA 105 having *gus* reporter gene. In various transformation studies in cucurbits, cotyledons have often been used as explants for *Agrobacterium* infection. This may be due to easy handling and the particular form of regeneration in this family. Since organogenesis is not restricted to one small area of the explant but to many small independent areas, the probability of the cells being competitive for both regeneration and transformation is high. Cotyledons were used for the transformation experiment since they showed better callus induction compared to the other explants. This is in accordance with the studies conducted by Awatef *et al* (2007) in Tunisian *Cucumis melo*.

Pre-culture of explants is a critical factor to achieve high frequency of transformation. It makes the explant tissue competent enough to withstand the bacterial infection and the related stress that followed it. In this study, 2 day old pre-cultured cotyledons were used for transformation. In the present study, EHA 105 strain was employed for transformation in pumpkin. Vasudevan *et al*. (2007) and Soniya and Das (2002) used the same strain for standardization of transformation experiments in cucumber. The selectable marker gene present in the T-DNA was *nptII* (neomycin phosphotransferase II), which confers resistance to kanamycin. The T-DNA also harbours genes conferring resistance to rifampicin and this act as bacteria selection agent to prevent contamination of bacterial strain. Once the T-DNA is transferred to the plant genome, the transformed plant cells are expected to develop resistance to kanamycin and will be capable of normal growth in the medium containing kanamycin, in contrast, the non-transformed cells will be susceptible to kanamycin. The transformed cells thus

developed will also show GUS activity, which can be detected by histochemical GUS assay.

The bacterial gene *gusA* encoding β-gucuronidase (GUS) is the most frequently used reporter gene for the analysis of plant gene expression. There is little or no detectable endogenous β glucuronidase activity in most of the higher plants allowing the easy detection of chimaeric gene expression. Glucuronidase activity can be detected easily and sensitively. Histochemical localization is possible in cells and tissues due to the blue colour developed with the substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide). The substrate is cleaved by the enzyme within the plant cells (transformed/transgenic), and the product is converted into an insoluble blue precipitate at the site of enzyme activity. The colour so developed is visible to the naked eye and even under microscope in sectioned tissues.

Agrobacterium strain EHA 105 contains the plasmid p35SGUSINT. Early detection of transformants is invariably based on the histochemical analysis of expression of the gus as a reporter gene. A major limitation to this approach is the expression of this reporter gene in *Agrobacterium* despite the use of plant promoters. The problem was overcome by inserting introns, which are processed in plants but not in *Agrobacterium.*

5.4.2.1 Standardization of inoculum density

Concentration of bacterial cells in the inoculation medium is an important factor to be considered for efficient transformation. Among the different inoculum densities tried, OD_{600}nm = 0.6 was found optimum. Transient GUS expression after 3 days of co-cultivation showed more blue areas when bacterial density was 0.6 (OD_{600}nm). But none of the transformed explants survived after 30 days (Fig.2). This is in contrast to the observations of Vasudevan *et al*. (2007) in which inoculum density of 1.0 (OD_{600}nm) was optimal for infection of explants. Explants inoculated with bacterial suspension diluted to 0.8 (OD_{600}nm) induced calli formation after a week in Tunisian *Cucumis melo* as reported by Awatef *et al* (2007). Very high bacterial density leads to necrosis and death of the

explant. This is due to the hypersensitive response of the tissue as a part of the plant defense against pathogen.

5.4.2.2 Standardization of infection time

The time of infection of hypocotyl explants of pumpkin var. Saras was standardized with *gus* construct. The infection times viz, 5, 10, 15 and 20 minutes were tried and 10 minutes was found optimum. This is in accordance with the report of Ganapathi and Perl-Treves (2000) in cucumber. Sarmento *et al*. (1992) reported that an infection time of 5 minutes was optimum for transformation of pickling cucumber. With the prolonged exposures, explants became necrotic and died or were colonized by *Agrobacterium* even in the presence of 500 mg l^{-1} cefotaxime. In the present study, there was a positive GUS expression in all the infection times tested. The low survival rate in an infection time of 5 minutes is due to the low rate of transformation. Low survival rate of the tissues in 15 and 20 minutes infection time is due to the hypersensitive response of the tissue (Fig.3). This was in contrast to the report by Awatef *et al* (2007).

5.4.2.3 Standardization of co-cultivation period

Co-cultivation period plays an important role in the success of the transformation. It is during this period that the *vir* genes are activated and the T-DNA transferred into the plant cell. However, increasing the co-cultivation period might lead to necrosis and death of the explant due to the hypersensitive response of the tissue. Hence the length of co-cultivation period should always be the shortest interval necessary to obtain the maximum frequency of transformation in terms of the number of transgenic plants recovered following co-cultivation.

In the present study, a co-cultivation period of 48 h was the best for transformation when compared to 0, 24, 72 and 96h. The co-cultivation period beyond 96 hrs, the calluses with free of *Agrobacterium* contamination were more difficult to obtain for cucumber (Raharjo *et al.,* 1996). These differences could be explained by the fact that the concentration of *Agrobacterium* was optimum during 48 h co-cultivation than after

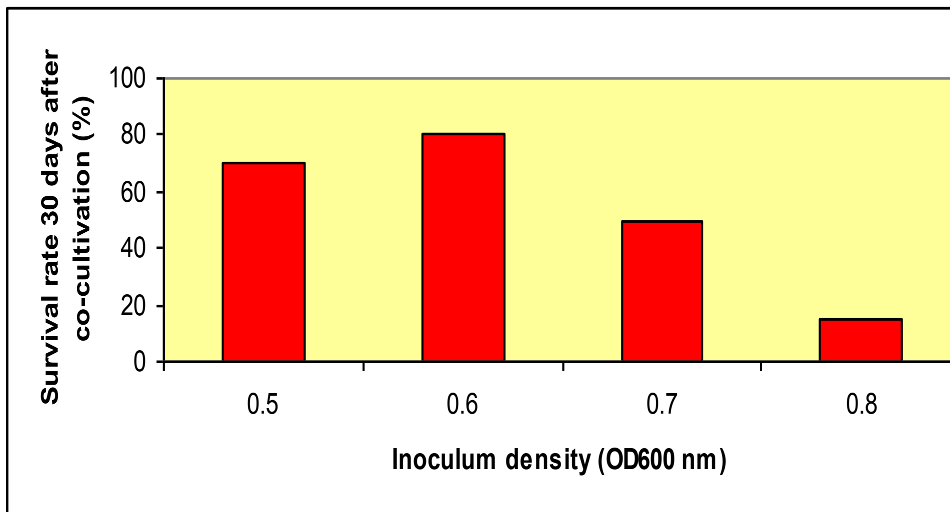


Fig.2. Effect of inoculum density on survival rate of transformed explants

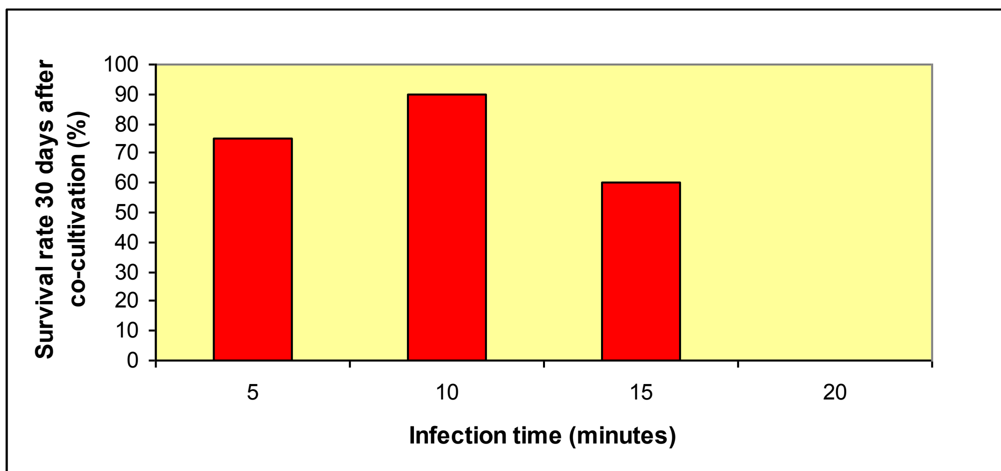


Fig.3. Effect of infection time on survival rate of transformed explants

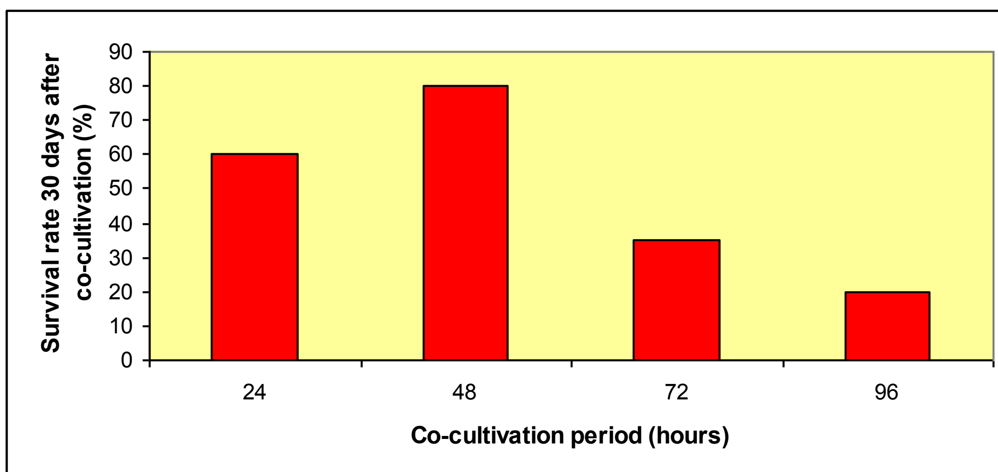


Fig.4. Effect of co-cultivation period on survival rate of transformed explants

24 h and that could considerably increase the probability of gene transfer (Fig.4). Similar results were also reported by Ganapathi and Perl-Treves (2000) in cucumber.

Bacterial cells multiplied in the co-culture medium and after 48 h, the optimum quantity of bacteria were available for transformation and hence a higher survival rate in the selection medium was achieved. After 48 h, the level of bacterial cells reached supra optimum level and competitive inhibition of competitive bacterial cells resulted in inhibition of transformation. This accounts for the low survival rate of explants after 48 h of co-cultivation.

5.4.2.4 Histochemical GUS assay

β-glucuronidase enzyme activity was used to monitor the transformation. Blue staining was evident when the explants were examined 3 days after co-cultivation with EHA 105. Microscopic examination showed that indigo dye precipitates were localized within cells.

5.4.2.5 Confirmation of transformation

To assess whether these cotyledon calli were transformed or not, PCR analysis of the *npt II* gene was done. Polymerase Chain Reaction (PCR) is a powerful technique for confirming DNA insertion in transgenic plants. Primers can be designed which simultaneously amplify specific regions on the T-DNA that are expected to be integrated into the genome of plants. Advantages include the rapid manner in which large collections of transgenic plants can be analysed and the very small amount of plant tissues required (Chawla, 2002). The forward and reverse primers of *npt II* gene were designed in such a manner that PCR amplification using these primers will amplify a 600bp fragment corresponding to *npt II* gene. The presence of a 600bp fragment confirms the transgenic nature. This indicated that the plasmid gene was successfully integrated into the genome of the pumpkin plant. The plasmid DNA was used as the positive control as it contained the construct having *npt II* gene.

The assay revealed that out of the seven calli only three showed the amplification of *npt II* gene. This indicates that even after growing the infected explant in selection media containing suitable selectable markers, some untransformed cells may also show growth (escapes or false positives). This could be because of the cross protection provided by the transformed cells. Hence all tissues showing growth in the selective medium cannot be considered as transgenic and further confirmatory tests are to be done to confirm the transfer and stable integration of the foreign genes.

The presence of a selectable marker allows selection of transgenic cells but does not guarantee 100% co-transmission and/or expression of the other genes on the same section of T-DNA. Independently selected transgenic calli often show varying degrees of gene expression. Several factors other than the absolute effects of the regulatory sequences can influence the magnitude of gene expression. These include copy number, position effects resulting from the site of insertion and methylation of the transferred genes. Hence molecular analysis of transformed plants using Southern hybridization, RT-PCR analysis and Western blotting are to be done to know the stable integration and expression of transgenes.

Summary

6. SUMMARY

Investigation on 'Development of *in vitro* regeneration and genetic transformation systems in pumpkin (*Cucurbita moschata* Poir.)' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara. The objective of the study was to standardize a viable regeneration and transformation system in pumpkin. The salient findings of the present study are stated below.

Mature seeds of pumpkin were collected, sufficient axenic seedlings were raised *in vitro* and explants were collected from axenic cultures for standardization of regeneration protocol and suitable explants for transformation work. Cotyledon explants were obtained from intact seeds of Pumpkin.

Surface sterilization procedure was standardized; the treatment involving 0.1 per cent mercuric chloride for 6 min, followed by washing in sterile water five times was effective in sterilizing the seeds.

Axenic seedlings were raised in MS basal medium. The youngest seedlings had unexpanded cotyledons, green at the proximal end with a short hypocotyl and one or more roots were used for collecting the explants.

MS medium supplemented with different concentrations of auxins and cytokinins such as 2,4 –D, NAA, BA, Picloram, TDZ, IAA, Casein hydrolysate and kinetin were tried on various explants such as cotyledonary leaf segments, hypocotyl segments, cotyledon segments and cotyledonary node segments.

Cotyledonary node segments produced multiple shoots in MS medium containing 1 mg l^{-1} BA and 1 mg l^{-1} Kinetin. The shoots were successfully rooted in MS + 1 mg l^{-1} IBA. The plants were successfully hardened and transferred to pots in the net house.

Among the different explants tried, cotyledons were found to be the best material for inducing callus. Cotyledons were dissected into well defined explant types, namely,

basal cotyledon explants, distal cotyledon explants and cotyledonary axis explants. MS medium supplemented with 5 mg l^{-1} 2,4 – D and 0.1 mg l^{-1} TDZ resulted in embryogenic callus initiation followed by callus proliferation within 2 weeks. Cotyledonary axis explants germinated *in vitro*.

Explant sensitivity to different concentrations of Kanamycin were tested. Pumpkin cotyledon explants were found to be sensitive to Kanamycin when used along with subculturing at 2 weeks interval. Callus induction was completely inhibited at a lower concentration and the explants turned brown when the concentration of Kanamycin reached 200 mg l^{-1}. Kanamycin 200 mg l^{-1} was used to discriminate between transformed and non-transformed cells.

The antibactericidal activity of Cefotaxime on *Agrobacterium tumefaciens* strain EHA 105 was studied. Cefotaxime was found to be effective for eliminating the bacteria from the cultures. Cefotaxime 250 mg l^{-1} added to the pre-selection and selection media to eliminate the bacteria was found to be effective.

Agrobacterium tumefaciens strain EHA 105 containing plasmid p35S GUSINT was used as the vector system for standardizing optimum conditions for affecting genetic transformation in pumpkin.

Tentative protocol for transformation in pumpkin include *Agrobacterium* inoculum density 0.6 OD_{600} nm, infection time 10 min and co-cultivation period of 48 h. Transient GUS assay revealed faint blue staining on the infected cotyledon explants.

Cotyledon explants showed callusing 4 weeks after co-cultivation. Bacterial overgrowth affects the survival of tissues. Transformed cotyledon explants induced callus on selection medium while normal control explants turned brown when they were transferred to selection medium containing Kanamycin 200 mg l^{-1} and Cefotaxime 250 mg l^{-1}.

Genomic DNA could be isolated from normal and transformed cotyledon explants using Doyle and Doyle method. Intact DNA bands were obtained for both explants.

PCR assays confirmed the presence of *npt* II coding sequence in DNA extracted from Kanamycin resistant calli expressing GUS activity.

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Appendices

APPENDIX

Appendix I.

1. Composition of different tissue culture media

Chemical	MS (mg l^{-1})
Inorganic constituents	
$(NH_4) NO_3$	1650
KNO_3	1900
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$(NH_4) H_2PO_4$	-
$CaCl_2.2H_2O$	440
Na_2 EDTA	37.3
$FeSO_4.7H_2O$	27.8
EDTA Na ferric salt	-
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic constituents	
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5

Thiamine HCl	0.1
Sucrose	30000
Myoinositol	100
PH	5.8

Half MS represents 50 percent concentration of inorganic constituents.

2. Chemical composition of Yeast Extract Mannitol (YEM) medium used for culturing *Agrobacterium tumefaciens*

Chemical	$g\ l^{-1}$
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Mannitol	10.0
Yeast Extract	1.0
Agar	20.0
pH	7.0

Appendix II

1. Reagents for plasmid DNA isolation

A. Solution I

Composition

Glucose	:	50 mM
EDTA	:	10 mM
Tris HCl	:	50 mM pH 8.0
Lysozyme	:	2 mg/ml
RNase	:	0.2 mg/ml

B. Solution II

Composition

NaOH	:	0.2 N
SDS	:	1%

This solution was prepared fresh, immediately before use.

C. Solution III

Composition

CH_3COOK	:	4 M pH 6.0

D. Solution IV

Composition

Na acetate	:	0.1 M
Tris HCl	:	50 mM pH 8.0

E. Solution V

Composition

CH_3COONH_4	:	7.5 M

F. TE Buffer

Composition

Tris HCl	:	10 mM pH 7.6
EDTA	:	1 mM

2. Reagents for plant DNA isolation

A. Extraction buffer (4x)

Composition

Sorbitol	:	2.5 g
Tris-HCl	:	4.8 g
EDTA 0.25M	:	0.74 g
Distilled water to	:	100 ml

The chemicals were dissolved in 60 ml sterile distilled water. The pH was adjusted to 7.5 and final volume was made up to 100 ml with distilled water and then autoclaved.

B. Lysis buffer

Composition

1M Tris-HCl (pH 8.0)	:	20 ml
0.25M EDTA	:	20ml
CTAB	:	2 g
NaCl 5 M	:	40 ml
Distilled water to	:	100 ml

Cetyl Trimethyl Ammonium Bromide (CTAB) was dissolved in 20 ml sterile distilled water. To this solution the required volumes of other stock solutions were added.

1M Tris-HCl (pH-8.0)

Tris-HCl 15.76g was dissolved in 60 ml sterile distilled water. The pH was adjusted to 8.0 and final volume was made up to 100 ml with distilled water and then autoclaved.

0.25 M EDTA

Ethylene Diamine Tetra Acetic acid (EDTA) 9.305 g was dissolved in 100 ml sterile distilled water and autoclaved.

5 M NaCl

Sodium chloride 29.22 g was dissolved in 100 ml sterile distilled water and autoclaved.

C. Sarcosine (5%)

Composition

Sarcosine	:	5 g
Distilled water to	:	100 ml

Sarcosine 5 g was dissolved in 100 ml sterile distilled water and autoclaved.

D. TE buffer

Composition

(Tris HCl -10.0 mM; EDTA -1.0 mM)

Tris-HCl 1.0 M (pH 8.0)	:	1.0 ml
EDTA 0.25 M (pH 8.0)	:	0.4 ml
Distilled water	:	98.6 ml

Autoclaved and stored at room temperature.

E. Chlroform: isoamyl alcohol mixture

Composition

Chloroform	:	24 ml
Isoamyl alcohol	:	1 ml

The mixture was stored in refrigerator before use.

F. Ice-cold Isopropanol

Isopropanol stored in refrigerator was used as such.

G. Ethanol 70 per cent.

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

DEVELOPMENT OF *IN VITRO* REGENERATION AND GENETIC TRANSFORMATION SYSTEMS IN PUMPKIN (*Cucurbita moschata* Poir.)

By

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ABSTRACT OF THESIS

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ABSTRACT

Investigation on 'Development of *in vitro* regeneration and genetic transformation systems in Pumpkin (*Cucurbita moschata* Poir.)' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, from October 2005 to July 2008. The study was undertaken to standardize a viable *in vitro* regeneration system and to develop protocol for genetic transformation in Pumpkin.

Axenic seedlings of Pumpkin variety Saras were raised under *in vitro* conditions to generate explants with reduced contamination for transformation. MS basal medium was found to be better compared to half MS basal with respect to the percentage of germination. Mercuric chloride (0.1%) treatment for 6 min was found to be effective in sterilizing the seeds.

In order to standardize a regeneration protocol, MS medium supplemented with varying concentrations of auxin and cytokinin were tried on different explants. Different explants such as cotyledonary leaf segments, hypocotyl segments, cotyledonary node segments from axenic seedlings and cotyledons from intact seeds.

Cotyledonary node explants produced multiple shoots in MS medium supplemented with 1 mg l^{-1} BA and 1 mg l^{-1} Kinetin. Shoot buds produced from cotyledonary node explants showed good multiplication in the same media. The shoots were successfully rooted in MS + 1 mg l^{-1} IBA. The plants were successfully hardened and planted out.

Embryogenic calli was induced from cotyledon explants cultured in MS medium containing 5 mg l^{-1} 2,4 – D and 0.1 mg l^{-1} TDZ.

Agrobacterium mediated transformation protocol was optimized considering all the factors for successful transformation. EHA 105 p35S GUSINT was used for

standardizing optimum conditions by comparing the levels of transient GUS expression in calli. The cotyledon explants were used for genetic transformation.

Optimum inhibitory concentration of selectable marker (Kanamycin: 200mg l^{-1}) was established. The antibiotic cefotaxime (250 mg l^{-1}) was selected for killing the bacteria. *Agrobacterium* strain EHA 105 harbouring the *gus* reporter gene was used for the standardization of transformation. Cotyledon explants of pumpkin were co-cultivated with *Agrobacterium* strain (EHA 105).

The influence of different parameters such as bacterial inoculum, co-cultivation periods and infection time effects on transformation frequency were studied. The inoculum density-0.6 OD_{600} nm, infection time-10 minutes and co-cultivation period-2 days were found optimum based on GUS assay and survival rate 30 days after co-cultivation.

Histochemical GUS assays were performed to study and compare the transient GUS expression from transformed tissues. Transient GUS assay revealed faint blue staining on the surface of the infected cotyledon explants. When these blue coloured areas were examined under microscope, clear blue specks were observed indicating transformation.

The explants on transfer to selection medium containing 200 mg l^{-1} Kanamycin and 250 mg l^{-1} Cefotaxime produced callus 4 weeks after co-cultivation. Normal explants turned brown when transferred to selection medium.

After selection of the transformants, the transformed cotyledon tissues were characterized employing molecular biology techniques viz. PCR-utilizing the gene specific primers of *npt II*. The presence of transgene was confirmed in the transformed tissues.