

ABSTRACT

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AGROBACTERIUM MEDIATED
TRANSFORMATION PROTOCOL IN
TOMATO (*Solanum lycopersicum* L.)
Cv. PKM-1.**

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The present investigation was carried out to standardize the *Agrobacterium* mediated transformation protocol in tomato and it is carried out at transgenic laboratory of Dept. of Plant Physiology, RARS, Tirupati and Dept. of Genetics and Plant Breeding, S.V. Agricultural College, Tirupati.

Seeds treated with 5% NaOCl for 20 min and inoculated on MS medium with out sucrose and incubation in dark for three days produced healthy and uniform seedlings without contamination. Explants i.e. cotyledons and hypocotyls isolated from 10 day old seedlings were found ideal for high frequency of regeneration compared to younger or older seedlings. Among the two explants i.e. cotyledon and hypocotyls, cotyledons showed better response compared to hypocotyls. Hence cotyledonary explants from 10 days old *in vitro* seedlings of PKM-1 were used for further studies on regeneration and transgenic protocols.

Among the various plant growth regulators combinations tried the best shoot regeneration was obtained when MS medium was supplemented with BAP 1.5 mg/L + Kinetin 1.0 mg/L and root regeneration was obtained when MS medium was supplemented with Kinetin 1.0 mg/L respectively.

Cotyledonary explants excised out from 10 days old seedlings were incubated for 10 min with over night grown *Agrobacterium* culture and co-cultivated for 2 days followed by transfer to media containing cefotaxime 500 mg/L for 4 days before transferring to the medium containing 75 mg/L kanamycin which was found to be optimum for checking the *Agrobacterium* growth.

Higher plantlet survival (86%) was obtained in soilrite mixture and 9.6 days has been taken for acclimatization.

The transformation was carried out using the *Agrobacterium* strain LBA4404 containing the binary vector pCAMBIA 2301 harboring *npt II* as selectable marker and GUS as reporter gene.

Confirmation of the transgene integration in the putative transformants was done by using the histochemical staining and PCR. The transformation efficiency of 44.4% was obtained in the cultivar PKM-1. The transformation frequency was 3.5% and the GUS gene transient expression level in transformants was 44.4%. Thus, the present study successfully demonstrated the indirect regeneration of transgenic plants from cotyledonary explants through *Agrobacterium* mediated genetic transformation approach in tomato Cv. PKM-1. The standardized protocols of present study may be utilized for further transgenics development in PKM-1 cultivar genetic background.

**STANDARDIZATION OF AGROBACTERIUM MEDIATED
TRANSFORMATION PROTOCOL IN TOMATO
(*Solanum lycopersicum* L.) Cv. PKM-1.**

Major Advisor: M. Shanti Priya

ABSTRACT

Seeds treated with 5% NaOCl for 20 min and inoculated on MS medium without sucrose and dark incubation for three days produced healthy and uniform seedlings without contamination. Cotyledons and hypocotyl explants isolated from 10 day old seedlings were found ideal for high frequency regeneration compared to younger or older seedlings. Comparatively cotyledons showed better explant response than hypocotyls. Hence, cotyledonary explants from 10 days old *in vitro* seedlings were used for further studies on regeneration and transgenic protocols. Best shoot and regeneration, was obtained when MS medium is supplemented with BAP 1.5 mg/L + Kinetin 1.0 mg/L and root regeneration, was obtained when MS medium is supplemented with Kinetin 1.0 mg/L respectively and higher plantlet survival (86%) was obtained in soilrite mixture with in 10 days. The transformation was carried out using the *Agrobacterium* strain LBA4404 containing the binary vector pCAMBIA 2301 harboring GUS as reporter gene. Cotyledonary explants excised out from 10 days old seedlings were incubated for 10 min with over night grown *Agrobacterium* culture and co-cultivated for 2 days followed by transfer to media containing cefotaxime 500 mg/L for 4 days before transferring to the medium containing 75 mg/L kanamycin was found to be optimum for checking the *Agrobacterium* growth. Stable integration of the transgene in the putative

transformants was confirmed by using the histochemical staining and PCR assay.

CHAPTER 1

INTRODUCTION

Tomato (*Solanum lycopersicum* L. 2n=24) is one of the most important vegetable crop and known as “protective food” because of its special nutritive value. Tomato is a rich source of minerals, vitamins and organic acids (healthy acids). Tomato fruit contains 3-4 % total sugars, 4-7 % total solids, 20-30 mg/100g fruit weight of lycopene and a range of 15-30 mg/100g fruit weight of vitamin-C. In addition the amino acid content ranges between 100-350 mg/100g of fresh weight. Tomato seed contains 24 % oil which is used as salad oil as well as in the manufacture of margarine.

In India, it is cultivated in an area of 5.20 Lakh ha with a production and productivity of 90.06 Lakh tonnes and 17,800 kg/ha respectively (Anonymous, 2007a). In Andhra Pradesh, it accounts for 0.765 Lakh ha with a production and productivity of 14.3 Lakh tonnes and 19,000 kg/ha, respectively (Anonymous, 2007b).

Tomato has been subjected to genetic breeding using classical methods for many years. Tomato is succumbed to several biotic and abiotic factors. Though heterosis was well exploited in this crop, resistance to several pests, diseases, and abiotic stresses were unanswered. The two factors which limit the progress of breeding efforts are the availability of source of interest in sexually related plants and the duration of the reproductive cycle. The wild relatives of cultivated tomato especially *L.peruvianum* are a rich source of

vitamin C. But, it is difficult to transfer these specific traits to cultivated tomato as they are governed by polygenes and existence of specific barriers in inter-specific hybridization with wild relatives.

The advent of genetic engineering techniques makes it possible to transfer gene of interest across all taxonomic boundaries into plants from other plants, animals and microbes. Gene transfer technology is the only alternative available to incorporate the traits required in modern tomato cultivars for which there are no available sources among the wild relatives of domesticated tomato or where the available resources are governed by complex genetic systems or existence of specific barriers for inter-specific hybridization.

Agrobacterium mediated gene transfer is the most widely used method for transformation in higher plants. The T-DNA of *Agrobacterium* has been suitably modified for the development of gene vectors to produce transgenic plants. Several workers reported the use of *Agrobacterium* mediated transformation and regeneration of variety of cultivars (Ieamkhang and Chatchawankanphanich, 2005 and Park *et al.*, 2003).

In this context, development of regeneration and transgenic protocols in any crop is a research priority. Tomato is very amenable to tissue culture and highly responsive to *in vitro* cultures. Standardization of both organogenesis and transformation protocol in this crop is a must for the development of efficient transformation procedures. However, regenerative

response is greatly dependent on the genotype. Though several protocols were developed for different varieties, work on regeneration protocol for PKM-1 is so far has not been accomplished. PKM-1 is an adaptable high yielding cultivar widely grown in A.P for its high acidity and is ideally suitable for long distance transport (Plate 1). It is also mostly used as a parent for the development of green shoulder hybrids. Development and standardization of transgenic protocol in this variety will lead to success in the production of transgenic tomato green shoulder hybrids resistant to biotic and abiotic stresses. Hence, keeping these points in view the present investigation has been taken up with the following objectives.

OBJECTIVES OF INVESTIGATION:

1. Optimization of seed germination *in vitro*.
2. Identification of suitable explant sources viz., hypocotyls and cotyledonary leaf yielding good regeneration potential.
3. Standardization of medium and *in vitro* culture conditions for efficient shoot and root regeneration.
4. To assess the transformation efficiency using GUS reporter gene employing *Agrobacterium*- mediated transformation system.
5. Standardization of acclimatization protocols.

CHAPTER-2

REVIEW OF LITERATURE

The beginning of plant tissue culture was made as early as 1898, when a German Botanist, G. Haberlandt, successfully cultured fully differentiated individual plant cells, isolated from different tissues in several plant species (Gupta, 2005). In 1902, he coined the concept of ‘**Totipotency**’ which is defined as the ability of plant cells to perform all the functions of development, which are characteristics of zygote, i.e. ability to develop in to a complete plant (Singh, 1998). For about thirty five years (up to 1934), a little progress in cell culture research was made, although culture of embryos, roots and other tissues was achieved in this period. During 1934-1939, due to the discovery of the importance of auxins and B-vitamins, the foundation of plant tissue culture was laid down by three scientists (Gautheret, White and Nobecourt), even though only small pieces of tissues and not individual differentiated cells could be grown in cultures. Media and culture techniques for a variety of plant materials became known and now plant tissue culture research has become a thrust area in all areas of crop improvement programmes (Gupta, 2005).

Tomato is an important vegetable crop and a model plant for cloning genetically important genes among dicotyledonous crop plants. Plant tissue or cell culture is a key facilitator component in genetic transformations using *Agrobacterium tumefaciens* (Bhatia *et al.*, 2004).

Success of gene transfer technology is greatly dependent on development of efficient regeneration protocol. The *in vitro* responses of cultured plant tissues are affected by different components of culture media and it is important to evaluate their effects on plant regeneration. So, a good regeneration system is essential for an effective genetic engineering that seeks to exploit genetically transformed plants for commercial applications. The studies on various aspects of tissue culture and transformation in tomato are reviewed below:

2.1 TOMAT TISSUE CULTURE

2.1.1 Mass propagation

Mass propagation of tomato has been attempted in tissue culture through the use of various techniques, including shoot tip culture (Mirghis *et al.*, 1995; Jabeen *et al.*, 2005), somatic embryogenesis (Newman *et al.*, 1996; Chandel and Khatiyar, 2000; Kaparaskis and Alderson, 2002), direct organogenesis from intact explants (Gubis *et al.*, 2003; Jabeen *et al.*, 2005) or protoplast culture (Chen and Adachi, 1994; Hossain *et al.*, 1995). In tomato, adventitious shoot regeneration can be achieved either directly (Bhatia *et al.*, 2005) or indirectly through an intermediate callus phase (Behki and Lesely, 1976). Indeed, both callus and shoots may be produced together (Bhatia, 2003).

2.1.2 Direct organogenesis

The primary mode of regeneration in tomato is via shoot organogenesis from callus that originates following dedifferentiation of leaf, hypocotyls or

cotyledon explants, or directly from a thin layer of cells of pedicel or peduncle (Compton and Veilleux, 1991). In tomato, the majority of tissue culture attempts have been made to obtain organogenesis rather than somatic embryogenesis or shoot tip culture (Gubis *et al.*, 2003; Rao *et al.*, 2007).

The success in tomato regeneration has been found to be largely dependent on genotype, explant and plant growth regulators used in culture medium (Gubis *et al.*, 2003).

Raj *et al.*, (2005) reported that direct shoot initiation was observed rather than callus at the cut edge of the proximal end of cotyledons.

2.2 FACTORS INFLUENCING REGENERATION

2.2.1 Genotypic factors

The *in vitro* development of a whole plant from a single cell is a characteristic feature of plants. The amenability of a plant to *in vitro* culture is influenced by the genotype and hence it has major importance in plant tissue culture response. Genotypic differences can be seen for the requirement of plant growth regulators and the type of explant. Most genotypes of tomato respond uniquely to plant growth regulators during regeneration (Kurtz and Lineberger, 1983; Rao *et al.*, 2007). Similarly Gubis *et al.* (2003) reported genotypic influences on regeneration.

Davis *et al.* (1994) reported that the cultivar ‘Better Boy’ regenerated only from hypocotyls, whereas ‘Spring giant’ regenerated from both hypocotyls and cotyledonary explants.

Rao *et al.* (2007) reported that Pusa Ruby regenerated from cotyledon and hypocotyl had the highest shoot regeneration frequency compared to S-22 followed by Pusa Early Dwarf. The *in vitro* regeneration of the genotypes differed significantly depending on the culture medium (Uddin *et al.*, 1988; Mirghis *et al.*, 1995).

2.2.2 Explant

The influence of explant on the growth and development of organs depends on several factors, including the genotype, the age of the explant, the size of explant and the method of inoculation (Bhatia *et al.*, 2005). El-Farash *et al.* (1993) found an interaction between genotype, explant type, and the age of the donor plant for shoot regeneration rate and the number of shoots produced per explant. Explants have shown variation in their regeneration capacity depending on the size and physiological state of the explant (Mc.Cormick *et al.*, 1986; Cheng *et al.*, 1998).

2.2.2.1 Explant type

Almost all the explants in tomato are amenable to regeneration. Success in callus cultures and plant regeneration have been reported from different explant sources such as cotyledons (Lima *et al.*, 2004 ; Gubis *et al.*, 2005; Bhatia *et al.*, 2005; Muthuvel *et al.*, 2005; Jabeen *et al.*, 2005; Rao *et al.*, 2007), hypocotyls (Pugliesi *et al.*, 1999; Rao *et al.*, 2007; Singh *et al.*, 2007), leaves (Soniya *et al.*, 2001 ; Sheeja and Mandal, 2003), stem (Alfonso and Alonso, 1981; Sheeja and Mandal, 2003), epicotyl (Filova, 2004), node (Pongtongkam *et al.*, 1993;

Filova, 2004), Shoot tips (Fari *et al.*, 1991; Izadpanah & Koshkhui, 1989; Mirghis *et al.*, 1995; Jabeen *et al.*, 2005), inflorescence (Compton and Veilleux, 1991), anthers (Brasiliero *et al.*, 1999), and roots (Moghaieb *et al.*, 2004). Similarly, regeneration of shoots from protoplast culture in *Lycopersicon esculentum* (Patil *et al.*, 1994; Chen and Adachi, 1994; Hossain *et al.*, 1995) as well as from wild species (Lefrancois and Chupeau, 1993; Imanishi and Suto, 1987) was reported.

The type of explant used not only determines the proportion of explants, which show organogenesis, but also the number of shoots produced per explant. Duzyaman *et al.* (1994) found that the degree of shoot regeneration was in the order of leaves \geq cotyledons \geq hypocotyls, in most of the cultivars, however, Plastira and Perdikaris (1997) reported differential regeneration frequency of various explants in the order of hypocotyl \geq cotyledon \geq leaf. Preferential regeneration was also demonstrated from hypocotyl explants better than from cotyledonary explants (Jabeen *et al.*, 2005; Gubis *et al.*, 2004; Borge *et al.*, 2005; Rao *et al.*, 2007).

In contrast to these findings, Garcia-Reina and Luque, (1988), Duzyaman *et al.* (1994), Muthuvel *et al.* (2005) and Grigoriadis *et al.* (2005) reported that *in vitro* shoot production from cotyledon explants was better than that from hypocotyl explants. Almost all the tissues in tomato were reported to have high totipotency, however, the choice of the right explant may vary with the genotype.

2.2.2.2 Effect of age, size and orientation of explant

The age of the explant influences the success of tissue culture. Young and soft tissues are generally more amenable to culture compared to old and woody tissues. However, Dai *et al.* (1988) reported that the regenerative capacity of tomato increased with an increase in the age of the explant.

Hamza and Chupeau (1993) and Gubis *et al.* (2004) reported that 8 day old seedlings gave better regenerants and transformants. But, Rao *et al.* (2007) reported that 12 day old seedlings were found ideal for higher frequency of regeneration compared to younger or older seedlings. However, several workers reported that 8-10 day old seedlings and cotyledons were found superior to other sources of explants (Hamza and Chupeau, 1993; Van Roekel *et al.*, 1993; Ling *et al.*, 1998).

Chandel and Katiyar (2000) reported that the ideal size for tomato explants for successful regeneration is 0.5 cm² for leaf explants and 1 cm long segments for shoot explants.

Explants can be inoculated on the culture media in polar or non polar orientation. The polar orientation generally regenerates roots and shoots more easily than non-polar orientation. More shoots were produced from leaf and cotyledon explants placed horizontally than from the ones placed vertically, and hypocotyl explants placed horizontally produce more shoots than those placed vertically or upside down (Duzyaman *et al.*, 1994).

Cotyledons placed in abaxial orientation consistently produced better shoot regenerative response and produced greater number and taller shoots compared to those inoculated in adaxial orientation (Bhatia *et al.*, 2005).

Grigoriadis *et al.* (2005) reported that proximal part of cotyledons have high shoot regenerative frequency in almost all varieties compared to middle and distal part on MS medium supplemented with Zeatin 0.5 mg/L and IAA 0.1 mg/L.

In contrast, Costa *et al.* (2000) found that the position of the cotyledon segments (apical or basal) did not result in significant differences in the average regeneration frequency nor shoot number. Usually medium size explants with right orientation could be a good choice to obtain high shoot regeneration in tomato.

2.3 PHYSICAL FACTORS

2.3.1 Effect of light and temperature

Light is an important factor, as the growth and differentiation of explants depends on the length of exposure and quality of light. The regeneration response of tomato explants to tissue culture depends on the quality and quantity of light used in raising the mother plant (Lercari *et al.*, 2002). Generally, the explants obtained from etiolated seedlings failed to show good regeneration response (Bertram and Lercari, 2000).

Light condition at the time of explant incubation also affects the explant response. Most of the studies on tomato regeneration have employed

16 hr light and 8 hr dark photoperiod. Bhatia *et al.* (2005) reported that light is essential for shoot regeneration. However, maximum shoot regeneration response (60%) could only occur in the explants exposed to 16 hr light and 8 hr dark. Comparable number of shoots (3.3) was produced when exposed to 16 hr light when compared to 24 hr light (2.7) and 24 hr dark (3.2).

2.4 CHEMICAL FACTORS

2.4.1 Nutrient media

Most researchers preferably used either MS or modified MS medium for successful regeneration of tomato (Chandel and Katiyar, 2000; Park *et al.*, 2003). B₅ vitamins along with MS basal major and minor nutrients were also successfully used by Kurtz and Lineberger (1983) and Raj *et al.* (2005).

2.4.2 Sugar concentration

Normally, it is essential to add a carbon source into the growth medium for the cell, tissue, or organ cultures. Sucrose is almost universally used for micropropagation purposes, as it is readily utilizable by cells. The optimum concentration of sucrose required to induce morphogenesis or growth differs among genotypes. Sucrose seems to be essential for the healthy growth of tomato cultures, and most researchers have used it as the sole source of 'carbon' (Costa *et al.*, 2000; Venkatachalam *et al.*, 2000; Bhatia *et al.*, 2005). However ribose, glucose, maltose, palatinose and furanose have also been tried by many researchers. Locy (1995) reported that when tomato callus or cell cultures were placed on media containing ribose as the sole carbon

source, the tissues turned dark brown and ceased growth. However, after about 60 days, bright green tissues emerged from about 3% of the brown necrotic callus tissue pieces. Gubis *et al.* (2005) found that sucrose conc. of 30 g/L (compared to 5, 10, or 20 g/L) was found to be optimal for the growth of tomato explants.

The majority of researchers have used 30 g/L sucrose conc. in their initiation and multiplication media (Costa *et al.*, 2000; Venkatachalam *et al.*, 2000). Bhatia *et al.* (2005) reported that sucrose at low concentrations 0.5-1.5% along with full strength media was found optimum for plant growth.

Sucrose was found essential for the development of chlorophyll. Chlorophyll content increased with an increase in sucrose concentration up to 3% and decreased at 5% sucrose.

2.4.3 Effect of Plant growth regulators

In addition to the nutrients and carbohydrates, it is necessary to include one or more growth substances such as auxins, cytokinins, and Gibberellins to support good growth of tissues and organs (Bhojwani and Razdan, 2005). Both organ differentiation and growth in tissue cultured plants are mediated by interplay of auxins and cytokinins (Miller and Skoog, 1957). The proper ratio of auxins and cytokinins in the plant system plays a vital role in proliferation, growth and development, and optimum balance determines whether explants will produce callus or differentiate in to shoots or roots.

Most of the reports on tomato regeneration through organogenesis of the seedling explants *viz.* cotyledon, hypocotyls etc. have been achieved with the incorporation of BAP and IAA (Duzyaman *et al.*, 1994; Shamshad *et al.*, 1999; Chandel and Katiyar, 2000; El-Bakry, 2002). Combinations of BAP and IAA have been found to be most suitable irrespective of explant and genotype by several workers (Dwivedi *et al.*, 1990; Vallejo and Polston, 1994). Park *et al.*, (2003) observed that the response of BAP and IAA combined was similar to Zeatin alone or in combination with IAA. Other cytokinins such as Kinetin (Locy, 1983; Uddin *et al.*, 1988; Chandra *et al.*, 1995), Zeatin (Filova, 2004; Gubis *et al.*, 2005; Grigoriadis *et al.*, 2005), Thiadiazuron (Zakir *et al.*, 1995; Rao *et al.*, 2007) alone or in combination with auxins such as IAA/NAA have been found to be effective in inducing regeneration.

The ratio of cytokinins to auxins depends on levels of endogenous plant growth regulators present in the plant and thus it varies with the plant species used. Pozueta-Romero *et al.* (2001) were able to regenerate multiple shoots in an explant consisting of radicle, hypocotyls and cotyledons after removing primary and axillary meristem when cultured on a medium with or without growth regulators. The growth regulator requirement was met through the explant as both roots and cotyledons of young seedlings are known to produce growth regulators actively (Hicks, 1994). Similarly, Fari *et al.* (1992) were able to regenerate shoots from decapitated seedlings grown on plant growth regulator free medium.

2.5 OPTIMIZATION OF SEED GERMINATION *IN VITRO*

In vitro seedling production in tomato is sensitive in terms of medium strength, sterilization protocols and the following workers reported several protocols.

Newman *et al.* (1996) reported that tomato seeds when placed in tea bag holder to prevent floating and surface sterilized for 15 min with 2.5% sodium hypochlorite with 2 drops of surfactant then, rinsed with sterile distilled water and cultured on MS basal media, MS basal along with 50 μ M and 80 μ M BA with 3% sucrose and 0.3% gelrite produced the seedlings.

Gubis *et al.* (2003) reported that *in vitro* seedlings were produced when tomato seeds were surface sterilized with 4% sodium hypochlorite for 15 min and then rinsed for 4 times with sterile distilled water and implanted on the half strength MS medium supplemented with 100 mg/L myo-inositol, 2mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 1% w/v sucrose and 0.6% agar produced seedlings. However, Bhatia *et al.* (2004) successfully produced *in vitro* seedlings with 1% sodium hypochlorite when used for surface sterilization.

Sheeja and Mandal (2003) reported another protocol for seedling production in which seeds were treated with 2% Bavistin solution and 5% teepol for 15 min and then surface sterilized with 0.1% mercuric chloride, and then rinsed with sterile distilled water and then implanted on half strength MS medium with 3% sucrose and 0.8% agar.

Reda *et al.* (2004) revealed successful seedlings production when tomato seeds were surface sterilized by immersion in 70% ethanol followed by immersion in 3% (v/v) sodium hypochlorite and rinsed twice in sterile distilled water and then the seeds were germinated in flasks of 0.8% (w/v) agar and cultures were incubated at 25°C with 16/8 hr (light/dark) photoperiod.

Bamel *et al.* (2007) adopted different sterilants in the protocol for seedling production of tomato. Seeds were soaked in sterile distilled water for an hour, surface sterilized with 1% (v/v) polysan and 0.1% (w/v) HgCl₂ followed by rinsing with rectified spirit and sterilized with distilled water and then seeds were germinated on Knop's medium containing 3% sucrose and 0.8% agar.

Singh *et al.* (2007) reported that seeds were thoroughly washed with tap water and soaked for 5-6 hr, then the seeds were surface sterilized with 0.1% mercuric chloride for 2 min and thoroughly washed 3-4 times with sterilized distilled water and then implanted on the half strength MS basal medium containing 0.5% sucrose produced the seedlings.

Rao *et al.* (2007) reported that seedling production is uniform and rapid only when implanted seeds were kept in dark for 4 days at 25 ± 2°C after following inoculation protocols.

2.6 SHOOT REGENERATION

Kurtz and Lineberger (1983) reported shoot regeneration on MS medium supplemented with 0.2 - 1.0 mg/L BA, Whereas, Soniya *et al.* (2001) observed multiple shoots induction on MS medium supplemented with 17.7 μ M BA alone in two weeks.

Izadpanah and Khoshkhui,(1989) reported highest shoot proliferation with 1.5 mg/L BA and 3 mg/L Kinetin in Cv. Cal-J and 2 mg/L BA and 5 mg/L Kinetin produced the highest shoot proliferation in Petomech and Redcloud.

Duzyaman *et al.* (1994) reported shoot regeneration from different explant sources viz. leaves, cotyledons, hypocotyls when MS medium was supplemented with 0.2 mg/L IAA + 2.3 mg/L BAP and 1.0 mg/L IAA + 1.2 mg/L Kinetin and the regeneration reached 3, 2.5, 1.7 shoots / callus for leaves, cotyledons and hypocotyls respectively.

Plastira and Perdikaris (1997) reported multiple shoots from both cotyledon and hypocotyl explants when cultured on MS medium supplemented with 0.1 to 10 mg/L BA and Zeatin. They also reported a maximum of 7.95 shootlets / explant.

Costa *et al.* (2000) reported high shoot regeneration when excised cotyledonary explants of tomato from 8-10 day old seedlings were cultured on MS medium supplemented with 1 mg/L Zeatin and 0.1 mg/L IAA. However, Dwivedi *et al.* (1990) observed that when leaf explants were

allowed to incubate on morphogenetic medium for full duration of 30 days, shoot buds grew in to shoots in the medium supplemented with 0.25 mg/L BA and 0.01 mg/L NAA.

Gubis *et al.* (2004) reported 100% adventitious shoot induction when MS medium was supplemented with 1 mg/L Zeatin and 0.1 mg/L IAA in the variety Premium, irrespective of the explant source. Similarly, Singh *et al.* (2007) reported the highest frequency of shoot regeneration on MS medium supplemented with 2 mg/L BAP and 1 mg/L IAA.

Garcia-Reina and Luque (1988) reported the highest organogenetic potential on MS medium supplemented with BAP 5 mg/L + IAA 0.5 mg/L, whereas Shamshad *et al.* (1999) reported that when shoot and leaf explants were cultured on MS medium supplemented with BAP 5.0 mg/L + IAA 0.5 mg/L produced 3.13 and 2.35 mean no. of shoots / explant respectively.

Pozueta-Romero *et al.* (2001) reported that multiple shoots regenerated on cut surface with a frequency average of 2.9 - 5.3 shoots/explant, when cotyledon and hypocotyl explants were grown on MS medium with out any growth regulators.

Bhatia *et al.* (2005) reported direct shoot regeneration from whole cotyledonary explants of 1 week old on MS medium supplemented with 15 μ M Zeatin.

Grigoriadis *et al.* (2005) reported that proximal part of cotyledons has high shoot regeneration frequency compared to middle part when MS medium was supplemented with Zeatin 0.5 mg/L and IAA 0.1 mg/L.

Rao *et al.* (2007) reported that TDZ supplied alone or in combination had taken comparatively less time for shoot regeneration and formed more no. of adventitious shoots (6.7 - 4.3) compared to BAP, Zeatin, Kinetin alone or in combinations.

2.7 ROOT REGENERATION

Kurtz and Lineberger (1983) reported that plants regenerated *in vitro* rooted on a medium supplemented with 0.2 - 2 mg/L IAA. Similarly, Majoul *et al.* (2001) observed that shoot buds after elongation rooted on MS medium supplemented with 0.1 mg/L IAA. Polevaya *et al.* (1988) achieved rooting on MS medium with added Kinetin and sucrose and plantlets were obtained in 4 - 5 weeks.

Mandal (1999) reported that *in vitro* developed tomato plantlets successfully produced roots when transferred to rooting medium consisting of half strength with 1.0 mg/L NAA and 0.5 mg/L IBA. Izadpanah and Khoshkhui (1989) reported the highest percentage of rooting on MS medium supplemented with 0.5 mg/L IBA and while Soniya *et al.* (2001) reported rooting of regenerated shoots on MS medium supplemented with 1 mg/L IBA.

Muthuvel *et al.* (2005) advocated that mostly IBA and IAA were used for rooting in tomato. On contrary, Dwivedi *et al.* (1990), Rao *et al.* (2007) and

Singh *et al.* (2007) reported rooting of regenerated shoots in tomato on MS medium supplemented with 0.1 mg/L NAA. Similarly, Moghaieb *et al.* (1999) reported root induction when regenerated shoots were transferred to half strength MS medium. Nambisan *et al.* (1992) observed that shootlets were rooted when cultured on MS medium with 1% sucrose and 0.1 mg/L NAA.

2.8 ACCLIMATIZATION

Chi Won Lee and Thomas (1985) observed that all healthy *in vitro* rooted cultures of buffalogourd survived when transferred in to a mixture of 1 peat : 1 Vermiculite : 1 Perlite and placed under mist for 1 week.

Rao *et al.* (2007) reported 75- 80% survival depending on the genotype in tomato when plants were acclimatized with sand and soil mix (1:1). The pots were covered with plastic sheets initially to maintain humidity and the nutrient solution was added daily. According to Dwivedi *et al.* (1990) the *in vitro* raised plants grew normally when potted in the soil. Izadpanah and Khoshkhui, (1989) reported that *in vitro* rooted plants in tomato were acclimatized in a 1/3 sterilized loam soil, 1/3 sand, 1/3 peat moss medium for 2 weeks.

2.9 AGROBACTERIUM MEDIATED TRANSFORMATION IN TOMATO

The bacterium *Agrobacterium tumefaciens* was found to infect tomato cells readily and proved to be an effective vehicle for delivering foreign DNA in to plant cells (Zambryski *et al.*, 1983). Therefore, *Agrobacterium* was embraced by tomato biologists and was developed as first and the most

favorable tool for the introduction of foreign genes in to the tomato genome. Genetic transformation of tomato via *Agrobacterium* was first reported in 1985 by Horsch *et al.* from Monsanto. Subsequently, a number of papers reporting successful transformation of tomato were published by several research workers.

2.9.1 Factors affecting transformation in Tomato

Many different factors were found to be substantially influence the efficiency of transformation *viz.*, over the years the genotype, explant type, the use of acetosyringone, the bacterial strain and type of helper plasmids employed in the co-cultivation procedure.

The transformation frequency of tomato was found to be affected by the genotype as it was reported by Mc Cormick *et al.* (1986), Davis *et al.* (1994), Agharbaoui *et al.* (1995) and Costa *et al.* (2000).

Agrobacterium mediated transformation systems using various tomato explants were well studied in cotyledons (Fillatti *et al.*, 1987; Park *et al.*, 2003) and hypocotyls (Reda *et al.*, 2004; Pozueta-Romero *et al.*, 2001; Frary and Eck, 2005).

Agrobacterium mediated transformation is also influenced by antibiotics employed for effective elimination of bacteria as it is necessary as soon as their presence in transformant cells is no longer required. Mostly carbenecillin/cefotaxime are used for suppression of *Agrobacterium*. Costa *et al.* (2000) found that higher regeneration frequencies could be obtained with timentin and this

has been suggested as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation (Cheng *et al.* 1998). Improved transformation frequencies were obtained with the use of tricarbencillin antibiotic compared to cefotaxime (Ling *et al.*, 1998; Hu and Phillips, 2001). Ieamkhang and Chatchawankanphanich (2005) suggested augmentin, an antibiotic for suppression of bacterial growth with comparative efficiency as timentin for tomato transformation.

Dillen *et al.* (1997) examined the effect of temperature during co-cultivation on the efficiency of T-DNA transfer from a binary plasmid into the plant. They showed that transient expression of β -glucuronidase (GUS) was greatly affected by the temperature during co-cultivation. The optimum temperature for co-cultivation was 22°C and incubations at higher or lower temperatures reduced GUS expression considerably. Incubation at 19 or 25°C reduced GUS activity by two to six folds.

Frary and Earle (1996) developed an improved transformation protocol for tomato cultivar 'Moneymaker'. They found that explant size, explant orientation, gelling agent and plate sealant affected the transformation frequency.

Wu *et al.* (2006) reported that explant type, explant size, explant source, the conc. of cytokinins, inoculation time, pH of inoculation and co-cultivation media, bacterial concentration, acetosyringone conc. and co-cultivation duration affected the transformation frequency.

2.10 SELECTABLE MARKERS FOR PLANT TRANSFORMATION

Gene expression and the selection of the genetic transformants requires the use of genes that function as reporters of gene expression and permit the recovery of transformant cells, tissues or organs. Selectable marker gene is an essential component of the modified T-DNA.

Several selectable marker genes are widely available today for plant transformation. Several requirements must be considered in the development of a truly useful selectable marker system. It is most critical that the selective agent be inhibitory to plant cells. However, not all compounds toxic to plant cells are necessarily useful as selective agents. Cells that are not transformed can be killed in such a manner that they become toxic to adjacent transformed cells. This presumably happens because of leakage of toxic compounds, such as phenols, from the dying cells. If this occurs, even high-level expression of a resistance gene in the transformed cells is insufficient to rescue these cells. The best selective agents are compounds that arrest the growth of non transformed cells or kill them.

For several reasons a single antibiotic resistant gene, even one as versatile as *npt II*, has not fulfilled all of the needs of plant molecular biologists. Probably the single most important reason is that this marker does not work in all plant species. This can be the consequence either of the lack of toxicity of kanamycin or of the failure of the enzyme to confer selectability in transformed cells and this led to the development of alternative markers.

Another useful component of the modified T-DNA is a reporter gene whose expression can be easily monitored upon transfer to plant cells. Features of a good reporter gene include no endogenous expression in plant cells, no expression in *Agrobacterium*, and an easily assayable product. The most common and versatile reporter gene is the β -glucuronidase (GUS) gene (*uid A*) from *E. coli*. The enzyme cleaves a wide range of β -glucuronidase substrates and the activity or expression can be conveniently measured using fluorometric assay (Miyoshi *et al.*, 1995), spectrophotometer or histochemical assays (Jefferson, 1987).

In most transformation studies, the constructs contained a *uid A* reporter gene driven by CaMV 35S promoter allowing detection of transient and eventually stable expression of the *uid A* gene in transgenic plants, regardless of the stage of plant development or tissue localization (Jefferson *et al.*, 1986). Several fruit ripening specific promoters such as E-4, E-8 and 2A11 were identified in tomato. They have been cloned, characterized and studied in relation to the effect of ethylene on fruit ripening (Nicholas *et al.*, 1995; Xu *et al.*, 1996). Krasnyanski *et al.* (2001) compared the promoters CaMV 35S promoter and an E-8 fruit ripening specific promoter in the expression of the *uid A* reporter gene in transgenic tomato plants. As expected, both vegetative and fruit tissues of transgenic T₀ and T₁ plants carrying the *uid A* gene under the control of CaMV 35S promoter showed varying levels of GUS activity, while no expression was observed in vegetative tissues of transgenic plants

carrying the *uid A* gene driven by E-8 promoter. However, the reporter gene expression was significantly higher when it was driven by CaMV 35S promoter. The *uid A* gene segregated in 3:1 ratio.

Janssen and Gardner (1990) developed a version of the GUS gene which lacks a bacterial ribosome binding site and shows negligible expression in *Agrobacterium*. The gene is especially useful when developing and optimizing *Agrobacterium* transformation protocols for specific plant genotypes or species.

2.11 CONFIRMATION OF GENE INTEGRATION

The integration of foreign genes into the plant nuclear genome can be determined via Southern nuclear DNA analysis and the use of polymerase chain reaction (PCR). Southern analysis allows determination of the number of copies and nature of the integration of specific genes or DNA regions. 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 DNA regions on the T-DNA that are expected to be integrated into the genome of plants. Advantages are that large collections of transgenic plants can be analysed rapidly and that only very small amounts of plant tissues are required.

In all the reports cited above, the most transformation protocols utilized to date, the bacterial *npt II* gene, which encodes for neomycin phosphotransferase, and a reporter gene GUS which encode β -glucoronidase

which consequently confer kanamycin resistance, has been used as the selective marker to isolate transgenic plants.

Histochemical staining for GUS gene transient expression has been reported by Gassama-Dia *et al.* (2004), Tripathi *et al.* (2005), Patnaik *et al.* (2006) and Cevik *et al.* (2006).

2.11.1 Transformation efficiency in Tomato

Hamza and Chupeau (1993) analysed the early events in the transformation of tomato cotyledons using *Agrobacterium tumefaciens* carrying binary vector with an *npt II* gene and GUS gene, and reported that sub-epidermal cells are more prone to transformation than epidermal cells.

Pozueta-Romero *et al.* (2001) reported transformation in cotyledon and hypocotyl explants of tomato with *A. tumefaciens* containing a 35S - GUS binary vector, which produced transgenic plants at the rate of 47%.

Park *et al.* (2003) developed a genotype independent *Agrobacterium* mediated transformation method for tomato and concluded that a pre-culture of the cotyledon and hypocotyls for one day on a medium with BA and NAA and 3 days co-cultivation with *A. tumefaciens* on the same medium followed by transfer to a medium with Zeatin and IAA resulted in a higher transformation frequency in all the cultivars tested.

Transformation efficiencies have ranged from 6% (Vidya *et al.*, 2000); 7-37% (Ling *et al.*, 1998); 9% (Van Roekel *et al.*, 1993); 11% (Frery and

Earle, 1996); 14% (Hamza and Chupeau, 1993); 20% (Park *et al.*, 2003) and 25% (Hu Wel and Phillips, 2001) in various studies.

Cortina, (2004) reported high transformation frequency when cotyledons infected by *Agrobacterium* strain harboring *npt II* gene were grown on increased concentrations of vitamins and phenolics.

Wu *et al.* (2006) reported that *Agrobacterium* strain LBA4404 carrying a binary vector PTOK 233 containing the GUS reporter gene and a kanamycin resistance gene *npt II* could be employed for optimizing the transformation efficiency as evaluated by a GUS gene transient expression level in tomato.

2.12 TRANSFORMATION OF GENES OF AGRONOMIC IMPORTANCE

Tomato is the major vegetable crop grown and consumed all over the world. Significant advances in yield and quality have been made through traditional breeding. Yet, improvement of some specific traits such as virus resistance (Toyoda, 1993), herbicide tolerance (Fillatti *et al.*, 1987), insect resistance (Fischhoff *et al.*, 1987), enhanced shelf life (Redenbarugh *et al.*, 1995), etc. have been addressed through genetic engineering more effectively. Recently, transgenic tomato plants with increased salt tolerance (Zhang and Blumwald, 2001) and nutritional quality in terms of high lycopene content (Mehta *et al.*, 2002), and virus resistance (Raj *et al.*, 2005) have been produced which further indicates the potential applications and future prospects of transgenic plants development in tomato genetic improvement.

CHAPTER-3

MATERIALS AND METHODS

The present study on transformation of tomato (*Solanum lycopersicum* L.) Cv. PKM-1 with GUS reporter gene was carried out at Transgenic Laboratory of Dept. of Plant Physiology, RARS, Tirupati and Dept of Genetics and Plant breeding S.V. Agricultural College, Tirupati.

Experimental materials used and research methodology adapted in the experiments are furnished in this chapter.

3.1 STANDARDIZATION OF *IN VITRO* REGENERATION PROTOCOL IN TOMATO Cv. PKM-1

3.1.1 Procurement of seed material

The seeds of PKM-1 procured from Dept. of Horticulture, TNAU, Coimbatore were used for further investigation.

3.1.2 Glassware

Glassware like petriplates, culture tubes, conical flasks, beakers, glass bottles, etc were of Borosil make.

3.1.2.1 Sterilization of glassware

Cleaned glassware were rinsed with double distilled water and dried in hot air oven at 80°C and then the surgical instruments and all other glassware were wrapped in aluminum foil and autoclaved at 121°C and at 15 lbs pressure for 20 min.

3.1.3 Chemicals

All the chemicals and growth regulators of analytical grade were procured from Himedia Laboratories, SD Fine, Qualigens, Fermentas etc.

3.1.4 Preparation of culture media

The culture medium used in the experiment was MS medium (Murashige and Skoog, 1962).

3.1.4.1 Preparation of stock solutions

The stock solutions were prepared by dissolving the chemicals of analytical grade in double distilled water and stored in reagent bottles at 4°C (Table 1).

There are four different stock solutions

1. MS-1 : Macro nutrients
2. MS-2 : Micro nutrients
3. MS-3 : Iron stock
4. MS-4 : Vitamin stock

Table 1: Preparation of stock solutions of Murashige and Skoog (MS) medium.

| Constituent | Conc. in MS medium 1962, (mg/L) | Conc. in the stock solution (mg/L) | Volume to be taken / litre of medium |
|--|---------------------------------|------------------------------------|--------------------------------------|
| Macro nutrients (20X) | | | |
| NH ₄ NO ₃ | 1650 | 33000 | 20 |
| KNO ₃ | 1900 | 38000 | |
| MgSO ₄ .7H ₂ O | 370 | 7400 | |
| KH ₂ PO ₄ | 170 | 3400 | |
| CaCl ₂ .2H ₂ O | 440 | 8800 | |
| Micronutrients (50X) | | | |
| MnSO ₄ .4H ₂ O | 22.3 | 1115 | 50 |
| ZnSO ₄ .7H ₂ O | 8.60 | 430 | |
| H ₃ BO ₃ | 6.20 | 310 | |
| KI | 0.83 | 41 | |
| CuSO ₄ .5H ₂ O | 0.025 | 1 | |
| NaMoO ₄ .2H ₂ O | 0.25 | 12 | |
| CoCl ₂ .6H ₂ O | 0.025 | 0.125 | |
| Iron source (50X) | | | |
| FeSO ₄ .7H ₂ O | 27.8 | 1390 | 50 |
| Na ₂ EDTA.2H ₂ O | 37.3 | 1850 | |
| Vitamin source (50X) | | | |
| Glycine | 2 | 100 | 50 |
| Thiamine HCl | 0.1 | 5 | |
| Pyridoxine HCl | 0.5 | 2.5 | |
| Nicotinic acid | 0.5 | 2.5 | |
| Myo-inositol | 100 | Added fresh | |
| Sucrose | 30000 | Added fresh | |
| Agar | 8000 | Added fresh | |

For iron stock preparation both the chemicals were dissolved separately, mixed together and boiled for few minutes until it turned into a clear solution. Then the volume was made up to one litre with distilled water and stored in amber coloured bottle. For vitamin stock preparation all the constituents were initially dissolved in few drops of dilute HCl (0.1N) and volume was made up with double distilled water up to one litre.

Carbon source:

Carbon is usually supplied as sucrose, fructose, maltose etc. Among these, sucrose is used as the carbon source in the present study.

3.1.4.3 Preparation of growth regulators stocks

Growth regulators used for standardizing the regeneration protocol were BAP, Zeatin, Kinetin, IAA and IBA.

The stock solutions of the growth regulators were prepared at a concentration of 10mg/100ml. They are prepared by dissolving in specific solvent (Table 1a) and making up the volume with double distilled water to a known quantity. The prepared stock solutions were stored at 4°C.

$$\text{Amount of hormonal Solution required} = \frac{\text{Concentration required X Volume to be made}}{\text{Concentration of stock}}$$

Table-1a: Solvents used for dissolving different plant growth regulators

| S.NO. | Plant growth regulator category | Name of the plant growth regulator | Solvent |
|-------|---------------------------------|------------------------------------|----------------------|
| 1 | Auxins | IAA | Ethyl alcohol |
| | | IBA | 1 N Sodium hydroxide |
| 2 | Cytokinins | BAP | 1 N Sodium hydroxide |
| | | Kinetin | 1 N Sodium hydroxide |
| | | Zeatin | 1 N Sodium hydroxide |

All the ingredients including growth regulators were dissolved in 800 ml of distilled water and the pH was adjusted to 5.8. Then the volume was made up to 1000 ml and the medium was autoclaved at 121°C temperature and 15 lbs pressure for 15 min.

3.1.5 Preparation of transfer area for aseptic culture

Maintenance of aseptic or sterile conditions is essential for successful tissue culture work. All the steps in the experiment were conducted under aseptic conditions in a laminar air flow cabinet. Before the use of the laminar air flow cabinet, the working surface of the cabinet was swabbed with 70% ethyl alcohol, prepared autoclaved culture media and vessels were arranged, then UV light was switched on for at least 30 minutes and then it was left for another 10 minutes without UV light followed by flow on. The surface of the chamber and the hands were sterilized with 70% alcohol before starting the experiment.

3.2 *IN VITRO* SEED GERMINATION

The seeds were immersed in sterile double distilled water for 15 minutes and treated with Bavistin 1% solution for 20 minutes followed by thorough rinsing with sterilized water. One drop of Tween-20 was added to the seeds and shaken thoroughly for 5 min and thoroughly rinsed with sterile distilled water for 4-5 times. The seeds were taken in to laminar air flow cabinet, and treated with different concentrations of various surface sterilants for different intervals of time (Table 2) with occasional swirling. They were washed with 4-5 changes of sterile distilled water and were treated with 70% ethyl alcohol for 30 sec followed by washing for 4-5 times with double distilled water.

Treated seeds were inoculated on

1. MS medium + light.
2. $\frac{1}{2}$ MS medium + light.
3. MS medium + Dark incubation.
4. $\frac{1}{2}$ MS medium + Dark incubation.
5. MS medium with out Sucrose + Light
6. $\frac{1}{2}$ MS medium without sucrose + Light.
7. MS medium without sucrose + Dark incubation.
8. $\frac{1}{2}$ MS medium without sucrose + Dark incubation.

The following observations were recorded after inoculation of seeds on the medium on visual basis:

1. No. of seeds germinated
2. Germination frequency (%)
3. Age of *in vitro* seedlings for high explant regeneration.

3.3 REGENERATION OF TOMATO PLANTS FROM COTYLEDON AND HYPOCOTYL EXPLANTS

Cotyledon and hypocotyl explants obtained from 8-10 days old seedlings were cultured on MS basal medium supplemented with BAP, Kinetin, Zeatin, IAA and IBA.

The following media combinations were tried on the explants obtained from 8, 10, 12 and 14 days of *in vitro* seedlings for identifying better explant regeneration:

Table 2a: List of various combinations used for better explant response of *Solanum lycopersicum* L. Cv. PKM-1

| S.No. | Concentrations (mg/L) |
|-------|-----------------------|
| 1 | MS + BAP 1.0 |
| 2 | MS + BAP 1.5 |
| 3 | MS + BAP 2.0 |
| 4 | MS + BAP 2.5 |
| 5 | MS + Kinetin 0.5 |
| 6 | MS + Kinetin 1.0 |
| 7 | MS + Kinetin 1.5 |
| 8 | MS + Kinetin 2.0 |

| | |
|----|----------------------------|
| 9 | MS + Zeatin 0.5 |
| 10 | MS + Zeatin 1.0 |
| 11 | MS + Zeatin 1.5 |
| 12 | MS + Zeatin 2.0 |
| 13 | MS + BAP 1.0 + Kinetin 0.5 |
| 14 | MS + BAP 1.5 + Kinetin 1.0 |
| 15 | MS + BAP 2.0 + Kinetin 1.5 |
| 16 | MS + BAP 2.5 + Kinetin 2.0 |
| 17 | MS + BAP 0.25 + IBA 0.1 |
| 18 | MS + BAP 0.5 + IBA 0.1 |
| 19 | MS + BAP 1.0 + IBA 0.1 |
| 20 | MS + BAP 2.0 + IBA 0.1 |
| 21 | MS + BAP 0.5 + IAA 0.1 |
| 22 | MS + BAP 1.0 + IAA 0.1 |
| 23 | MS + BAP 1.5 + IAA 0.5 |
| 24 | MS + BAP 2.0 + IAA 0.5 |
| 25 | MS + Zeatin 0.5 + IAA 0.1 |
| 26 | MS + Zeatin 1.0 + IAA 0.1 |
| 27 | MS + Zeatin 1.5 + IAA 0.5 |
| 28 | MS + Zeatin 2.0 + IAA 0.5 |

The same combinations mentioned in the Table 2a were tried for shoot initiation, multiplication and elongation for cotyledonary explants.

The inoculated cultures were incubated in culture rack provided with white fluorescent tubes with a light intensity of 30-40 μ moles under a 16 hour light and 8 hr dark photoperiod regime in a culture room whose temperature was maintained at $25 \pm 2^\circ\text{C}$.

The following observations were recorded after the shoot buds / shoot lets initiated:

- a) Time taken for shoot bud initiation: The time (no. of days) taken for shoot bud initiation in the culture tubes was recorded visually.
- b) No. of explants producing shoot buds: It is the no. of explants producing shoot buds from the total number of explants.
- c) Shooting frequency (%) = $\frac{\text{Number of explants producing shoots}}{\text{Total number of explants}} \times 100$
- d) Mean length of shoots: The mean shoot length was arrived by taking the average length of the shoots in each treatment.
- e) No. of days taken for shooting: These observations are taken visually.

The multiple shoot buds that initiated from the small areas on the cut surfaces of cotyledons were excised and sub cultured on to fresh medium periodically until they grow to a length of 3-4 cm. Then, the elongated shoots were transferred to the rooting medium.

3.4 ROOT REGENERATION

The shootlets obtained were transferred to combinations mentioned in Table 2b for root regeneration:

Table 2b: List of various combinations used for root regeneration of *Solanum lycopersicum* L. Cv. PKM-1

| S.No. | Concentrations (mg/L) |
|-------|---------------------------|
| 1 | MS + IBA 0.1 |
| 2 | MS + IBA 0.2 |
| 3 | MS + IBA 0.3 |
| 4 | MS + IBA 0.4 |
| 5 | MS + Kinetin 0.5 |
| 6 | MS + Kinetin 1.0 |
| 7 | MS + Kinetin 1.5 |
| 8 | MS + Kinetin 2.0 |
| 9 | MS + BAP 0.25 + IBA 0.1 |
| 10 | MS + BAP 0.5+ IBA 0.1 |
| 11 | MS + BAP 1.0 + IBA 0.5 |
| 12 | MS + BAP 1.5 + IBA 0.5 |
| 13 | MS + Kinetin 0.5+ IBA 0.1 |
| 14 | MS + Kinetin 1.0+ IBA 0.1 |
| 15 | MS + Kinetin 1.5+ IBA 0.5 |
| 16 | MS + Kinetin 2.0+ IBA 0. |

The following observations were recorded:

- a. No. of shoots producing roots: It is the no. of shoots producing roots from the total number shoots taken for each treatment.
- b. Rooting frequency (%) = $\frac{\text{Number of plants producing roots}}{\text{Total number of shoots taken}} \times 100$
- c. Mean no. of roots / shoot: Mean no. of roots produced per shoot in a treatment was arrived by counting the no. of roots in each treatment and dividing with the total no. of rooted shoots.
- d. Mean length of roots: The length of roots produced from each shoot was recorded and divided with total no. of roots in all the treatments.
- e. No. of days taken for rooting: These observations are taken on visual basis.

3.5 EX-VITRO ESTABLISHMENT

The *in vitro* rooted plantlets were removed from the culture vessels and the agar on the roots was gently washed off under tap water without damaging the roots.

3.6 ACCLIMATIZATION

These plantlets were then transplanted to polythene bags containing autoclaved soil, sand, soilrite, and soil, sand and soilrite in the ratio of 1:2:1 and watered to field capacity. The bags were stapled with pin at the top to maintain high humidity and kept under white fluorescent lights at room temperature. After two weeks, the bags were opened and kept under the same

conditions for another week. Then the plants were transferred to pots in the glass house.

3.7 STATISTICAL ANALYSIS

In the experiment on better explant response and shoot regeneration there were 28 treatments and for root regeneration there were 16 treatments. For each treatment, 10 bottles/plates/test-tubes constituting 4 replications were made in a completely randomized design. Each bottle/Petri plate contained 10 explants and each test tube with one explant. The data were analysed for variance.

3.8 TRANSFORMATION

3.8.1 Kanamycin sensitivity test

Kanamycin is the most popular selectable marker gene used in plant transformation vectors. For successful selection, the target plant cells must be susceptible to antibiotics. In this experiment, kanamycin sensitivity test was carried out to find out the concentration of kanamycin required for selection of nontransformed plants. This was done by culturing the cotyledonary explants (without co-cultivation) on MS regeneration medium containing kanamycin at 0, 25, 50, 75, 100 mg/L concentration and the lethal dosage at which there was no regeneration was identified.

3.8.2 Maintenance and growth of *Agrobacterium* cultures

The *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pCAMBIA-2301 harboring GUS as a reporter gene under the control of CaMV 35S promoter was used for transformation (Fig 1).

3.8.2.1 Maintenance of bacterial culture

The *Agrobacterium* culture was maintained on the semi solid LB medium containing 50 mg/L kanamycin. Sub-culturing was done every one month in fresh media containing kanamycin.

Preparation of LB medium

| Constituents | (Quantity g/L) |
|---------------------|----------------|
| 1. Hive hydrosylate | 10.0 |
| 2. Yeast extract | 5.0 |
| 3. Sodium chloride | 5.0 |
| 4. Agar | 15.0 |

For broth, agar was excluded. After autoclaving, the medium was cooled and 50 mg/L of kanamycin was added under sterile conditions in the laminar hood.

3.8.2.2 Growth of *Agrobacterium*

Single bacterial colony was transferred from the LB plate containing *Agrobacterium* culture into a 25 ml LB liquid medium containing 50 mg/L kanamycin and was kept in a shaking water bath overnight at 28°C. The culture that had absorbance of 1.0 at 600 nm was chosen for transformation. The overnight grown culture was centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in half strength MS medium and diluted four times with half strength MS basal medium.

3.9 TRANSFORMATION OF TOMATO WITH GUS GENE

The most responsive media for regeneration of shoots was used for transformation.

1. The cotyledons from *in vitro* germinated (8-10 days old) seedlings were cut at the both ends.
2. The cotyledons were then inoculated with *Agrobacterium* culture for various periods of time (5 - 20 min).
3. Later cotyledons were taken out of the bacterial suspension and the excess bacterial culture was blotted using sterile blotting paper.
4. The cotyledons were then placed on regeneration medium for co- cultivation for different periods of time (1 - 3 days).
5. The cotyledons were then transferred to regeneration medium containing cefotaxime (500 mg/L) for different periods of time (1 - 5 days).
6. The optimum time of inoculation, co-cultivation and presence in cefotaxime medium was identified by looking for healthy explants without bacterial overgrowth.

3.10 CONFIRMATION OF THE PRESENCE OF TRANSGENE

To confirm the presence of transgene,

- a. Histochemical staining for the GUS gene was done.
- b. PCR amplification of GUS gene through polymerase chain reaction (PCR) in both control and transformed plants was carried out.

3.10.1 Histochemical staining for presence of GUS gene

The expression of the GUS gene was tested by histochemical staining of tissue. The best substrate for histochemical localization of β -glucuronidase activity in tissues and cells is 5-bromo-4-chloro-3 indolylglucuronide (X-gluc). The substrate gives a blue precipitate at the site of enzymatic activity.

The product of glucuronidase action of X-gluc is not coloured. The indoxyl derivative produced must undergo an oxidative dimerization to form the insoluble and highly coloured indigo dye. This dimerization is stimulated by atmospheric oxygen, and can be enhanced by using potassium ferricyanide, an oxidative catalyst (Jefferson, 1987).

Staining solution

1. 50mM sodium phosphate buffer (pH 7.0)
 2. 1mM X-gluc
 3. 0.1% Triton X-100
 4. 4 mM Potassium ferricyanide
 5. 100 μ g/ml Chloramphenicol
- Leaf bits (0.5 cm²) obtained from the putative transformed shootlets were immersed in GUS staining solution and incubated overnight at 37°C.
 - Next day the staining solution was decanted and 1 ml of absolute alcohol was added and kept at room temperature to remove the chlorophyll.

- The GUS expressing cells were detected microscopically by the distinct blue colour which is a result of enzymatic cleavage of X-gluc.

Observations recorded:

The transformation frequency of explants was determined in terms of transient GUS expression and was calculated and expressed in percentage using the formulae.

$$\text{Transient GUS expression (\%)} = \frac{\text{Number of explants showing blue colour}}{\text{Total number of explants incubated}} \times 100$$

3.10.2 PCR amplification of GUS gene through polymerase chain reaction (PCR)

For PCR amplification, DNA from the plasmid, control and transformed plants was isolated as follows.

3. 10.2.1 Isolation of plasmid DNA from Agrobacterium (QIAGEN KIT)

1. A single *Agrobacterium* colony was picked up aseptically using a sterile inoculation needle and was grown overnight in 1ml LB medium containing kanamycin (50 mg/L) in a sterile microfuge tube.
2. Overnight grown 1 ml culture was added to 25 ml LB medium containing kanamycin and again grown overnight.
3. One ml of culture was taken and centrifuged at 5000 rpm for 2 minutes.

4. The cell pellet was collected by removing supernatant and the pellet was resuspended in 250 μ l buffer P₁ (Resuspension Buffer) and transferred to a microcentrifuge tube.
5. To this 250 μ l buffer, P₂ (Lysis Buffer) was added and mixed thoroughly by inverting the tube for 4-6 times.
6. Then 350 μ l of buffer, N₃ (Neutralization Buffer) was added and mixed immediately and thoroughly by inverting the tube for 4 times.
7. The suspension was centrifuged for 10 min at 13,000 rpm in a table top microcentrifuge.
8. The supernatant was collected and applied to the QIA prep spin column by decanting or pipetting.
9. Centrifugation was done at 10000 rpm for 30 sec and the flow was discarded.
10. Then QIA prep spin column was washed by adding 0.75 ml buffer PE (wash buffer) and centrifuged for 60 s and flow was discarded.
11. Again discarded the flow through, and centrifuged for 1 min at 10000 rpm to remove the additional wash buffer.
12. To elute the DNA, QIA prep column was placed in a clean 1.5 ml micro centrifuge tube. And then, to this 50 μ l of buffer, EB (Elution Buffer) was added to the center of each QIA prep spin column, and allowed to stand for 1 min and then centrifuged for 1min.

3.10.2.2 Isolation of genomic DNA by CTAB method (N-Cetyl-N, N, N-Trimethyl Ammonium Bromide)

This is an efficient method for isolating plant genomic DNA from leaf tissues. It provides high quality preparation of high molecular DNA. CTAB is used to liberate the nucleic acid from the cell, which is then further purified by phenol-chloroform to remove proteins and other contaminating plant debris.

Reagents required

- 1) Extraction buffer: For 50ml

| | |
|-----------------------|------|
| 1M Tris | 5ml |
| 5M Nacl | 14ml |
| 0.5M EDTA | 2ml |
| 2 % CTAB | 1g |
| 1 % PVP | 0.5g |
| 0.1 % Mercaptoethanol | 50µl |

- 2) Chloroform:Isoamylalcohol (24:1)

- 3) RNase - 10mg/ml (1 µl /100 µl)

- 4) Ice cold Isopropanol

- 5) 5M Sodium acetate

- 6) 70% Ethanol

7. 10X TBE :

| | | |
|------------|---|-------|
| Tris base | : | 54g |
| Boric acid | : | 27.5g |
| EDTA | : | 4.65g |

Procedure

1. 0.5 g of leaf tissue was weighed from each putatively transformed plant and a control.
2. The leaves were cut into pieces and ground well in liquid nitrogen using pestle and mortar to powder form and transferred to sterile eppendorf tube.
3. One ml of extraction buffer was added and then tubes were incubated at 60° C water bath for 1 hour with intermittent shaking for every 10 minutes.
4. Tubes were removed from water bath and cooled, centrifugation was done at 10,000 rpm for 10 minutes at room temperature and the supernatant was collected into new eppendorf tube.
5. Equal volumes of Chloroform and Isoamyl alcohol (24:1) and RNase (1µl/100µl) was added and incubated at room temperature for 10 minutes.
6. Centrifugation was done at 10,000 rpm for 10 minutes, supernatant was separated and to this, 0.6 volume of Ice cold isopropyl alcohol + 0.1 volume of sodium acetate was added and incubated at -20°C for overnight.
7. After incubation, the tubes were taken out and centrifuged at 13,000 rpm for 20 minutes at 4°C.
8. Supernatant was discarded and the pellet was washed with 70% alcohol.
9. The pellet was air dried and then dissolved in 100 µl of distilled water.

3.10.2.3 Quantification of DNA

It is crucial to know the exact quantity of DNA present in the solution before carrying out experiments on gene manipulations or cloning. DNA concentrations can be accurately measured by U.V absorbance spectrophotometry (Nanodrop). The amount of UV-radiation absorbed at 260 nm by the solution of DNA is directly proportional to the amount of DNA present in the sample. Usually the absorbance is measured at wavelength of 260 nm and 280 nm which allows the calculation of DNA concentration in the sample. An absorbance of 1 at 260 nm corresponds to 50 µg/ml of double stranded DNA. UV-absorbance can also be used to check the purity of the DNA preparation. For a pure sample of DNA, the ratio of the absorbance at 260 nm to 280 nm, A_{260}/A_{280} is 1.8 - 2.0. Ratios less than 1.8 indicate that the preparation is contaminated either with phenol or proteins.

3.10.2.4 Confirmation of the presence of GUS gene in the putative transformants by PCR

This was done using PCR and primers specific to GUS. Polymerase chain reaction is a very simple method for *in vitro* amplification of specific nucleic acids using Hot start *Taq* DNA polymerase and short stretch of oligonucleotides (primers) which are specific to the DNA to be amplified.

DNA amplification involves repeated rounds of DNA synthesis, which is based on the following three simple steps:

1. Denaturation of the template DNA into single strands.
2. Annealing of primers to each original strand for new strand synthesis.
3. Extension of the new DNA strands from the primers with the use of Hot start *Taq* polymerase.

Materials

1. DNA amplification reagent kit (FERMENTAS)
 - a. 10X assay buffer for Hot start *Taq* DNA polymerase with 25 mM MgCl_2
 - b. Hot start *Taq* DNA polymerase (5 U / μl)
 - c. Deoxynucleotide triphosphates dNTPS -25 mM
2. GUS primers (from MWG Biotech, Bangalore) for specific amplification of GUS gene.

Forward primer : - (5' ACG TCC TGT AGA AAC CCC AA 3')

Reverse primer : - (3' CCC GCT TCG AAA CCA ATG CC 5')

3. DNA samples to be amplified: plasmid DNA (positive control) and genomic DNA samples (transformed and control plants).
4. Sterile PCR tubes.
5. Micropipettes and sterile tips.
6. Eppendorf Master Cycler.

Procedure

1. 2.5 μl of assay buffer for Hot start *Taq* polymerase containing 25 mM MgCl_2 was taken in 0.2 ml PCR tubes.
2. 0.2 μl of 25 mM dNTPs mix was added to the tubes.

3. 2 μl (0.6 pmol / μl) of each forward and reverse primers and 0.06 μl of Hot start *Taq* polymerase (5 U / μl) were added
4. 1 μl of sample DNA (template) was added (50 ng / μl)
5. Finally 14.7 μl of sterile distilled water was added to make up the volume to 25 μl .
6. The contents were mixed and spun for 2-3 seconds.
7. The tubes were placed firmly in the wells of the Eppendorf thermocycler.

Conditions for amplification were as follows

- Step I : Initial denaturing at 95°C for 4 minutes
- Step II : Denaturing at 94°C for 1 minute
- Step III : Primer annealing at 60°C for 1min
- Step IV : Primer extension at 72°C for 2 min
- Step V : Go to, Step II 35 cycles
- Step VI : Final extension at 72°C for 5 min
- Step VII : 4°C pause

3.10.2.5 Electrophoresis and visualization of amplified products

Electrophoresis is the widely used technique for characterization of purified compounds and to test the homogeneity of nucleic acids and proteins. Separation is based on the electrical charge carried by the compound. When these charged molecules are subjected to an electrical field, they move towards opposite charge.

DNA molecules carry a net negative charge and therefore, when placed in an electrical field, they migrate towards the positive electrode (anode).

Electrophoresis is usually carried out in solid matrix like agarose or polyacrylamide gels. In a gel, the shape and size of the DNA fragment to be separated and the concentration of the agarose used influence the migration rate. Smaller the DNA fragment, higher the concentration of the agarose to be used in the gel for good separation.

The size of the fragment is determined by comparing mobility of DNA fragments of known size and mobility, such as 1 kilo base pair DNA ladder (marker).

Materials

1. TBE buffer (10X stock): Tris base (54 g), boric acid (27.5 g), EDTA 4.65 g, (pH 8.0) 500 ml, diluted to 1X for running the gel.
2. Loading buffer: 0.25% Bromophenol blue + 30% glycerol + 0.25% xylene cyanol.
3. 1.0% Agarose gel.
4. Gel frames and comb.
5. Ethidium bromide (10 mg/ml final concentration).
6. UV-Transilluminator (302-365 nm).

Procedure

1. The frame of the gel casting unit was cleaned and sealed with a tape to form a mould. The frame was placed on a flat platform to ensure a flat and level base. The comb was then positioned parallel to the open edge of the frame about 2 mm above the surface.

2. Agarose powder was dissolved in TBE buffer by melting at 100°C. The solution was cooled to 50°C and 4µl of ethidium bromide (10 mg/ml stock) was added to 100 ml of the solution, poured into the gel frame and allowed to set.

3. After setting the gel, the comb was removed and the gel was transferred to the gel tank such that the wells were towards the negative electrode. The gel tank was filled with 1X TBE buffer just enough to cover the surface of the gel.

4. Amplified DNA samples of 25 µl were mixed with 4µl of loading buffer (6X) and loaded in the wells of the submerged gel using a micro-pipette. 10 µl of 1 kb DNA ladder (Fermentas, USA) was also mixed with loading buffer and loaded on to one of the wells.

5. The electrophoresis apparatus was connected to the power supply and electrophoresis was carried out at 100 V for 2 hours.

6. The gel was then visualized on UV - transilluminator and the picture was taken from (Alpha innotech) Gel documentation system.

Observations recorded:

1. Transformation efficiency: It was calculated as the number of putative transformants shown positive for reporter gene over the number of putative transformants obtained.

2. Transformation frequency: It was calculated as the number of positive transformants over the number of co-cultivated explants.

CHAPTER 4

RESULTS

Gene transfer technology in plants holds excellent promise in crop improvement. It will enhance the efforts of traditional breeders in the generation of new crops. These new molecular techniques provide opportunities to alter specific characteristics such as pest and disease resistance. Although there are several methods of gene transfer, *Agrobacterium* mediated gene transfer is the preferred transformation technique. It has several distinct advantages over the other methods such as introduction of gene in to whole plant tissue with precise and stable integration. Major advantages in this technology are however, dependent on the efficient regeneration and transformation system.

Keeping this in view, the present study was carried out with the objective of developing efficient regeneration protocol and to study transformation efficiency in a widely adapted cultivar PKM-1 genetic background. The results obtained in the present investigation are presented below:

4.1 OPTIMIZATION OF SEED GERMINATION *IN VITRO*

4.1.1 Efficacy of different surface sterilants on contamination during *in vitro* seedling production

Surface sterilization of seeds is an essential pre-requisite for seedling production in any tissue culture experiment to minimize the contamination. The three sterilants evaluated for their efficacy are mercuric chloride (HgCl₂),

Hydrogen peroxide (H_2O_2) and Sodium hypochlorite (NaOCl) with different concentrations and for different durations (Table 3).

All the concentrations of HgCl_2 used for the surface sterilization of seeds *viz.*, 0.1, 0.2, 0.3, 0.5% for 5 minutes inhibited germination completely, except at 0.1% treatment which resulted in poor germination and infection.

Among different concentrations of H_2O_2 *viz.*, 10, 15, 20 per cent concentrations treated for 15 min, 10 and 15 per cent concentrations resulted in 80 and 70 per cent germination respectively but the contamination of cultures was observed. Whereas seed treated with 20 per cent H_2O_2 resulted in contamination free cultures but the germination per cent was reduced (70%) with non uniform seedling growth.

When NaOCl was used as surface sterilant for 15 min treatment at 3, 4, 5, 6 per cent concentrations, all the cultures were contaminated and when it was used at same concentrations but for 20 min duration resulted in higher germination rate with uniform healthy seedlings. Among the durations treated, soaking for 20 minutes with occasional swirling produced higher germination per cent (95 %) followed by treatment for 15 minutes.

Among the above mentioned three sterilants used, surface sterilization of seeds with 5 % NaOCl for 20 minutes was found to be more effective resulting in high germination rate and contamination free cultures. Hence, the 5 per cent NaOCl was used for surface sterilization of seeds through out the experiment.

1.2 Effect of media and culture conditions on *in vitro* seedling production

MS basal medium with half strength and full strength without dark incubation (exposure to light) and with dark incubation (exposure to darkness for 3 days before exposed to light) were experimented to standardize better media for optimum seedling production in terms of time taken for initiation of germination and per cent of germination (Table 4). The data revealed significant differences between the treatments.

Among the different media used, MS medium with out sucrose with dark incubation for three days recorded lower no. of days (3.3 days) for initiation of germination with 96 % germination (Plate 2 and Plate 3) followed by ½ MS without sucrose with dark incubation recorded 4.3 days for initiation with 83% germination and MS with dark incubation for three days recorded 4.6 days for initiation with 83% germination. All the treatments without sucrose with dark incubation for three days recorded lower no. of days taken for initiation of germination and highest percentage of germination with uniform and healthy seedlings.

4.2 EFFECT OF AGE OF *IN VITRO* SEEDLINGS ON REGENERATIVE RESPONSE OF EXPLANTS

Different concentrations of BAP, Kinetin, Zeatin alone and BAP, Kinetin, Zeatin, IAA, IBA in combinations were evaluated for better explant response in terms of age of the explant for 8, 10, 12, 14 days both in

cotyledons and hypocotyls mentioned in Table 5. Explant response was recorded visually based on bulging or becoming flaccid on transfer to the

ium (Table 5). The results indicated that the significant differences between treatments were observed only with cotyledonary explants of age 8, 10, 12 days and hypocotyls of 10 days old.

Among different combinations used, cotyledons of 10 days old seedlings showed the highest explant response followed by 8 days, 12 days and least response has been observed at 14 days. In terms of hormonal treatments, MS medium + BAP 1.5 mg/L + Kinetin 1.0 mg/L reported high explant response (8.6) when 10 days old cotyledons were used as explant followed by MS medium + BAP 1.0 mg/L + Kinetin 0.5 mg/L (7.3), MS medium + BAP 0.25 mg/L + IBA 0.1 mg/L (6.33) (Plate 4). However there was no significant difference between these treatments.

In case of hypocotyls, also 10 days old showed better explant response followed by 8 days, 12 days and 14 days. In terms of treatments MS medium supplemented with Kinetin 1.0 mg/L and BAP 1.5 mg/L + Kinetin 1.0 mg/L (3.6) showed better explant response when 10 days old hypocotyls were used as explant followed by Zeatin 1.0 mg/L + IAA 0.1 mg/L (3.0) and Kinetin 2.0 mg/L (3.0) (Plate 5). However, there was no significant difference between these treatments.

Based on the results of this experiment, cotyledons from 10 days old seedlings were used as explants in the successful medium for developing further work i.e. regeneration and transgenic protocols.

4.3 SHOOT INITIATION, MULTIPLICATION AND ELONGATION IN COTYLEDONARY EXPLANTS

MS basal medium with different hormonal concentrations of BAP, Kinetin, Zeatin or in combination with IAA and IBA were evaluated for their effect on days taken for shoot bud initiation, No. of explants producing shoot buds, shooting frequency, mean no. of shoots / explant and Length of the shoots (Table 6). The data revealed significant differences between the treatments.

Among different combinations, MS medium + BAP 1.5 mg/L+ Kinetin 1.0 mg/L recorded less no. days taken for shoot bud initiation (12.3 days) followed by MS medium + BAP 0.5 mg/L + IAA 0.1 mg/L (12.6 days), MS medium + BAP 1.0 mg/L + Kinetin 0.5 mg/L (13.3 days) compared to all other hormonal combinations (Plate 6). However, there was no significant difference between these treatments.

For the parameter no. of explants producing shoot buds, the combination of MS medium + BAP 1.5 mg/L + Kinetin 1.0 mg/L recorded 5.3 out of 7 explants with shooting frequency of 76.18 % followed by MS medium + BAP 1.0 mg/L + Kinetin 0.5 mg/L and MS medium + Zeatin 1.0 mg/L + IAA 0.1 mg/L (4.3 explants/7 explants) with frequency of 61.90%

compared to other treatments. However, there was no significant difference between these treatments.

The hormonal concentration giving more mean no. of shoots/explant was MS medium + BAP 1.5 mg/L + Kinetin 1.0 mg/L (4.3 shoots) followed by MS medium + BAP 1.0 mg/L + Kinetin 0.5 mg/L (3.3 shoots), MS medium + BAP 2.0 mg/L + Kinetin 1.5 mg/L (2.6 shoots) (Plate 7). When the sub cultures were done in the same media, no. of shoots and the length of the shoots has been increased (Plate 8). Significant difference has been observed between these treatments.

Among different combinations used in the experiment, the highest shoot length was recorded when MS medium was fortified with BAP 2.0 mg/L + IBA 0.1 mg/L (3.5 cm) followed by BAP 0.5 mg/L + IBA 0.1 mg/L (3.43 cm) and BAP 1.0 mg/L + IBA 0.1 mg/L (3.40 cm) compared to other hormonal combination (Plate 9). However, there was no significant difference between these treatments.

Among the various treatments evaluated, the MS medium supplemented with BAP 1.5 mg/L + Kinetin 1.0 mg/L had taken lower no. of days for shoot bud initiation, high shooting frequency, higher mean no. of shoots/explant with higher length of the shoot followed by BAP 1.0 + Kinetin 0.5 mg/L compared to all other hormonal combinations.

4.4 ROOT INDUCTION

Different combinations of Kinetin, IAA, IBA, NAA alone and Kinetin, IBA, BAP combinations were used with MS medium for root induction. However, IAA and NAA did not show any response on root induction in the present study. The treatments of MS medium with different concentrations of IBA, Kinetin alone and BAP + IBA, Kinetin + IBA in combination as mentioned in Table 7 were used to study their effects on root induction. Significant differences were observed among the treatments for all the rooting parameters presented in the Table 7.

Mean no. of days taken for root initiation was significantly lower when MS medium was supplemented with IBA 0.3 mg/L (14.3 days) compared to all other combinations followed by IBA 0.2 mg/L (15.0 days) and BAP 0.25 mg/L + IBA 0.1 mg/L (15.3 days). However there was no significant difference between these treatments.

MS medium with Kinetin 1.0 mg/L resulted in the highest no. of shoots (3.5 Shoots/5 Shoots) producing roots with the highest frequency of 71.6 % compared to all other treatments followed by BAP 0.25 mg/L + IBA 0.1 mg/L with rooting frequency 70.6 %, Kinetin 1.5 mg/L with rooting frequency 65 %. However, there was no significant difference between these treatments.

Among the various treatments, the combination i.e. MS medium + BAP 0.25 + IBA 0.1 mg/L recorded high mean no. of roots (8.6) followed by MS medium + BAP 0.5 mg/L + IBA 0.1 mg/L (7.9), MS medium + BAP 1.0

mg/L + IBA 0.5 mg/L (7.4) compared to other treatments (Plate 10 and Plate 11).

However, there was no significant difference between these treatments.

Similarly, MS medium fortified with 1.0 mg/L Kinetin recorded higher mean length of root of 6.33 cm followed by Kinetin 1.5 mg/L (5.26 cm) and Kinetin 2.0 mg/L (4.96 cm) (Plate 12). Significant differences were observed between these treatments.

Among the different treatments evaluated for root induction, MS medium + Kinetin 1.0 mg/L was proved to be more successful combination recording more no. of shoots producing roots (3.3 Shoots/5 Shoots), maximum rooting frequency (71.6 %) with mean no. of roots/shoot (7.0) and high mean length of roots (6.3 cm) although it has taken 19 days for root initiation.

4.5 ACCLIMATIZATION

Among the different soil mineral mixtures evaluated, *in vitro* rooted plants established with 86 % success in poly bags containing soilrite mixture (Table 8) and days taken for acclimatization is 9.6 (Plate 13 and Plate 14) followed by Soil : Sand : Soilrite (1:2:1) with 63 % and 12.3 days for acclimatization. Then the plants were subsequently transferred to earthen pots. Significant differences were observed between these treatments.

4.6 TRANSFORMATION OF TOMATO

Transformation of tomato was carried out using cotyledon explants of Cv. PKM-1. The explants were co-cultivated with *Agrobacterium* strain

LBA4404 containing the binary vector pCAMBIA 2301 harboring the reporter gene GUS. GUS gene was used as a selectable marker.

4.6.1 Kanamycin sensitivity test

In order to avoid the possibility of selecting the non-transformed plants, the concentration of kanamycin at which there was no regeneration in non-transformed cotyledons was identified by adding kanamycin to the regeneration media. Kanamycin concentration of 50 mg/L was found to be effective in inhibiting the regeneration totally in non-transformed plants (Table 9). Application of kanamycin to media in general inhibited the development of multiple shoots. For further studies, kanamycin at 75 mg/L was used to select the transformed plants from non-transformed plants.

4.6.2 Transformation of tomato Cv. PKM-1

Several conditions were standardized to develop an efficient transformation protocol for the cultivar PKM-1. An experiment was conducted to optimize several factors such as duration of inoculation with *Agrobacterium* culture, co-cultivation period, concentration of cefotaxime and culture period of co-cultivated explants on cefotaxime medium to prevent bacterial growth.

4.6.1.1 Inoculation and co-cultivation

Based on the results of present investigation cotyledons alone with successful hormonal combination i.e. MS medium + BAP 1.5 mg/L + Kinetin

1.0 mg/L was used for developing transgenic protocol. Hence, cotyledons from *in vitro* raised 10 days old seedlings were used.

Concentration of *Agrobacterium* and duration of co-cultivation were standardized. Among the various time periods tried, incubation for 10 min with the *Agrobacterium* culture diluted in the ratio of 1:4 in distilled water followed by co-cultivation for 2 days proved to be optimum compared to lower duration of 5 min and higher duration of 15 min and 20 min. Co-cultivation for 5 min showed no bacterial growth and retained green explant colour. However, higher durations i.e. 15 - 20 min of co-cultivation resulted in overgrowth of bacterium, softening and blackening of tissues (Table 10).

4.6.3 Selection of transformants

4.6.3.1 Prevention of *Agrobacterium* overgrowth

Co-cultivation followed by culture on MS medium + BAP 1.5 mg/L + Kinetin 1.0 mg/L with two different concentrations of cefotaxime were studied to restrict *Agrobacterium* overgrowth completely (Table 11). Among the two concentrations, 500 mg/L for four days was found to be more effective in checking the bacterial over growth.

4.6.3.2 Selection of putative transformants

Selection of putative transformants is the prime step in the process of developing transformed plants. After 4 days of culturing on MS medium + BAP 1.5 mg/L + Kinetin 1.0 mg/L + 500 mg/L cefotaxime the explants were

transferred to the same medium containing 75 mg/L kanamycin for selection as it is proved that non transformed shootlet regeneration was minimum. The shoots which regenerated on this medium were considered as putative transformants and on an average the number of putative transformants obtained in the present study were 9 out of 112 explants and the per cent putative transformants was 8.03 % (Plate 15).

4.6.4 Confirmation of the integration of transgene

4.6.4.1 *Histochemical staining for GUS assay*

In order to confirm the presence of gene at an early stage itself, histochemical assay was carried out for GUS expression from the leaf bits of putatively transformed *in vitro* shoots. The leaf bits from putatively transformed shoots of Cv. PKM-1 were picked up and stained with GUS staining solution for one day, followed by treatment with absolute alcohol. The distinct blue colour was viewed microscopically (Plate 16) and 44.4% of transient GUS gene expression level was observed (Table 12).

4.6.4.2 *Using PCR technique*

PCR amplification of GUS gene was done to confirm the stable integration of transgene in the putative transformants. Plasmid DNA isolated from *Agrobacterium*, genomic DNA from both control and putatively transformed plants were subjected to PCR amplification of GUS gene using the specific primers. Out of 9 putative transformants subjected to PCR

analysis, 4 showed amplification of GUS gene in Cv. PKM-1. An 1100 bp band was found amplified in both plasmid DNA and two of putative transformants tested where as the same band was found absent in control plants (Plate 17).

The putative transformants were checked for the presence of the transgene by GUS assay and PCR. The transformation efficiency of 44.4 % and transformation frequency of 3.5% was observed (Table 12).

The histochemical staining also gave the similar results as that of PCR analysis with 4 shoots in staining blue Cv. PKM-1.

CHAPTER - 5

DISCUSSION

The techniques of plant tissue culture have proved to be a practical tool in paving a new avenue for the application of biotechnology in agriculture. The past few years have witnessed dramatic increase in our ability to manipulate and study plant cell and tissues in culture. The fact that a whole plant could be regenerated from a single cell has created an exciting scenario in the field of genetic manipulation and crop improvement. The growing realization of the potentialities of plant cell and tissue culture for plant breeding has itself provided a substantial impetus for research related to generation of variability and selection of variants.

Agrobacterium mediated transformation offers an exciting proven approach for genetic manipulation of crop plants. Plants transformed with *Agrobacterium* have been obtained in a wide range of crop species. Major advances in this technology were however dependent on an efficient explant based regenerative and transformation system.

The present investigation was carried out with the objectives of developing suitable and efficient protocol for regeneration and transformation of tomato Cv. PKM-1. The results of present study have been discussed in this chapter.

5.1 OPTIMIZATION OF SEED GERMINATION *IN VITRO*

5.1.1 Efficacy of different surface sterilants on contamination during *in vitro* seedling production:

Establishment of efficient tissue cultures involves the surface sterilization or disinfection of explants that carry a wide range of microbial contaminants. Removing contaminants from the surface of the explant is of prime concern (Hartmann *et al.*, 1997). Disinfection requires the use of chemicals that are toxic to microorganism but non toxic to plant material. Tissue culture became possible with the use of convenient and effective disinfectants such as ethanol, mercuric chloride and sodium hypochlorite (Krikorian, 1982).

In order to find an optimized protocol for sterilization of a specific tissue, three factors have to be taken into consideration *viz.*, sterilizing chemical, its concentration and the treatment duration. A series of experiments were conducted using Mercuric chloride, Hydrogen peroxide and Sodium hypochlorite to optimize a protocol for surface sterilization of seeds, as it is an essential pre-requisite for seedling production.

Application of mercuric chloride at different concentrations and time duration for surface sterilization of explants resulted in contamination free cultures and the germination was inhibited completely with increased concentrations. Such response is due to bleaching action of two chloride atoms and also ions that combines strongly with proteins causing the death of organisms (Pauling, 1955). All the treatments except 0.1 % resulted in

contamination free cultures and the germination was inhibited. In contrast Sheeja and Mandal (2003), Bamel *et al.* (2007) and Singh *et al.* (2007) reported seedling production by using 0.1 % HgCl₂.

Among different combinations of H₂O₂ viz., 10, 15, 20% treated for 15 min, lower concentrations i.e. 10 and 15% resulted in 80 and 70% germination but contamination of cultures was observed. Increased concentration i.e. 20% H₂O₂ resulted in contamination free cultures with 70% germination but seedling growth was not uniform. However, this survival rate was lower than the rate which could guarantee an aseptic culture establishment. The failure of above sterilants could be attributed to resistance or tolerance of microbial agents present on the surface of explant or due to the high load of contaminants on the explant surface. Hence, the above sterilants are not suitable for sterilizing the seed.

Almost all the concentrations of sodium hypochlorite for 15 min resulted in contaminated cultures with lower rates of germination. But when the seeds were soaked for 20 min with occasional swirling in 5% concentration of sodium hypochlorite resulted in higher germination rate (95 %) with uniform seedling growth and reduced levels of contamination followed by 4 per cent for 20 min. When NaOCl was used as surface sterilant at lower concentration than 4 per cent resulted in contaminated cultures and when used at higher concentration than 5%, it resulted in the inhibition of germination. The reports of Newman *et al.* (1996) used NaOCl 2.5 per cent

for 15 min; Gubis *et al.* (2003) 4 per cent sodium hypochlorite for 15 min; Reda *et al.* (2004) 3 per cent (v/v) sodium hypochlorite, played a role in deciding sodium hypochlorite as effective surface sterilant for sterilization of tomato seeds.

5.1.2 Effect of media and culture conditions on *in vitro* seedling production

The culture conditions and media not only affect germination, but also effect the uniform growth of seedlings. The prime conditions selected for optimized seedling production are light, darkness, medium with or with out carbon source.

A series of experiments were conducted where MS full strength medium or $\frac{1}{2}$ strength MS medium, light incubation (exposure to 16 hr light and 8 hr dark till the growth of seedlings) or dark incubation (complete darkness for 3 days followed by 16 hr light and 8 hr dark) and MS medium or MS medium with out sucrose were adopted to optimize a protocol for healthy and uniform seedling production.

When the seeds were surface sterilized and implanted on full strength MS medium with out sucrose followed by dark incubation had taken lesser no. of days (3.3) for initiation of germination with 96 % germination followed by $\frac{1}{2}$ strength MS medium with out sucrose followed by dark incubation.

The superiority of full strength MS medium over half strength MS medium was due to quick depletion of the nutrients which are relatively low in full strength MS medium when compared to $\frac{1}{2}$ strength MS medium. The

results also revealed that seeds were germinated on MS medium with out carbon source i.e. sucrose, denoting the less importance of sucrose for germination.

In contrast, Bhatia (2005) reported that use of lower concentrations of sucrose (0.5-1.5%) along with full strength MS medium was optimum for plant growth in tomato.

From the data it is evident that tomato *in vitro* seed germination and seedling growth is influenced by dark or light conditions. In seeds which were exposed to normal growth room conditions i.e. 16 hr light and 8 hr darkness till the growth of seedlings, both germination per cent as well as seedling growth was affected. However, when seeds were dark incubated for 3 days followed by exposure to normal growth room conditions showed higher germination per cent as well as seedling growth. The results are in accordance with Rao *et al.* (2007) who reported uniform and rapid seedling production when the seeds were kept in dark for 4 days compared to complete light incubation.

5.2 EFFECT OF AGE OF IN VITRO SEEDLING ON REGENERATIVE RESPONSE OF EXPLANTS

In vitro plant regeneration frequency depends on the age of the explant, type of explant and culture conditions. Response of seedling age in terms of explant response is known to be existed and it has been reported by many workers (Hamza and Chupeau, 1993, Gubis *et al.*, 2004 and Rao *et al.*, 2007). In the present study also cotyledons and hypocotyls of 10 days old were

found to be superior compared to explants collected from 8, 12, 14 days old seedlings when MS medium is supplemented with BAP 1.5 mg/L + Kinetin 1.0 mg/L.

Among the cotyledons and hypocotyls better regenerative response has been observed in case of cotyledons. In terms of explant response Duzyaman *et al.* (1994), Muthuvel *et al.* (2005) and Grigoriadis *et al.* (2005) reported the superiority of cotyledon explants over the hypocotyl explants in tomato. In contrast, Jabeen *et al.* (2004), Gubis *et al.* (2004), Borge *et al.* (2005) and Rao *et al.* (2007) reported that hypocotyls were superior to cotyledon explants in tomato.

The present investigation revealed that genotypic variations existed in the regenerative response of explants and the age of the seedlings. Since, seedling vigour and uniformity of germination depends on the seed vigour of the genotype, these results vary with the genotype selected for the study. Similarly variability in regenerative response of the explants were already reported by several workers and also recorded in this study. Hence, these results further support the fact that the regeneration protocols are strictly genotype specific one and the tissue culture regeneration responses vary from genotype to genotype to a greater extent.

For further study on regeneration and transgenic protocols, cotyledonary explants collected from 10 days old *in vitro* seedlings of Cv. PKM-1 were used.

5.3 SHOOT INITIATION, MULTIPLICATION AND ELONGATION IN COTYLEDONARY EXPLANTS

Plant growth regulators are known to play a crucial role in regeneration response of the explants. The response of a plant species to an exogenous growth regulator would depend mainly on the endogenous level of that growth regulator (and of other growth regulators as well) in that species (Singh, 1998). Accordingly, different workers have used different cytokinins *viz.*, BAP (Chandel and Katiyar, 2000; Rao *et al.*, 2007), Kinetin (Uddin *et al.*, 1988; Chandra *et al.*, 1995), Zeatin (Filova, 2004; Gubis *et al.*, 2005; Grigoriadis *et al.*, 2005), Thiadiazuron (Zakir *et al.*, 1995; Rao *et al.*, 2007) alone or in combination with auxins such as IAA / NAA and have been found to be effective in inducing regeneration. Hence, in the present investigation, different combinations of hormones were used *viz.*, cytokinins like BAP, Zeatin, Kinetin alone or along with the combination of auxins like IBA and IAA.

Best regenerative response in terms of days taken for shoot bud initiation, no. of explants producing shoot buds, shooting frequency (%), mean no. of shoots/explant were obtained when MS medium was supplemented with both cytokinins *viz.*, BAP 1.5 mg/L and Kinetin 1.0 mg/L. This hormonal combination recorded 12.3 days for shoot bud initiation, shooting frequency of 76.18% and the highest mean no. of shoots/explant (4.3) compared to all other plant hormonal combinations. Similar results were reported by Izadpanah and Khoshkui (1989) in tomato.

The length of the shoot was high when MS medium was fortified with BAP 2.0 mg/L + IBA 0.1 mg/L followed by BAP 0.5 mg/L + IBA 0.1 mg/L. When two cytokinins were used, the length of the shoot was comparatively less when compared to combination of auxins and cytokinins because auxins are essential for cell elongation. When sub cultures are done in weekly intervals in the same shoot initiation medium, the no. shoots/explant and length of shoot has been increased.

In contrast, Kurtz and Lineberger (1983) reported shoot regeneration on MS medium supplemented with 0.2 - 1.0 mg/L BA. Whereas, Soniya *et al.* (2001) observed multiple shoots when MS medium was supplemented with 17.7 μ M BA alone in two weeks. Bhatia *et al.* (2005) reported direct shoot regeneration from whole cotyledonary explants of 1 week old on MS medium supplemented with 15 μ M Zeatin. Similarly, Singh *et al.* (2007) reported the highest frequency of shoot regeneration when MS medium was supplemented with 2 mg/L BAP + 1 mg/L IAA. On contrary, Rao *et al.* (2007) reported that TDZ supplied alone or in combination had taken comparatively less time for shoot regeneration and formed more no. of adventitious shoots (6.7 - 4.3) compared to BAP, Zeatin, Kinetin alone or in combinations. In contrast to these, Pozueta-Romero *et al.* (2001) reported that multiple shoots regenerated on cut surface with a frequency average of 2.9 - 5.3 shoots/explant, when cotyledons of tomato were grown with out any growth regulators. Similarly Shamshad *et al.* (1999) reported 3.13 and 2.35 mean no. of shoots/explant

when MS medium supplemented with high conc. of BAP 5.0 mg/L and low conc. of IAA 0.5 mg/L.

The results of the present study further lead support to the fact that shoot regeneration and its influence by hormonal concentrations are genotype dependent and they vary with the type of genotype selected for the study.

5.4 STUDIES ON ROOT INITIATION AND ELONGATION

Rooting in shootlets is a very important part of any *in vitro* propagation scheme, and usually it is necessary to adopt a separate rooting procedure using special media or methods to induce roots to form. Rhizogenesis usually follows treatment with auxin and cytokinin (George, 1993).

Mostly in all rhizogenesis studies, auxins are mostly used due to their regeneration response. However, in the present study, both auxins (IBA) and cytokinins (Kinetin) were used alone or in combinations.

In the present study, MS medium + Kinetin 1.0 mg/L was proved to be more successful treatment compared to all other hormonal combinations in inducing high rooting frequency (71.6 %), and high mean length of roots (6.33 cm) Similar kind of results on rooting induction were reported by Polevaya *et al.* (1988) in tomato. Different workers have used different auxins for root induction with full strength MS medium (Mandal, 1999 and Soniya *et al.*, 2001).

In contrast, Moghaieb *et al.* (1999) reported successful root regeneration when regenerated shootlets were transferred to half strength MS medium with 1% sucrose and 0.1 mg/L NAA. Kurtz and Lineberger, (1983) and

Majoul *et al.* (2001) reported rooting with IAA. However, such rooting response with IAA and NAA was not observed in Cv. PKM-1 possibly due to the specific nature of genotype and it seems to be non-responsive to IAA and NAA treatments.

5.5 ACCLIMATIZATION

Successful establishment of *in vitro* grown plantlets in the soil constitutes the ultimate success for any micropropagation technique. It is very difficult to recover the plants upon transfer to soil under external conditions. These may be attributed to the fact that the *in vitro* grown plantlets have been continuously exposed to unique micro environment that has been selected to provide a minimal stress and optimal conditions for plant growth. So, the *in vitro* grown plantlets require an acclimatization process in order to ensure better survival upon transfer to field conditions.

The results of present investigation revealed soilrite as the most suitable substrate for acclimatization of tomato plantlets recording the highest percentage of survival (86%) with least duration (9.6 days) followed by soil : sand : soilrite (1 : 2 : 1) with 63% survival and 12.3 days for acclimatization.

In contrast, Rao *et al.* (2007) reported 75-80% survival when plants were acclimatized with sand and soil mix (1:1) while Dwivedi *et al.* (1990) revealed that the *in vitro* raised tomato plants grew normally when potted in soil.

In conclusion, the cotyledon regeneration system was proved to be an excellent method, as it has produced large number of regenerated tomato

plants (86%) over a relatively shorter period i.e. (3 months). In cotyledons, shoot formation was rapid and prolific and a large proportion of these shoots were developed in to phenotypically normal fertile plants. This protocol is a genotype dependent one and it has provided a way for transformation of plants with desired gene of interest.

5.6 TRANSFORMATION

Following the development and standardization of an efficient regeneration system, transformation work was initiated in Tomato Cv. PKM-1 at this lab. Cotyledons from 10 days old seedlings were alone used for transformation work. Other workers also reported that cotyledon is the preferred explant in tomato for transformation (Shahin *et al.*, 1984; Fillatti *et al.*, 1987; Park *et al.*, 2003).

5.6.1 Kanamycin sensitivity test

The presence of GUS gene confers the ability on transformed cells to grow on a medium containing kanamycin. The concentrations of selective agent vary widely depending on the sensitivity of plant species. In tomato, kanamycin concentrations ranging from 50-100 mg/L have been reported to show sensitivity (Agharbaoui *et al.*, 1995; Frary and Earle, 1996; Pozueta-Romero *et al.*, 2001). In some instances, it was found to inhibit shoot regeneration and it was necessary to omit or reduce the concentration of selective agent (Conner *et al.*, 1991). Hence, in the present study, thorough experimentation was done to prevent non transformants. For this purpose

50 mg/L and 75 mg/L of kanamycin were found to be lethal conc. where there was no regeneration of non transformed cotyledonary explants was observed in the present study. However, higher concentration i.e. 75 mg/L of kanamycin was used for selecting the transformants.

5.6.2 Inoculation and co-cultivation

Standardization of optimum co-cultivation period of the tissues with *Agrobacterium tumefaciens* is very essential for efficient gene transfer. In this study, inoculation of cotyledons for 10 min with *Agrobacterium tumefaciens* culture (1.0 OD at 600 nm) and diluted for 1 : 4 times with distilled water followed by co-cultivation on regeneration media for 2 days was found to be optimum. Co-cultivation for 5 min showed no bacterial growth and higher durations i.e. 15 to 20 min of co-cultivation resulted in over growth of bacterium, softening and blackening of tissues. The results are in accordance with Frary and Earle, 1996 and Park *et al.* 2003. They also reported higher transformation rates when tomato cotyledons were co-cultivated with *Agrobacterium* for 2 days and transformation rates were decreased when co-cultivated for either less than or more than 2 days.

5.6.3 Selection of transformants

After co-cultivation, the explants were transformed to the medium containing cefotaxime to check the bacterial growth of explants. A cefotaxime conc. ranging from 200 mg/L to 500 mg/L has been reported by different workers in tomato transformation to check over growth of

Agrobacterium after co-cultivation (Hamza and Chupeau, 1993; Pozueta-Romero *et al.*, 2001). Culturing of explants on a cefotaxime conc. of 500 mg/L for 4 days was recorded to check the bacterial over growth in the present study.

5.7 CONFIRMATION OF TRANSGENE INTEGRATION

Selection and growth of plant cells on selective media provides 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, the most commonly used selection agent for plant transformation. There are reports that even though plants seem to be transformed on selection medium, they might have not transformed, having escaped the selection pressure (Frary and Earle, 1996). Therefore, biochemical and molecular evidence is essential to confirm expression and integration of transferred genes.

5.7.1 Histochemical staining for GUS gene

The commonly and extensively used method for measuring GUS gene was histochemical assay (Jefferson *et al.*, 1987). In the present study, all the putatively transformed shootlets were assayed for GUS activity and the results of histochemical staining were confirmed with the PCR analysis (Gassama-Dia *et al.*, 2004; Tripathi *et al.*, 2005; Patnaik *et al.*, 2006 and Cevik *et al.*, 2006).

Dillen *et al.* (1997) found that the expression of GUS was greatly affected by the temperature during co-cultivation. However, in the present study, co-cultivation was performed better at around 26°C. Since the GUS results matched with the PCR analysis, it is felt that optimum transformation efficiency was obtained at this temperature.

Per cent transient GUS gene expressed (no. of plants giving blue colour over the plants tested) was 44.4 % in the present study. Such study will help in the study of expression of gene of interest in the future work. Wu *et al.* (2006) reported that *Agrobacterium tumefaciens* strain LBA4404 carrying a binary vector PTOK 233, which contained the GUS reporter gene and a kanamycin resistance gene *npt II* could be employed for optimizing the transformation efficiency evaluated by a GUS gene transient expression level in tomato.

5.7.2 PCR Analysis

It gives an indirect indication of the presence of transgene. Besides, PCR technique has the advantage of screening large collection of transgenic plants using small amounts of DNA.

In the present study, PCR analysis was carried out with the DNA isolated from well established putatively transformed plant leaves, plasmid DNA and control plants. Evidence for the presence of transgene *viz.*, reporter gene (GUS gene) was confirmed indirectly by amplification of the GUS gene using specific primer sequences for the GUS coding region. Out of nine putatively transformed plants tested for PCR amplification, four showed

bands of expected size of 1100 bp for the GUS fragments at the same position as the positive control (Plasmid DNA). Ling *et al.* 1998 confirmed the transformed plants of tomato through the use of PCR.

For the transformation experiment, transformation frequency was calculated as the percentage explants that produced a plantlet determined to be positive by PCR analysis. In the present study a transformation frequency of 3.5 % was obtained for the tomato cultivar PKM-1.

The major stumbling block in most of the genetic transformation experiments is the low transformation frequencies that vary considerably between the species. Reports published so far on transgenic tomato showed a wide range of transformation frequencies from 6% (Vidya *et al.*, 2000); 9% (Van Roekel *et al.*, 1993); 11% (Frery and Earle, 1996); 14% (Hamza and Chupeau, 1993); 20% (Park *et al.*, 2003) and 25% (Hu Wel and Phillips, 2001).

Van Roekel *et al.* (1993) have described several factors influencing transformation frequency in tomato and their results have shown that transformation frequencies vary depending up on plant genotypes and bacterial stains used.

Transformation frequencies were also reported to depend on factors such as bacterial concentration, length of co-cultivation and kind of growth hormones used and hence, optimization of these conditions is very important to get higher transformation rates (Fillatti *et al.*, 1987).

Transformation efficiency in tomato (number of putative transformants tested positive for reporter gene over the no. of putative transformants) was

reported to range from 10-23 % (Frary and Earle, 1996; Ling *et al.*, 1998). However, in the present investigation, higher transformation efficiency of 44.4% was achieved.

Transformation efficiency has been known to be influenced by the antibiotic which is used for suppressing the growth of *Agrobacterium* (Cheng *et al.*, 1998; Ling *et al.*, 1998; Costa *et al.*, 2000). There are several factors known to influence transformation efficiency viz., explant, explant type, explant orientation, plate sealant, *Agrobacterium* strain and genotype.

FUTURE LINE OF WORK

The cultivar PKM-1 is most widely grown green shoulder tomato with high acidity. This is also a best donor parent for developing green shoulder hybrids. The results of the present investigation paved a way for transferring gene of interest in to this variety with highest transformation efficiency. Hence, using these techniques, PKM-1 transgenic plants can be developed for desired traits viz. insect resistance, viral resistance and abiotic tolerance in tomato.

CHAPTER – 6

SUMMARY

Tomato (*Solanum lycopersicum* L.) is the world's largest crop next to potato cultivated for fleshy fruits. During last few decades, considerable efforts have been made towards the development of efficient regeneration and transformation protocol for tomato. The crop is succumbed to several biotic and abiotic stresses. Due to the agricultural importance of this crop, any biotechnological improvement would have significant agronomic and economic benefits. Further more, biotechnological approaches would complement the efforts of traditional breeders in the generation of improved varieties.

Keeping this in view, the present investigation was carried out with the objective of developing regeneration and transgenic protocol via *Agrobacterium* mediated GUS gene transfer.

The highlights of present investigation are as follows:

- An efficient and reproducible regeneration protocol for tomato Cv. PKM-1 was developed.
- Sterile seedlings for explant source were established by treating the seeds with 5 % sodium hypochlorite for 20 min.
- Based on the studies on optimization of media and cultural conditions for seedling growth, the best media and cultural conditions identified was MS medium with out sucrose and dark incubation for three days which recorded lower no. of days (3.3 days) for initiation with highest germination (96%).

- Among the different ages of explants studied 10 days old cotyledons and hypocotyls showed better explant response compared to 8 days, 12 days and 14 days old explants.
- Among the two different explants i.e. cotyledons and hypocotyls, cotyledons showed better explant response compared to hypocotyls. So the regeneration and transgenic protocol has been developed by using cotyledons as explant source.
- Among the various plant growth regulator combinations tried for shoot regeneration, the best shoot regeneration response was obtained in MS medium supplemented with BAP 1.5 mg/L + Kinetin 1.0 mg/L.
- Among the various plant growth regulator combinations tried for root regeneration, maximum rooting frequency (71.6%) was obtained with Kinetin 1.0 mg/L.
- The rooted plantlets were established well in the polybags containing soilrite mixture as medium with 86 % success and days taken for acclimatization were 9.6 days.
- The transformation was carried out using the *Agrobacterium* strain LBA4404 containing the binary vector pCAMBIA 2301 harboring *npt II* as selectable marker and GUS as reporter gene.
- Kanamycin sensitivity test was conducted to ascertain the conc. of kanamycin at which transformants could be selected. It was found that kanamycin @ 50 mg/L inhibited regeneration totally in non transformed plants. Therefore, kanamycin conc. of 75 mg/L was used for selection of transformants.

- Incubation for 10 min with over night grown *Agrobacterium* culture (1.0 OD at 600 nm) diluted (1:4 times) and co-cultivation for 2 days followed by transfer to cefotaxime medium (500 mg/L) for 4 days before transferring to selection medium containing kanamycin 75 mg/L was found to be the optimum for effectively checking the growth of *Agrobacterium* and retention of green colour of explant.
- The putative transformants recorded in the cultivar PKM-1 was 8.03%.
- There was no discrepancy in the results for the confirmation of transgene carried out either through GUS assay or PCR analysis. All the GUS assay positive shoots showed PCR amplification for reporter gene i.e. GUS gene.
- Among the putatively transformed shoots the transformation efficiency obtained was 44.4% with transformation frequency of 3.5%, and the GUS gene transient expression level of 44.4%.

The results of the present study established the genotype dependent regeneration protocol with high shoot and rooting frequencies in tomato Cv. PKM-1. It took 3 months from *in vitro* seed germination to development of acclimatized plants. *Agrobacterium* mediated transfer of GUS gene into PKM-1 cultivar genetic background has been proved to be simple and efficient, which can be further exploited for the development of green shoulder transgenic plants with desired traits *viz.*, insect resistance, viral resistance and abiotic stress resistance in tomato.

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* Originals not seen

Table 7: Effect of different hormonal treatments on Rooting in Tomato Cv. PKM-1

| S. NO. | Concentration | Days taken for root initiation | No. of shoots producing roots | Frequency of rooting (%) | Mean no. of roots / shoot | Mean length of roots(cm) |
|---------------|-------------------------|---------------------------------------|--------------------------------------|---------------------------------|----------------------------------|---------------------------------|
| 1 | MS + IBA 0.1 | 0 | 0 | 0 | 0 | 0 |
| 2 | MS + IBA 0.2 | 15.0 | 0.6 | (21.38) 13.3 | 3.3 | 2.2 |
| 3 | MS + IBA 0.3 | 14.3 | 1.3 | (31.04) 26.6 | 3.8 | 2.26 |
| 4 | MS + IBA 0.4 | 15.6 | 1.0 | (26.56) 20.0 | 2.6 | 2.43 |
| 5 | MS + Kinetin 0.5 | 0 | 0 | 0 | 0 | 0 |
| 6 | MS + Kinetin 1.0 | 19 | 3.58 | (57.79) 71.6 | 7.0 | 6.33 |
| 7 | MS + Kinetin 1.5 | 20.6 | 3.25 | (53.72) 65.0 | 7.0 | 5.26 |
| 8 | MS + Kinetin 2.0 | 21.3 | 3.08 | (51.70) 61.6 | 6.3 | 4.96 |
| 9 | MS + BAP 0.25 + IBA 0.1 | 15.3 | 3.53 | (57.17) 70.6 | 8.6 | 3.83 |

| | | | | | | |
|----|---------------------------|------|------|--------------|------|------|
| 10 | MS + BAP 0.5+ IBA 0.1 | 16.0 | 3.16 | (52.71) 63.3 | 7.9 | 3.50 |
| 11 | MS + BAP 1.0 + IBA 0.5 | 16.0 | 2.64 | (46.89) 53.3 | 7.4 | 3.23 |
| 12 | MS + BAP 1.5 + IBA 0.5 | 15.6 | 2.52 | (45.11) 50.2 | 6.9 | 2.93 |
| 13 | MS + Kinetin 0.5+ IBA 0.1 | 20.3 | 1.6 | (35.24) 33.3 | 4.3 | 2.80 |
| 14 | MS + Kinetin 1.0+ IBA 0.1 | 21.3 | 1.3 | (31.04) 26.6 | 5.3 | 2.76 |
| 15 | MS + Kinetin 1.5+ IBA 0.5 | 22.3 | 0.6 | (21.38) 13.3 | 4.0 | 2.93 |
| 16 | MS + Kinetin 2.0+ IBA 0.5 | 21.0 | 0.3 | (14.88) 6.6 | 3.3 | 2.80 |
| | (±) S.Em | 0.35 | 0.32 | 4.49 | 0.34 | 0.10 |
| | C.D. at 5 % | 1.01 | 0.93 | 12.96 | 0.98 | 0.29 |

Note: **Figures in parentheses represent arc sine transformed values.**

Observations were taken from five shootlets.

Table 6: Shoot initiation, multiplication and elongation in cotyledonary explants of tomato Cv. PKM-1

| S. No. | Concentrations | Days taken for shoot bud initiation | No. of explants producing shoot Buds | Shooting frequency (%) | Mean no. of shoots / explant | Length of shoots (cm) |
|---------------|----------------------------|--|---|-------------------------------|-------------------------------------|------------------------------|
| 1 | MS + BAP 1.0 | 15.6 | 3.3 | (43.63) 47.61 | 1.6 | 2.32 |
| 2 | MS + BAP 1.5 | 16.0 | 4.3 | (51.88) 61.90 | 1.3 | 2.31 |
| 3 | MS + BAP 2.0 | 15.3 | 3.6 | (46.35) 52.37 | 1.0 | 2.10 |
| 4 | MS + BAP 2.5 | 18.0 | 2.3 | (35.26) 33.33 | 1.3 | 2.30 |
| 5 | MS + Kinetin 0.5 | 14.6 | 3.3 | (43.63) 47.61 | 1.6 | 2.74 |
| 6 | MS + Kinetin 1.0 | 16.0 | 3.6 | (46.35) 52.37 | 2.3 | 2.86 |
| 7 | MS + Kinetin 1.5 | 15.3 | 2.3 | (35.26) 33.33 | 1.3 | 2.51 |
| 8 | MS + Kinetin 2.0 | 16.3 | 2.0 | (32.31) 28.57 | 0.6 | 2.72 |
| 9 | MS + Zeatin 0.5 | 15.0 | 3.3 | (43.63) 47.61 | 0.6 | 2.50 |
| 10 | MS + Zeatin 1.0 | 15.3 | 2.6 | (38.10) 38.09 | 1.3 | 2.43 |
| 11 | MS + Zeatin 1.5 | 15.6 | 2.3 | (35.26) 33.33 | 1.6 | 2.50 |
| 12 | MS + Zeatin 2.0 | 16.0 | 1.6 | (29.19) 23.80 | 0.6 | 2.36 |
| 13 | MS + BAP 1.0 + Kinetin 0.5 | 13.3 | 4.3 | (51.88) 61.90 | 3.3 | 2.36 |

| | | | | | | |
|----|----------------------------|------|------|---------------|------|------|
| 14 | MS + BAP 1.5 + Kinetin 1.0 | 12.3 | 5.3 | (60.78) 76.18 | 4.3 | 2.70 |
| 15 | MS + BAP 2.0 + Kinetin 1.5 | 14.3 | 3.6 | (46.35) 52.37 | 2.6 | 2.40 |
| 16 | MS + BAP 2.5 + Kinetin 2.0 | 15.0 | 3.3 | (43.63) 47.61 | 1.6 | 2.30 |
| 17 | MS + BAP 0.25 + IBA 0.1 | 14.6 | 3.3 | (43.63) 47.61 | 2.0 | 2.26 |
| 18 | MS + BAP 0.5 + IBA 0.1 | 15.6 | 2.6 | (38.10) 38.09 | 1.6 | 3.43 |
| 19 | MS + BAP 1.0 + IBA 0.1 | 16.0 | 2.0 | (32.31) 28.56 | 1.0 | 3.40 |
| 20 | MS + BAP 2.0 + IBA 0.1 | 16.0 | 1.6 | (29.19) 23.80 | 1.0 | 3.50 |
| 21 | MS + BAP 0.5 + IAA 0.1 | 12.6 | 3.3 | (43.63) 47.61 | 0.6 | 3.15 |
| 22 | MS + BAP 1.0 + IAA 0.1 | 13.6 | 3.6 | (46.35) 52.37 | 1.0 | 3.03 |
| 23 | MS + BAP 1.5 + IAA 0.5 | 14.6 | 3.0 | (40.87) 42.83 | 1.3 | 3.33 |
| 24 | MS + BAP 2.0 + IAA 0.5 | 14.0 | 2.0 | (32.29) 28.54 | 2.0 | 3.33 |
| 25 | MS + Zeatin 0.5 + IAA 0.1 | 13.6 | 3.6 | (46.35) 52.37 | 1.0 | 2.80 |
| 26 | MS + Zeatin 1.0 + IAA 0.1 | 14.6 | 4.3 | (51.88) 61.90 | 1.6 | 2.96 |
| 27 | MS + Zeatin 1.5 + IAA 0.5 | 14.0 | 3.3 | (40.64) 47.61 | 1.0 | 2.83 |
| 28 | MS + Zeatin 2.0 + IAA 0.5 | 15.3 | 3.0 | (40.87) 42.83 | 1.0 | 3.00 |
| | (±) S.Em | 0.39 | 0.37 | 3.22 | 0.34 | 0.05 |
| | C.D at 5% | 1.12 | 1.06 | 13.4 | 0.97 | 0.16 |

**Note: Figures in parentheses represent arc sine transformed values
Observations were taken from seven explants.**

Table 4 : Effect of media and culture conditions on *in vitro* seedling production

| S. NO. | Medium / Condition | Mean time taken for initiation of germination in days | Mean no. of seeds germinated (Out of 10 seeds / bottle) | Per cent of germination | Nature of response |
|--------|--|---|---|-------------------------|------------------------------------|
| 1 | MS medium + Light | 5.6 | 8.0 | (63.43) 80 | Healthy and non uniform seedlings. |
| 2 | ½ MS medium + Light | 6.3 | 7.3 | (58.69) 73 | Healthy and non uniform seedlings. |
| 3 | MS medium + Dark incubation | 4.6 | 8.3 | (65.64) 83 | Healthy and uniform seedlings. |
| 4 | ½ MS medium + Dark incubation | 5.3 | 7.6 | (60.66) 76 | Healthy and uniform seedlings. |
| 5 | MS medium with out sucrose+ Light | 5.0 | 8.3 | (65.64) 83 | Healthy and non uniform seedlings. |
| 6 | ½ MS medium with out sucrose + Light | 5.3 | 8.0 | (63.43) 80 | Healthy and non uniform seedlings. |
| 7 | MS medium with out sucrose + Dark incubation | 3.3 | 9.6 | (78.46) 96 | Healthy and uniform seedlings. |
| 8 | ½ MS medium with out sucrose + Dark incubation | 4.3 | 8.3 | (63.43) 83 | Healthy and uniform seedlings. |
| | (±) S.Em | 0.3 | 0.19 | 3.11 | |
| | C.D at 5% | 0.97 | 0.60 | 9.42 | |

- Note:**
1. The treatment of dark adoption means, media with seed kept for three days in dark followed by exposure to light until full seedling growth
 2. Figures in parentheses represent arc sine transformed values.

**Table 2: List of various sterilants used for sterilization of seeds of
Solanum lycopersicum L. Cv. PKM-1**

| S.NO. | Sterilant | Concentration in per cent | Duration in min |
|-------|-------------------------------|------------------------------|-----------------|
| 1 | HgCl ₂ | 0.1 | 5 |
| 2 | HgCl ₂ | 0.2 | 5 |
| 3 | HgCl ₂ | 0.3 | 5 |
| 4 | HgCl ₂ | 0.5 | 5 |
| 5 | H ₂ O ₂ | 10 | 15 |
| 6 | H ₂ O ₂ | 15 | 15 |
| 7 | H ₂ O ₂ | 20 | 15 |
| 8 | NaOCl | 3 | 15 |
| 9 | NaOCl | 4 | 15 |
| 10 | NaOCl | 5 | 15 |
| 11 | NaOCl | 6 | 15 |
| 12 | NaOCl | 3 | 20 |
| 13 | NaOCl | 4 | 20 |
| 14 | NaOCl | 5 | 20 |
| 15 | NaOCl | 6 | 20 |

Table 5: Effect of Age of *in vitro* seedlings on higher explant response (No. of explants bulged or flaccid) in different hormonal media.

| S. NO | Hormonal combination | Cotyledons | | | | Hypocotyl | | | |
|----------|----------------------|------------|---------|---------|---------|-----------|---------|---------|---------|
| | | 8 days | 10 days | 12 days | 14 days | 8 days | 10 days | 12 days | 14 days |
| 1 | MS + BAP 1.0 | 4.0 | 5.0 | 3.3 | 1.0 | 1.6 | 2.0 | 1.6 | 0.3 |
| 2 | MS + BAP 1.5 | 5.0 | 6.0 | 4.0 | 1.0 | 1.6 | 2.3 | 1.3 | 0.3 |
| 3 | MS + BAP 2.0 | 6.0 | 5.0 | 4.6 | 1.0 | 1.6 | 1.6 | 1.3 | 0.6 |
| 4 | MS + BAP 2.5 | 5.3 | 3.6 | 4.3 | 0.6 | 1.3 | 1.3 | 1.3 | 0.3 |
| 5 | MS + Kinetin 0.5 | 3.3 | 4.3 | 2.3 | 0.6 | 1.3 | 2.0 | 1.6 | 1.0 |
| 6 | MS + Kinetin 1.0 | 3.0 | 4.0 | 3.0 | 0.6 | 2.3 | 3.6 | 2.0 | 0.6 |
| 7 | MS + Kinetin 1.5 | 5.0 | 6.0 | 4.0 | 1.0 | 1.6 | 2.6 | 1.3 | 1.3 |
| 8 | MS + Kinetin 2.0 | 3.6 | 4.6 | 3.3 | 0.6 | 1.6 | 3.0 | 1.3 | 0.6 |
| 9 | MS + Zeatin 0.5 | 3.6 | 4.6 | 2.6 | 1.0 | 1.6 | 2.0 | 1.3 | 0.6 |
| 10 | MS + Zeatin 1.0 | 3.6 | 4.6 | 3.3 | 1.3 | 2.0 | 2.6 | 1.3 | 1.0 |
| 11 | MS + Zeatin 1.5 | 3.6 | 5.0 | 3.6 | 0.3 | 1.6 | 2.6 | 1.3 | 1.0 |
| 12 | MS + Zeatin 2.0 | 4.0 | 4.0 | 3.0 | 1.0 | 1.6 | 3.0 | 1.6 | 1.0 |

| | | | | | | | | | |
|----|----------------------------|------|------|------|------|------|------|------|------|
| 13 | MS + BAP 1.0 + Kinetin 0.5 | 6.3 | 7.3 | 5.3 | 1.3 | 1.6 | 2.0 | 1.3 | 1.3 |
| 14 | MS + BAP 1.5 + Kinetin 1.0 | 7.6 | 8.6 | 5.6 | 1.6 | 1.6 | 3.6 | 1.6 | 0.6 |
| 15 | MS + BAP 2.0 + Kinetin 1.5 | 5.3 | 5.3 | 4.3 | 1.0 | 2.0 | 2.0 | 1.3 | 1.0 |
| 16 | MS + BAP 2.5 + Kinetin 2.0 | 6.0 | 5.6 | 5.0 | 0.6 | 1.0 | 1.6 | 1.3 | 0.6 |
| 17 | MS + BAP 0.25 + IBA 0.1 | 5.0 | 6.3 | 4.6 | 1.0 | 2.0 | 2.0 | 1.6 | 0.3 |
| 18 | MS + BAP 0.5 + IBA 0.1 | 4.3 | 5.6 | 4.0 | 0.6 | 1.6 | 1.3 | 1.3 | 0.6 |
| 19 | MS + BAP 1.0 + IBA 0.1 | 3.6 | 4.0 | 3.3 | 0.6 | 1.3 | 1.6 | 1.3 | 0.6 |
| 20 | MS + BAP 2.0 + IBA 0.1 | 3.3 | 3.3 | 2.3 | 0.3 | 1.3 | 1.6 | 1.3 | 0.6 |
| 21 | MS + BAP 0.5 + IAA 0.1 | 4.3 | 5.0 | 4.3 | 1.0 | 1.3 | 2.6 | 1.6 | 0.3 |
| 22 | MS + BAP 1.0 + IAA 0.1 | 4.0 | 5.6 | 3.6 | 0.6 | 2.0 | 2.0 | 2.3 | 1.0 |
| 23 | MS + BAP 1.5 + IAA 0.5 | 3.6 | 4.3 | 3.6 | 0.3 | 1.6 | 1.6 | 1.3 | 0.6 |
| 24 | MS + BAP 2.0 + IAA 0.5 | 3.3 | 4.0 | 2.6 | 0.6 | 1.3 | 1.3 | 1.3 | 1.0 |
| 25 | MS + Zeatin 0.5 + IAA 0.1 | 4.3 | 5.3 | 3.6 | 1.0 | 1.6 | 2.0 | 1.3 | 0.6 |
| 26 | MS + Zeatin 1.0 + IAA 0.1 | 5.0 | 6.0 | 4.3 | 1.0 | 2.0 | 3.0 | 2.0 | 1.0 |
| 27 | MS + Zeatin 1.5 + IAA 0.5 | 4.0 | 4.6 | 3.3 | 0.6 | 1.6 | 2.3 | 1.3 | 0.6 |
| 28 | MS + Zeatin 2.0 + IAA 0.5 | 3.6 | 3.6 | 2.3 | 1.0 | 1.6 | 2.0 | 1.3 | 1.0 |
| | (±) S.Em | 0.44 | 0.47 | 0.40 | 0.45 | 0.38 | 0.41 | 0.36 | 0.35 |
| | C.D at 5% | 1.25 | 1.34 | 1.14 | NS | NS | 1.18 | NS | NS |

Note: Observations were taken from ten explants.

Table 8: Effect of different soil mineral mixtures on Acclimatization and survival of regenerated plantlets in Tomato Cv. PKM-1

| S.NO | Soil mineral mixture | No. of days taken for acclimatization | Survival percentage |
|------|------------------------|---------------------------------------|---------------------|
| 1 | Soilrite | 9.6 | (68.02) 86 |
| 2 | Sand | 14.6 | (30.0) 25 |
| 3 | Soil | 13.3 | (40.39) 42 |
| 4 | Soil : Sand : Soilrite | 12.3 | (52.53) 63 |
| | (±) S.Em | 0.38 | 0.43 |
| | C.D at 5% | 1.3 | 1.51 |

Note: Figures in parentheses represent arc sine transformed values

Table 10: Effect of inoculation time (min) and period of Co-cultivation (days) on growth of *Agrobacterium* (\pm) and health of explants (%) recorded in tomato Cv. PKM-1

| S. No | Time (min) | Co- cultivation period (days) | | | Mean |
|-------|------------|------------------------------------|-------------------------------------|-------------------------------------|------|
| | | 1 Day Health of explants (%) | 2 Days Health of explants (%) | 3 Days Health of explants (%) | |
| 1. | 5 | 100 - | 100 - | 100 - | 100 |
| 2. | 10 | 100 - | 100 - | 75 + | 92 |
| 3. | 15 | 100 - | 100 - | 50 ++ | 83 |
| 4. | 20 | 100 - | 90 + | 25 +++ | 72 |

Indications of *Agrobacterium* over growth on plants

- +++ - High
- ++ - Low
- + - Very Low
- - No

Table 9: Effect of Kanamycin on regeneration response of non transformed cotyledon explants of tomato Cv. PKM-1

| S. No | Concentration of Kanamycin (mg/L) | Regeneration response |
|--------------|--|------------------------------|
| 1. | 0 | + |
| 2. | 25 | + |
| 3. | 50 | - |
| 4. | 75 | - |
| 5. | 100 | - |

+ Normal shoot let Regeneration
- No regeneration

Table 11: Effect of cefotaxime concentration (mg/L) and culture period (days) on suppression of *Agrobacterium* growth in co-cultivated explants of tomato Cv. PKM-1

| S.No. | Concentration of cefotaxime (mg/L) | Percent explants found free from <i>Agrobacterium</i> growth during Culture period(days) | | | | |
|-------|------------------------------------|--|-------|-------|-------|-------|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| 1. | 400 | 45 | 60 | 65 | 70 | 90 |
| 2. | 500 | 55 | 70 | 85 | 100 | 100 |

Table 12: Transformation frequency of cotyledonary explants of tomato Cv. PKM-1 for GUS gene assay

| S.No | Genotype | PKM-1 |
|-------------|-----------------------------------|--------------|
| 1. | No. of cultivated explants | 112 |
| 2. | No. of putative transformants | 9 |
| 3. | Putative transformants (%) | 8.03 |
| 4. | No. of GUS positive shoots | 4 |
| 5. | No. of PCR positive shoots | 4 |
| 6. | Transformation efficiency (%) | 44.4 |
| 7. | Transformation frequency (%) | 3.5 |
| 8. | Transient GUS gene expression (%) | 44.4 |

Table 3: Effect of various sterilants on sterilization of seeds of *Solanum lycopersicum* L. Cv. PKM-1

| S.NO. | Sterilant | Concentration | Treatment duration min | Per cent of germination | Nature of response |
|-------|-------------------------------|---------------|------------------------|-------------------------|---|
| 1 | HgCl ₂ | 0.1 | 5 | (50.76) 60 | Germination delayed and contaminated. |
| 2 | HgCl ₂ | 0.2 | 5 | 0 | No germination |
| 3 | HgCl ₂ | 0.3 | 5 | 0 | No germination |
| 4 | HgCl ₂ | 0.5 | 5 | 0 | No germination |
| 5 | H ₂ O ₂ | 10 | 15 | (63.43) 80 | Cultures contaminated |
| 6 | H ₂ O ₂ | 15 | 15 | (56.78) 70 | Cultures contaminated |
| 7 | H ₂ O ₂ | 20 | 15 | (56.78) 70 | No contamination but no uniform germination |
| 8 | NaOCl | 3 | 15 | (45.0) 50 | Cultures contaminated |
| 9 | NaOCl | 4 | 15 | (33.21) 30 | Cultures contaminated |
| 10 | NaOCl | 5 | 15 | (39.23) 40 | Cultures contaminated |

| | | | | | |
|----|-----------|---|----|------------|--|
| 11 | NaOCl | 6 | 15 | (39.23) 40 | Cultures contaminated |
| 12 | NaOCl | 3 | 20 | (63.43) 80 | Cultures contaminated |
| 13 | NaOCl | 4 | 20 | (71.56) 90 | Seedlings healthy and uniform and no contamination |
| 14 | NaOCl | 5 | 20 | (77.07) 95 | Seedlings healthy and uniform and no contamination |
| 15 | NaOCl | 6 | 20 | (65.64) 83 | Seedlings healthy and uniform and no contamination |
| | (±) S.Em | | | 1.93 | |
| | C.D at 5% | | | 7.43 | |

Note: Figures in parentheses represent arc sine transformed values

Fig 1: Diagram of T-DNA of the plasmid pCambia- 2301.

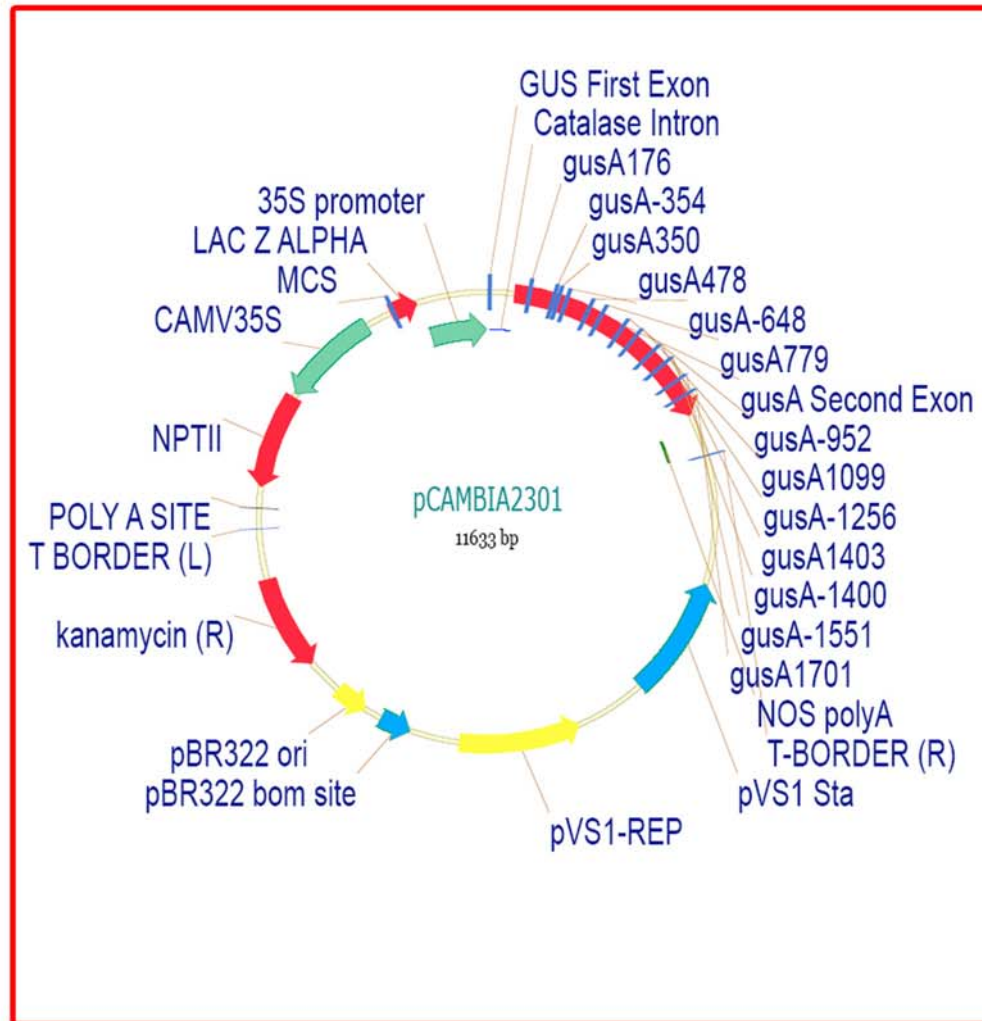


Plate 1: Selected cultivar PKM-1

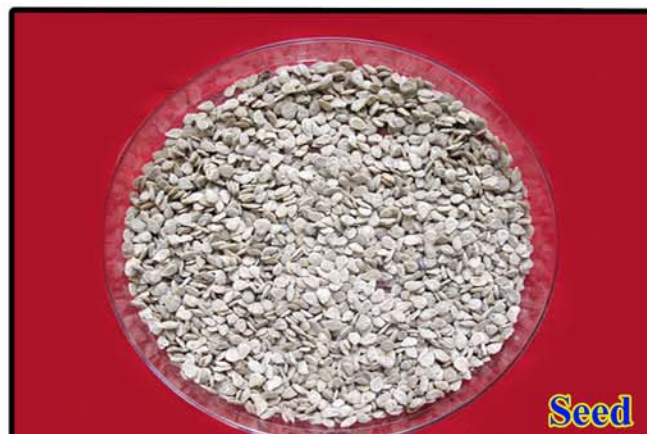
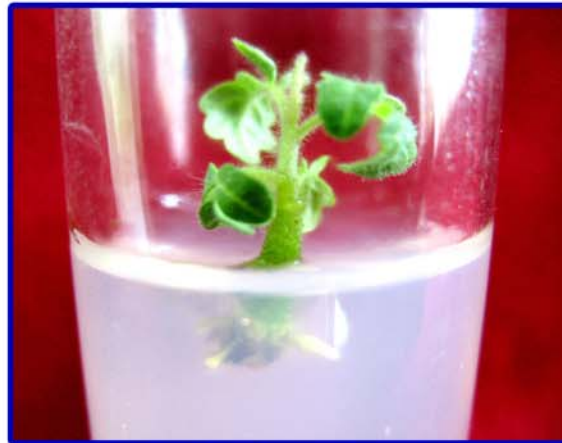


Plate 10: Extent of root initiation from the regenerated shoots of cotyledonary explants of PKM-1 when MS medium is supplemented with



BAP 0.25 + IBA 0.1 mg/L



BAP 0.5 + IBA 0.1 mg/L

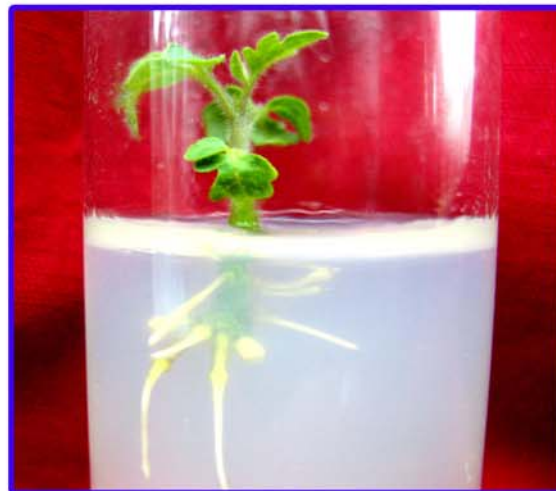


BAP 1.0 + Kinetin 0.5 mg/L.

Plate 11: Extent of root induction along with shoot regeneration in cotyledonary explants of PKM-1 when MS medium is supplemented with



BAP 0.25 mg/L + IBA 0.1 mg/L



BAP 0.5 mg/L + IBA 0.1 mg/L



BAP 1.0 mg/L + IBA 0.5 mg/L

Plate 5: Better explant response in hypocotyls of 10 days old seedlings when MS medium is supplemented with



Kinetin 1.0 mg/L



BAP 1.5 + Kinetin 1.0 mg/L



Zeatin 1.0 + IAA 0.1 mg/L

Plate 6: Extent of Shoot initiation from cotyledonary explants when MS medium is supplemented with



BAP 1.5 +Kinetin 1.0 mg/L



BAP 0.5 + IAA 0.1 mg/L



BAP 1.0 +Kinetin 0.5 mg/L

Plate 6: Extent of Shoot initiation from cotyledonary explants when MS medium is supplemented with



BAP 1.5 +Kinetin 1.0 mg/L



BAP 0.5 + IAA 0.1 mg/L



BAP 1.0 +Kinetin 0.5 mg/L

Plate 7: Multiple shoot induction from cotyledonary explants when MS medium is supplemented with



BAP 1.5 + Kinetin 1.0 mg/L



BAP 1.0 + Kinetin 0.5 mg/L



BAP 2.0 + Kinetin 1.5 mg/L

Plate 7: Multiple shoot induction from cotyledonary explants when MS medium is supplemented with



BAP 1.5 + Kinetin 1.0 mg/L



BAP 1.0 + Kinetin 0.5 mg/L



BAP 2.0 + Kinetin 1.5 mg/L

Plate 8: Proliferation of multiple shoots from cotyledonary explants up on sub culturing when MS medium is supplemented with



BAP 1.5 + Kinetin 1.0 mg/L



BAP 1.0 + Kinetin 0.5 mg/L



BAP 2.0 + Kinetin 1.5 mg/L

Plate 9: Extent of Shoot elongation from cotyledonary explants when MS medium is supplemented with



BAP 2.0 + IBA 0.1 mg/L



BAP 0.5 + IBA 0.1 mg/L



BAP 1.0 + IBA 0.1 mg/L