IN VITRO REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) IN RELATION TO DISEASE RESISTANCE AGAINST GROUNDNUT BUD NECROSIS VIRUS

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DECLARATION

I hereby declare that this thesis entitled **"*in vitro* regeneration and *Agrobacterium* mediated transformation in tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against groundnut bud necrosis virus"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis "***in vitro* regeneration and *Agrobacterium* mediated transformation in tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against groundnut bud necrosis virus"** is a record of research work done independently by Ms. **Ramjitha P.** (2003-11-58) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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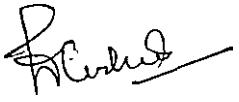

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Dedicated to my beloved Parents

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LIST OF ABBREVIATIONS

BA	Benzyl adenine
IBA	Indole 3 butyric acid
IAA	Indole 3 acetic acid
DMF	Dimethyl formamide
gus	β-D-Glucuronidase
M	Molar
μM	Micromolar
MS medium	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
npt	Neomycin phosphotransferase
PCR	Polymerase Chain Reaction
RT-PCR	Reverse transcription- Polymerase Chain Reaction
pH	Per Hydrogen
vir	Virulence genes
X-gluc	5-Bromo-4-chloro-3-indolyl β-D glucuronide
YEP	Yeast Extract Peptone
OD	Optical Density
ELISA	Enzyme Linked Immunosorbent Assay

Introduction

1. INTRODUCTION

Tomato is a major vegetable crop grown in almost every country of the world. In India, tomato has wider coverage, compared to other vegetables, especially during the last 20 years. The leading tomato growing states are Uttar Pradesh, Karnataka, Maharashtra, Haryana, Punjab, Bihar and Tamil Nadu. In Kerala the farmers are cultivating tomato in large scale in certain parts of the state. The quality of fruit picked in the garden when fully ripe far surpasses anything available on the market, even in season. The fruit is available in the market almost all the year around.

The tomato plant is a tender and warm-season perennial. A ripe tomato contains 94 per cent water, it has nutritive value, being a rich source of minerals, a good source of Vitamin A and B and an excellent source of Vitamin C. It is very appetizing, removes constipation and has a pleasing taste. It adds variety of colours and flavours to the food.

Diseases are the major constraints for the cultivation of tomato. In Kerala bacterial wilt and viral diseases are major problems. Kerala Agricultural University has released two bacterial wilt resistant varieties Sakthi and Mukthi suitable for cultivation in our agro climatic condition. The viral diseases are still a major threat for tomato cultivation. Viruses are known to occur and infect tomato at all stages of its growth. Its effects can be devastating. It is a constraint to increase in production. Among the viral diseases, the groundnut bud necrosis virus (GBNV) causing disease is a great problem, which is caused by a type member of the genus tospovirus. The tospo viruses are highly cosmopolitan group. Many viruses present in this genus have very wide host ranges and can infect groundnut, potato, tobacco, vegetables and ornamental plants.

The infection of the virus leads to drastic decrease in the production of tomato in our state. At present, the major share of requirement of tomato is met

from the neighbouring states. The reduction in yield due to viral disease stresses the need to evolve tomato varieties resistant to viral infection.

The present study aims at standardization of procedure for *in vitro* regeneration and efficient genetic transformation in tomato variety Mukthi using *Agrobacterium tumefaciens* for transferring desirable genes and isolation of coat protein gene of the GBNV. The study may help in evolving GBNV resistant varieties of tomato through pathogen derived genes in the long run.

Review of Literature

2. REVIEW OF LITERATURE

2.1 TOMATO TISSUE CULTURE

2.1.1 Response of different explants

2.1.1.1 Cotyledonary leaves

Regeneration of hypocotyl, cotyledon and leaf explants of tomato was reported from two week old seedlings germinated *in vitro* were cultured on agar solidified MS medium containing various concentrations and combinations of BA, kinetin and zeatin (Plastira *et al.*, 1996). Callus formation rate was 100 per cent from cotyledonary and hypocotyl explants. Pozueta *et al.* (2001) reported seedling explants, consisting of radicle, hypocotyl and cotyledonary leaves of tomato and bell pepper (*Capsicum annum*) were cultured on solid MS medium without growth regulators, explants of both species regenerated multiple shoots on the cut surface (2.9 - 5.3 shoots per explants for tomato and 1.2 - 2.2 for bell pepper cultivars). Gubis *et al.* (2003) studied shoot regeneration from aseptically grown hypocotyls and cotyledonary explants of different tomato cultivars Premium, Hana and UC 82. Among the different concentrations 1.0, 2.0 and 3.0 per cent of sucrose, glucose and maltose respectively, sucrose 3.0 per cent induced the highest number of shoots from both types of explants. Among the different cultivars Premium showed the best regeneration capacity (0.23 shoots per explant) when hypocotyls explants are used, whereas in cv. Hana the best regeneration was obtained when cotyledon was used as explant. Muthuvel *et al.* (2005) reported organogenic callus induction and plant regeneration using hypocotyl, leaf and cotyledonary segments of tomato as explants. Among the different forms of callus, only the green compact and nodular callus produced multiple shoots.

Cotyledon explants were cultured on liquid MS medium containing 10 g myo-inositol, 3 per cent sucrose, 0.1 mg l^{-1} IAA and 1 mg l^{-1} zeatin (Ichimura *et al.*, 1995). Cotyledon segments of tomatoes were cultured on MS liquid medium

containing 0.1 mg l^{-1} IAA, 1.0 mg l^{-1} zeatin and 3 per cent sucrose (Ichimura and Oda, 1998). Ren-ChunMei *et al.* (2002) reported that tomato cotyledons have more regeneration and increased survival rate after successful transformation. Gubis *et al.* (2004) reported that the regeneration frequency of cotyledons was from 67 to 100 per cent on the media supplemented with different BA and NAA concentrations and from 75 to 100 per cent on the medium supplemented with zeatin. Bhatia *et al.* (2005) reported direct shoot regeneration of tomato cv. Red Coat from cotyledonary explants on MS medium supplemented with 15 µM Zeatin.

2.1.1.2 Leaf segments

Padmanabhan *et al.* (1974) reported that morphogenesis of cultured leaf explants from three different tomato genotypes varied in their response to 64 combinations of IAA and kinetin. Callus proliferation began by eight to ten days and shoot formation occurs within 30 days. Coleman and Greyson (1977) observed that shoot and root formation from leaf callus was totally dependent on some external factors, particularly auxins. After continuous sub culturing Meredith *et al.* (1979) observed that the established callus show high shoot regeneration from callus at higher concentration of zeatin in combination with lower auxins level. Behki and Lesley (1980) found that the shoot regeneration is rapid when the callus derived from leaf bits in MS media coupled with 2, 4-D and BA, were transferred to a medium containing zeatin alone at higher concentration. Kurtz and Lineberger (1983) evaluated 12 cultivars of tomato leaf for regeneration using different combinations of IAA (0.0 - 2.0 mg l^{-1}) and BA (0.0 - 10 mg l^{-1}). They observed that morphogenic response were cultivar dependent.

Selvi and Khader (1993) cultured leaf disc of tomato cv. PKM-I leaf on half or full strength MS medium supplemented with various growth regulators, the best callus and shoot formation responses were observed on MS medium with 0.2 mg l^{-1} IAA and 2.5 mg l^{-1} BA. Leaf explants of tomato were cultured on agar - solidified MS medium containing various concentrations and combinations of BA, kinetin and zeatin. Appropriate combinations of plant growth regulators promoted

the formation of green, compact callus with numerous shoot primordia after 15-20 days. Callus growth continued and new shoot formation was observed for more than seven weeks (Plastira *et al.*, 1997). Mandal (1999) reported profuse callusing from leaf disc explants of tomato, within 10 to 12 days after inoculation. Calli derived from leaf disc were greenish yellow and relatively less nodular in appearance and showed maximum shoot regeneration in MS basal medium containing 2 mg l^{-1} BAP and 1mg l^{-1} NAA. Chandel and Katiyar (2000) reported that 0.5 cm^2 leaf explants of tomato produced callus. MS medium supplemented with 1.5 mg l^{-1} BAP and 1.5 mg l^{-1} IAA was the most responsive for formation of shoot buds from 8 to 12 week old callus culture. Soniya *et al.* (2001) inoculated the leaf explants of tomato var. Sakthi on MS basal medium. Green morphogenic calli were induced on leaf explants within three weeks of culture on MS medium supplemented with BA (8.9 µM) and Picloram (4.13 µM). Muthuvel *et al.* (2005) reported callus induction and plant regeneration hypocotyl, leaf and cotyledonary segments of tomato. Among the different forms of callus, only the green compact and nodular callus produced multiple shoots. More calli were obtained in MS medium supplemented with 2.0 mg l^{-1} IAA and 1.0 mg l^{-1} kinetin. The induced calli produced multiple shoots on the same medium after 3-4 weeks.

2.1.1.3 Nodal segments

Pongtongkam *et al.* (1993) reported the regeneration from nodal segments of tomato cultivars Seedat hip1, Seedat hip 91, VF-134-1-2 and Peto-86 on MS with 0.5 mg l^{-1} BA. Paranhos *et al.* (1996) reported regeneration from long nodal segments of tomato cvs. Empire and Monte Carlo on MS medium with 0.05, 0.1 and 0.2 mg l^{-1} NAA in combination with 0.5, 1.0 and 0.2 mg l^{-1} kinetin. Nodal segments of both cultivars regenerated plantlets.

2.1.2 Standardization of tissue culture media

2.1.2.1 Callus induction

Behki and Lesley (1976) observed that the combination of NAA and BA induced callus and shoot formation in tomato. Selvi and Khader (1993) reported

that when leaf disc, stem and shoot tip explants of tomato cultivar PKM-1 were cultured on half or full strength MS, the best callus induction were observed on MS with 0.2 mg l^{-1} IAA and 2.5 mg l^{-1} I BA.

Ali and Li (1994) cultured leaf explants of tomato and *L. pimpinellifolium* on MS medium in presence of growth regulators. They found that callus initiation was at greater rate and largest on MS medium supplemented with 3 mg l^{-1} zeatin or 0.6 mg l^{-1} 1AA and 2 mg l^{-1} BA in all genotypes and callus differentiated at the highest rate on MS medium supplemented with 0.6 mg l^{-1} IAA and 2 mg l^{-1} BA. Plastira and Perdikaris (1997) reported culturing of leaf explants of cvs. ACE 55 VF, Pakmor, San Marzano nano, San Marzano, Principe Borghese and Toper. Leaf explants from six week old green house grown explants were cultured in MS medium containing various concentrations and combinations of BA, kinetin and zeatin. Appropriate combinations of plant growth regulators promoted the formation of green, compact callus with numerous shoot primordia after 15 to 20 days. Jawahar *et al.* (1998) observed that when tomato (cv. PKM-1) cotyledonary explants were cultured on MS medium supplemented with different concentrations of NAA, IAA or 2,4-D or combinations of NAA and kinetin, white greenish and friable callus was induced on MS medium supplemented with NAA, IAA and 2, 4-D alone. White green compact callus was obtained in the presence of NAA and kinetin. Geetha *et al.* (1998) observed that callus were initiated from leaf explants of 10-15 day old seedlings of tomato cv. CO-1 after two weeks of culture on MS medium supplemented with different combinations of NAA (3 or 6 µM) and BA (2, 4, 6 or 8 µM).

The hypocotyl explants of *L. esculentum* cv. PKM were cultured on MS medium supplemented with different concentration and combinations of various auxins and cytokinins. Among the combinations used NAA (1 mg l^{-1}) and kinetin (0.1 mg l^{-1}) were found to be the best highest frequency callus induction (Vehkatachalam *et al.*, 2000). Soniya *et al.* (2001) reported calli were induced from leaf explants of tomato cv. Sakthi on MS medium supplemented with 8.88 µM BA and 4.13 µM picloram.

2.1.2.2. Organogenesis

After callusing, the regeneration occurs as a result of organogenesis which is stimulated by endogenously synthesized auxins and cytokinins or by exogenous addition of auxin and cytokinins to the regeneration medium. Cotyledons from 10 to 12 day old seedlings produced shoots on MS medium with 1 mg l^{-1} IAA and 1mg l^{-1} zeatin (Ye *et al.*, 1994). Jawahar *et al.* (1998) observed that when cotyledonary explants from tomato (cv. PKM-1) were cultured on MS medium, the maximum shoot regeneration was achieved on MS medium supplemented with NAA at 2.5 mg l^{-1} and kinetin at 1.5 mg l^{-1}. Bhatia *et al.* (2005) used MS medium supplemented with 15 µM zeatin for direct shoot regeneration of tomato cv. Red Coat from cotyledonary explants.

Padmanabhan *et al.* (1974) reported shoot initiation from the tomato leaf callus in the medium containing IAA and kinetin. Kartha *et al.* (1976) reported the regeneration of tomato from leaf explants of tomato on MS medium supplemented with 0.22 mg l^{-1} zeatin. Plastira and Perdikaris (1997) reported regeneration of tomato leaf explants of cvs. ACE 55 VF, Pakmor, San Marzano nano, San Marzano, Principe Borghese and Toper. Green compact callus with numerous shoot primordia was observed after 15 to 20 days. Capote *et al.* (2000) observed the effect of different culture media on the regeneration of leaf tissues of five lines, the best results were obtained with MS medium supplemented with 0.175 mg l^{-1} NAA and 1.5 mg l^{-1} BA. Veatachalam *et al.* (2000) reported that in *L. esculentum* cv. PKM.-1, BAP was found to be more suitable for maximum shoot bud differentiation as well as multiple shoot induction.

Fari *et al.* (2000) evaluated the regeneration capacity of the IPA-5 and IPA-6 Brazilian industrial tomato cultivars. In the case of IPA-5, the number of shoots was higher when the induction of shoot buds was accomplished in culture medium containing 2.5 mg l^{-1} BAP and 0.2 mg l^{-1} IAA followed by three subcultures in medium containing zeatin 0.5 mg l^{-1}.

Locy *et al.* (1983) obtained regeneration from stem bits using various concentrations of IAA ranging from 0.1 mg l^{-1} to 10.0 mg l^{-1}. Pongtongkam *et al.* (1993) cultured excised leaf and stem explants of five tomato cultivars cultured on various media, maximum regeneration was obtained on MS medium supplemented with 1 mg l^{-1} or 2 mg l^{-1} kinetin. Mandal (1999) reported that when somatic callus derived from stem and leaf explants of four tomato varieties, BWR-1, BWR-6 and PKM-1 were cultured on MS basal medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA. This medium was found to be the most efficient in generating maximum plantlets.

Cappadocia and Sree Ramalu (1980) used 0.05 mg l^{-1} BAP for the regeneration of tomato. Locy (1983) used a higher concentration of kinetin.10.0 mg l^{-1}. Kurtz *et al.* (1983) stated that the amount of callus produced and shoot regeneration efficiency of tomato explants is dependent on types and concentration of growth regulators in the culture medium, growth condition of donor plant, age of donor plant, explant of choice, portion and age of donor tissue and genotype of donor plant. Tomato cv. PKM-1 leaf disc, stem and shoot tip explants were cultured on half or full strength MS medium supplemented with various growth regulators. Selvi and Khader (1993) reported that shoot proliferation was high in MS medium supplemented with 0.2 mg l^{-1} IAA and 2.5 mg l^{-1} kinetin.

El-Farash *et al.* (1993) cultured hypocotyl and cotyledonary explants from 6, 12, 18 and 24 day old seedlings on MS medium containing 6 mg l^{-1} IAA and 5 mg^{-1} kinetin, and after 1 month callus and shoot formation rates were recorded. Callus formation rate was 100 per cent from cotyledonary and hypocotyl explants from 12, 18 and 24 day old seedlings.

Lich *et al.* (1996) reported the regeneration capacities of two tomato cvs. Potent and Rutgers and three accessions of wild tomato. Completely regenerated plants were obtained from all the tested species, but organogenesis occurred almost two weeks earlier in wild tomatoes than cultivated varieties. Chandel and Katiyar (2000) standardized media and cultural conditions for plant regeneration

from tomato *via.* embryogenesis and organogenesis. Effect of different concentrations and combinations of phytohormones (2, 4-D, kinetin, BAP, NAA and IAA) were also examined and plant lets were regenerated.

2.1.2.3 Rooting

Selvi (1993) reported rooting of tomato cv. PKM-1 on medium containing half strength MS with B5 vitamins and 2.0 mg l^{-1} IAA. According to Plastira and Pudikaris (1997) *in vitro* propagated shoots were rooted within seven to ten days on basal MS medium and grew into phenotypically normal plants when transferred to soil. Jawahar *et al.* (1997) observed regenerated shoots from hypocotyls and cotyledon explants of tomato cv. PKM-1. They rooted on MS medium containing 2.0 mg l^{-1} IBA. According to Geetha *et al.* (1998) elongated shoots of leaf bit explants produced shoots when sub cultured on medium supplemented with IBA (5μM). Mandal (1999) reported that half strength MS medium supplemented with 1.0 mg l^{-1} NAA was most effective in inducing rooting. Rooting of the regenerated shoots of PKM-1 was observed on half strength MS medium supplemented with IBA (0.1 to 0.5 mg l^{-1}) (Venkatachalam *et al.,* 2000). Soniya *et al.* (2001) reported rooting of tomato var. Sakthi on MS medium supplemented with 10.0 μM IBA.

2.1.3 Hardening and *Ex vitro* establishment

Direct transplanting of regenerated plants to non-aseptic conditions without prior hardening resulted in poor survival (Ziv *et al.*, 1970). Hu and Wang (1983) reported that light, temperature and relative humidity are the major factors to be controlled during acclimatization. A period of humidity acclimatization is required for newly transformed plants.

2.2 AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION

Agrobacterium tumefaciens is a soil-borne phytopathogenic bacteria that uses genetic engineering processes to subvert the host plant cell metabolic machinery. Parasitized cells are also induced to proliferate and the resulting crown gall tumour disease is a direct result of the incorporation of a region of transfer

DNA, T-DNA, from a large (150-250 kb) circular Ti (tumour inducing) plasmid, carried by *A. tumefaciens*, into the host plant genome. An understanding of this natural transformation process, together with the realization that any foreign DNA placed between the T-DNA border sequences can be transferred to plant cells, led to the construction of the first vector and bacterial strain systems for plant transformation (Hooykaas and Shilperoort, 1992).

Since the first record on a transgenic tobacco plant expressing foreign genes (Fraley *et al.*, 1983), great progress in understanding *Agrobacterium* mediated gene transfer at the molecular level has been achieved.

Agrobacterium tumefaciens naturally infects only dicotyledonous plants and methods for *Agrobacterium* mediated gene transfer into monocotyledonous plants were also developed (Smith and Hood., 1995).

Success of *Agrobacterium* mediated transformation has been reported to be dependent on the variety, type of explants, delivery system, *Agrobacterium* strain, conditions of co-cultivation, selection method and mode of regeneration (Mathis and Hinchee, 1994)

2.2.1 Agrobacterium Mediated Genetic Transformation of Tomato

Tomato (*Lycopersicon esculentum*) is one of the most important vegetable crop and a genetic model for improving other dicotyledonous crop plants (Mc Cormic *et al.*, 1986; Ling *et al.*, 1998). Therefore, the development of an efficient and genotype independent tomato transformation method is crucial. The first report on tomato transformation was by Mc Cormic *et al.* (1986). Since then there have been numerous publications of transformation in various species and cultivars of tomato (Chyi and Philip, 1987; Fillatti *et al.*,1987; Delannay *et al.*, 1989; Van Roekel *et al.*, 1993; Agharbaoui *et al.*, 1995; Fraay and Earle, 1996, Ling *et al.*, 1998; Tabaeizadeh *et al.*, 1999; Vidya *et al.*, 2000; Hu and Philips, 2001).

2.2.1.1 Explants for Genetic transformation

Agrobacterium mediated transformation is most widely used method of gene transfer in tomato. A wide range of explants are successfully transformed (Davis *et al.*, 1991; Oktem *et al.*, 1999; Pozurta Romero, 2001). Norova *et al.* (1991) reported *Agrobacterium* mediated genetic transformation of tomato.

Conditions for plant regeneration from explants of the tomato cv. UC82B were studied with a view to optimizing transformation procedure (Hamza and Chupeau, 1993). The best regeneration rate was obtained from Cotyledon explants from eight to ten day old seedlings. Zhang *et al.* (1999) used cotyledons of 5 tomato cultivars for genetic transformation of *ipt* gene to tomato and regeneration of transgenic plants. Cotyledonary leaves of tomato cv. pusa Ruby were transformed by co-cultivation with *Agrobacterium tumefaciens* strain LBA 4404 (Vidya *et al.*, 2000). Cotyledon explants from *Lycopersicon esculentum* cv. UC82B were infected by *Agrobacterium tumefaciens* strain LBA 4404 harbouring the neomycin phosphotransferase (*npt II*) reporter gene (Cortina *et al.*, 2004). Park *et al.* (2003) reported an efficient method to transform five cultivars of tomato Micro Tom, Red cherry, Rubion, Piedmont and E6203. A comparison was made of leaf, cotyledon and hypocotyls explants.

Lipp and Brown (1993) reported *Agrobacterium* mediated transformation in root of tomato. Fray and Earle (1996) developed an improved protocol for *Agrobacterium* mediated transformation of tomato by examining the factors like explant size, explant orientation and gelling agent on the efficiency of transformation.

2.2.1.2 Strain specificity

Agrobacterium mediated transformation had been successful with a broad range of dicotyledonous plants and few monocotyledons. There are difference in the susceptibility between species and even between cultivars and genotype of the species. The efficiency of transformation is depending upon the strain used for

transformation. The strain harbouring good selectable marker should be best for the transformation.

Hoekema *et al.* (1983) reported *Agrobacterium* mediated transformation in five tomato (*Lycopersicon esculentum* Mill.) cv. Micro Tom, Red cherry, Rubion, Piedmont and E6203 by using *Agrobacterium tumefaciens* strain LBA 4404. Cotyledonary explants of tomato cultivar UC82B were inoculated with *Agrobacterium* strain LBA 4404 and transformants were selected (Murray *et al.*, 1998). Cotyledonary leaves of tomato cv. pusa Ruby were transformed by *Agrobacterium tumefaciens* strain LBA 4404 (Vidya *et al.*, 2000). Tomato transformation was analysed from cotyledon explants from *Lycopersicon esculentum* cv. UC82B by *Agrobacterium tumefaciens* strain LBA 4404 harbouring *npt II* gene (Cortina, 2004).

2.2.1.2.1 Bacterial density

Concentrations of bacterial cells in the induction medium is an important factor for efficient transformation. Very low density of bacterial population could lead to ineffective transformation, where as very high density may lead to necrosis and death of explants. So optimum density is essential for efficient transformation.

Hoekema *et al.* (1983) reported that *Agrobacterium tumefaciens* was grown for two days in YEP medium containing appropriate antibodies at 28°C on a rotary shaker (220 rpm) until an OD_{600} of 1.0 was obtained. Murray *et al.* (1998) reported that the transformation efficiency was high and tomato transformation was best if cell density was between 20 and 390 x 106 cells ml^{-1}.

A single colony of *Agrobacterium tumefaciens* was taken from an agar plate and grown in 10 ml Luria-Bertani (LB) liquid medium containing 50 mg l^{-1} ampicilin at 28°C, overnight. Bacterial cells were then collected by centrifugation at 1500 g for 5min at room temperature and resuspended in liquid MS medium to a final OD_{600} of approx 0.3 (Ouyang *et al.*, 2005). Park, S.M (2005) reported transformed *Agrobacterium* grown to log phase in YEP liquid medium (OD_{600} 0.7

– 0.9) was centrifuged at 3500 rpm for 10 min and the pellets so obtained were resuspended in MS medium.

2.2.1.3 Co-cultivation

The explants used for transformation is exposed to the bacterial culture media in the induction with optimum cell density. Both the composition of the media and the time of induction will decide the efficiency of transformation. MS medium is used for the induction, segregation and culturing of bacteria. For inoculation, the explants are immersed in induction medium for a specific period of time, which depends on bacterial population, type of vector and explant used for transformation.

Park *et al.* (2003) reported transformation methodology using explants cultured on MS media with growth regulators and three days of co-cultivation with the *Agrobacterium* on this same medium.

Tomato explants were isolated and cultured onto the precultured media for one day. At the end of the day, the explants were dipped in an *Agrobacterium* culture, blotted and recultured on the same media for a three day co-cultivation period. The explants were then transferred to fresh media (Park, S.H, 2003). Ouyang *et al.* (2005) reported two days of co-cultivation of tomato cotyledons on MS medium supplemented with 0.2 mg l^{-1} zeatin and 1.0 mg l^{-1} IAA showed high efficiency of transformation.

2.2.1.4 Use of Acetosyringone during transformation

Transformed root clones of tomato treated with 20 µM acetosyringone and untreated controls also were selected on kanamycin medium. Root clones derived from acetosyringone treatment grew more vigorously in the presence of kanamycin. It was concluded that acetosyringone treatment enhanced the transformation process, possibly by stimulating multiple insertions of the T-DNA into the host genome (Norova *et al*, 1993). Transformed root clones of explants treated with 20 µM acetosyringone and untreated controls of cv. Ailsa Craig were selected on kanamycin medium and the presence of the *nptII* gene (Joao and

Brown, 1993). Murray *et al.* (1998) compared two treatments before and during *Agrobacterium* co-cultivation, using acetosyringone at various concentrations (0, 20, 50 and 100 mM). Acetosyringone treatments of 50 and 100 mM produced more shoot regeneration. Using the 50 mM acetosyringone treatment, it was found that tomato transformation was best. Cortina *et al.* (2004) reported that when the phenolic acetosyringone was present in the co-culture medium at 200 µM, concentration confirmed transgenic lines reached 50 per cent of antibiotic resistant shoots and the transformation efficiency reached 12.5 per cent.

2.2.1.5 Elimination of bacteria after co-cutivation

Manoharan *et al.* (1998) used 400 mg l^{-1} cefotaxime for killing the *Agrobacterium tumifacience* after transformation of tomato. Maneekard *et al.* (2003) reported that the transformed cotyledon explants of tomato were cultured on MS medium containing various concentrations of either cefotaxime or timentin (0, 100, 250, 500 and 1000 mg l^{-1}) .These antibiotics can suppress *Agrobacterium* growth very effectively and promote the regeneration of transformed tomato cells into mature plantlets simultaneously.

2.2.1.6 Selection of transformed cells

Fari *et al.* (1995) developed protocol for plant regeneration and genetic transformation from cotyledons of *in vitro* grown seedlings of tomato cv. Kecskemeti lila. After co-cultivation with *Agrobacterium tumefaciens* strain C58C1 RifR harbouring a multifunctional vector carrying *nptII* and *gus* genes, transformants were selected on media containing 100 mg l^{-1} kanamycin. Yasseen *et al.* (1998) cultured the explants (cotyledonary leaves and hypocotyls) from tomato cultivars VF36, Beef Master, Money Maker, Lazy and Craig Alisa on MS medium supplemented with 1.5 mg l^{-1} BA, 0.5 mg l^{-1} IAA and 50 mg l^{-1} kanamycin per for selection for four to six weeks. Vidya *et al.* (2000) cultured the cotyledonary explants after transformation on regeneration medium supplemented with 50 mg l^{-1} kanamycin and 400 mg l^{-1} cefotaxime. After selection the putative transformants were transferred to a rooting medium containing 25 mg l^{-1} kanamycin, where successful rooting was obtained.

Manoharan *et al.* (1998) selected the transformed cotyledonary leaves of chilly in regeneration medium containing 50 mg l^{-1} kanamycin and 400 mg l^{-1} cefotaxime. The *nptII* gene, driven by the nopaline synthase promoter and terminator sequences, that provides resistance to kanamycin, was used as a selectable marker.

2.2.1.6 Histochemical GUS Expression

Some reporter gene products can be detected in intact plant tissue. The most popular of these systems is the *E.coli gus* gene. It encodes a stable enzyme that is not normally present in plants and that catalyses the cleavage of a range of β- glucoronidase. The *gus* activity in transformed plant tissue can be localized by observing the blue colour that is formed after hydrolysis of the uncoloured substrate 5- bromo 4-chloro 3-indolyl β-D-glucoronic acid. Hamza and Chupeau, (1993) observed that *gus* activity appeared specifically at the cut surface cotyledonary leaves of tomato. They found that sub epidermal cells were more susceptible to transformation than epidermal cells. Murray *et al.* (1998) developed an improved tomato transformation protocol using *A. tumefaciens* LBA 4404 (p35S GUS INT), which regenerated putative transgenic tomato plantlets. PCR analysis and a GUS assay showed incorporation and expression of the *gus-intron* marker gene.

Manoharan *et al.* (1998) reported that in chilly, the transgenic nature of the regenerated plants was confirmed by the histochemical staining of *gus.*

2.3 IMMUNODETECTION AND CHARECTERISATION OF GBNV

Avila *et al.* (1990) conducted serological differentiation of 20 isolates of tomato spotted wilt virus. The results indicated that, ELISA employing polyclonal and monoclonal antisera could be used to differentiate TSWV isolates and to detect defective forms. Resende *et al.* (1991) found that, the virus could efficiently be detected in high dilutions of sap from infected plants, at low concentration of purified virus and nucleocapsid protein preparations in cocktail ELISA and DAS ELISA. Porter *et al.* (1991) reported TSWV in peanuts and virus

identification was confirmed by standardized ELISA. Disease symptoms in the field and serological tests (ELISA) were used to determine the incidence of diseases Wongkaew and Chuapong (1995). Cho *et al.* (1986) reported ELISA as a method of choice for diagnosis and detection of tospo viruses and thrips in plants. Serological differentiation of Irish yellow spot virus (IYSV) from other tospo viruses was proved using DAS- ELISA (Cortes *et al.*, 1998). Singh *et al.* (1996) suggested ELISA tests for TSWV-W is serologically related to the TSWV-W isolate from Taiwan, but not to tomato spotted wilt tospovirus (TSWV) or impatiens necrotic spot tospovirus (INSV). Xia *et al.* (1998) developed an ELISA for detection of watermelon silver mottle virus and groundnut bud necrosis virus.

The sunflower tospovirus showed positive serological relationship with groundnut bud necrosis virus (GBNV) and watermelon silver mottle virus (WSMV) in direct antigen-coated (DAC)-ELISA using polyclonal antisera directed against nucleocapsid protein (Jain *et al.*, 2000). Bhat *et al.* (2001) tested the tospovirus in black gram, cowpea, green gram and soyabean with seven different tospovirus antisera against these isolates. In direct antigen-coated ELISA, positive reactions were seen only with groundnut bud necrosis virus (GBNV) and watermelon silver mottle virus (WSMV). Umamaheswaran *et al.* (2003) reported that the host range studies indicated that the tomato tospovirus isolate was closely related to groundnut bud necrosis virus (GBNV). Sequence analyses of nucleocapsid protein (N) gene showed that the amino acid sequence identity with GBNV was 96 per cent.

2.4 MOLECULAR CHARACTERIZATION OF GROUND NUT BUD NECROSIS VIRUS BY RT-PCR

Pappu *et al.* (1993) performed RT-PCR using the primer pair derived from the nucleocapsid protein (N) gene sequence of groundnut bud necrosis virus. Cortes *et al.* (1998) purified the nucleocapsid of tospo virus (Irish yellow spot virus (IYSV) and physalis severe mottle virus (PSMV)) and amplification of the full length sRNA using PCR. Eirans *et al.* (1998) developed a fast and sensitive molecular detection method for universal tospo virus detection. Jain *et al.* (2004)

was cloned and sequenced the nucleocapsid protein (N) gene of the tospovirus causing potato stem necrosis disease. Sequence analyses showed that the N gene was most closely related to groundnut bud necrosis virus (GBNV). The amino acid sequence identity with GBNV was 98-99 per cent, confirming that the potato stem necrosis disease is caused by a strain of GBNV.

Materials and Methods

3. MATERIALS AND METHODS

The experiments on *Agrobacterium* mediated genetic transformation of tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against *groundnut bud necrosis virus* were carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004 to 2006.

3.1 TISSUE CULTURE OF TOMATO

3.1.1 Variety

Tomato (*Lycopersicon esculentum* Mill.) variety Mukthi released from Kerala Agricultural University was used for the study. The seeds were collected from the Department of Olericulture, College of Horticulture, Vellanikkara.

3.1.2 Type of Explants used

Cotyledonary leaves, leaves and nodes were used as explants. They were collected from both *in vitro* and *ex vitro* established plants.

3.1.3 Surface Sterilization

Explants were surface sterilized with 0.08, 0.09 and 1.0 per cent mercuric chloride for seven minutes or sodium hypochlorite one per cent for 10, 15 and 20 minutes, followed by three washes with sterile distilled water.

3.1.4 Culture media

3.1.4.1 Preparation of medium

Standard procedures (Murashige and Skoog, 1962) were followed for the preparation of the plant tissue culture media. After mixing appropriate quantities of the stock solutions and making up the volume using double distilled water, the pH of the medium was adjusted to 5.7 using 0.1 N NaOH/HCl . Agar was added at the rate of 6.3 g l^{-1} and the medium was heated to mix agar. Then the medium was dispensed to the culture vessels at the rate of 15 ml culture tube. The test tubes were plugged with non absorbent cotton. Medium was sterilized by

autoclaving at 121 °C and 1.06 $kgcm^{-2}$ pressure for 20 minutes to sterilize the medium. It was allowed to cool to room temperature and stored in culture room until used.

3.1.4.2 Standardization of Media for Callusing and Regeneration

MS medium (Murashige and Skoog, 1962) with three different combinations of BA and IAA were used for callusing and regeneration. The composition of MS media is given in Appendix I.

The compositions of different media used for callusing and regeneration of tomato were listed below.

Treatments:

T-1: MS + 1.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA

T-2: MS + 2.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA

T-3: MS + 2.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA

T-4: MS + 1.5 mg l^{-1} BA + 1.0 mg l^{-1} IAA

T-5: MS + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA

T-6: MS + 2.5 mg l^{-1} BA + 1.0 mg l^{-1} IAA

T-7: MS + 1.5 mg l^{-1} BA + 1.5 mg l^{-1} IAA

T-8: MS + 2.0 mg l^{-1} BA + 1.5 mg l^{-1} IAA

T-9: MS + 2.5 mg l^{-1} BA +1.5 mg l^{-1} IAA

Leaf, node and cotyledonary leaf explants were cultured on regeneration media for 16 h at 26 °C in a plant growth chamber. After 2-3 weeks of culturing, the explants were sub cultured on same regeneration medium at 15 days interval.

3.1.5 Induction Medium for Rooting

Half strength MS basal medium supplemented with 1.0 mg l^{-1} IBA was used for root induction. Shoots having 4-5 cm height alone were used for rooting.

3.1.6 *Ex vitro* Establishment

The *in vitro* rooted plantlets having normal growth were taken out with the help of forceps. The agar adhering to the roots were completely removed by thorough washing with running tap water. The plantlets were then treated with Indofil M-45 (0.3 per cent) solution for 30 min before planting out. Then they are planted out in small pots containing sterile sand. The percentage of survival was recorded after two weeks.

3.2 AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION OF TOMATO

3.2.1 Source of Explants

The *in vitro* raised callus from cotyledonary leaves were used as explants for transformation experiment.

3.2.2 Culture Media

3.2.2.1 Chemicals

All the chemicals used for the preparation of the culture media were of analytical grade and procured from Sisco Research Laboratories (SRL) India. The antibiotics and plant growth substances were obtained from Himedia laboratories, India.

3.2.2.2 Glass ware, Plastic ware and Other items

Borosilicate glass ware of Borosil brand and disposable sterile petri dishes of Tarsons and membrane filters from Sartories, Germany were used for the experiments.

3.2.2.3 Composition of Media

Basal MS medium (Murashige and Skoog., 1962) and modification of the media supplemented with various plant growth substances were used for plant tissue culture experiments. Yeast extract peptone (YEP) medium (An *et al.*, 1998) and AB minimal medium were used for bacterial culture during the study. The composition of these media were given in Appendix I and II

3.2.2.4 Preparation of Media

Standard procedures (Murashige and Skoog, 1962) were followed for the preparation of the plant tissue culture media. For bacterial culture, the pH of the medium was adjusted to 5.7. Solidification of the medium was done using agar at the rate 20 gl^{-1}. The medium was prepared in conical flask and stored in culture room, after sterilization.

3.2.2.5 Preparation of Stock Solutions

3.2.2.5.1 Kanamycin

The stock solution was prepared at 10^4 mg l^{-1} concentration. The required quantity of kanamycin was weighed and dissolved in sterile water. Then it was filter sterilized.

3.2.2.5.2 Cefotaxime

The 10^4 mg l^{-1} concentrations of cefotoxime were prepared by dissolving it in sterile water and then filter sterilized.

3.2.2.6 Agrobacterium strain

Agrobacterium strain LBA4404 containing binary vector pCAMBIA 2301 was used for transformation.

3.2.2.6.1 pCAMBIA2301 (Hajdukiewicz *et al.*, 1994)

The T-DNA of this binary vector contains the *gusA* gene and the *nptII* (kanamycin resistance) gene under the control of CaMV35 S promoter. Sequance data of the vector is given in Appendix IV.

3.2.2.6.2 Maintenance of *Agrobacterium* strain

The *Agrobacterium* strains were maintained in plates and as stab culture in YEP medium. For long term storage it was maintained in glycerol stock.

3.3 INOCULATION AND OTHER ASEPTIC MANIPULATION

All the aseptic measures were carried out under laminar air flow cabinet (Thermodyne) fitted with UV lamp. Sterile forceps and loops are used for transferring the explants and bacteria.

3.4 CULTURE CONDITIONS

The cultures were incubated in culture racks at 26 °C in an air conditioned culture room with 16 hr photoperiod (1000 lux) supplied by cool white florescent tubes. The humidity in the culture room varied between 60 and 80 per cent.

3.5 PRODUCTION OF CALLUS

The explants, for callusing, were collected from *in vitro* raised seedlings. Cotyledonary leaves, leaves and internodes were used as explants. These explants were inoculated on MS medium, supplemented with different concentrations of BA and IAA. The cultures were incubated in the culture room, in darkness. Observation on callus initiation and number of days taken for callusing were recorded.

3.6 REGENERATION OF CALLUS

The regeneration of callus was also obtained from the MS medium supplemented with different concentrations of BA and IAA. Sub culturing of the callus was done periodically and kept in culture room.

3.7 EVALUATION OF TOMATO CULTURES FOR SENSITIVITY TO ANTIBIOTICS

Sensitivity of tomato cultures to antibiotics was evaluated to utilize it as a selectable marker system for the selection of transformants. The tomato calli were tested for antibiotics like kanamycin and cefatoxime. The culture response to different concentrations of kanamycin (5, 10, 25, 50, 75, 100 and 150 mg l^{-1}) and cefotaxime (5, 10, 25, 50, 75, 100 and 150 mg l^{-1}) was evaluated at weekly intervals.

3.8 SCREENING OF AGROBACTERIUM STRAIN FOR SENSITIVITY TO ANTIBIOTICS

Screening of *Agrobacterium* strain for sensitivity to antibiotics was essential for the selection of transformants and removing the bacteria after co-cultivation. This screening was done in YEP medium supplemented with different concentrations of kanamycin (5, 10, 25, 50, 75, 100, 150, 200, 300, 400, 500mg l^{-1}) and cefotaxime (5, 10, 25, 50, 75, 100, 150, 200, 300, 400, 500 mg l^{-1}) and the growth of bacteria was recorded within two days.

3.9 GENETIC TRANSFORMATION OF TOMATO

3.9.1 Preparation of *Agrobacterium* Strains for Co-cultivation

The *Agrobacterium* strain was grown in AB minimal medium in sterile Petri plates. They were kept in culture room for allowing the growth of bacteria. For preparing *Agrobacterium* suspensions, a single colony from the plate was picked up and inoculated in 1ml AB liquid medium containing required antibiotics. This liquid medium was kept in a shaker for overnight at 28°C. Then the bacterial suspension was spun in a centrifuge at 5000 rpm at 4°C for 5 min. The pellet obtained was resuspended in half strength 1ml MS medium with 200 μM acetosyringone.

3.9.2 Preparation of Plant Material

The cotyledonary leaves were used as explants for the transformation experiment. These explants were precultured fifteen days before co-cultivation for callusing. This newly produced callus in active cell division was used for co-cultivation. Ten to fifteen explants were co-cultured in a single petri plate.

3.9.3 Infection Process

The explants were taken from the culture plate and cut into small pieces by sterile knife. These explants were mixed thoroughly with the prepared *Agrobacterium* suspension by gentle swirling for 10 to 20 minutes. This may facilitate the infection process of *Agrobacterium* on the explants.

3.9.4 Co-cultivation

After infection, *Agrobacterium* strain with the binary vector and the plant tissues were co-cultivated in dark for two, three, four and five days at 26 °C in a culture room

3.9.5 Incubation on bacteriostatic medium

After co-cultivation the tissues were washed in half strength liquid MS medium containing 75 mg l^{-1} cefotaxime. Tissues were blot dried on sterile filter paper and were transferred to half strength MS medium with supplemented with 75 mg l^{-1} cefotaxime for elimination of excess *Agrobacterium*. This plate was kept in dark for five days.

3.9.6 Selection of Putative Transformants

The transformed tissues were selected on MS medium containing 100 mg l^{-1} kanamycin. The bacteriostatic agent cefotaxime 75 mg l^{-1} was also added in the medium for complete eradication of bacteria. Tissues were kept in the selection media for four weeks. Only the transformed tissues will survive in the media and the untransformed tissues will be bleached.

3.10 GUS HISTOCHEMICAL ASSAY

Jafferson *et al.* (1987) reported a histochemical assay to detect the expression of the *gus* gene (β- Glucuronidase). The transformed tissues were incubated in GUS substrate X-gluc for 24 h at 37°C in dark. The X-gluc was prepared in 100μM dimethyl formamide, 50mM sodium phosphate, 0.1% (v/v) Triton X100. X-gluc is a well established chromogenic substrate used for measuring gus activity. The transient expression of the *gus* gene was visualized 24 to 30 hours after staining the transformed tissues for GUS activity. The enzyme converts this substrate to an insoluble, intense indigo blue chromophore. The GUS expressions in transformed cells were detected as blue spots on the explants under microscope. Each spot was scored as one transformation event, regardless of its size (Puddephat *et al.*, 1999).

3.11. IMMUNODETECTION OF THE TOSPO VIRUS IN TOMATO

Direct antigen coating – Enzyme Linked Immunosorbent Assay (DAC-ELISA)

ELISA for the detection of ground nut bud necrosis virus in Tomato was done. Antigen was obtained from diseased samples from the field. The serodiagnosis of the virus was carried out following procedure described by Hugenot *et al.* (1992).

The healthy and diseased leaf samples were ground separately in coating buffer (Carbonate buffer) in the ratio 1:5 (w/v). The homogenate obtained after grinding, was centrifuged at 5000 rpm for 10 min at 4°C. 100 µl of this homogenate was added to the wells in the ELISA plate. After incubation for 2 hours at 37°C, the wells were washed with PBS-T, 3 times each for duration of 3 min by using ELISA plate washer. Blocking was done with 100µl of 1 per cent Bovine Serum Albumin (BSA) for 30 min at 37°C. After incubation blocking agent was removed, plates were washed with PBS-T as before. Antibody specific for tospo virus was used. The antibodies at 1:250, 1:500, 1:1000 dilutions in PBS-TPO were added and incubated over night at 4°C. The plates were washed with PBS-T and treated with 100µl of alkaline phosphatase conjugated anti-rabbit immunoglobulin, diluted in PBS–T (10^{-4}) and incubated for 2 hrs at 37°C. Wells were washed with PBS-T as before. The substrate *para*- nitro phenyl phosphate (p-NPP) in diethanol amine buffer (1mg ml^{-1}) was added to each well (100 µl/ well) and incubated for 1 hr at 37°C. Reaction was stopped by adding 500 µl of 4% sodium hydroxide. The absorbance was read at 405 nm in an ELISA reader (ECIL, MS5608).

3.12. MOLECULAR CHARACTERIZATION OF THE TOSPO VIRUS IN TOMATO

3.12.1 RNA Isolation

Total RNA from the infected tissues (10 mg) was extracted using RNA isolation kit of M-BIO according to the manufacturer's instructions and was used as a template in the reverse transcription - polymerase chain reaction (RT-PCR).

3.12.2 PCR amplification

RT-PCR was carried out using the RT-PCR kit (Bangalore GENEI, KT-24). Amplification was performed in an automated thermal/cycler (PTC 150, M.J. Research).

The cDNA synthesis was performed at 42 °C for one hour. PCR amplification of the cDNA was carried out using the primer pair derived from the N gene sequence of GBNV. The genome sense primer, 5'ATGTCTAACGT(C/T) AAGCA (A/G) CTC 3' was derived from the beginning of first 21 bases of the coding region. The genome antisense primer 5'TTACAATTCCAGCGAAGGACC 3' represented the last 21 bases of the coding region of N gene. PCR programme includes, one cycle of 94 °C for 2 minutes for denaturation, 1 minute of annealing at 56 °C and 1 minute of extension at 72 °C followed by 1 cycle of final extension for 60 minutes at 72 °C.

The PCR products were analysed in 1.0 per cent Agarose gel. The amplicon (800bp) was visualized and documented using gel-documentation system (BIO-RAD). 1kb DNA ladder was used as molecular weight marker.

Results

4. RESULTS

Investigations on *in vitro* regeneration and *Agrobacterium* mediated transformation in tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against groundnut bud necrosis virus were carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004 to 2006. The results of the study are presented in this chapter.

4.1 ESTABLISHMENT OF *IN VITRO* CULTURE OF TOMATO

4.1.1 Surface Sterilization

Surface sterilization of cotyledonary leaves, leaves and nodal segment were tried with mercuric chloride for seven minutes and sodium hypo chlorite for 15 minutes (Table 1).

In the case of cotyledonary leaves, surface sterilization using mercuric chloride at a concentration of 0.08 per cent for seven minutes was found to be most effective and gave a survival per cent of 96.23. The other treatments like concentrations at 0.09 and 0.1 per cent mercuric chloride for seven minutes, the survival per cent were 87.5 and 72.03, respectively. The survival per cent of 75.68 was obtained when the treatment was carried out at one per cent sodium hypo chlorite for 15 minutes, while surface sterilization with sodium hypo chlorite at 0.8 and 1.2 per cent for 15 minutes gave 45.9 and 35.8 per cent of survival.

When leaves were used as explants 95 per cent of survival was obtained using the treatment with mercuric chloride at a concentration of 0.08 per cent for seven minutes. The other treatments using 0.09 and 0.1 per cent for seven minutes gave the survival per cent of 83.3 and 70.2, respectively. The treatment of leaves with a concentration of one per cent sodium hypo chlorite for 15 minutes gave a survival per cent of 82.3. The survival per cent obtained with sodium hypo

chlorite at a concentration of 0.8 and 1.2 per cent for 15 minutes was 62.5 and 72.03, respectively

The highest culture establishment of 90.05 per cent from nodes was recorded using mercuric chloride at a concentration of 0.08 per cent for seven minutes. Concentration of 0.09 and 0.1 per cent of mercuric chloride for seven minutes showed a decreased survival per cent of 87.5 and 70.1 respectively. A survival per cent of 89.12 was observed when one per cent of sodium hypo chlorite for 15 minutes was given. A lower survival per cent of 60.7 and 75.0 was recorded when nodes were treated with 0.8 and 1.2 per cent sodium hypo chlorite respectively, for 15 minutes.

4.1.2 Standardization of Medium for *in vitro* Establishment of tomato

Nine treatments involving three different combinations of IBA and IAA were tried to study their effect on callusing and shoot proliferation from cotyledonary leaves, leaves and nodal segments (Table 2, Table 3 and Table 4) and (Plate 1-10).

Treatments T-1, T-4, T-7 with 1.5 mg l^{-1} BA and varying concentration of IAA (0.5 mg l^{-1}, 1.0 mg l^{-1}, 1.5 mg l^{-1}) gave the callus induction per cent of 49.04, 47.92 and 46.80, respectively with in a period of 16.86, 17.66 and 18.47 days. The regeneration per cent was 68.06, 66.20 and 66.34 in duration of 24.12, 24.97 and 25.46 days, respectively.

Treatments T-2, T-5, T-8 with a BA concentration of 2 mg l^{-1} along with varying concentration of IAA (0.5 mg l^{-1}, 1.0 mg l^{-1}, 1.5 mg l^{-1}) was tested. Among these T-5 with BA concentration of 2 mg l^{-1} and IAA concentration of 1.0 mg l^{-1} recorded the highest callus induction (78.08%) within a short period of 14.02 days. The regeneration per cent of 78.00 was obtained from the same medium within 21.52 days. But in the case of T-2 and T-8 the per cent of callus induction was 66.33 and 62.16, respectively within a period of 15.42 and 16.08 days. The regeneration per cent recorded from these treatments was 66.72, 62.78 within a time period of 21.44 and 24.04 days.

Table 1. Effect of surface sterilents on different explants of tomato

Sl.No.	Explants	Surface sterilents	Concentration (%)	Duration of treatment (min)	Survival (%)
1	Cotyledonary leaves	1. $HgCl_2$	0.08 0.09 0.10	7 7 7	96.23 87.50 72.03
		2.NaOCl	0.80 1.00 1.20	15 15 15	45.90 75.68 35.80
2.	Leaves	1. $HgCl_2$	0.08 0.09 0.10	7 7 7	95.00 83.30 70.20
		2. NaOCl	0.80 1.00 1.20	15 15 15	62.50 82.30 72.03
3.	Nodes	1. $HgCl_2$	0.08 0.09 0.10	7 7 7	90.05 87.50 70.10
		2. NaOCl	0.80 1.00 1.20	15 15 15	60.70 89.12 75.00

Treatments T-3, T-6, T-9 with combinations of 2.5 mg l^{-1} BA and varying concentration of IAA (0.5 mg l^{-1}, 1.0 mg l^{-1}, 1.5 mg l^{-1}) gave the callus induction per cent of 62.74, 64.14, 65.54 respectively within a duration of 15.06, 15.76 and 16.46 days, while the regeneration per cent was 78.08, 79.42 and 78.66, respectively within a period of 22.47, 21.02 and 22.08 days.

This result revealed that T-6 with a combination of 2.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA is the best treatment for the regeneration from cotyledonary leaves.

The effect of different concentration of BA and IAA were also tried on the *in vitro* regeneration of tomato from leaves.

The treatments like T-1, T-4, T-7 recorded the callus induction per cent of 38.47, 64.44 and 62.72, respectively within a period of 19.72, 18.33 and 18.46 days. Treatment T-1 showed the least per cent of callus induction. The shoot regeneration per cent of T-1, T-4, T-7 was obtained as 24.86, 54.72 and 60.47, respectively within a time limit of 23.48, 25.46, and 25.02 days.

T-2, T-5, T-8 showed the per cent of callus induction as 42.82, 68.00, 40.44 within a time period of 17.76, 16.42 and 17.08 days. The per cent regeneration from the same treatments was 40.42, 62.04 and 42.46, respectively within 24.72, 22.14 and 23.86 days. This result revealed that T-5 is the best medium for callus induction.

T-3, T-6, T-9 gave the callus induction per cent of 48.96, 54.02 and 52.66, respectively within a period of 17.84, 16.58 and 17.92 days and the regeneration per cent of 58.06, 64.66 and 60.06 were obtained within 22.42, 21.07 and 21.64 days. Among these nine treatments T-6 with 2.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA showed the maximum regeneration per cent (64.60) within a short period of 21.07 days.

The nine treatments were tried with nodal segments also. The T-1, T-4 and T-7 gave percentage of regeneration of 28.62, 32.46 and 35.48, respectively within a time limit of 19.14, 18.04 and 18.86 days. The treatments T-2, T-5 and T-8 recorded the regeneration percentage of 52.08, 51.68 and 49.42, respectively in

Table 2. Effect of Plant growth regulators on *in vitro* regeneration of tomato from cotelydonary leaves.

Treatment No.	Plant growth regulator (mgl^{-1})		Days for callusing	Survival of callus (%)	Days for shoot initiation	Survival of shoots (%)
	BA	IAA				
T1	1.5	0.5	16.86	49.04	24.12	68.06
T2	2.0	0.5	15.42	66.33	21.44	66.72
T3	2.5	0.5	15.06	62.74	22.47	78.08
T4	1.5	1.0	17.66	47.92	24.97	66.20
T5	2.0	1.0	14.02	78.08	21.52	78.00
T6	2.5	1.0	15.76	64.14	21.02	79.42
T7	1.5	1.5	18.47	46.80	25.46	66.34
T8	2.0	1.5	16.08	62.16	24.04	62.78
T9	2.5	1.5	16.46	65.54	22.08	78.66

Culture medium: MS

Table 3. Effect of plant growth regulators on *in vitro* regeneration of tomato from leaves.

Treatment No.	Plant growth regulator (mgl^{-1})		Days for callusing	Survival of callus (%)	Days for shoot initiation	Survival of shoots (%)
	BA	IAA				
T1	1.5	0.5	19.72	38.47	23.48	24.86
T2	2.0	0.5	17.76	42.82	24.72	40.42
T3	2.5	0.5	17.84	48.96	22.42	58.06
T4	1.5	1.0	18.33	64.44	25.46	54.72
T5	2.0	1.0	16.42	68.00	22.14	62.04
T6	2.5	1.0	16.58	54.02	21.07	64.66
T7	1.5	1.5	18.46	62.72	25.02	60.47
T8	2.0	1.5	17.08	40.44	23.86	42.46
T9	2.5	1.5	17.92	52.66	21.64	60.02

Culture medium: MS

Table 4. Effect of plant growth regulators on *in vitro* regeneration of tomato from nodal segments.

Treatment No.	Plant growth regulator (mgl^{-1})		Days for shoot initiation	Survival of shoots (%)
	BA	IAA		
T1	1.5	0.5	19.14	28.62
T2	2.0	0.5	16.74	52.08
T3	2.5	0.5	15.45	50.12
T4	1.5	1.0	18.04	32.46
T5	2.0	1.0	16.74	51.68
T6	2.5	1.0	14.72	74.62
T7	1.5	1.5	18.86	34.48
T8	2.0	1.5	17.46	49.42
T9	2.5	1.5	15.68	58.74

Culture medium: MS

Plate1, plate2 and Plate3: Callus induction from cotyledonary leaves of tomato

Plate4, Plate5 and Plate6: Regeneration from cotyledonary leaves of tomato

Plate 1

Plate 2

Plate 3

Plate 4

Plate 5

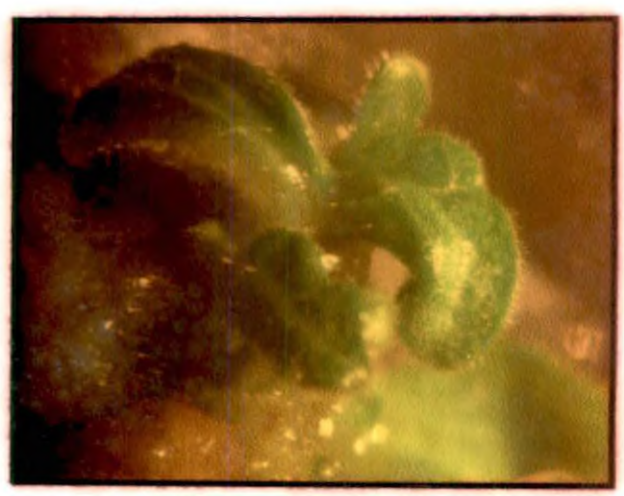


Plate 6

Plate7, plate8, Plate9 and Plate10: Regeneration from cotyledonary leaves of tomato

Plate 7

Plate 8

Plate 9

Plate 10

a period of 16.74, 16.94, and 15.68 days. T-3, T-6 and T-9 recorded the regeneration percentage of 50.12, 74.62 and 58.74, respectively in 15.45, 14.72 and 15.68 days, respectively.

This result revealed that T-6 with 2.5 mg l^{-1} BA and 1 mg l^{-1} IAA was the best regeneration medium for the nodal segments.

The root induction was observed in 62.4 per cent of cultures. The regenerated shoots were transferred to rooting media containing half strength MS supplemented with 1 mg l^{-1} IBA.

After rooting the plants were planted in sterile sand. After two weeks, it was observed that 60 per cent of the rooted plants were established *ex vitro*.

4.2 SENSITIVITY OF TOMATO TISSUES TO ANTIBIOTICS

Experiments were carried out to evaluate the tomato tissues to different doses of kanamycin and cefotaxime (Table 5 and Table 6, Figures 1 and 2).

4.2.1 Kanamycin

The calli showed slight discolouration on 7th and 8th week, respectively when kept at 5 and 10 mg l^{-1} kanamycin. At a concentration 25 mg l^{-1} kanamycin, partial discolourations of the calli were observed in 5th week and bleached appearance in 8th week. The tissues showed partial discolouration in 4th week, bleaching in 6th week and browning in 8th week in 75 mg l^{-1} kanamycin. At concentration 100 mg l^{-1} kanamycin the tissues showed partial discolouration in 2nd week, bleaching in 4th week, browning in 6th week and death of the calli in 8th week. The tissues started partial discolouration from 1st week onwards in 150 mg l^{-1} kanamycin. Then it started bleaching in 2nd week, browning and death of the tissues were observed in 5th week (Plates 11-16).

4.2.2 Cefotaxime

Sensitivity of tomato cultures to different doses of cefotaxime was studied. All the calli remained green in control. At 5 and 10 mg l^{-1} cefotaxime,

slight discolouration was recorded in 8th week. Slight discolouration recorded in 7th week at 25 mg l^{-1} cefotaxime.

At concentration 50 mg l^{-1} cefotaxime slight discolouration was observed in calli in 8th week. At 75 mg l^{-1} cefotaxime partial discoloration was recorded at 5th week and it was slightly bleached in 8th week. At 100 mg l^{-1} cefotaxime calli showed discolouration from 4th week onwards and at the 8th week, bleaching of the calli were observed and at 150 mg l^{-1} cefotaxime the calli bleached from 6th week onwards (Plates 23-26).

4.3. ESTABLISHMENT OF BACTERIAL CULTURE

The *Agrobacterium* strain LBA4404 harbouring pCAMBIA 2301 was selected for the study.

4.3.1 Growth of *Agrobacterium*

The *Agrobacterium* strain LBA4404 harbouring pCAMBIA 2301 produced white smooth and shiny colonies at 30 hours on culture plate containing YEP medium.

4.3.2 Screening of Agrobacterium strains for Antibiotic sensitivity

4.3.2.1 Kanamycin

The sensitivity of bacterial strain LBA 4404 harbouring plasmid pCAMBIA 2301 to different doses of kanamycin was tested. The bacterial growth was recorded from 5 to 500 mg^{-1} kanamycin. The bacteria produced colonies up to 350 mg l^{-1} kanamycin. Concentration 350 mg l^{-1} and above there was no bacterial growth (Table 7).

4.3.2.2 Cefotaxime

The sensitivity of bacterial strain LBA 4404 containing plasmid pCAMBIA 2301 to different doses of cefotaxime ranging from 5 to 500 mg l^{-1} was tested. At concentrations 75 mg l^{-1} and above there was no bacterial growth was recorded. (Table 8)

Table.5 Sensitivity of tomato callus to different doses of cefotaxime

Sl. No	Cefotaxime (mgl^{-1})	Sensitivity (weeks)								Survival (%)
		I	II	III	IV	V	VI	VII	VIII	
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++	100.00
2.	5	++++	++++	++++	++++	++++	++++	++++	+++	93.50
3.	10	++++	++++	++++	++++	++++	++++	++++	+++	90.62
4.	25	++++	++++	++++	++++	++++	++++	+++	+++	79.40
5.	50	++++	++++	++++	++++	++++	+++	+++	+++	69.60
6.	75	++++	++++	++++	++++	+++	+++	+++	++	60.70
7.	100	++++	++++	++++	+++	+++	+++	++	++	58.20
8.	150	++++	++++	+++	+++	+++	++	++	++	49.60

Mean of three replications

++++ Fully green
+++ Partial discolored
++ Bleached
+ Turning brown and dead.

Sensitivity of tomato calli to different concentrations of cefotaxime

Plate11: Sensitivity of tomato callus to cefotaxime 5 mg l^{-1}

Plate 12: Sensitivity of tomato callus to cefotaxime 25 mg l^{-1}

Plate13: Sensitivity of tomato callus to cefotaxime 50 mg l^{-1}

Plate14: Sensitivity of tomato callus to cefotaxime 75 mg l^{-1}

Plate15: Sensitivity of tomato callus to cefotaxime 100 mg l^{-1}

Plate16: Sensitivity of tomato callus to cefotaxime 150 mg l^{-1}

Plate 11

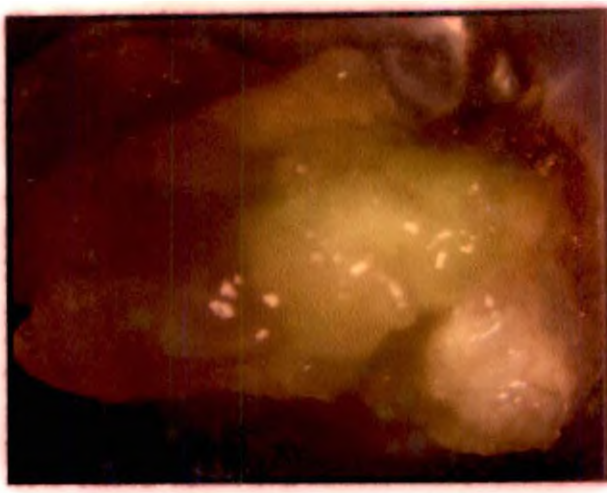


Plate 12

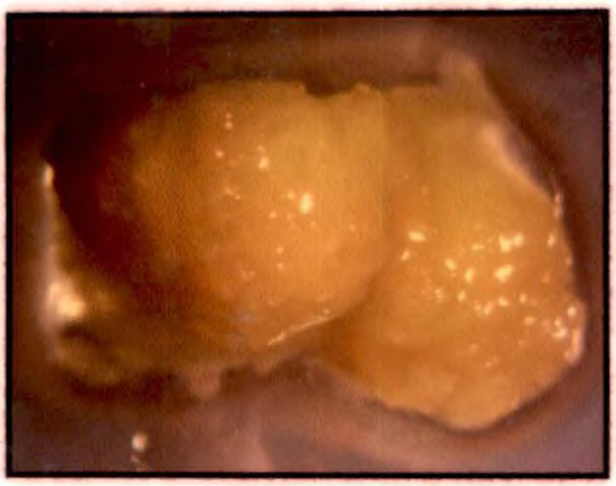


Plate 13

Plate 14

Plate 15

Plate 16

Table.6 Sensitivity of tomato callus to different doses of kanamycin

Sl. No	Kanamycin (mgl^{-1})	Sensitivity (weeks)								Survival (%)
		I	II	III	IV	V	VI	VII	VIII	
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++	100.00
2.	5	++++	++++	++++	++++	++++	++++	++++	+++	65.60
3.	10	++++	++++	++++	++++	++++	++++	+++	+++	62.70
4.	25	++++	++++	++++	++++	++++	+++	+++	+++	58.70
5.	50	++++	++++	++++	++++	+++	+++	+++	++	46.50
6.	75	++++	++++	++++	+++	+++	++	++	+	32.90
7.	100	++++	+++	+++	++	++	+	+	+	22.00
8.	150	+++	++	++	++	+	+	+	+	15.10

Mean of three replications

++++ Fully green
+++ Partial discolored
++ Bleached
+ Turning brown and dead.

Sensitivity of tomato calli to different concentrations of kanamycin

Plate17: Sensitivity of tomato callus to kanamycin 5 mg l^{-1}

Plate18: Sensitivity of tomato callus to kanamycin 25 mg l^{-1}

Plate19: Sensitivity of tomato callus to kanamycin 50 mg l^{-1}

Plate20: Sensitivity of tomato callus to kanamycin 75 mg l^{-1}

Plate21: Sensitivity of tomato callus to kanamycin 100 mg l^{-1}

Plate22: Sensitivity of tomato callus to kanamycin 150 mg l^{-1}

Plate 17

Plate 18

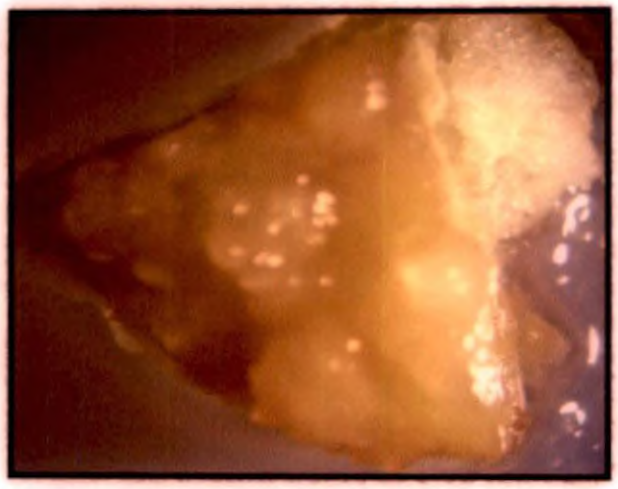


Plate 19

Plate 20

Plate 21

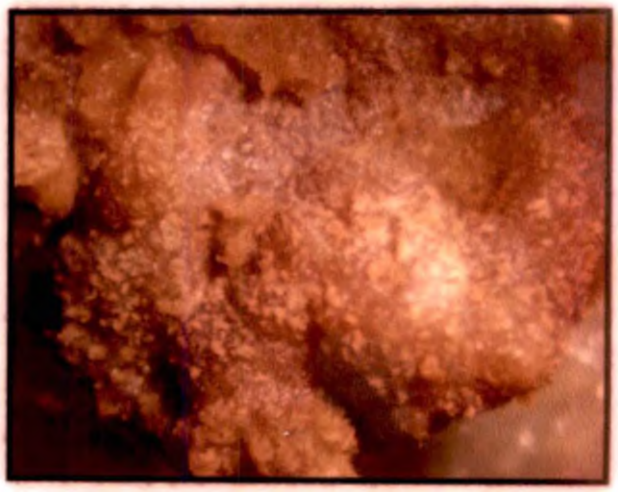


Plate 22

Fig. 1. Sensitivity of tomato calli to different doses of kanamycin

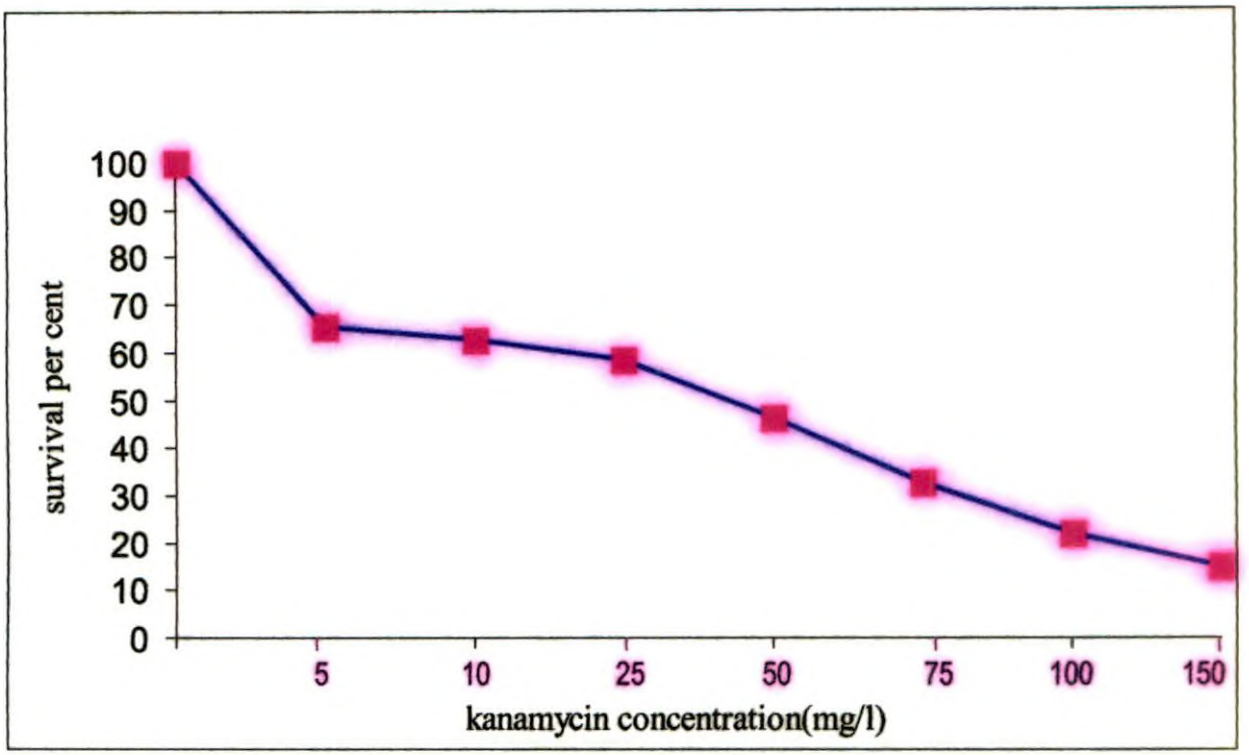


Fig. 2. Sensitivity of tomato calli to different doses of cefotaxime

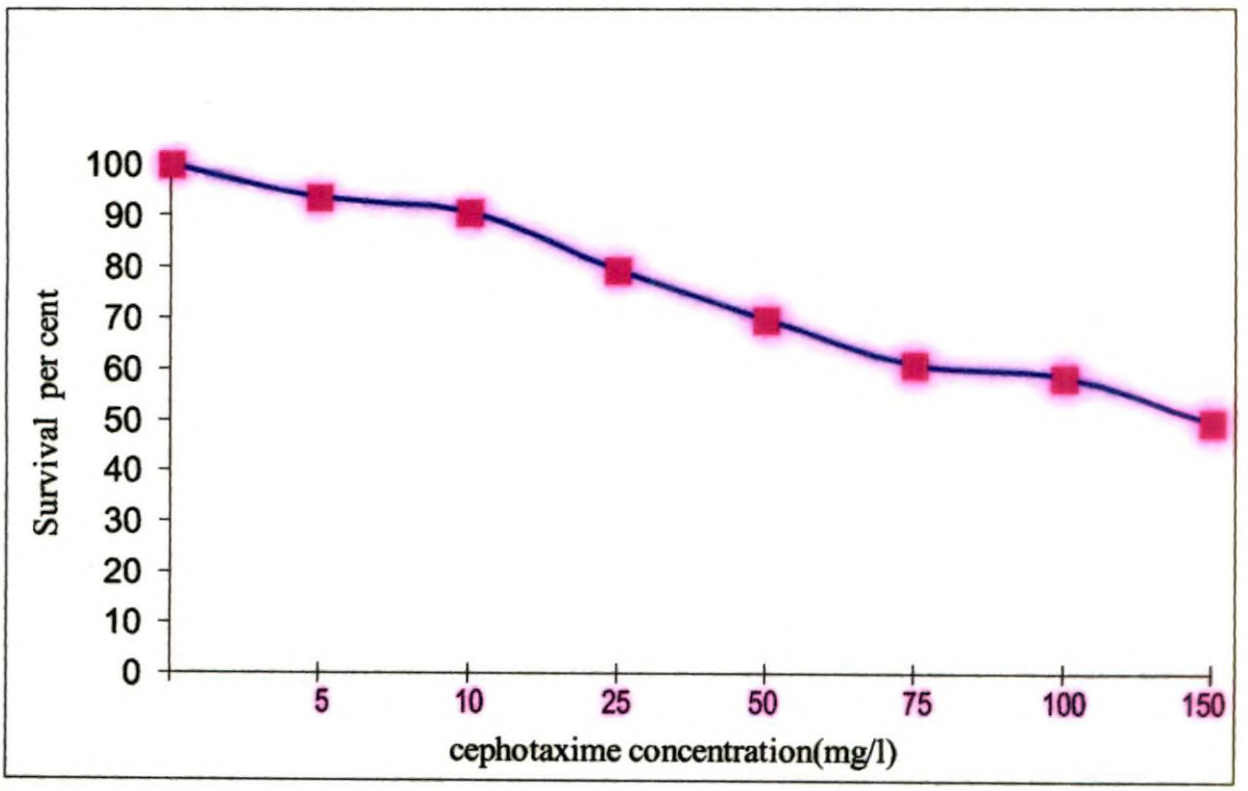


Table.7 Sensitivity of *Agrobacterium* to different doses of kanamycin

Sl. No	Kanamycin (mgl^{-1})	Bacterial growth
		Strain LBA 4404 with plasmid pCAMBIA 2301
1.	0	+
2.	5	+
3.	10	+
4.	25	+
5.	50	+
6.	75	+
7.	100	+
8.	200	+
9.	300	+
10.	350	–
11.	400	–
12.	500	–

Mean of three replications

\+ Bacterial growth
– No bacterial growth

Table.8 Test for bactericidal activity of cefotaxime

Sl. No	Cefotaxime (mgl^{-1})	Bacterial growth
		Strain LBA 4404 with plasmid pCAMBIA 2301
1.	0	+
2.	5	+
3.	10	+
4.	25	+
5.	50	+
6.	75	–
7.	100	–
8.	200	–
9.	300	–
10.	350	–
11.	400	–
12.	500	–

Mean of three replications

\+ Bacterial growth
– No bacterial growth

4.4 GENETIC TRANSFORMATION OF TOMATO

4.4.1 Explant Used for Transformation

Callus tissues were used for the genetic transformation of tomato with *Agrobacterium* strain LBA 4404 harbouring plasmid pCAMBIA 2301.

4.4.2 Effect of Infection Time

The infection was carried out for 10, 15 and 20 minutes so as to standardize the optimum time required for infection (Table 9). Among different treatments, the explants immersed in bacterial suspension for 15 minutes gave maximum transformants of 83.33 per cent.

4.4.4 Effect of Co-cultivation Period

Agrobacterium strain with the binary vector and the plant tissues were co-cultivated in dark for two, three, four and five days at 26 °C in a culture room. The effect of number of days of co-cultivation on transformation efficiency was recorded (Table 10). The calli were co-cultivated with bacteria for two, three and four days. Highest per cent 84.68 of transformants were obtained from explants co-cultivated with *Agrobacterium* in dark for three days.

4.4.2 Effect of Number of Explants

The number of callus kept per Petri dish for co-cultivation ranged between 10 and 20. The highest transformation efficiency was recorded in plates with 12 calli. In plates with more than 12 calli the overgrowth of bacteria was recorded on the callus.

4.4.5 Elimination of Bacteria

The growth of bacteria after co-cultivation was eradicated with the addition of cefotaxime. The effect of the cefotaxime was tested is three, four and five days. Washing the tissues after co-cultivation with 75 mg l^{-1} cefotaxime and addition of 75 mg l^{-1} cefotaxime in culture plate reduced the bacterial growth. The best transformation efficiency was obtained when the tissues were incubated for

Table: 9 Effect of *Agrobacterium* infection time on tomato transformation

SL. NO.	Infection time (min)	Tissues retained after selection (%)
1	10	66.66
2	15	83.33
3	20	34.64

Table: 10 Effect of co-cultivation period on tomato transformation

SL. NO.	Co-cultivation period (days)	Tissues retained after selection (%)
1	2	78.20
2	3	84.68
3	4	73.40
4	5	54.92

five days. The complete eradication of bacteria was observed after five days. Bacterial growth was observed in other two treatments.

4.4.7 Effect of Acetosyringone

To study the effect of transformation acetosyringone was added in the co-cultivation medium. The co-cultivation was done with and without acetosyringone. Compared to control, the transformation efficiency was high in medium with acetosyringone (200μM).

4.4.8 Selection of Putative Transformants

The putative transformants were selected in MS medium with 100 mg l^{-1} kanamycin and 75 mg l^{-1} cefotaxime. 85 per cent kanamycin resistant transformants were selected after four weeks.

4.5 GUS STAINING

For the selection of transformants GUS staining was done. Out of the total kanamycin resistant tissues 95 per cent showed GUS activity. The X-gluc staining pattern varied among the callus from those partially stained and stained as blue spots in the callus (plates 23-26). The distribution of GUS expression (blue spots and blue patches) on the surface of the callus was even. The callus from the non co-cultivated cells (control) did not stain blue.

4.6 TRANSFORMATION EFFICIENCY

The transformation efficiency was 96.6 per cent based on GUS staining.

4.7 IMMUNO DETECTION OF GROUNDNUT BUD NECROSIS VIRUS

Enzyme linked immunosorbant assay of the virus

DAC- ELISA

A polyclonal antibody for tospo virus was used for detecting the tospo virus (GBNV) in tomato. The antibody with various dilutions and cross absorbed antibody with various dilutions was tried. The results of the experiment revealed that the antibody gave high reactivity towards the virus isolates. The absorbance

Plate23, Plate24, Plate25, Plates26: GUS Stained Transformed Tissues of Tomato

Plate 23

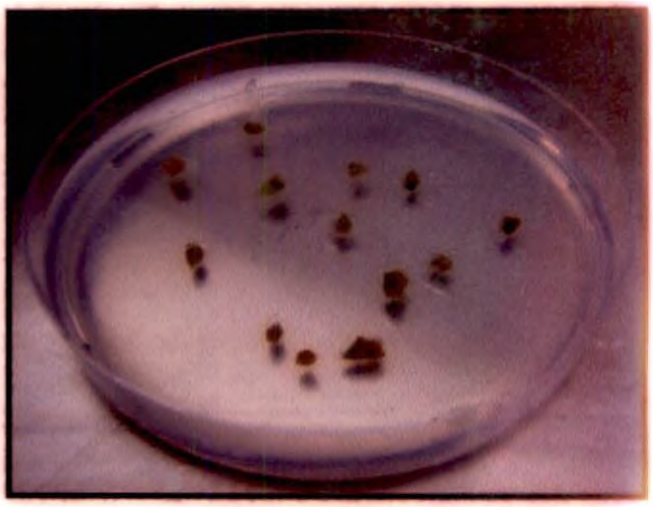


Plate 24

Plate 25

Plate 26

of the diseased sample (0.625) against the anti-serum was more than three times that of healthy sample (0.262) in 1:250 dilution of the antibody. The similar result was also recorded in other dilutions also (Table11). This result revealed that the association of tospo virus with bud necrosis disease of tomato (Plate 30).

4.8. MOLECULAR CHARACTERIZATION OF THE TOSPO VIRUS (GBNV) IN TOMATO

Total RNA extracted from the infected tissues (100 mg) used as a template in the reverse transcription - polymerase chain reaction (RT-PCR). RT-PCR was carried out using the RT-PCR kit. The amplicon (800bp) was visualized on 1 per cent agarose gel and documented using gel-documentation system. 1kb DNA ladder was used as molecular weight marker (Plate 31).

Plate 27

Plate 28

Plate 29

Table: 11 Reaction of tospovirus (GBNV) isolate from tomato using DAC- ELISA

Samples		Dilutions of antibody	Absorbance at 405nm
Healthy	Antibody	1: 250	0.262
		1: 500	0.133
		1:1000	0.038
	Cross absorbed antibody	1: 250	0.033
		1: 500	-0.008
		1:1000	0.009
Diseased	Antibody	1: 250	0.625
			0.567
		1: 500	0.359
			0.367
		1:1000	0.200
			0.170
	Cross absorbed antibody	1: 250	0.102
			0.001
		1: 500	0.094
			0.079
		1:1000	0.028
			0.038

Plate30:	Immuno detection of tospo virus (GBNV) isolate from tomato using DAC-ELISA
Buffer	B2, C2, D2, E2, F2 and G2
Healthy	
1:250	B3
1:250 c	C3
1:500	D3
1:500 c	E3
1:1000	F3
1:1000 c	G3
Diseased	
1:250	B5, C5
1:250 c	E5, F5
1:500	B7, C7
1:500 c	E7, F7
1:1000	B9, C9
1:1000 c	E9, F9

c- Cross absorbed antibody

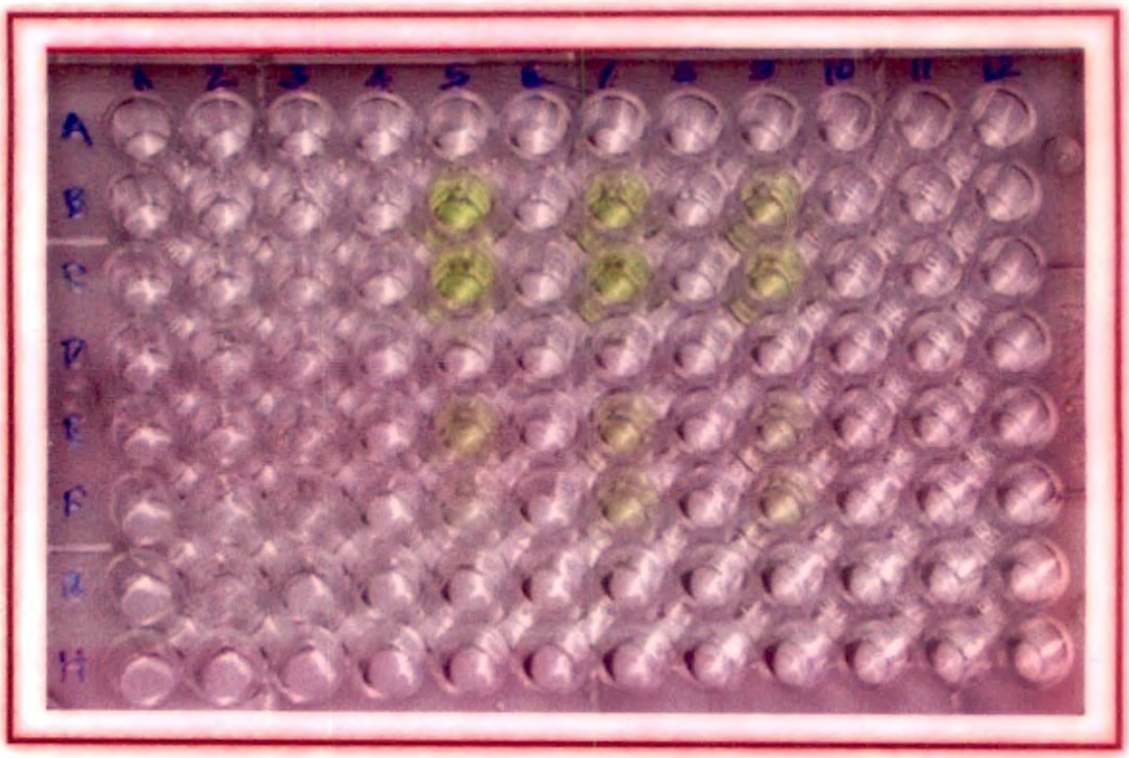


Plate 30

Plate 31: Molecular characterization of coat protein gene of GBNV by RT-PCR

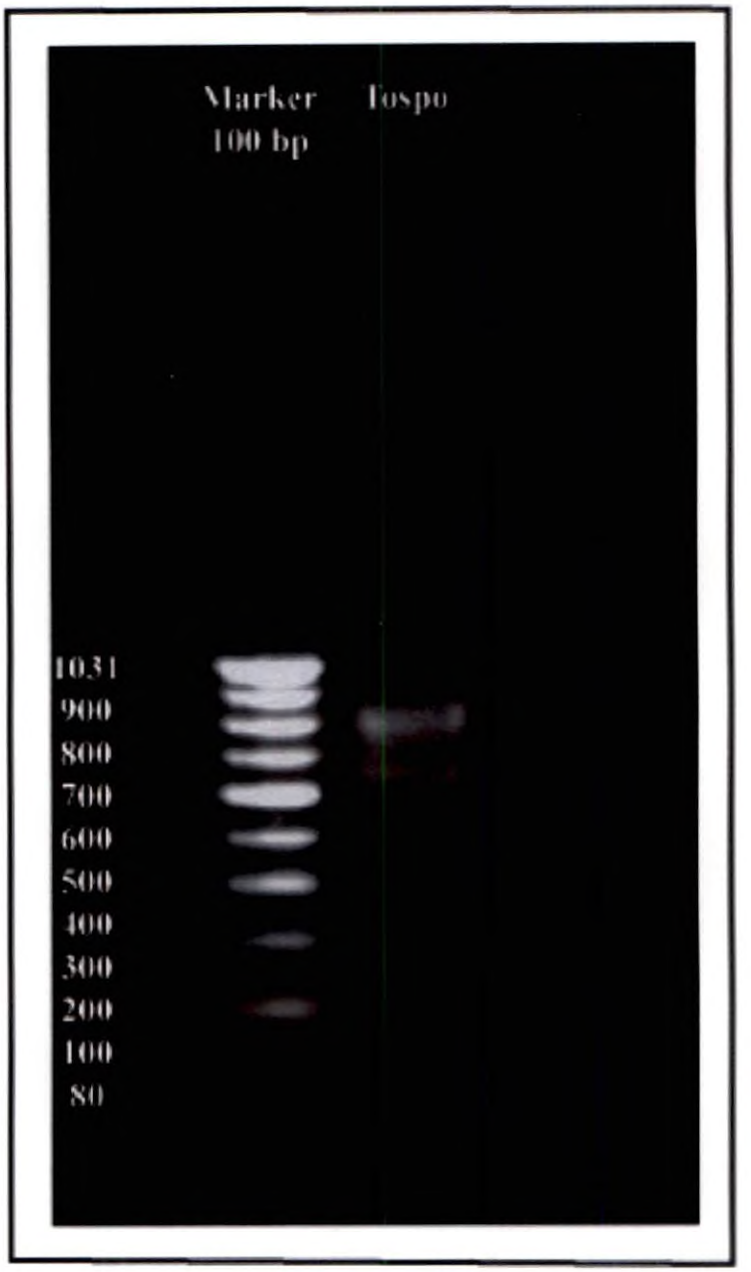


Plate 31

Discussion

5. DISCUSSION

Tomato (*Lycopersicon esculentum* Mill.) is the most widely grown vegetable in the world. Diseases are the major constraints for the cultivation of tomato. The reduction in yield due to viral disease stress needs to evolve tomato varieties resistant to viral infection. Among the viral diseases, the bud necrosis disease caused by groundnut bud necrosis virus is a major threat to tomato cultivation. Evolving virus resistance varieties through traditional breeding is a time consuming process. Successful transformation is essential to impart virus resistance genes to cultivated varieties.

The two types of plant transformation are direct gene transfer techniques and the most widely used, *Agrobacterium* mediated genetic transformation. The natural DNA transfer capacity of *Agrobacterium tumefaciens* is exploited for the introduction of new genes into plants. *Agrobacterium* mediated transformation of plant tissue generally results in a low transgene copy number, minimal rearrangements, and higher transformation efficiency than direct DNA delivery techniques. Success of *Agrobacterium* mediated transformation has been reported to be dependent on the variety, type of explants, delivery system, *Agrobacterium* strain, conditions of co-cultivation, selection method and mode of regeneration (Mathis and Hinchee, 1994). This method is widely used to introduce foreign genes into dicots and in some monocots. Hence this method was selected in the present study.

Tomato transformation is crucial for incorporating novel genes. The first report of tomato transformation was by Mc Cormic *et al.* (1986) in leaf disc transformation of tomato. Since then there have been numerous reports on transformation in various species and cultivars of tomato (Fraay and Earle, (1996) Ling *et al.*, 1998; Tabaeizadeh *et al.*, 1999; Vidya *et al.*, 2000; Hu and Philips, 2001).

The present study was undertaken at the Department of Plant Biotechnology, College of Agriculture, Vellayani to standardize the protocol for *Agrobacterium* mediated genetic transformation in tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against groundnut bud necrosis virus. The variety Mukthi is resistant to bacterial wilt, but highly susceptible for GBNV causing bud necrosis disease. So this variety was chosen for the transformation experiment for transferring the resistance imparting genes.

The first requirement for molecular breeding is that the genetically engineered tissue must regenerate plants, for which the *in vitro* regeneration was standardized with cotyledonary leaves, leaves and nodes.

Different surface sterilization treatments were tried to minimize the contamination. Surface sterilization was found to be most effective when cotyledonary leaves were surface sterilized with 0.08 per cent mercuric chloride for seven minutes. The per cent of aseptic cultures obtained from this treatment was 96.23, followed by one per cent sodium hypochlorite for 15 minutes (75.68). The culture establishments from leaves, surface sterilized with 0.08 per cent mercuric chloride for seven minutes and one per cent sodium hypochlorite for 15 minutes was 95.00 per cent and 82.30 per cent, respectively. The culture establishment was low with 0.09 and 0.1 per cent mercuric chloride and 0.08 and 1.2 per cent of sodium hypochlorite. The survival of aseptic cultures from nodes treated with 0.08 per cent mercuric chloride for seven minutes was 90.05 per cent and with one per cent sodium hypochlorite for 15 minutes were 89.12. It was very high when compared with the survival rate from the nodes sterilized with 0.09 per cent and one per cent mercuric chloride for seven minutes and 0.08 and 1.2 per cent sodium hypochlorite for 15 minutes.

The highest survival per cent of cultures were observed with 0.08 per cent mercuric chloride for seven minutes, followed by one per cent sodium hypochlorite for 15 minutes. The survival per cent was comparatively low at higher and lower concentrations of mercuric chloride and sodium hypochlorite. And with lower concentrations the contamination rate was high. This was

adversely affecting the growth of the culture. This result revealed that treatment with 0.08 per cent mercuric chloride was found to be the most effective surface sterilent for cotyledonary leaves, leaves and nodal explants of tomato, followed by one per cent sodium hypochlorite for 15 minutes. Mercuric chlorite is the preferred surface sterilent for the crops grown in humid tropical conditions.

In the case of cotyledonary leaves among the three different combinations of BA and IAA tested, T-5 with BA concentration of 2 mg l^{-1} and IAA concentration of 1.0 mg l^{-1} recorded the highest callus induction (78.08 %) within a short period of 14.02 days. The regeneration per cent of 78.00 was obtained from the same medium within 21.52 days. But highest regeneration (79.42 %) within a short period of 21.02 was obtained from T-6 with a combination of 2.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA.

In the case of leaves, the highest callus induction (68.00 %) within 16.42 days in T-5. But regeneration was maximum in T-6 with 2.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA showed the maximum regeneration per cent (64.60) within a short period of 21.07 days. The result revealed that the percentage of regeneration from cotyledonary leaves and leaves was high in medium with higher concentrations of BA. Ali and Li (1994) reported that callus initiation was at greater rate and largest on MS medium supplemented with 3 mg l^{-1} zeatin or 0.6 mg l^{-1} 1AA and 2 mg l^{-1} BA from the leaves. Selvi and Khader (1993) also reported the best shoot formation from the leaf disc cultured on MS medium with 0.2 mgl^{-1} IAA and 2.5 mgl^{-1} BA.

The nine treatments were tried with nodal segments also. Among these treatments T-6 with 2.5 mg l^{-1} BA and 1 mg l^{-1} IAA was found to be the best regeneration medium for the nodal segments. The regeneration observed from this media was 74.62 per cent in 14.72 days. Pongtongkam *et al.* (1993) reported the regeneration from nodal segments of tomato on MS with 0.5 mgl^{-1} BA.

In vitro rooting (62.4) was observed in plants in MS medium supplemented with IBA 1 mg l^{-1}. More rooting was observed in media supplemented with IBA. Venkatachalam *et al.* (2000) observed the rooting of the

regenerated shoots of tomato on half strength MS medium supplemented with IBA (0.1 to 0.5 mg l^{-1}). Soniya *et al.* (2001) reported rooting of tomato var. Sakthi on MS medium supplemented with 10.0 µM IBA. After rooting the plants were successfully established (60%) *ex vitro* in sterile sand. Jager *et al.* (1998) reported the successful hardening of *in vitro* plantlets in sand: soil (2:1) mix.

The callus tissues were selected as explant for genetic transformation in tomato. Once an efficient *in vitro* regeneration protocol from callus was standardized, the next requirement for a successful genetic transformation is the selection of an efficient gene delivery system. *Agrobacterium tumefaciens* mediated transformation was selected for the transformation experiment. The *Agrobacterium* strain LBA 4404 harbouring plasmid pCAMBIA 2301 was used for the study. Several reports are available on genetic transformation of tomato with the same strain. Hoekema *et al.* (1983) reported *Agrobacterium* mediated transformation in tomato (*Lycopersicon esculentum* Mill.) cv. Micro Tom, Red cherry, Rubion, Piedmont and E6203 using *Agrobacterium tumefaciens* strain LBA 4404 and Vidya *et al.* (2000) reported the transformation of cotyledonary leaves of tomato cv. pusa Ruby with *Agrobacterium tumefaciens* strain LBA 4404.

The pCAMBIA 2301 contains the kanamycin resistance gene. This kanamycin resistance gene was taken as marker for the selection of transformants. For screening of the transformed cells evaluation of sensitivity of plant tissues and bacterial strains to different concentration of kanamycin was necessary. It will help to select the optimum concentration of antibiotic that is required to inhibit the growth of untransformed tissues.

The bacterial growth was recorded from 5 to 500 mg^{-1} kanamycin. The bacteria produced colonies up to 350 mg l^{-1} kanamycin. Concentration 350 mg l^{-1} and above there was no bacterial growth. But bleaching of callus was started with kanamycin 75 mg l^{-1}. Hence kanamycin was used at strength of 100 mg l^{-1} for selection of tomato transformants. Fari *et al.* (1995) also screened the tomato transformants with 100 mg l^{-1} kanamycin.

Cefotaxime had been successfully used for elimination of bacteria in transformation work with a number of crops. Elimination of bacteria from plant tissues after co – cultivation is an essential step. For that sensitivity of tissues and bacterial strain to different doses of cefotaxime were tested to record the optimum dose at which the tissues can thrive and bacteria will not survive. At concentrations 75 mg l^{-1} and above there was no bacterial growth was recorded. So cefotaxime 75 mg l^{-1} was selected for the complete eradication of *Agrobacterium*. At this concentration, the tomato tissues showed normal growth. Manoharan *et al.* (1998) used 400 mg l^{-1} cefotaxime for killing the *Agrobacterium tumifacience* after transformation of tomato.

Wounding of the callus is a prerequisite for the infection of *Agrobacterium* was done with sterile knife. Infection was carried out using overnight grown bacterial suspension. The number of *Agrobacterium* in the inoculum was a critical factor for the transformation process. For assessing the inoculum density, OD value of the overnight grown bacterial suspension was checked and the highest transformation was obtained when the OD at 600 nm was 0.1. This was also reported by Hoekema *et al.* (1983).

During the infection process, the *Agrobacterium* gets attached to the plant cell. Experiments were undertaken to standardize the optimum time required for the infection process. Among different treatments, the explants immersed in bacterial suspension for 15 minutes gave maximum transformants of 83.33 per cent. The length of the time that the explants were immersed in the bacterial suspension influenced transformation. In the other two treatments the transformation was low.

To standardize the number of days required for the co-cultivation, experiments were done. Highest per cent 84.68 of transformants were obtained from explants co-cultivated with *Agrobacterium* in dark for three days. The transformation was highest in co –cultivation medium was 12 calli in a Petri plate. In plates with more than 12 calli as the distance between the explants is less there was rapid spread of *Agrobacterium* which led to overgrowth of bacteria on callus.

Ouyang *et al.* (2005) reported two days of co-cultivation of tomato cotyledons showed high efficiency of transformation. When co-cultivated for more than three days overgrowth of bacteria was noticed. The tissues with such bacterial overgrowth were washed in liquid MS medium or sterile water with Cefotaxime 75 mg l^{-1}. This washing reduced the number of explants with bacterial overgrowth. After co – cultivation, the callus were blotted dry on sterile filter papers. The undried calli were lost due to contamination by fungal and bacterial pathogens.

The wounding of the callus produces a phenolic compound known as acetosyringone. Acetosyringone plays a key role in the gene transfer process by *Agrobacterium.* These small molecules induce activity of virulence (*vir*) genes that are encoded on the plasmid. The induced *vir* genes were responsible for the 'T DNA' (which contain gene of interest) transfer to plant cell. Addition of acetosyringone in the co-cultivation medium.might has acted synergistically to increase the infection and the transformation efficiency. Compared to control (without acetosyringone) the addition of acetosyringone (200μM) in co-cultivation medium enhanced the transformation efficiency. The similar result was also reported by Cortina *et al.* (2004).

Selection of transformed cells is a key factor in developing successful method of genetic transformation. In this study the pCAMBIA 2301 harbors the kanamycin resistance gene, which was taken as marker for the selection of transformants. The selection of transformed tissues was done in selection medium (MS medium) with 100 mg l^{-1} kanamycin and 75 mg l^{-1} cefotaxime. The highest transformants with 85 per cent kanamycin resistance were selected after four weeks. The selection of tomato transformants in MS medium with100 mg l^{-1} kanamycin was reported by Fari *et al.* (1995).

The transformants were also selected based on GUS staining. The distribution of GUS expression (blue spots and blue patches) on the surface of the callus was even. The callus from the control did not stain blue. Murray *et al.*

(1998) also reported the expression of *gus* gene in transformed tomato by using *A. tumefaciens* LBA 4404 (p35S GUS INT).

DAC-ELISA was carried out for immuno detection of tospo virus (ground nut bud necrosis virus) in tomato. A polyclonal antibody for tospo virus was used for detecting the virus. The absorbance of the diseased sample (0.625) against the anti-serum was more than three times that of healthy sample (0.262) in 1:250 dilution of the antibody. Result revealed the association of tospo virus with bud necrosis disease of tomato. Cho *et al.* (1986) reported ELISA as a method of choice for diagnosis and detection of tospo viruses in plants. Bhat *et al.* (2001) tested the tospovirus in black gram, cowpea, green gram and soyabean with seven different tospovirus antisera against these isolates in direct antigen-coated ELISA, positive reactions were seen only with groundnut bud necrosis virus (GBNV) and watermelon silver mottle virus (WSMV).

Molecular characterisation coat protein of tospo virus (ground nut bud necrosis virus) in tomato was done by RT-PCR. The amplicon (800bp) was visualised in 1 per cent agarose gel and documented using gel-documentation system. 1kb DNA ladder was used as molecular weight marker. The presence of excess band may be due to unspecific primer binding. Pappu *et al.* (1993) also carried out RT-PCR using the primer pair derived from the nucleocapsid protein (N) gene sequence of groundnut bud necrosis virus.

The recent progress in genetic engineering has openedup many avenues to develop viral disease-resistant plants. Different genetic engineering techniques applied to achieve viral disease resistance are cloning and expression of antisense RNA, plantibody, coat protein gene, viral polymerase, etc. Coat protein-mediated protection (CPMP) is potentially useful for developing virus resistant plants. In the long run, this study may help in evolving GBNV resistant transgenic varieties of tomato through coat protein mediated resistance.

Summary

6. SUMMARY

A study on *in vitro* regeneration and *Agrobacterium* mediated transformation in tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against groundnut bud necrosis virus was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004 to 2006. The salient findings of the above studies are summarized in this chapter.

Surface sterilization of cotyledonary leaves, leaves and nodal segment with 0.08 per cent mercuric chloride for seven minutes, followed by one per cent sodium hypochlorite for 15 minutes were the most effective treatments for the establishment of the cultures of tomato.

The explants cotyledonary leaves and leaves initiated callus and formed adventitious shoots on all nine combinations. Highest callus induction was obtained from cotyledonary leaves (78.00%) and leaves (68.00%) in MS medium supplemented with 2 mg l^{-1} BA and 1 mg l^{-1} IAA and regeneration from cotyledonary leaves (79.42 %) and leaves (78.00%) was highest in medium containing 2.5 mg l^{-1} BA and 1 mg l^{-1} IAA. The MS medium 2.5 mg l^{-1} BA and 1 mg l^{-1} IAA was the best for regeneration (74.62%) for the nodal segments. Rooting was obtained in MS medium with 1 mg l^{-1} IAA. Among the rooted plants 66.8 per cent of plants were successfully established *ex vitro*.

The sensitivity of tomato callus to different doses of kanamycin was studied. Partial discoloration was observed in the tissues treated with kanamycin 150 mg l^{-1} in the first week. In the second week, partial discoloration was observed from kanamycin 100 mg l^{-1} and bleached appearance was observed in the tissues with 150 mg l^{-1} kanamycin. Third week onwards bleaching was observed in the tissues with kanamycin 100 mg l^{-1}. In the fourth week partial discoloration was seen in tissues with 75 mg l^{-1} kanamycin. In the fifth week, bleaching was seen in tissues with 75 mg l^{-1}, 50 mg l^{-1} and 150 mg l^{-1} kanamycin.

From the sixth week onwards the tissues with 50 mg l^{-1} and 75 mg l^{-1} kanamycin turned brown and died.

Sensitivity of tomato calli to different doses of cefotaxime was studied to use it as bacteriostatic agent. The calli were not very much affected by the cefotaxime up to 100 mg l^{-1} concentrations.

The *Agrobacterium tumefaciens* strain LBA4404 harbouring pCAMBIA 2301 was used for the study. It produced white smooth and shiny colonies in 30 hours on culture plate containing YEP medium.

The sensitivity of bacterial strain to different doses of kanamycin was studied (5-500 mg l^{-1}). At a concentration of 350 mg l^{-1} and above there was absence of bacterial growth.

The bactericidal activity of cefotaxime was also studied (5-500 mg l^{-1}). It was observed that the *Agrobacterium* was effectively killed by cefotaxime at 75 mg l^{-1}.

The transformation efficiency (83.33%) was high when the infection time was 15 minutes. The optimum number of tissues that should be kept for co-cultivation in a single Petri dish was 12.

The highest percentage (84.68) of transformation was obtained from the explants co-cultivated with *Agrobacterium* for three days. The bacteria after co-cultivation were eradicated with the addition of cefotaxime in media at 75 mg l^{-1} for five days. Washing the tissues after co-cultivation with 75 mg l^{-1} concentration cefotaxime and addition of 75 mg l^{-1} cefotaxime in culture plate reduced the bacterial growth.

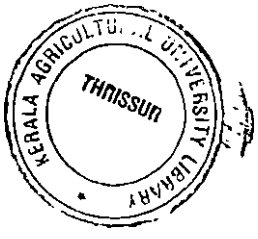
The transformation efficiency was increased with the addition of 200 µM acetosyringone in the co-cultivation media, compared to control.

Selection of transformed tissues was done in selection media with 100 mg l^{-1} kanamycin and 75 mg l^{-1} cefotaxime. 85 per cent kanamycin resistant transformants were selected after four weeks.

Out of the total kanamycin resistant tissues 95 per cent showed GUS activity. The distribution of GUS expression (blue spots and blue patches) on the surface of the callus was even. And the transformation efficiency obtained was 96.6 per cent based on the GUS staining.

A polyclonal antibody for tospo virus was used for detecting the GBNV virus in tomato. The antibodies with various dilutions and cross absorbed antibodies with various dilutions were tried. The results of the experiment revealed that the antibody gave high reactivity towards the virus isolates. The absorbance of the diseased sample against the anti-serum was more than three times that of healthy sample in each dilutions of the antibody.

For the molecular characterization of the coat protein gene of the GBNV using RT-PCR, the primer pair derived from the N gene sequence of GBNV. The amplicon (800bp) was visualized and documented using gel-documentation system.



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Appendices

APPENDIX I

Chemical composition of media employed for the *in vitro* culture of tomato.
MS medium

Macro-nutrients (mgl^{-1})

$Mg\ SO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170

Micro-nutrients (mgl^{-1})

$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$AlCl_3$	0.025
KI	0.83
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25

Iron Source (mgl^{-1})

$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25

Vitamins (mgl^{-1})

Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

Amino acid source (mgl^{-1})

Glycine	2.0
Inositol (mgl^{-1})	100
Sucrose (gl^{-1})	30
Agar (gl^{-1})	6

APPENDIX II

Chemical composition of media employed for the culture of *Agrobacterium tumefaciens*

YEP medium (per l)

Yeast extract	-	10g
Peptone	-	10g
Sodium chloride	-	5.0g
pH	-	7.0

AB Minimal Medium

AB salts (gl^{-1}) 20 X stock

NH_4Cl	-	20
$MgSO_4.7H_2O$	-	25
KCl	-	3
$CaCl_2$	-	0.2
$FeSO_4.7H_2O$	-	0.05
pH	-	7

AB buffer (gl^{-1}) 20 X stock

K_2HPO_4	-	60
NaH_2PO_4	-	23

Glucose	-	0.5%
Agar	-	1.5%

Working solution 1X

APPENDIX III

Buffers for ELISA

1.- Phosphate buffer saline(1X PBS) pH 7.4

NaCl	- 8 g
$Na_2HPO_4.2H_2O$	- 1.44 g
KH_2PO_4	- 0.2 g
KCl	- 0.2 g
Distilled water	- 1 litre

2. Wash buffer (PBS - T)

3. Coating buffer (Carbonate buffer pH 9.6)

Na_2CO_3	- 1.59 g
$NaHCO_3$	- 2.93 g
Distilled water	- 1 litre

4. Enzyme conjugate diluent buffers (PBS-TPO)

   Add 20 g PVP and 2 g ovalbumin to 1 litre PBS-T

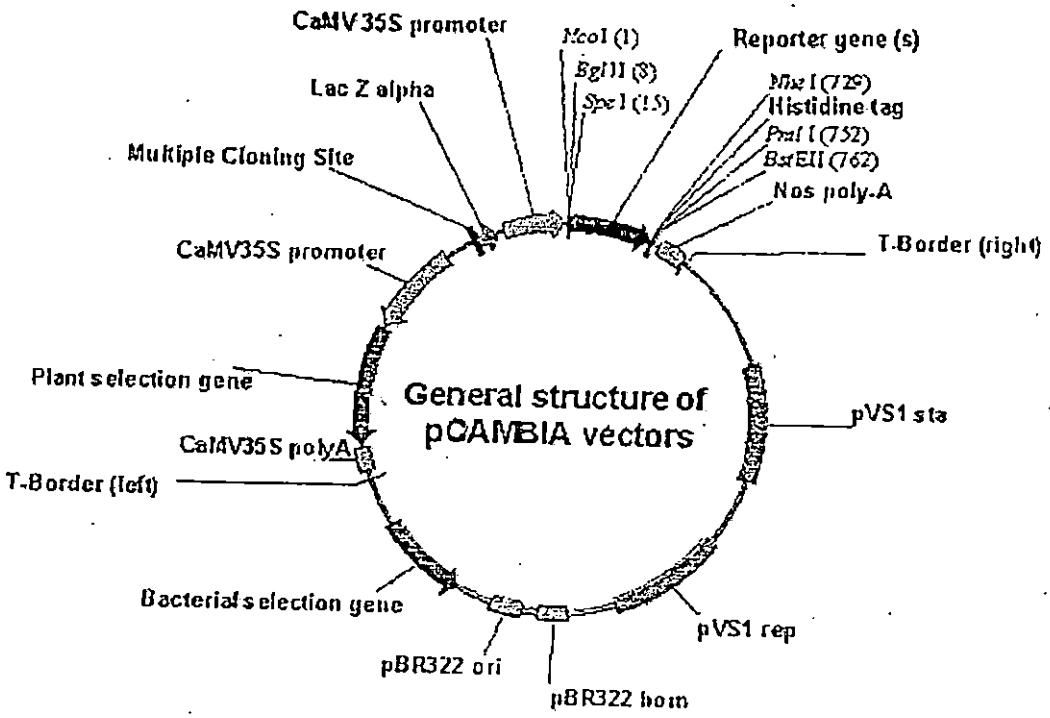
5. Antibody diluent buffer
   Same as PBS-TPO

6. Substrate buffer (pH 9.8)

Diethanol amine	- 97 ml
Distilled water	- 800 ml

Make up to 1 litre

APPENDIX IV



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  TITLE     A comprehensive set of modular vectors for advanced manipulations
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  REMARK    Full description of constructs
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IN VITRO REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) IN RELATION TO DISEASE RESISTANCE AGAINST GROUNDNUT BUD NECROSIS VIRUS

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ABSTRACT

A study on *in vitro* regeneration and *Agrobacterium* mediated transformation in tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against groundnut bud necrosis virus (GBNV) carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004 to 2006.

Irrespective of explants of tomato 0.08 per cent mercuric chloride for seven minutes and one per cent sodium hypochlorite for 15 minutes were found to be the most effective treatment for surface sterilization.

Highest callus induction was obtained from cotyledonary leaves (78.00%) and leaves (68.00%) in MS medium supplemented with BA 2 mg l^{-1} and IAA 1 mg l^{-1} and regeneration from cotyledonary leaves (79.42 %) and leaves (78.00%) was highest in medium containing 2.5 mg l^{-1} BA and 1 mg l^{-1} IAA. The MS medium with 2.5 mg l^{-1} BA and 1 mg l^{-1} IAA was the best regeneration (74.62%) medium for the nodal segments.

Rooting was obtained on MS medium with 1 mg l^{-1} IAA. Among the rooted plants 66.8 per cent of plants were successfully established *ex vitro*.

Experiments were conducted to evaluate the tomato calli to different doses of antibiotics *viz.* kanamycin and cefotaxime. It was observed that kanamycin 75 mg l^{-1} induced bleaching of callus. At concentration of 100 mg l^{-1} and above there was complete browning of callus. The callus was not seriously affected by cefotaxime up to 150 mg l^{-1}.

The *Agrobacterium* strain LBA 4404 containing plasmid pCAMBIA 2301 was used for the experiment.

Effect of kanamycin and cefotaxime on bacterial growth was studied. It was observed that no colonies were produced after 350 mg l^{-1} kanamycin and 75 mg l^{-1} cefotaxime.

The transformation efficiency was high with the infection process for 15 minutes using the bacterial suspension. Highest transformants was recovered only with the wounded callus in dark for three days. The transformation efficiency was very high with the addition of 200 µM acetosyringone. The bacteria after transformation were killed with 75 mg l^{-1} Cefotaxime.

The selection of transformants was done by using 100 mg l^{-1} kanamycin. Out of the total kanamycin resistant tissues 95 per cent showed GUS activity. The distribution of GUS expression (blue spots and blue patches) on the surface of the callus was even. The transformation efficiency obtained was 96.6 per cent based on the GUS staining.

DAC-ELISA was done for the immunological detection of GBNV in tomato. A polyclonal antibody for tospo virus was used for detecting the GBNV virus in tomato. The results of the experiment revealed that the antibody gave high reactivity towards the virus isolates.

The molecular characterization of the coat protein gene of the GBNV was done by RT-PCR, with the primer pair derived from the N gene sequence of GBNV. The amplicon (800 bp) was visualized and documented using gel-documentation system.