GENETIC TRANSFORMATION AND EXPRESSION ANALYSIS OF COLD TOLERANT GENE IN TOMATO (*Solanum lycopersicum* Mill.)

BY

SABIR HUSSAIN SHAH

A dissertation submitted to The University of Agriculture, Peshawar
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
(PLANT GENOMICS & BIOTECHNOLOGY)

DEPARTMENT OF PLANT GENOMICS & BIOTECHNOLOGY
PARC INSTITUTE OF ADVANCED STUDIES IN AGRICULTURE
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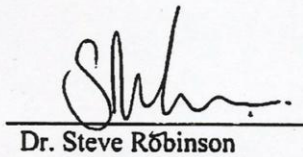

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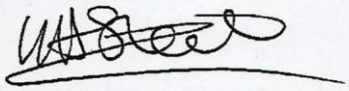

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(Sabir Hussain Shah)

ABBREVIATIONS

%	Percent
µl	Micro litre
$^1O_2$	Singlet oxygen
2, 4-D	2, 4-dichlorophenoxyacetic acid
ABA	Abscisic acid
$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AS	Acetosyringone
AsA	Ascorbic acid
BA	6-Benzyl adenine
BAP	6-benzylaminopurine
CaMV 35S	Cauliflower mosaic virus 35 S
CaMV	Cauliflower mosaic virus
CAT	Catalase
CBF3	Cold responsive-element binding factor 3
CBFs	C-repeat binding factors
CCM	Co-cultivation medium
Cf	Cefotaxime
CIM	Callus induction medium
$CoCl_2$	Cobalt chloride
CRD	Completely randomized design
CS	Carbon sources
CTAB	Cetyl trimethyl ammonium bromide
Cvs	Cultivars
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRE/CRT	Dehydration responsive element/C-repeat
DREB1A	Dehydration responsive element binding factor 1A
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
g	Gram
GA_3	Gibberellic acid
GB	Glycinebetaine
GM	Germination medium
GUS	β-glucuronidase gene
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
hpt	Hygromycin phosphotransferase
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IM	Inoculation medium
Kin	Kinetin
LB	Luria broth

Lip	Lipase
LSD	Least significant difference
MDA	Malondialdehyde
mg/l	Milli gram per litre
mM	Milli mole
MS	Murashige and Skoog (basal medium)
NAA	Naphthalene acetic acid
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NaOCl	Sodium hypochlorite
NOS	Nopaline synthase
NPT II	Neomycin phosphotransferase gene
NT	Non-transgenic
O_2^-	Superoxide radical
OD	Optical density
OH	Hydroxyl radical
PCM	Pre-culture medium
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGRs	Plant growth regulators
pH	-log H^+
POD	Peroxidase
PPO	Polyphenol oxidase
PSM	Pre-selection medium
RIM	Root induction medium
RM	Regeneration medium
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	Relative water contents
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Shoot elongation medium
SIM	Shoot induction medium
SM	Selection medium
SOD	Superoxide dismutase
Taq	*Thermus aquaticus*
T-DNA	Transfer DNA
TE	Transformation efficiency
T-G7	Terminator of gene 7 of *Agrobacterium*
Ti-plasmid	Tumour inducing plasmid
TSS	Total soluble sugars
Vir-gene	Virulence gene
WM	Washing medium
YEP	Yeast extract peptone
ZEA	Zeatin

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GENETIC TRANSFORMATION AND EXPRESSION ANALYSIS OF COLD TOLERANT GENE IN TOMATO (*Solanum lycopersicum* Mill.)

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ABSTRACT

Chilling stress severely reduces the productivity of tomato as it is a cold sensitive plant. *CBF3/DREB1A* plays a key role in generating cold tolerance in tomato by regulating the response of multiple genes under chilling stress. In this study, cold tolerant gene (*DREB1A*) driven by Lip9 promoter, was transformed in three tomato genotypes (Rio Grande, Moneymaker and Roma) through *Agrobacterium tumefaciens*, employing tissue culture dependent and tissue culture independent transformation strategies. For tissue culture dependent transformation strategy, the effects of various PGRs (IAA, NAA, ZEA, Kin, BAP and GA_3) and two ethylene inhibitors ($AgNO_3$ and $CoCl_2$) were investigated on callus induction and *in vitro* shoot regeneration. The maximum callus induction frequency (67.48%) was recorded on MS basal media enriched with 2.0 mg/l IAA, 2.5 mg/l BAP in cv. Rio Grande followed by Roma (62%) and Moneymaker (58.23%). Supplementation of $AgNO_3$ (10-15 mg/l) in MS basal media along with PGRs (2.0 mg/l IAA, 2.5 mg/l BAP) significantly yielded the highest callus induction frequency (91.83%) in cv. Rio Grande, followed by Moneymaker (82.66%) and Roma (88.33%). Similarly, *in vitro* shoot regeneration frequency on MS media fortified with 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP significantly enhanced with the addition of 8-10 mg/l $AgNO_3$ in all the cultivars i.e. in cv. Rio Grande (96.65%) followed by Roma (92.66%) and Moneymaker (90%). Likewise, the highest callogenesis (75.65%) was recorded in cv. Rio Grande on callus induction medium (CIM) supplemented with $CoCl_2$ (5.5 mg/l), IAA (2.0 mg/l) and BAP (2.5 mg/l) followed by cv. Roma whose best callus induction (73.66%) was obtained on CIM supplemented with $CoCl_2$ (4.5 mg/l), IAA (1.0 mg/l) and BAP (2.5 mg/l). In case of cv. Moneymaker the best callogenesis (68%) was secured on CIM having $CoCl_2$ (3.5 mg/l), NAA (2.0 mg/l) and BAP (2.0 mg/l). The

highest *in vitro* shoot regeneration (85%, 81% and 78%) was recorded in Rio Grande, Moneymaker and Roma, respectively on shoot induction media supplemented with $CoCl_2$ (4.25 – 5.0 mg/l). During this study, various concentrations of sucrose and sorbitol were scrutinized on *in vitro* shoot culture. The highest *in vitro* shoot regeneration frequency (100, 97.69 and 99%) was recorded in Rio Grande, Moneymaker and Roma with accumulative effect of sucrose and sorbitol (30: 30 g/l). Subsequently, transformation experiments were conducted by optimizing various factors both for tissue culture based and *in planta* techniques. For tissue culture based method of transformation; fifteen days old *in vitro* seedlings, forty-eight hours pre-culture period, bacterial density ($OD_{600\ nm}$ = 0.2), three minutes infection period, 60 µM acetosyringone, forty-eight hours co-cultivation period, pH 5.6 of co-cultivation media, six days pre-selection duration, cefotaxime (500 mg/l) and hygromycin (35 mg/l) as lethal dose were found optimum. For *in planta* technique of transformation, various factors such as growing medium; soil: vermiculite (1: 1), optical density ($OD_{600\ nm}$ = 1.0) and incubation period (20 min) were found optimum for efficient transformation efficiency. Polymerase chain reaction, Multiplex polymerase chain reaction, Southern blotting and Reverse transcriptase PCR confirmed the presence, integration and expression of *DREB1A* in T_0 - T_2 transgenic lines. Physiological and biochemical analyses of T_2 transgenic plants depicted that after various chilling stresses; stomatal conductance, transpiration rate, CO_2 concentration rate, photosynthetic rate, relative water contents, proline contents, total soluble sugar contents, chlorophyll contents, carotenoid contents and ascorbic acid contents of transgenic lines were significantly higher than those of NT plants. These findings clearly indicate that transgenic tomato plants over-expressing *Arabidopsis CBF3* gene enhanced protection and provided cold tolerance under controlled conditions in transgenic containment.

I. INTRODUCTION

Low temperature is the most critical factor limiting the productivity and distribution of many plants. In nature, low temperature (0 – 15 ºC) stress is common that directly inhibits metabolic reactions by preventing the full expression of plant genetic capability and generates various stresses such as osmotic stress by inhibiting water uptake, oxidative stress and dehydration (Chinnusamy *et al.,* 2007; Jewell *et al.,* 2010; Theocharis *et al.,* 2012). Among them, dehydration causes severe losses to crop plants as it produces cellular changes such as disruption of membrane structure, denaturation of proteins and the production of huge amount of toxic solutes (Byun *et al.,* 2009). Moreover during cold, reactive oxygen species are yielded that rupture cell membranes and as a result, gene expression and protein synthesis are altered (Wang *et al.,* 2003; Nishiyama *et al.,* 2006). Chilling stress loses membrane integrity which results in the leakage of solutes. Due to this leakage, integrity of cellular organelles and proper functioning of photosynthesis are interrupted because chilling destroys the electron transport chain and activity of Rubisco protein (Mahajan and Tuteja, 2005; Huang *et al.,* 2012). Phenotypically, chilling stress exhibits various symptoms such as smaller leaf expansion, wilting and necrosis which lead to death of tissues and in severe cases, reproductive growth of plants may also be hampered leading to the sterility in flowers (Jiang *et al.,* 2002).

Tomato (*Solanum lycopersicum* Mill.) is the most economical cash crop in fresh vegetable market and also in food-processing-industry (Hendelman *et al.,* 2013). It is a tremendous source of lycopene and β-carotene. Lycopene has been found protective against prostate cancer, while β-carotene works as a precursor for vitamin A (Romer *et al.,* 2000). Minute quantity of flavonoids is also present in their peels (5-10 mg/kg fresh weight) that are used as antioxidants to reduce the risk of cardiovascular diseases in humans (Muir *et al.,* 2001). It has also been extensively used for domestic purposes, breeding studies and fruit development (Huang *et al.,* 2012). It has been widely used experimentally due to small genome size (0.7-1.0 pg) and diverse germplasm (Kumar *et al.,* 2012). In Pakistan, area under tomato cultivation was 52,300 hectares with an annual

production of 530,000 tons during the year 2011-12 (Agricultural Statistics of Pakistan, 2011-12).

The first report about genetic transformation in tomato via *Agrobacterium* was given in 1986 (McCormick *et al*., 1986). Since then, a lot of reports have been found in literature about successful tomato transformation for multiple purposes such as production of glyphosate herbicide tolerant plants (Fillatti *et al.,* 1987), delay in ripening of fruits (Janssen *et al.* 1998), improved protocols for genetic transformation (Park *et al.*, 2003), release of resistant plants against fungal and bacterial diseases (Lin *et al.*, 2004; Afroz *et al.,* 2011), improved quality of fruits (Davuluri *et al.,* 2005), production of human vaccines (Youm *et al.,* 2008), production of male sterile plants (Sinha and Rajam, 2013), production of salt tolerant plants (Alvarez Viveros, 2013) and modulation of plant defense system (Xin *et al.,* 2014).

Tomato serves as an ideal plant for learning plant defenses against different stresses (Tian *et al.,* 2012). However, there has not been much known about its stress responses (Hsieh *et al.,* 2010). The plants that are susceptible to temperature below 12 ºC are said to be chilling-sensitive. Tomato is a chilling-sensitive plant as its growth, respiration and photosynthesis are severely affected by low temperature (below 12 ºC) (Garstka *et al.,* 2007; Sui *et al.,* 2007). In tomato, about 70% production losses have been reported only due to cold (Lysak, 2010). Therefore, the plants must adapt the specific tolerance mechanisms in order to maintain their growth and productivity under cold stress conditions. Plant modification has been done by manipulating the genes to defend and retain the proper structure and functions of various parts of a cell. (Wang *et al.,* 2003). Currently plant biotechnology strategies are helpful by the introduction of genes regulating the membrane perpetuity, enhancement of antioxidative mechanism for scavenging ROS, accumulation of soluble sugars, low molecular weight cryoprotectants such as proline, glycine betaine and other solutes, maintaining ion homeostasis and stabilizing proteins for stress tolerance have become vital in defending plant cells from chilling stress (Yang *et al.,* 2005; Byun *et al.,* 2009; Janska *et al.,* 2010).

Chilling tolerance is controlled by multiple genes and it is difficult to engineer multigenic traits for resistance in tomato like that of monogenic traits of resistance for

herbicides and pests (Foolad, 1999; Roy *et al.,* 2006). Therefore, the transformation of transcriptional regulatory genes in place of one or a few genes has been well-liked among researchers (Singh *et al.,* 2011). It has been documented that the capability to cold acclimatize is associated with definite signal transduction pathways ensuring in the commencement of numerous cold-regulated (COR) genes that develop tolerance in plants (Luo *et al.,* 2012; Muniz Garcia *et al.,* 2012; Castonguay *et al.,* 2013). C-repeat binding factors (*CBF/DREB*) have been reported in previous studies to induce the expression of a group of genes related to increased stress tolerance (Wisniewski *et al.,* 2011; Yang *et al.,* 2011; Rae *et al.,* 2011). The subfamily *DREB* can be divided into further six small classes termed as A-1 to A-6 (Liu *et al.,* 2007). Among them, *DREB1A* (A-1) and *DREB2A* (A-2) are two larger groups of *DREB*/*CBF* family of proteins induced by chilling and drought stress, respectively and express multiple genes which are implicated in stress tolerance in various plants (Sengupta and Majumder, 2009). Dehydration-responsive element binding factor 1A/cold responsive-element binding factor 3 (*DREB1A*/*CBF3*) binds to a DNA regulatory element termed as CRT/DRE containing the same motif (CCGAC) only, regulating the expression of cold stress-responsive genes and enhances chilling tolerance in tomato (Wang *et al.,* 2003; Xiong and Fei, 2006; Hsieh *et al.,* 2010). In previous research study, it has also been reported that *CBF3*/*DREB1A* is induced by chilling stress at transcriptional level in an ABA-independent manner encoding proteins that save plants against chilling injury (Kim, 2007).

The degree of cold tolerance in tomato is correlated with its tendency to stomatal closure during chilling stress (Aroca *et al.,* 2001, 2003; Bloom *et al.,* 2004). The stomatal behavior during chilling is related to water status within the leaf and integrity of membrane system. The flow of water declines during cold stress due to which stomata become closed (Hubbard *et al.,* 2001; Matzner and Comstock, 2001). Photosynthesis is greatly influenced by stomatal closure under cold stress due to which CO_2 supply to the leaf becomes limited and ultimately electron transport chain of photosynthesis is reduced, resulting in the production of reactive oxygen species (ROS) (Gill *et al.,* 2013). It has been reported that the first symptom of low temperature stress occurs as a stomatal closure that inhibits photosynthesis (Zhang *et al.,* 2012). The transpiration rate is an

essential phenomenon that affects the water relation in plants. Water acts as a solvent and medium for many physiological and biochemical activities such as photosynthesis, uptake of nutrients, transportation of materials and cooling effects that have direct influence on plant growth and development (Farooq *et al.,* 2009; Guana *et al.,* 2011). The high transpiration rate increases water uptake and turgor pressure that allow more supply of CO_2 to the leaf due to greater stomatal aperture as well as rapid cell division (Brini *et al.,* 2007). Relative water contents (RWC) is an essential parameter for screening cold tolerance in plants because more RWC indicates that plants have more water holding capacity that makes them more adaptive in cold environment (Gupta *et al.,* 2012). Chilling stress reduces the membrane viscosity and decelerates metabolism leading to the production of free radicals and oxidative stress that change the normal activities of macromolecules and decrease the osmotic potential (Xiong *et al.,* 2002). Chilling stress also affects the water relations in a cell as well as in the whole plant that creates ice crystals, due to which dehydration stress is also produced (Beck *et al.,* 2007). In order to maintain water potential, the plant cell must regulate its internal osmotic potential to enhance the mechanism of stress tolerance (Khare *et al.,* 2010).

The most common method of gene transfer in tomato is via *Agrobacterium* because it is superior to other direct methods including PEG-mediated gene transfer, electroporation, microinjection and gene gun system in a sense that it is an economical and efficient method that ensures stable transformation of desired genes (Rai *et al.,* 2012). This method utilizes natural potential of *Agrobacterium* cells for transferring T-DNA into host genome; therefore it avoids special equipment and gene silencing during transformation (Yenchon and Te-chato, 2012). Presently, a novel method of gene transfer is being employed in various crop species that avoids tissue culture steps and can offer efficient transformation by producing transgenic plants directly from infected tissues (Supartana *et al.,* 2005). This *Agrobacterium*-mediated *in planta* transformation technique is better than that of tissue culture based method because it is simple, quick, less labour and expertise incentive. It limits the chances of accidental mutagenesis and also minimizes the somaclonal variations that occurred through *in vitro* system (Mehrotra and Goyal, 2012; Subramanyam *et al.,* 2013).

Efficient gene transfer by *Agrobacterium tumefaciens* necessitates efficient regeneration system for transformed cells because there is no warranty that a transformed plant cell will be proved to be a regenerable (Ijaz *et al.,* 2012). Therefore, *in vitro* development of plants is a prime requirement of plant biotechnology because different tomato genotypes possess diverse morphogenetic capabilities (Tomsone *et al.,* 2004; Vasil, 2008). Genetic make-up of plants is not an absolute factor for controlling their morphogenesis but plant growth regulators also have a greater influence on morphogenesis (El-Bakry, 2002). *In vitro* development of calli, shoots and roots mainly depend upon the ratio of hormones especially auxins and cytokinins supplemented to media also on type of explants (Tantikanjana *et al.,* 2001). A standard *in vitro* shoot regeneration protocol is crucial for the development of stress tolerant cultivars through genetic transformation (Godishala *et al.,* 2011). Unfortunately, the regeneration system of tomato is several times lower than those of other Solanaceae family members (Venkatesh and Park, 2012).

The phenomenon of ethylene mechanism of action in *in vitro* culturing of plant studies is not clear, but its effect has been reported in many aspects of callus induction and regeneration (Ptak *et al.,* 2010). Silver nitrate ($AgNO_3$) is an important ethylene inhibitor (Kanwar *et al.,* 2010). The stimulatory effect of $AgNO_3$ has been reported in many studies and it has been concluded that it greatly improves regeneration ability of many monocots and dicots inhibiting ethylene synthesis (Parimalan *et al.,* 2011). Cobalt chloride is another chemical that strongly inhibits the production of ethylene and hence improves growth and development of plants (Ascough *et al.,* 2007; Kothari-Chajer *et al.,* 2008).

Carbon sources are crucial ingredients for tissue culture media, and sucrose is frequently used as a carbon source (Yaseen *et al.,* 2013). It is because of its some important characteristics such as it is highly soluble in water, lack of adverse effects on many biochemical processes. The various *in vitro* studies have revealed that sucrose enhances the optimum growth and is relatively cheaper carbon source (Mello *et al.,* 2001). In spite of extensive use of sucrose in tissue culture studies, other sugars such as sorbitol also acts as a primary source of carbon for the enhancement of organogenesis

frequency and also as an osmotic regulator for improving the potential of regenerating calli (Geng *et al.*, 2008; Kumar *et al.*, 2010; Shahsavari, 2011). These sugars are translocated into plants and increase the process of cell differentiation (Jain and Babbar, 2003). The sufficient osmotic potential can't be created by the addition of carbohydrates in culture medium for the enhancement of somatic embryos. This enhancement can be provoked by the supplementation of polyalcohol such as sorbitol (Hita *et al.,* 2003).

Taking into account the ever increasing requirements of tomato in Pakistan, it has become important to produce tomato under open field conditions in winter season. Therefore, the current research had following objectives:

1. To establish various tissue culture protocols for different genotypes of tomato.
2. To optimize various transformation factors both for tissue-culture dependent and tissue-culture independent (*in planta*) methods.
3. To incorporate *CBF3*/*DREB1A* gene using *Agrobacterium*-mediated tissue culture dependent and *in planta* transformation methods.
4. To evaluate cold tolerance in tomato transgenic lines through transformation of *CBF3* gene on the basis of molecular, physiological and biochemical studies.

II. REVIEW OF LITERATURE

The cultivated tomato is more sensitive to chilling stress (0 to 12 ºC) at all developmental stages from germination to fruit setting (Liu *et al.,* 2012). The injurious effects of chilling stress can be minimized by developing chilling tolerant tomato cultivars. The chilling tolerance is a complex phenomenon that depends upon various physiological and biochemical changes that occur during cold acclimation (Heidarvand and Maali Amiri, 2010). It has been well documented that CBFs pathway regulates multiple cold inducible genes for achieving cold tolerance in tomato (Chinnusamy *et al.*, 2007; Zhou *et al.,* 2011). Therefore, for the production of cold tolerant tomato cultivars, the comprehensive literature review about various tissue cultures, genetic transformation, physiological and biochemical studies in tomato have been systemized below:

2.1 Tissue culture studies

Khuong *et al.* (2013) optimized *in vitro* shoot regeneration system in tomato cultivar, Micro Tom using leaf explants and concluded that microshoot induction was obtained on MS basal media enriched with zeatin (1.0 mg/l) and IAA (0.1 mg/l). During this study, regeneration was improved by transferring microshoots on MS basal media having 2.0 mg/l GA_3 and the highest root formation frequency (97%) was secured on MS media enriched with 1 mg/l IAA.

Vinoth *et al.* (2013) scrutinized the effect of phytohormones and sea weed extracts in tomato and reported that the highest callus induction frequency (97.8%) was secured on MS medium augmented with picloram (0.6 mg/l) and kinetin (0.3 mg/l) using leaf explants and maximum number of embryos were obtained on MS medium fortified with 10% extract of *Gracilaria corticata* sea weed.

Ali *et al.* (2012) studied the interaction of various concentrations of two cytokinins (BA and Kinetin) for callus induction and proliferation followed by regeneration in four tomato genotypes. The maximum frequency (100%) of calli induction and *in vitro* shoot regeneration was recorded for all the genotypes tested from cotyledon and hypocotyl explants. The cotyledons generated more multiple shoots per

explant compared to hypocotyls. MS medium enriched with kinetin (1.0 mg/l) and BAP (1.0 mg/l) was found to be optimal for multiple shoots from both types of explants.

Vikram *et al. (*2012) reported an efficient regeneration and multiple shoot induction protocol for a tomato genotype (S-22) using cotyledon as explants. The explants produced the highest number of multiple shoots on MS media enriched with IAA (0.2 mg/l) and zeatin (1.2 mg/l). The efficient shoot elongation percentage was recorded on MS medium fortified with GA_3 (0.6 mg/l) and zeatin (1.0 mg/l). The elongated shoots were shifted to a rooting medium along with IAA (0.5 mg/l), IBA (0.5 mg/l) and NAA (0.5 mg/l) combination.

Ashakiran *et al.* (2011) developed an efficient tissue culture protocol for the tomato cultivar Shalimar. The best callus induction frequency was obtained from cotyledonary explants with MS media having 2, 4-D (2 mg/l). The protocol for direct embryogenesis was also optimized where somatic embryos were produced directly on MS medium fortified with 2, 4-D (5 mg/l). The fresh globular and torpedo embryos were achieved with MS medium having sorbitol (0.4 M) and ABA (0.25 mg/l).

Cruz-Mendivil *et al.* (2011) reported that the maximum regeneration frequency (82%) and the highest number of shoots (5.6) were achieved with MS basal medium fortified with 2.0 mg/l zeatin, 0.1 mg/l IAA and 300 mg/l timentin in a tomato cultivar (Micro-Tom) using leaf as explants. The developed shoots were then transferred to ½ MS media having 15 g/l sucrose for efficient shoot regeneration.

Osman and Khalafalla (2010) scrutinized the effect of $AgNO_3$ and $CoCl_2$ on *in vitro* regeneration of shoots in tomato cv. Omdurman using shoot tip explants and pointed that efficient shoot regeneration and more shoots were recorded on MS basal media fortified with kinetin (4.0 mg/l), $AgNO_3$ (5.0 mg/l) and $CoCl_2$ (3.0 mg/l). Similarly, the longest roots were found on MS media having 0.1 - 0.5 mg/l IBA.

Rashid and Bal (2010) investigated the direct shoot regeneration in two tomato genotypes; Punjab Upma and IPA-3 using hypocotyls explants and concluded that the highest regeneration frequency (82.57 and 86.02%) was obtained in IPA-3 and Punjab Upma on MS medium augmented with 0.5 mg/l Kin and 0.5 mg/l BAP. The shoot

elongation was done on MS medium fortified with BAP (0.3 mg/l) and then developed shoots were shifted to ½ MS basal media and hundred percent rooting was noticed in both the genotypes. After proper rooting, the plantlets were transferred to moist cotton as hardening that showed efficient plants survival rate in tomato.

Harish *et al*. (2010) found maximum calli induction (90%) on hormonal regimes of NAA (0.5 mg/l) and BAP (2.0 mg/l) using leaf, hypocotyls and stem from 10-day-old *in vitro* seedlings in tomato. Similarly, the efficient *in vitro* shoot regeneration was secured on MS basal media along with 3.0 mg/l BAP. During this study, the hypocotyls performed better in producing shoots compared to leaf and stem in all the genotypes and treatments tested.

Ishag *et al*. (2009) investigated *in vitro* shoot regeneration in tomato using shoot tip and cotyledon explants from eight to ten day-old *in vitro* produced seedlings and narrated that explants, types and varying levels of PGRs mutually affected the *in vitro* shoot induction. In this experiment, shoot tip and kinetin were found to be more efficient in producing multiple shoots as compared to cotyledon and BAP. While multiple shoot induction was adversely affected when NAA was added in MS medium having kinetin and BAP.

Devi *et al.* (2008) conducted a series of research tests for *in vitro* morphogenic characteristics in four tomato genotypes under distinct concentrations and in combination of plant synthetic hormones in MS media. Among these hormones, BAP @ 3 mg/l and IAA @ 2.5 mg/l in MS medium showed best callus induction, regeneration and shoots formation. The efficient rhizogenesis was recorded on ½ strength MS basal media fortified with IBA (0.2 mg/l).

2.2 Transformation studies

Vinoth *et al.* (2013) optimized a novel approach for the production of transgenic tomato using *in planta* technique. Germinated seeds (one-day-old) were microinjected with bacterial inoculums having $OD_{600\ nm}$ = 0.6 and co-cultivated with MS basal medium enriched with acetosyringone (100 mM) and kanamycin (30 mg/l) that improved the transformation efficiency (46.28%) in tomato.

Rizwan and Bal (2012) developed an efficient transformation protocol for a tomato cultivar Punjab Upma. The *Agrobacterium tumefaciens* strain GV3101 carrying kanamycin as a selection agent controlled by 35 S promoter employed for transformation. Various factors such as *Agrobacterium* density ($OD_{600\ nm}$ = 1.0), acetosyringone concentration (25 µM) and kanamycin concentration (30 mg/l) was found to be optimal for securing the highest transformation efficiency.

Guo *et al.* (2012) established a quick transformation protocol for Micro-Tom tomato. Various factors including *Agrobacterium* optical density, infection time, co-cultivation period and optimum dose of carbancilin antibiotic for selection were investigated. The explants morphology after infection was also studied. Results revealed that bacterial cell density ($OD_{600\ nm}$ = 0.5), infection time (5 min), addition of 500 mg/l carbancilin in selection media increased the production of transgenic plants. The overall transformation efficiency (5.1%) was recorded in this study.

Islam *et al.* (2010) established a new transformation protocol in tomato using cotyledonary explants and outlined that bacterial optical density ($OD_{600\ nm}$) of 0.8 and 3 days co-cultivation period increased transformation efficiency. The resulted tranformants were selected on kanamycin concentration (200 mg/l) and screened by PCR for the presence of *GUS* gene.

Paramesh *et al.* (2010) reported a rapid transformation system for a tomato cultivar L15. An *Agrobacterium* strain GV 2260 carrying GUS gene along with hygromycin phosphotransferase (*hptI*) gene for selection was used. Various factors such as co-cultivation period of forty-eight hours and 300 mg/l claforan® rapidly enhanced the transformation efficiency.

Gao *et al.* (2009) optimized an efficient and reproducible transformation system in tomato. The overnight *Agrobaterium* culture was diluted ten times in LB media and $OD_{600\ nm}$ = 0.1 was used for infection. The results showed that $OD_{600\ nm}$ = 0.1 increased transformation efficiency up to 44.7 %.

Siddig *et al.* (2009) reported a transformation protocol for tomato cv CastleRock. The results showed that cotyledonary explants were infected with bacterial culture having

optical density ($OD_{600\ nm}$ = 0.8) for 30 minutes and hgromycin @ 25 mg/l increased transformation efficiency. After selection, hygromycin resistant cotylydonary explants were moved to MS basal media containing 5 mg/l BA and 1.0 mg/l IAA for regeneration.

Sharma *et al.* (2009) described a rapid transformation protocol in tomato by optimizing co-cultivation period, and *Agrobacterium* optical density. Explants were co-cultivated with *Agrobacterium* density (10^8 cells/ml) for 3 d on MS media containing 2.0 mg/l BAP and were regenerated with MS media having 1 mg/l zeatin. The resulted transgenic plants were screened through GUS assay showing 41.4 % transformation frequency while stable integration was accomplished through Southern blotting.

Yasmeen *et al.* (2009) established a new *in planta* transformation protocol via floral dip and fruit injection method. The transformation frequency varied with different constructs used. The two days incubation period was found to be optimal for the highest transformation efficiency. The transformation frequency was greater for mature red fruit compared to green fruit. The transformation frequency was higher with floral dip method compared to fruit injection method. Moreover, infection of mature red fruits performed better than that of infecting the green fruits.

Maruyama *et al.* (2009) reported CBF3/DREB1A a transcription factor interacted with a DRE; involved in expression of cold responsive genes in *Arabidopsis* and improved the freezing tolerance in transgenic plants due to overexpression of *DREB1A* accumulating important metabolites such as monosaccharides, disaccharides, trisaccharides, and sugar alcohols etc.

2.3 Physiological studies on cold tolerance

Huang *et al.* (2012) asserted that CO_2 concentration was limited by low temperatures due to stomatal closure and therefore, generation of $NADP^+$ was reduced in Calvin cycle, accordingly, electron transport chain of photosynthesis was over-reduced. All these changes resulted in the production of reactive oxygen species such as superoxide radical and singlet oxygen that impaired the chloroplast.

Zhang *et al.* (2012) reported significant chilling tolerance in cotton transferring *betA* gene that enhanced glycinebetaine synthesis. Due to overexpression of *betA* gene,

transgenic plants exhibited higher transpiration rate, stomatal conductance and net photosynthesis than that of wild type plants.

Singh *et al.* (2011) attempted to improve cold tolerance in tomato and engineered tomato plants over-expressing *At-CBF1* gene. The transformants in T_1 generation were marker-free that showed inducible expression only under cold stress (4 ºC) for three days and more survival rate (50%) was noticed in transgenic lines compared to non-transgenic plants (10%). The transgenic plants had higher relative water contents than that of NT plants after exposing to chilling stress and indicated better adaptive capability under chilling stress.

Khare *et al.* (2010) conducted a study on tomato by transferring *mtlD* gene for the improvement of cold tolerance and reported that tomato transgenic plants survived for two days when exposed to chilling stress (4 ºC) while control plants gradually died. They also narrated that transgenic plants accumulated mannitol which increased leaf osmotic potential to retain water potential in surroundings and remained protected from dehydration stress induced by cold.

Pino *et al.* (2008) examined the role of *AtCBF1* in potato under chilling stress and their findings implied that overexpression of *AtCBF1* enhanced cold tolerance in transgenic plants when they were exposed to 2 – 4 ºC chilling stress. They also reported that transgenic plants were darker green in color due to more chlorophyll contents and greater stomata than their wild type counterparts and showed higher photosynthetic rate, signifying greater potential for productivity.

Garstka *et al.* (2007) narrated that chilling disrupted photosynthesis by interrupting stomatal conductance, thylakoid electron transport, Rubisco and other enzymes activities in tomato. The primary response of chilling resulted in the reduction of stomatal conductance, due to which intercellular CO_2 concentrations was reduced as stomata might be a primary target of chilling and ultimately, photosynthesis was declined.

2.4 Biochemical studies on cold tolerance

Liu *et al.* (2013) improved chilling tolerance in tomato by transforming *Lefad7* gene and reported that transgenic tomato plants exhibited higher degree of cold tolerance by regulating trienoic fatty acids and accumulating increased chlorophyll contents than wild type plants under low temperature (4 °C) stress. They inferred that chilling tolerance was generated in tomato by overexpressing *Lefad7* with alteration in membrane lipids composition.

Zhou *et al.* (2013) isolated *LeLUT1* gene from tomato, transformed this gene in tobacco and concluded that transgenic tobacco plants improved chilling tolerance overexpressing *LeLUT1* gene. They also reported that transgenic plants had higher carotenoid contents and lower levels of relative electrical conductivity and reactive oxygen species including H_2O_2, O_2^-, malondialdehyde contents compared to control plants under cold stress and ultimately photosynthesis sensitivity to cold stress was alleviated in transgenic plants.

Gupta *et al.* (2012) developed cucumber transgenic plants by transferring *Arabidopsis DREB1A* gene under the control of an inducible promoter RD29 and reported that T_2 transgenic plants enhanced the activities of antioxidants such as SOD and CAT under cold stress (4 °C) with improved chilling tolerance.

Miura *et al.* (2012) transformed *SlICE1* gene in tomato and claimed that antioxidants produced due to overexpression of *SlICE1* gene, protected the plant cells against free radicals during cold stress. They inferred that overexpression of *SlICE1* gene improved chilling tolerance in tomato accumulating antioxidants including ascorbic acid.

Movahed *et al.* (2012) improved potato transgenic plants against freezing stress overexpressing *DREB1B* and reported that transgenic plants conferred a considerable freezing tolerance accumulating higher proline contents and more relative water contents under cold stress conditions. They concluded that *DREB1B* stimulated the expression of several genes for the accumulation of improved proline contents associated with chilling tolerance in transgenic plants.

Yuanyuan *et al.* (2009) studied thoroughly the role of total soluble sugars in cold stress and ascertained that soluble sugars exerted their functions in cold acclimation and protected the plant cells from cold injury through various ways, including osmoprotectants, acting as nutrients and intruding with lipid bilayer and the most important one is to act like hormones in signal transduction pathways.

Zhang *et al.* (2009) generated tomato transgenic plants tolerant to cold transferring *AtIpk2b* gene from *Arabidopsis* and concluded that no significant differences were found between transformed and wild type plants with respect to electrolyte leakage under normal conditions but after exposure to chilling stress (4 ºC) for one day, the rates of membrane leakage wer greater in wild type plants as compared to transformed plants.

Sui *et al.* (2007) investigated the response of antioxidant enzymes in chilling stress by engineering tomato plants overexpressing the tomato *LeGPAT* gene. They concluded that elevated levels of two antioxidants such as SOD and POD were recorded in transgenic tomato plants that limited the production of free radicals such as O_2^- and H_2O_2 during cold stress (4 °C) resulting in the improvement of tolerance against cold stress.

In previous research reports, it has been documented that constitutive promoters especially CaMV35S decreases growth and productivity of plants, while lip9 inducible promoter shows the best results in producing stress tolerance without any negative impact on growth of plants (Takasaki *et al.,* 2010; Nakashima *et al.,* 2007, 2014). Moreover, the overexpression of *Arabidopsis DREB1A* gene in tomato under lip9 inducible promoter has not been reported so far. Therefore, in this study choice of lip9 inducible promoter was due to the reason that continuous expression of transgenes due to 35S promoter throughout life cycle of plants may cause changes in physiology as well as phenotypes of plants. This may arise biosafety issues and concerns.

The construct pBIH was provided by NIGAB, NARC Islamabad. It contained *Arabidopsis DREB1A* gene under the control of an inducible promoter lip9. Due to non-inhibitory effects of overexpression of *AtDREB1A* on plant growth, we used this construct to produce cold tolerance tomato transgenic lines. As in Pakistan, cold

drastically affects tomato production and up till now, no research work has been done on tomato to improve its cold tolerance.

From detailed literature survey, we selected the most cold sensitive Rio Grande, Moneymaker and Roma tomato cultivars to conduct the present study. These cultivars have determinate type of growth, early maturity and high yield. Moreover, these have large sized red fruit which is an excellent source of fresh market and processing. Since tissue culture and genetic transformation protocols are genotype dependent. Therefore, revise and optimized protocols in this study were attempted for efficient transformation system in all the genotypes tested.

III. MATERIALS AND METHODS

The transformation experiments and molecular analyses of cold tolerant gene (*CBF3*) were performed at Genetic Transformation Lab, National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (Islamabad), Pakistan during the year 2010-12, while morphological, physiological and biochemical analyses of transgenic and NT tomato lines were conducted at Post Harvest Laboratory, Department of Horticulture, PMAS-Arid Agriculture University (Rawalpindi), Pakistan during the year 2013-14.

3.1 Techniques employed in tissue culture experiments

3.1.1 Plant material

Seeds of tomato (*Solanum lycopersicum* Mill. cvs. Rio Grande, Moneymaker and Roma) were provided by Horticultural Research Institute (HRI), NARC Islamabad, Pakistan. The mature and healthy seeds of these cultivars were drenched in sterilized water for 24 hrs at 4 $^{\circ}$C for breaking seed dormancy. The seeds were disinfected with 70% (v/v) ethyl alcohol for 1 min and then in 5.25% sodium hypochlorite (NaOCl) at 40% (v/v) with 2 drops/100 ml of Tween-20 for 20 min. Subsequently, the seeds were washed 5 times with sterilized water to remove the traces of clorox from the seeds. The seeds were dried on autoclaved filter paper for 15 min and cultured on Murashige and Skoog (MS) (1962) medium supplemented with 30 g/l sucrose and 7 g/l agar (Sigma, USA). The pH of the medium was maintained at 5.7 with 1.0 N NaOH or 1.0 N HCl before autoclaving. The cultures were kept in the dark conditions for about 5 days (until germination) and then put under 16 hrs' photoperiod, 25 ± 2 ºC temperature, 50 µmolm^{-2}s^{-1} fluorescence light and 65-70% relative humidity.

3.1.2 Evaluation of the best responsive explants in tomato tissue culture studies

In this experiment, 5 explants namely hypocotyls, epicotyls, internodes, leaf discs and cotyledons were appraised for their ability to produce shoots. Hypocotyls and epicotyls (1-2 cm), cotyledon (0.5-1.0 cm), internodes (4-7 mm) and leaf discs (5×5 cm^2) were cut from 15-d-old *in vitro* seedlings under sterile conditions and used as explants.

These were cultured on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA. Data were collected after 1 month of culturing.

3.1.3 Culture of explants on callus induction medium fortified with silver nitrate

Hypocotyl and leaf discs were cut from 15-d-old *in vitro* seedlings under sterile conditions, used as explants and were put on callus induction medium (CIM). The effects of various PGRs were evaluated on callus induction (Table 3.1). The cytokinin; BAP (0.5 – 2.5 mg/l) and kinetin (2.0 mg/l) in combination with auxins; IAA (0.5 – 2.0 mg/l), NAA (1.0 – 2.0 mg/l) and 2, 4-D (3.0 – 4.0 mg/l) were put in Erlenmeyer flasks containing 25 ml MS medium. Hereafter, the explants were cultured on CIM supplemented with an ethylene inhibitor, $AgNO_3$ @ 2.5 – 20.0 mg/l for investigating the effect of $AgNO_3$ on callus induction in tomato (Table 3.2). In all callus induction media, 30 g/l sucrose was added and pH was set to 5.7 with HCl (1.0 N) or NaOH (1.0 N) before putting 7 g/l agar (Sigma, USA) and autoclaving was done at 121 ºC for 15 min. $AgNO_3$ in aqueous solution was sieved through filters (0.22 µm Millipore, Sartorius Germany Ltd) and then supplemented it to sterilized CIM after cooling up to 50 ºC and stored at 4 ºC. The flasks were shifted to a culture room at 25 ± 2 ºC in dark condition for 15 d and then put in 50 $\mu molm^{-2}s^{-1}$ fluorescent light with 16 hrs light and 8 hrs dark photoperiod and 65-70% relative humidity. For all the genotypes, 4 explants per jar and 3 repetitions for each genotype and treatment were investigated. After 30 d of culture, the callus induction frequency was recorded for each treatment combination.

3.1.4 Evaluation of CIM fortified with cobalt chloride on *in vitro* callus induction

Hypocotyls and leaf discs were cultured on CIM supplemented with cobalt chloride ($CoCl_2$) @ 0.5 – 10.0 mg/l to assess its efficacy on callus induction in three tomato cultivars (Table 3.3). The same amount of sugar (30 g/l) was added to the tested media of callus induction and pH was adjusted at 5.7 prior to addition of 7 g/l of agar (Sigma, USA). The media were autoclaved for 15 minutes at 121 ºC. At the same time $CoCl_2$ was filter-sterilized using a filter (0.22 µm Millipore) and then poured it into cooled media up to 50 ºC. The flasks were shifted to a culture room at 25 ± 2 ºC in dark condition for fifteen days and then put in 50 $\mu molm^{-2}s^{-1}$ fluorescent light with sixteen

hours light and eight hours dark and 65-70% relative humidity. For all the genotypes, 4 explants per jar and 3 repetitions for each genotype and treatment were investigated. The callus induction frequency was recorded for each treatment after thirty days of culturing.

3.1.5 *In vitro* shoot regeneration from embryogenic callus culture via hormonal combinations and $AgNO_3$

The hypocotyls and leaf discs-derived embryogenic calli were cultured on various shoot induction media (SIM) (MS salts, sucrose 3%, plant agar 0.7% and different hormonal regimes). During this study, the influence of cytokinins, auxins and gibberellin were investigated on shoot organogenesis and the number of primordial shoots per explants. The cytokinins; BAP (0.5 – 3.0 mg/l), kinetin (1.0 – 2.5 mg/l) and zeatin (1.0 mg/l) alone or in combination with auxins; IAA (0.5 – 1.0 mg/l) and NAA (0.5 mg/l) and gibberellin; GA_3 (0.5 mg/l) were put in jars (height; 12 cm & diameter; 8 cm) (Table 3.4). The proliferated calli were then shifted to these jars having different SIM for shoot organogenesis. The impact of $AgNO_3$ was also explored on shoot organogenesis and the number of primordial shoots per explant. The compact calli were cultured on SIM enriched with $AgNO_3$ @ 2.0 – 15.0 mg/l (Table 3.5). $AgNO_3$ was sieved through filters (0.22 µm Millipore) and then supplemented to sterilized shoot induction media. For all the genotypes, 4 explants per jar and 3 repetitions for each genotype and treatment were investigated. The data about the frequency of explants showing shoot regeneration percentage and the mean number of shoots per calli clumps were recorded weekly until 60^{th} day of culture.

3.1.6 Assessment of $CoCl_2$ on shoot organogenesis from calli clumps

The embryogenic calli from both explants were transferred on various shoot induction media (SIM) (MS basal salts, 3% sucrose, 0.7% plant agar) and diverse hormonal combinations i.e. cytokinins (0.5 – 3.0 mg/l BAP), (1.0 – 2.5 mg/l kinetin) and (1.0 mg/l zeatin) alone or combined with auxins (0.5 – 1.0 mg/l IAA), (1.0 mg/l NAA) and gibberellins (0.5 mg/l GA_3) along with $CoCl_2$ (0.5 – 10.0 mg/l) were taken in jars (height; 12 cm & diameter; 8 cm) for *in vitro* shoot regeneration under same cultural

Table 3.1 Type I callus induction media used for calli proliferation in tomato with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Media	Composition
CIM_1	MS, 3.0 mg/l 2,4-D
CIM_2	MS, 4.0 mg/l 2,4-D
CIM_3	MS, 0.5 mg/l IAA, 0.5 mg/l NAA, 0.5 mg/l Kin, 0.5 mg/l BAP
CIM_4	MS, 2.0 mg/l NAA, 2.0 mg/l BAP
CIM_5	MS, 1.0 mg/l NAA, 2.5 mg/l BAP
CIM_6	MS, 2.0 mg/l IAA, 2.5 mg/l BAP
CIM_7	MS, 1.0 mg/l IAA, 1.0 mg/l NAA, 2.0 mg/l Kin
CIM_8	MS, 0.5 mg/l IAA, 2.0 mg/l 2,4-D

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), 2, 4-D; 2, 4 dichlorophenoxy acetic acid, IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin

Table 3.2 Type II callus induction media used for calli proliferation in tomato with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Media	Composition
CIM_{1A}	MS, 3.0 mg/l 2,4-D, 2.5 mg/l $AgNO_3$
CIM_{2A}	MS, 4.0 mg/l 2,4-D, 5.0 mg/l $AgNO_3$
CIM_{3A}	MS, 0.5 mg/l IAA, 0.5 mg/l NAA, 0.5 mg/l Kin, 0.5 mg/l BAP, 7.5 mg/l $AgNO_3$
CIM_{4A}	MS, 2.0 mg/l NAA, 2.0 mg/l BAP, 10.0 mg/l $AgNO_3$
CIM_{5A}	MS, 1.0 mg/l NAA, 2.5 mg/l BAP, 12.5 mg/l $AgNO_3$
CIM_{6A}	MS, 2.0 mg/l IAA, 2.5 mg/l BAP, 15.0 mg/l $AgNO_3$
CIM_{7A}	MS, 1.0 mg/l IAA, 1.0 mg/l NAA, 2.0 mg/l Kin, 17.5 mg/l $AgNO_3$
CIM_{8A}	MS, 0.5 mg/l IAA, 2.0 mg/l 2,4-D, 20.0 mg/l $AgNO_3$

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), 2, 4-D; 2, 4 dichlorophenoxy acetic acid, IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, $AgNO_3$; Silver nitrate

Table 3.3 Type III callus induction media used for calli proliferation in tomato with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Media	Composition
CIM_{1B}	MS, 3.0 mg/l 2,4-D, 0.5 mg/l $CoCl_2$
CIM_{2B}	MS, 4.0 mg/l 2,4-D, 1.5 mg/l $CoCl_2$
CIM_{3B}	MS, 0.5 mg/l IAA, 0.5 mg/l NAA, 0.5 mg/l Kin, 0.5 mg/l BAP, 2.5 mg/l $CoCl_2$
CIM_{4B}	MS, 2.0 mg/l NAA, 2.0 mg/l BAP, 3.5 mg/l $CoCl_2$
CIM_{5B}	MS, 1.0 mg/l NAA, 2.5 mg/l BAP, 4.5 mg/l $CoCl_2$
CIM_{6B}	MS, 2.0 mg/l IAA, 2.5 mg/l BAP, 5.5 mg/l $CoCl_2$
CIM_{7B}	MS, 1.0 mg/l IAA, 1.0 mg/l NAA, 2.0 mg/l Kin, 7.5 mg/l $CoCl_2$
CIM_{8B}	MS, 0.5 mg/l IAA, 2.0 mg/l 2,4-D, 10.0 mg/l $CoCl_2$

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), 2, 4-D; 2, 4 dichlorophenoxy acetic acid, IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, $CoCl_2$; Cobalt chloride

Table 3.4 Type I shoot induction media used for *in vitro* shoot regeneration in tomato with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Media	Composition
SIM_1	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP
SIM_2	MS, 0.2 mg/l IAA, 1.5 mg/l Kin, 1.0 mg/l BAP
SIM_3	MS, 0.2 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP
SIM_4	MS, 0.1 mg/l IAA, 1.0 mg/l Kin, 1.0 mg/l BAP
SIM_5	MS, 0.1 mg/l IAA, 2.0 mg/l Kin, 1.0 mg/l BAP
SIM_6	MS, 0.1 mg/l IAA, 3.0 mg/l BAP
SIM_7	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP
SIM_8	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP
SIM_9	MS, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA_3
SIM_{10}	MS, 1.0 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP, 1.0 mg/l ZEA, 0.5 mg/l NAA
SIM_{11}	MS, 2.0 mg/l BAP

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, ZEA; Zeatin

Table 3.5 Type II shoot induction media used for *in vitro* shoot regeneration in tomato with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Media	Composition
SIM_{1A}	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 2.0 mg/l $AgNO_3$
SIM_{2A}	MS, 0.2 mg/l IAA, 1.5 mg/l Kin, 1.0 mg/l BAP, 3.0 mg/l $AgNO_3$
SIM_{3A}	MS, 0.2 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP, 4.5 mg/l $AgNO_3$
SIM_{4A}	MS, 0.1 mg/l IAA, 1.0 mg/l Kin, 1.0 mg/l BAP, 6.0 mg/l $AgNO_3$
SIM_{5A}	MS, 0.1 mg/l IAA, 2.0 mg/l Kin, 1.0 mg/l BAP, 7.5 mg/l $AgNO_3$
SIM_{6A}	MS, 0.1 mg/l IAA, 3.0 mg/l BAP, 8.5 mg/l $AgNO_3$
SIM_{7A}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP, 10.0 mg/l $AgNO_3$
SIM_{8A}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP, 12.0 mg/l $AgNO_3$
SIM_{9A}	MS, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA_3, 13.0 mg/l $AgNO_3$
SIM_{10A}	MS, 1 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP, 1.0 mg/l ZEA, 0.5 mg/l NAA, 14.0 mg/l $AgNO_3$
SIM_{11A}	MS, 2.0 mg/l BAP, 15.0 mg/l $AgNO_3$

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, ZEA; Zeatin, $AgNO_3$; Silver nitrate

Table 3.6 Type III shoot induction media used for *in vitro* shoot regeneration in tomato with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Media	Composition
SIM_{1B}	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 0.5 mg/l $CoCl_2$
SIM_{2B}	MS, 0.2 mg/l IAA, 1.5 mg/l Kin, 1.0 mg/l BAP, 1.5 mg/l $CoCl_2$
SIM_{3B}	MS, 0.2 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP, 2.25 mg/l $CoCl_2$
SIM_{4B}	MS, 0.1 mg/l IAA, 1.0 mg/l Kin, 1.0 mg/l BAP, 3.0 mg/l $CoCl_2$
SIM_{5B}	MS, 0.1 mg/l IAA, 2.0 mg/l Kin, 1.0 mg/l BAP, 3.75 mg/l $CoCl_2$
SIM_{6B}	MS, 0.1 mg/l IAA, 3.0 mg/l BAP, 4.25 mg/l $CoCl_2$
SIM_{7B}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP, 5.0 mg/l $CoCl_2$
SIM_{8B}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP, 6.0 mg/l $CoCl_2$
SIM_{9B}	MS, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA_3, 7.5 mg/l $CoCl_2$
SIM_{10B}	MS, 1.0 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP, 1.0 mg/l ZEA, 0.5 mg/l NAA, 8.5 mg/l $CoCl_2$
SIM_{11B}	MS, 2.0 mg/l BAP, 10.0 mg/l $CoCl_2$

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, ZEA; Zeatin, $CoCl_2$; Cobalt chloride

Table 3.7 Type IV shoot induction media used for *in vitro* shoot regeneration by assessing various concentrations of sucrose and sorbitol individually and accumulatively in MS basal medium

Media	Composition
RMS_1	4.3 g/l MS salts and vitamins, 5 g/l sucrose ± 5 g/l sorbitol
RMS_2	4.3 g/l MS salts and vitamins, 10 g/l sucrose ± 10 g/l sorbitol
RMS_3	4.3 g/l MS salts and vitamins, 15 g/l sucrose ± 15 g/l sorbitol
RMS_4	4.3 g/l MS salts and vitamins, 20 g/l sucrose ± 20 g/l sorbitol
RMS_5	4.3 g/l MS salts and vitamins, 25 g/l sucrose ± 25 g/l sorbitol
RMS_6	4.3 g/l MS salts and vitamins, 30 g/l sucrose ± 30 g/l sorbitol
RMS_7	4.3 g/l MS salts and vitamins, 35 g/l sucrose ± 35 g/l sorbitol
RMS_8	4.3 g/l MS salts and vitamins, 40 g/l sucrose ± 40 g/l sorbitol

MS; 4.3 g/l MS salts and vitamins ((Murashige and Skoog, 1962). Each media was supplemented with 7.0 g/l agar and pH was adjusted 5.7

Table 3.8 Type V shoot induction media used for *in vitro* shoot regeneration by assessing various concentrations of sucrose and sorbitol individually and accumulatively in N6 basal medium

Media	Composition
RMN_1	4.0 g/l N6 salts and vitamins, 5 g/l sucrose ± 5 g/l sorbitol
RMN_2	4.0 g/l N6 salts and vitamins, 10 g/l sucrose ± 10 g/l sorbitol
RMN_3	4.0 g/l N6 salts and vitamins, 15 g/l sucrose ± 15 g/l sorbitol
RMN_4	4.0 g/l N6 salts and vitamins, 20 g/l sucrose ± 20 g/l sorbitol
RMN_5	4.0 g/l N6 salts and vitamins, 25 g/l sucrose ± 25 g/l sorbitol
RMN_6	4.0 g/l N6 salts and vitamins, 30 g/l sucrose ± 30 g/l sorbitol
RMN_7	4.0 g/l N6 salts and vitamins, 35 g/l sucrose ± 35 g/l sorbitol
RMN_8	4.0 g/l N6 salts and vitamins, 40 g/l sucrose ± 40 g/l sorbitol

N6; 4.0 g/l N6 basal salts and vitamins (Chu, 1978). Each media was supplemented with 7.0 g/l agar and pH was adjusted 5.7

conditions as in callus induction (Table 3.6). The filters (0.22 µm Millipore) were used to sieve the aqueous solution of $CoCl_2$ and later it was enriched with sterilized SIM. Four to five explants were transferred to each jar for all the genotypes and each treatment was repeated three times. The data was recorded about *in vitro* shoot regeneration frequency and the average no. of primordial shoots per calli clumps on week basis until 60^{th} day of culturing. The *in vitro* shoot regeneration frequency (%) was determined as no. of calli clumps regenerating shoots divided by the total no. of calli clumps cultured on SIM and multiplied by hundred.

3.1.7 Evaluation of individual and accumulative effects of sucrose and sorbitol in MS and N6 basal media on shoot organogenesis

Hypocotyls and leaf discs (1-2 cm) were excised from 15-d-old *in vitro* seedlings under aseptic conditions and used as explants sources. These were cultured on MS basal medium amalgamated with various concentrations of carbon sources (sucrose and sorbitol) individually (Table 3.7). Similarly, the explants were cultured on N6 basal media with diverse concentrations of sucrose and sorbitol in separate combinations (5 – 40 g/l) (Table 3.8). The hypocotyls and leaf discs were cultured on regeneration media (MS basal medium supplemented with various combinations of sucrose: sorbitol (Table 3.7). Likewise, the explants were cultured on another regeneration media (N6 basal medium fortified with various combinations of sucrose and sorbitol (5: 5 – 40: 40) (g/l) (Table 3.8). The pH of all media was set to 5.7 with HCl (1.0 N) or NaOH (1.0 N) and autoclaving was done at 121 ºC for fifteen minutes. The culture was shifted to growth room and after twenty days, the *in vitro* shoot regeneration was compared on the basis of organogenesis frequency (%) and the *in vitro* shoot length (cm) for each treatment combination using both types of explants in three tomato genotypes.

3.1.8 *In vitro* shoot regeneration on MS and N6 media having sucrose and sorbitol along with various PGRs

During this study, the influence of various ratios of sucrose and sorbitol in MS and N6 basal media along with different hormonal regimes were investigated on shoot organogenesis and the number of shoot primordial. The embryogenic calli obtained on

CIM (MS salts, sucrose 3%, IAA 2.0 mg/l and BAP 2.5 mg/l) from hypocotyls and leaf discs were cultured on various shoot induction media. The cytokinins (BAP, 0.5 – 2.0 mg/l), kinetin (1.0 – 2.5 mg/l) and zeatin (1.0 mg/l) alone or in combination with auxins (IAA, 0.1 – 1.0 mg/l) and gibberellins; (GA_3, 0.5 mg/l) were added in MS and N6 media with sucrose, sorbitol and put in jars (height; 12 cm & diameter; 8 cm) (Tables 3.9 & 3.10). For all the genotypes, four explants per jar and 3 repetitions for each genotype and treatment were investigated. The data about the frequency of explants showing shoot regeneration percentage and the mean number of shoots per calli clumps was recorded weekly until day 60 of culture. The shoot organogenesis frequency was computed as the number of regenerated explants per total number of cultured explants multiplied by 100.

3.1.9 Measurement of shoot length and number of shoot primordial

In case of direct shoot regeneration, all of the plantlets were taken out from the jars after 15 days of culturing and placed on autoclaved petri plate with adhered graph paper and shoot length was measured and recorded. Similarly, in case of indirect shoot regeneration, the plantlets were taken out after 40 days of culturing and number of primordial shoots were measured and recorded. All the procedures were performed under aseptic conditions. After measurements, all of the plantlets were transferred to jars having respective fresh medium.

3.1.10 Root formation

The regenerated shoots about 3-5 cm in length obtained by hormonal regimes, $AgNO_3$, $CoCl_2$ and carbon sources were excised from calli clumps and washed with sterilized water to remove the agar. Subsequently, they were transferred to root induction medium (RIM) (4.3 g/l MS salts, 30 g/l sucrose, Nitsch vitamins, IBA (0.4 mg/l), pH 5.7 and solidified with agar 3.0 g/l in sterilized jars (12 × 8 cm).

3.1.11 Acclimatization of plantlets

After four weeks of culturing on RIM, the plantlets with well-developed roots were transferred to pots (75 mm) containing vermiculite and soil sterilized mixture (1: 1). The transparent polythene bags were placed on the plantlets to maintain high humidity, kept in a growth chamber (50 $\mu molm^{-2}s^{-1}$ fluorescent light with sixteen hours light and

eight hours dark period and 65-70% relative humidity). The plantlets were irrigated at 2-3 days interval until for 3-4 weeks. The plantlets were then transferred to larger pots and maintained in a greenhouse under normal conditions until they reached maturity stage and set fruits.

3.2 Techniques employed in transformation experiments

3.2.1 Cloning vector

The cloning vector pBIH used in the present study was provided by NIGAB, NARC (Islamabad), Pakistan. pBIH; a binary vector had *Arabidopsis thaliana DREB1A* gene (cold tolerant) driven by lip9 (an inducible promoter) and NOS terminator. The plant selectable marker gene (*hpt*; hygromycin phosphotransferase from *Escherichia coli*) under the control of P-NOS (nopaline synthase promoter) and T-G7 (terminator of gene 7 of *Agrobacterium tumefaciens*) for the regulation of *hpt* gene were also present in T-DNA region of the construct (Fig. 3.1).

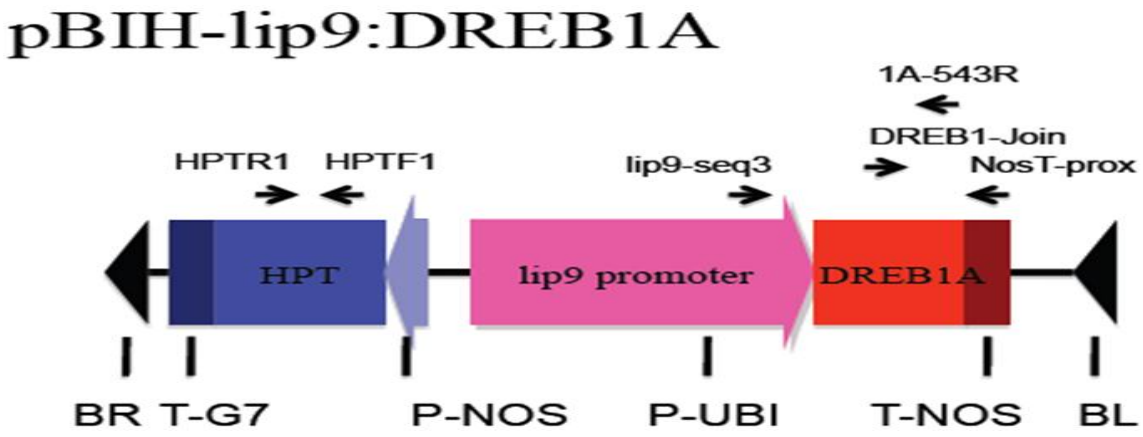

Fig. 3.1 Schematic diagram of the structure of the T-DNA region in the plasmid pBIH-lip9:DREB1A. LB; Left border, RB; Right border, T-G7; Terminator, *hpt*; hygromycin phosphotransferase gene, T-NOS and P-NOS (terminator and promoter sequences of nopaline synthase gene, Lip9; Lipase 9 promoter

Table 3.9 Type VI shoot induction media used for *in vitro* shoot regeneration in tomato, 7.0 g/l agar and pH 5.7

Media	Composition
RM_{1A}	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 5: 5 (g/l) sucrose: sorbitol
RM_{2A}	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 10: 10 (g/l) sucrose: sorbitol
RM_{3A}	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 15: 15 (g/l) sucrose: sorbitol
RM_{4A}	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 20: 20 (g/l) sucrose: sorbitol
RM_{5A}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP, 25: 25 (g/l) sucrose: sorbitol
RM_{6A}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP, 30: 30 (g/l) sucrose: sorbitol
RM_{7A}	MS, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA3, 35: 35 (g/l) sucrose: sorbitol
RM_{8A}	MS, 2.0 mg/l BAP, 40: 40 (g/l) sucrose: sorbitol

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), IAA; Indole-3-acetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, ZEA; Zeatin

Table 3.10 Type VII shoot induction media used for *in vitro* shoot regeneration in tomato, 7.0 g/l agar and pH 5.7

Media	Composition
RM_{1B}	N6, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 5: 5 (g/l) sucrose: sorbitol
RM_{2B}	N6, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 10: 10 (g/l) sucrose: sorbitol
RM_{3B}	N6, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 15: 15 (g/l) sucrose: sorbitol
RM_{4B}	N6, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 20: 20 (g/l) sucrose: sorbitol
RM_{5B}	N6, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP, 25: 25 (g/l) sucrose: sorbitol
RM_{6B}	N6, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP, 30: 30 (g/l) sucrose: sorbitol
RM_{7B}	N6, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA3, 35: 35 (g/l) sucrose: sorbitol
RM_{8B}	N6, 2.0 mg/l BAP, 40: 40 (g/l) sucrose: sorbitol

N6; 4.0 g/l N6 basal salts and vitamins (Chu, 1978), IAA; Indole-3-acetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, ZEA; Zeatin

3.2.2 Transformation of *Escherichia coli* (DH5α)

The *Escherichia coli* strain (DH5α) was transformed by a plasmid (pBIH) under aseptic conditions by following procedures:

3.2.2.1 *E. coli* DH5α competent cells preparation and transformation

The DH5α strain of *E. coli* was employed to get the multiple copies of the desired plasmid. For this purpose, the competent cells of DH5 alpha (without its plasmid) were prepared by following protocol devised by Sambrook and Russell (2001). The *E. coli* cells were streaked from glycerol stock on LB media (solid) plate with chloramphenicol (34 mg/l), then allowed them to grow overnight at 37 ºC. A single colony of DH5α was isolated from the plate and inoculated into five milliliters liquid LB medium (Table 3.11) and the cells were grown overnight at 250 revolutions per minute (rpm) at 37 °C. The bacterial culture was diluted hundred times (0.5 ml culture was supplemented to fifty milliliters LB media). Then it was centrifuged at 250 rpm for two to three hours at 37 ºC until optical density ($OD_{600\ nm}$) was reached 0.3 to 0.4. The culture was dispensed in 2 autoclaved conical centrifuge tubes (50 ml) (Gilson, France) (15 ml in each tube) and centrifuged at 8000 rpm with four degree centigrade for ten minutes. The pellet was resuspended and dissolved in ten milliliter doubled distilled water. Centrifugation was repeated second time at the same rate and pellet was dissolved in sterilized distilled water (10 ml). Centrifugation was done third time at the same rate as above and pellet was put in ten percent glycerol. The falcon tubes were centrifuged fourth time. Now supernatant was removed and pellet was dissolved in four hundred microliter of ten percent glycerol. Now these competent cells of bacterial culture were ready for incorporating plasmid by electroporator (Eppendorf 2510, Thermo Scientific, Germany)

Fifty microliter of these competent cells and three microliter of plasmid construct were mixed in an autoclaved electroporator cuvette. This cuvette was kept in electroporator and subjected to 1400 volts electric shock for three seconds. Subsequently, the cuvette mixture was taken in an eppendorf tube (Gilson, France) containing one ml YEP medium (Table 3.11). The culture was incubated at 150 rpm with thirty seven degree Celsius for a period of two hours for recovery of cells (after electric shock). The

selection was performed by plating the bacterial culture on yeast extract peptone media containing 50 mg/l (kanamycin sulphate) and 250 mg/l (tetracycline) incubated at twenty-eight degree Celsius for two days for the formation of colonies. An isolated colony was assessed by PCR for desired *CBF3* with a set of gene specific primers.

3.2.2.2 Extraction of recombinant plasmid (pBIH) from DH5α

The bacterial culture (*E. coli*; DH5α) was grown overnight in Luria Broth medium (3 ml) by inserting single colony from selection plates. The LB medium was also fortified with kanamycin sulphate (50 mg/l). The recombinant plasmid DNA from DH5α was isolated and purified from the culture by using a Miniprep method (Birnboim and Doly, 1979).

The microfuge tube (1.5 ml) was filled with overnight bacterial suspension and the cells were condensed by centrifuging the tube at fourteen thousand revolutions per minute for one minute at room temperature. The supernatant was eliminated and again 1.5 ml bacterial culture was mixed with the pellet and centrifugation (14000 rpm) was done for one minute. Then pellet was cleaned with 100 µl of alkaline lysis solution-I (Tris HCl (25 mM; pH 8.0), EDTA (10 mM; pH 8.0) by vortexing. In this step the glucose and Tris HCl acted as osmotic pressure and pH stabilizers, respectively, while EDTA limited the degradation of DNA by attaching Mg++ ions that acted as co-factor for nucleases of bacteria. Now this mixture was incubated on ice for five minutes. Then alkaline lysis solution-II [NaOH (0.2 N), SDS 1% (w/v)] was supplemented with the mixture and mixed thoroughly by vortexing or inverting gently for five seconds. In this step NaOH denatured both DNA (plasmid and chromosomal) into single strands while sodium dodecyl sulphate dissolved the lipids of plasma membrane and cellular proteins. Hence, the destruction of cells occurred by this alkaline solution. The alkaline lysis solution-III (100 µl) (Potassium acetate (3M; pH 3.8), Glacial acetic acid (11.5 ml for 100 ml solution), ddH_2O (The overall pH of the solution-III was maintained at 4.8-5.0) was added to microfuge tube and vortexing was done gently for making homogenous mixture. The glacial acetic acid made the pH neutral and thus DNA strands renatured. While the potassium acetate precipitated the sodium dodecyl sulphate from solution that brought together the chromosomal DNA into a single mass that pelleted after

centrifugation and thus only plasmid DNA and RNA remained in the solution. The eppendorf tube was put on ice for five minutes and centrifugation was done at 14000 rpm for five minutes. The supernatant was shifted to new autoclaved tubes and chloroform: isoamylalcohol (300 µl) (24: 1) was fortified and dissolved by vortexing. The centrifugation was done. The supernatant was put in fresh tubes and hundred percent ethanol in equal volume was added to the tubes and incubated at fifteen minutes at 25 ºC. The ethanol in this step helped in removing the salts and traces of SDS to avoid restriction digestion interference. Afterwards, the supernatant was discarded after centrifuging the tubes at fourteen thousand revolutions per minute and 500 µl of seventy percent ethanol was rinsed and mixed by vortexing. The tubes were spun at 14000 rpm for five minutes at 25 ºC and discarded the supernatant. Finally pellet (plasmid) was dried and dissolved in 15 µl doubled distilled water and was stored at – 20 ºC. The plasmid DNA concentration was calculated using spectrophotometer (Optima SP 3000 Plus, Japan) and its size was confirmed by agarose gel electrophoresis by using gel documentation machine (Alpha innotech, Germany).

3.2.3 *Agrobacterium-tumefaciens* transformation with recombinant plasmid

The plasmid pBIH containing *DREB1A* gene was transformed in a super virulent *Agrobacterium tumefaciens* strain, EHA105 under aseptic conditions by the following procedure:

3.2.3.1 *Agrobacterium* (EHA105) electro-competent cells preparation

The electro-competent cells of *Agrobacterium tumefaciens* strain (EHA105) were made ready by the procedure followed by Sambrook and Russell (2001) same to the protocol developed for electro-competent cells preparation of *E. coli* as described in 3.2.2.1.

3.2.3.2 Transformation of *Agrobacterium* through electroporation

The electroporator cuvette was filled with a mixture of 50 µl electro-competent cells and 3 µl plasmid construct and put into electroporator. It was given an electric shock of 1400 volts for 3 seconds. After shock, the mixture was transferred into one milliliter yeast extract peptone medium in an eppendorf tube. Now this mixture was incubated at

one hundred and sixty revolutions per minute and twenty-eight degree Celsius for two hours. Subsequently, the transformed cells were spread on solid YEP medium having appropriate amount of antibiotics (Kanamycin sulphate, 50 mg/l and tetracycline, 250 mg/l) for selection. A colony PCR was performed for the confirmation of target gene (*DREB1A*) with specific set of primers.

3.2.4 Genetic transformation of tomato via *Agrobacterium tumefaciens*

The genetic transformation of three tomato genotypes through *Agrobacterium*-mediated method was performed by optimizing the following parameters for improving overall genetic transformation efficiency in tomato.

3.2.4.1 Bacterial culture preparation

Agrobacterium tumefaciens culture was prepared by scrapping a single colony from selection plate with the help of a flame-sterilized metal loop and put in 5 ml liquid YEP medium having kanamycin sulphate (50 mg/l). The bacterial suspension was incubated at 160 revolutions per minute with constant temperature (28 ºC) for 24 hours. The suspension was spun at 8000 revolutions per minute for ten minutes in a large centrifuge tube. The pellet was dissolved in sterilized water and effects of various cell densities of *Agrobacterium* ($OD_{600\ nm}$ = 0.1, 0.2, 0.3, 0.4 and 0.5) were evaluated on transformation efficiency. Acetosyringone (AS) solution was prepared by dissolving in DMSO or ethanol to requisite stock concentration, sieved through filter and then preserved at – 20 ºC. Different concentrations of AS (0, 20, 40, 60, 80 and 100 µM) were added in two experiments for the improvement of transformation efficiency. In one experiment, acetosyringone (0-100 µM) was added in inoculation medium (Table 3.11; IM) and its effect on transformation efficiency was investigated. The hypocotyls and leaf discs were excised to about 0.5 cm segments from different ages of *in vitro* seedling (5, 10, 15, 20 and 25 days) to check the influence of seedling age on transformation efficiency. Before co-cultivation, the explants were pre-cultured on pre-culturing media (Table 3.11; PCM) for various time periods (0, 24, 48, 72 and 96 hrs) and their impact on transformation efficiency was examined.

3.2.4.2 Co-cultivation following co-infection

In first experiment, pre-cultured explants were immersed in *Agrobacterium* culture for 2, 3, 5, 8 and 10 min in order to optimize the co-infection duration. In second experiment, the influence of different co-cultivation period (24, 48, 72 and 96 hrs) on transformation efficiency was studied. After co-infection, the hypocotyls and leaf discs were blotted dry on sterilized filter papers in order to avoid surplus bacteria. The explants were then transferred on co-cultivation media (Table 3.11; CCM) having various AS concentrations (0, 20, 40, 60, 80 and 100 µM) with different pH (5.3, 5.4, 5.5, 5.6, 5.7 and 5.8) of co-cultivation media were also optimized in transformation experiments. The co-cultivation media plates were covered with filter papers to control the bacterial overgrowth. Each petri plate was wrapped with parafilm and incubated in dark at twenty-eight degree Celsius. The explants without co-infected were also treated with the same growth conditions and used as control. During co-cultivation period the material was monitored regularly for any contamination.

3.2.4.3 Pre-selection following washing

After exposure to co-cultivation duration hypocotyls and leaf discs were washed with ddH_2O four to five times followed by four times washing with washing medium (Table 3.11; WM) fortified with different concentrations of claforan® (cefotaxime sodium) (Sanofi-aventis, Pakistan) @ 200, 300, 400, 500, 600 and 700 mg/l for about 40 minutes to limit the *Agrobacterium* growth in co-infected explants during callus induction and morphogenesis phases. The explants were dried by blotting on autoclaved filter papers and transferred to pre-selection medium (Table 3.11; PSM) for 0, 2, 4, 6 and 8 days in order to optimize the pre-selection period. In this medium hygromycin was not added in order to prevent the direct shock of antibiotics. The explants after washing were also shifted directly to selection medium and used as control.

3.2.4.4 Optimization of suitable marker (Hygromycin) concentration

The effects of various concentrations (0, 10, 25, 35, 45 and 60 mg/l) of hygromycin B (Phyto Technology, USA) were investigated on *in vitro* shoot regeneration frequency in tomato. In order to determine the lethal dose of hygromycin for maximum

survival of transformants, the explants transferred to shoot elongation media (Table 3.11; SEM) supplemented with various levels of hygromycin and kept at 25 ºC under 16 hrs light photoperiod, 50 µmolm^{-2}s^{-1} fluorescence light and 65-70% relative humidity for four to six weeks. After two weeks interval, the explants were shifted to fresh medium to improve the *in vitro* shoot regeneration potential of tomato genotypes.

3.2.4.5 Transformants selection

After pre-selection, the explants were moved to selection media fortified with hygromycin and cefotaxime (Table 3.11; SM) and incubated at 25 ºC in dark in order to select the transformants. After 4-5 weeks, the resistant calli were initiated. These calli were transferred to shoot induction medium (Table 3.11; SIM) and were sub-cultured twice after every two weeks. The shoots were regenerated from resistant calli clumps on shoot induction medium within 3-4 weeks. The regenerated shoots (2-3 cm) were separated from calli clumps, washed with tap water and shifted to root induction medium (Table 3.11; RIM). However, the smaller shoots were removed and cultured on a shoot elongation media (Table 3.11; SEM). Transformation efficiency was measured by the number of hygromycin resistant explants showing regeneration divided by the total number of explants cultured on selection medium, expressed in percentage. The plantlets with efficient roots were acclimatized in culture tubes having tap water for five days at 25 ºC. Subsequently, the plantlets were shifted to mini plastic bags having a mixture of vermiculite and soil (1: 1) and maintained in glasshouse. The plantlets survival rate was recorded periodically for each experiment and they were allowed to grow until maturity to bear fruit and seeds.

Table 3.11 Different types of culture media used for regeneration and transformation of three genotypes of tomato (*Solanum lycopersicum* Mill.)

Culture media	Composition
Germination medium (GM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, 3.0 g/l phytagel, pH 5.7
YEP medium	10 g/l yeast extract, 10 g/l bacto-peptone, 5 g/l NaCl, pH 7.0, ± 7.0 g/l phytagel (for solid and liquid medium) (Sambrook and Russell, 2001)
LB medium	5 g/l yeast extract, 10 g/l bacto-tryptone, 10 g/l NaCl, pH 7.0, ± 7.0 g/l phytagel (for solid and liquid medium) (Sambrook and Russell, 2001)
Inoculation medium (IM)	MS basal medium, 30 g/l sucrose, 1.0 g/l casein hydrolysate, 60 µM acetosyringone, 3.0 g/l phytagel, pH 5.7
Pre-culture medium (PCM)	MS basal medium, 30 g/l sucrose, 2.0 mg/l IAA, 2.5 mg/l BAP, 1.0 g/l myo-inositol, 3.0 g/l phytagel, pH 5.7
Co-cultivation medium (CCM)	N6 basal medium, 30 g/l sucrose, 2.0 mg/l IAA, 2.5 mg/l BAP, 0.5 g/l cysteine, 60 µM acetosyringone 3.0 g/l phytagel, pH 5.7
Washing medium (WM)	N6 basal medium, 30 g/l sucrose, 1.0 mg/l IAA, 1.5 mg/l BAP, 0.5 mg/l cysteine, 500 mg/l cf, 3.0 g/l phytagel, pH 5.7
Pre-selection medium (PSM)	N6 basal medium, 30 g/l sucrose, 1.0 mg/l IAA, 2.0 mg/l BAP, 0.5 mg/l cysteine, 1.0 g/l myo-inositol, 30 g/l sorbitol, 500 mg/l cf, 3.0 g/l phytagel, pH 5.7
Selection medium (SM)	N6 basal medium, 30 g/l sucrose, 1.0 mg/l IAA, 2.0 mg/l BAP, 0.5 g/l cysteine, 1.0 g/l myo-inositol, 30 g/l sorbitol, 500 mg/l cf, 35 mg/l hygromycin, 3.0 g/l phytagel, pH 5.7
Shoot induction medium (SIM)	N6 basal medium, 30 g/l sucrose, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP, 0.5 g/l cysteine, 1.0 g/l myo-inositol, 30 g/l sorbitol, 500 mg/l cf, 35 mg/l hygromycin, 3.0 g/l phytagel, pH 5.7
Shoot elongation medium (SEM)	N6 basal medium, 30 g/l sucrose, 0.5 g/l cysteine, 1.0 mg/l glycine, 1.0 g/l myo-inositol, 30 g/l sorbitol, 500 mg/l cf, 35 mg/l hygromycin, 3.0 g/l phytagel, pH 5.7
Root induction medium (RIM)	MS basal medium, 30 g/l sucrose, 0.4 mg/l IBA, 500 mg/l cf, 35 mg/l hygromycin, 3.0 g/l phytagel, pH 5.7

N6; 4.0 g/l N6 salts and vitamins (Chu, 1978), IAA; Indole-3-acetic acid, BAP; 6-benzylaminopurine, cf; cefotaxime

3.2.5 Genetic transformation of tomato via *Agrobacterium-mediated in planta* method

During this study, a novel *in planta* method via *Agrobacterium tumefaciens* was also developed for three tomato cultivars namely Rio Grande, Moneymaker and Roma using shoot apical meristem from 3 days old seedlings as a source of explants for all the genotypes.

3.2.5.1 Plant material and seed germination

Seeds of three tomato cultivars namely Rio Grande, Moneymaker and Roma were immersed in ddH_2O for 24 h at 4 °C to limit seed dormancy. Seeds were surface sterilized with 70% ethyl alcohol for one minute only and then with 40% clorox (Sodium hypochlorite, NaOCl) (5.25%) for twenty minutes and then cleaned with ddH_2O three to five times for the removal of bleach traces. Then the seeds were put on filter papers saturated with sterilized water in petriplates to allow germination at 28 °C in dark conditions. The shoot apical meristem from 3-days old seedlings were used as explant sources for co-infection with *Agrobacterium*.

3.2.5.2 Transformation strategy

Agrobacterium strain EHA105 (Hood *et al.,* 1993) harboring pBIH was streaked on YEP solid medium having 50 mg/l kanamycin sulphate. Single colony was taken and grown in liquid Luria Broth medium (Table 3.11) supplemented with 50 mg/l kanamycin sulphate at twenty-eight degree Celsius for 24 hours. The cell suspension was spun at 8000 rpm to make the pellet. The pellet was put into MS (1962) liquid medium and volume of competent cells was increased ($OD_{600\ nm}$ = 0.5, 1.0, 1.5 and 2.0), so that the optical density of *Agrobacterium* was maintained to be optimum. The culture was also supplemented with different concentrations of acetosyringone (0, 20, 40, 60, 80 and 100 µM). The shoot apical meristem from 3 days old seedlings were pierced with sterilized needle of syringe (3cc) and inserted a drop of bacterial suspension (Fig. 4.17A) and subsequently immersed in *Agrobacterium* culture for 10, 20, 30 and 40 min (Fig. 4.17B). The seeds were washed briefly with ddH_2O and dried on autoclaved filter papers for fifteen minutes to get rid of excess bacteria. The seedlings co-infected in this way were

placed on filter paper wet with sterilized water in dark conditions for a co-cultivation period of 1, 2, 3 and 4 days. After co-cultivation, the infected seedlings were moved to different growth media (sterilized soil, vermiculite and soil: vermiculite) in order to find out the optimum growth media for maximum seedling establishment frequency. The seedlings (along with seeds and roots) were covered with soil except the shoot. The plates were then incubated at 4 ℃ for 3 weeks in dark conditions. Afterwards, various concentrations of hygromycin (0, 10, 25, 35, 45 and 60 mg/l) were directly applied on 3-week old seedlings. The pots were irrigated regularly and maintained in glasshouse at twenty-five degree Celsius for further growth.

3.3 Techniques employed in molecular analysis

The transgenic tomato plants of all the genotypes developed by both tissue culture-dependent and tissue culture-independent methods in T_0 – T_2 generations were confirmed by the following molecular techniques.

3.3.1 Genomic DNA extraction

The genomic DNA was extracted by CTAB (Cetyltrimethyl ammonium bromide) method from transgenic and NT fresh leaves following the protocol devised by Murray and Thomson (1980). The 0.1 g fresh leaf sample was crused with liquid nitrogen to a fine powder and added 0.7 ml of pre-heated (60 °C) 2X CTAB extraction buffer (Murray and Thomson, 1980). The plant extract mixture was moved to a microfuge tube and kept at 60 ℃ in water bath for 30 minutes. During incubation, the samples were shaken 2-3 times to take away any solid material. The extract mixture was spun at 12000 rpm for ten min to lower down cell debris. The supernatant was shifted to a new microfuge tube. The mixture was alloyed with 250 µl of chloroform: isoamyl alcohol (24: 1) and mixed by inversion. After mixing, the tubes were spun at 12000 revolutions per minute at 20 ℃ for 10 min. The upper fluent material was poured carefully into a new microfuge tube and 0.8 volume (aqueous phase) of chilled isopropanol was supplemented. The tubes were inverted slowly for several times and incubated at - 20 ℃ for one hour to precipitate DNA. The precipitate was isolated by spinning tubes at 12000 rpm for 10 minutes and aqueous phase was removed out. The seventy percent ethanol (300 µl) was alloyed to

wash pellet. Subsequently, the tubes were spun at 12000 rpm for 10 min. The supernatant was removed out and pellet was air-dried by inverting the tubes on sterilized tissue paper. The over-drying of DNA was avoided and dehydrated the pellet with absolute alcohol. The pellet was resuspended in 50-100 µl of 0.1xTE buffer (pH 8.0) and treated with RNase A (1 µl/100 ml TE buffer) to remove RNA. Subsequently, the DNA was kept at 40 °C for 20 min to get rid of DNases. The concentration of DNA was confirmed by spectrophotometer (IMPLEN, Germany), while its integrity was monitored on agarose gel electrophoresis.

3.3.2 PCR analysis

The targeted genes in transgenic tomato were examined through PCR assay. The primers sequences used to amplify 649 bp fragment of *DREB1A* gene were: forward primer: *DREB1A*–F 5'TGAACTCATTTTCTGCTTT-3'and reverse primer: *DREB1A*–R 5'-TAATAACTCCATAACGATA-3'. While the primers sequences used to amplify the 399 bp fragment of hygromycin phosphotrasferase (*hpt*) gene were: forward primer: *hpt*–F 5'TCGTGCTTTCAGCTTCGATG-3' and reverse primer: *hpt*–R 5'TCCATCACAGTTTGCCAGTG-3'. The total volume in PCR reactions system was 20 µl containing 2 µl of a 10 × Taq DNA Buffer (Fermentas), 2.4 µl of 50 mM $MgCl_2$ (Fermentas), 0.4 µl of 10 mM dNTPs (Fermentas), 1.0 µl of *DREB1A* forward primer, 1.0 µl of *DREB1A* reverse primer, 0.3 µl of Taq DNA polymerase (Fermentas), 10.9 µl of double distilled water (nuclease free) and 2.0 µl of DNA template. These reactions were done in PCR machine (Applied Biosystem). The PCR profile of *DREB1A* and *hpt* was optimized as: initial denaturation at 94 ºC for five minutes, denaturation at 94 ºC for one minute, annealing at 50 ºC for one minute, extension at 72 ºC for one minute and final extension at 72 ºC for ten minutes followed by 35 cycles. The amplified products were confirmed by electrophoresis on 1% agarose gels, stained with 5.0 µl/100 ml ethidium bromide.

3.3.3 Multiplex PCR analysis

Multiplex PCR was run after optimizing various PCR ingredients. The ingredients were optimized for *DREB1A* and *hpt* genes separately in simple PCR and then the same

concentrations of PCR reaction was used for Multiplex PCR, which yielded the uneven amplification. To avoid this ambiguity, following PCR reaction mixture was optimized for Multiplex PCR. The total volume in PCR reaction system was 20 µl containing 3.0 µl of a 10 × Taq DNA Buffer (Fermentas), 2.8 µl of 50 mM $MgCl_2$ (Fermentas), 0.6 µl of 10 mM dNTPs (Fermentas), 0.8 µl of *DREB1A* forward primer, 0.8 µl of *DREB1A* reverse primer, 1.2 µl of *hpt* forward primer, 1.2 µl of *hpt* reverse primer, 0.5 µl of Taq DNA polymerase (Fermentas), 7.1 µl of double distilled water (nuclease free) and 2.0 µl of DNA template. These reactions were done in PCR machine (Applied Biosystem). The PCR profile of *DREB1A* and *hpt* genes for Multiplex PCR was optimized as: initial denaturation at 94 ºC for five minutes, denaturation at 94 ºC for one minute, annealing at 50 ºC for one minute, extension at 72 ºC for one minute and final extension at 72 ºC for 12 minutes followed by 35 cycles at 4 ºC cover temperature. The primers sequences used to amplify 649 bp fragment of *DREB1A* gene were: forward primer: *DREB1A*–F 5'TGAACTCATTTTCTGCTTT-3'and reverse primer: *DREB1A*–R 5'-TAATAACTCCATAACGATA-3'. While the primers sequences used to amplify the 399 bp fragment of hygromycin phosphotrasferase (*hpt*) gene were: forward primer: *hpt*–F 5'TCGTGCTTTCAGCTTCGATG-3' and reverse primer: *hpt*–R 5'TCCATCACAGTTTGCCAGTG-3'.The amplified products were confirmed by electrophoresis on 1% agarose gels, stained with 5.0 µl/100 ml ethidium bromide.

3.3.4 RNA extraction

RNA was extracted from transgenic and NT tomato plants by the following two methods:

3.3.4.1 RNA extraction by TRI Reagent

RNA was extracted following the method devised by Chomczynski and Sacchi (2006). The tomato leaf tissues (50-100 mg) were ground by liquid nitrogen to a fine powder in autoclaved mortar pestle and homogenized adding 1 ml TRI Reagent (Invitrogen, USA) and then the homogenate was transferred to eppendorf tubes and kept at 25 ºC for five min. The chloroform (200 µl) was fortified and mixed with the samples by vigorous hand-shaking for about 20 seconds. The samples were kept at 25 ºC for five min followed by spinning at 12000 rpm for fifteen minutes at 4 ºC. The upper colorless

phase (RNA) was taken into fresh tubes and 0.5 ml of 100% isopropanol was supplemented into aqueous phase. The mixture was kept at room temperature for fifteen minutes and spun at 12000 rpm for ten min at 4 ºC. RNA pellet was cleaned with one ml of 70% ethyl alcohol by vortexing, after discarding supernatant. The centrifugation of tubes was done for 5 min at 4 ºC and then supernatant was removed and remained only RNA pellet which was air-dried for 5-10 minutes. Subsequently, the pellet was dissolved in 40 µl of ddH_2O (nuclease free) and RNA was stored at – 80 ºC.

3.3.4.2 RNA extraction by NucleoSpin® RNA kit

Total RNA from transgenic and NT tomato plants was extracted using NucleoSpin RNA kit (MACHEREY-NAGEL GmbH, Germany) following manufacturer's instructions. The isolated RNA by this method was quantified using spectrophotometer (IMPLEN, Germany) at 260 nm.

3.3.5 Expression analysis of *CBF3* gene through two steps RT-PCR

Two steps reverse-transcriptase PCR was performed to detect the expression of desired gene quantitatively. In first step, the complementary DNA was synthesized from good quality RNA using a kit (RevertAid Reverse Transcriptase, Thermo Scientific, Catalog # EP0442). Briefly, 4.0 µl of RNA template was mixed with 1.0 µl of Oligo $(dT)_{18}$ primer (#SO131) and 9.5 µl DEPC-treated H_2O (#R0601). This material was mixed gently, centrifuged briefly and incubated at 65 ºC for five minutes with cover temperature of 70 ºC and then chilled on ice for 2 – 3 min. Subsequently, 4.0 µl of RT buffer, 0.5 µl of reverse transcriptase (RevertAid) and 1.0 µl of dNTP mix were added in the above mixture making a total volume of 20 µl. Now the mixture was centrifuged briefly and incubated at 42 ºC for 80 min. Then the reaction was ceased by incubating at 70 ºC for ten min. In second step, 2.0 µl of cDNA from reverse transcription was used as a template and *DREB1A* gene specific primers were employed to amplify the desired fragment through conventional PCR with optimized conditions as initial denaturation at 94 ºC for five minute, denaturation at 94 ºC for one minute, annealing at 50 ºC for one minute, extension at 72 ºC for one minute and final extension at 72 ºC for ten minutes followed by 35 cycles. The end product of RT-PCR was separated running on 1%

agarose gel by the process of electrophoresis and visualized on Alpha innotech gel documentation system.

3.3.6 Southern blot analysis

Genomic DNA was isolated following CTAB method (Murray and Thomson, 1980). For Southern blot of *CBF3* gene, the protocol devised by Southern (2006) was followed. A good quality DNA (10 µg) was digested using 2 µl BamHI and incubated at a specific temperature (37 ºC) overnight. The DNA after digestion was fractionated on 1.5% agarose gel enriched with 0.5 µg/ml ethidium bromide in TAE buffer at 40 volts and 35 mA current for 6-16 hrs by electrophoresis. The gel was immersed in 1.5 M NaCl and 0.5 M NaOH solution in a plastic container for a period of 15–30 min as denaturation. Subsequently, the denaturation solution was replaced with another solution of 3.0 M NaCl and 0.5 M Tris HCl (with pH 7) and the gel was incubated in this solution for further 15-30 min as neutralization. The DNA was transferred from gel to nylon membrane (SensiBlot™ Plus Nylon-Membrane; catalogue #M1001, #M1002, Fermentas) overnight by the process of capillary action. For this blotting, one piece of Whatmann paper (3 MM) and one piece of nylon membrane was soaked well in 20× SSC and 2× SSC solutions, respectively. The wet nylon membrane with DNA was placed on equal-sized paper towel and then UV cross linked using CL-1000 UV Crosslinker at 254 nm to control the partial digestion of DNA. Then the membrane was properly dried, followed by incubating in prehybridization solution at 42 ºC for 1-2 hrs in order to block the non-specific binding of probe to nucleic acids. The amplified product (649 bp) of *CBF3* gene was purified following DNA elusion process (Appendix 66), labelled with biotin-11-dUTP using a kit (Biotin DecaLabel DNA-Labeling; Fermentas, Germany) and denatured it by incubating at 100 ºC for five minutes, immediately chilled on ice. The denatured probe was added to pre-hybridization solution and probing was done incubating the hybridization solution at 42 ºC for 14 hrs.

3.4 Techniques employed in analyzing morphological characteristics

The experiment was conducted in Post-Harvest Laboratory, Department of Horticulture, Pir Mehr Ali Shah Arid Agriculture University (Rawalpindi), Pakistan. Three tomato (*Solanum lycopersicum* Mill.) transgenic lines (Rio Grande, Moneymaker and Roma) (T_1 generation) maintained in greenhouse conditions and were allowed to self-pollinate for bearing fruit. The seeds of each line were harvested separately and sown in nine centimeter petri plates in a growth room (with 16 hours light period at twenty-two degree Celsius, 8 hours dark period at eighteen degree Celsius and 75% relative humidity that germinated within 5-6 days and yielded T_2 generation. Six days after germination, the well-developed seedlings were moved to small plastic bags having equal amount of vermiculite and soil (1: 1). The plantlets were watered regularly after every three days for about three weeks and then transferred to pots and were kept under normal growth conditions in glasshouse. Total plant DNA was extracted by CTAB method and PCR analysis was performed for the confirmation of *CBF3* and *HPT* genes. Thirty-days-old transgenic plants that followed Mendelian segregation ratio (3: 1) were chosen for morphological analysis, compared with their isogenic NT plants. Plant height of 12 randomly selected transgenic and NT plants was measured as the distance from ground level to upper level of main photosynthetic tissues using a meter rod and recorded in centimeters. Then transgenic and NT plants of all the lines were uprooted cautiously and root length was measured in cm using the meter rod. Fresh weight was computed immediately after harvesting transgenic and NT plants. Subsequently, these plants were incubated at 80 ºC in an oven for two days and dry weight was recorded. The fresh mean fruit weight (g) at maturity was also calculated harvesting 12 mature fruits and then their average was recorded. The diameter of twelve mature fruits from different plants was measured in centimeter using measuring tape and then their average was recorded for statistical analysis. Morphological data on no. of leaves/plant, days to flowering, no. of flowers/plant, first fruit set, no. of fruit/plant and no. of seeds/fruit were also recorded.

3.5 Techniques employed in analyzing physiological characteristics

The T_2 transgenic plants that followed Mendelian segregation ratio (3: 1) were chosen for cold tolerance analysis. Three weeks old transgenic and NT plants (obtained from normal seeds) of all the genotypes were exposed to different low temperature stresses (4, 6 and 8 °C) under controlled environment conditions (16/8 hrs photoperiod and 50 µmole $m^{-2}s^{-1}$ fluorescence light) for seven days and then returned to 25 °C for recovery as described by Hsieh *et al.,* 2002; Singh *et al.,* 2011; Zhang *et al.,* 2011. The plant survival rate was determined after seven days of stress when plants regained their growth under normal conditions. Subsequently, physiological characteristics such as stomatal conductance (gs), transpiration rate (E), CO_2 concentration rate (Ci), photosynthetic rate (A), relative water contents (%) and leaf osmotic potential (MPa) were assessed in transgenic and NT lines under cold stressed as well as normal conditions.

3.5.1 Gas exchange parameters

Photosynthetic parameters including stomatal conductance, transpiration rate, CO_2 concentration rate and photosynthetic rate of the youngest, fully expanded attached leaves of three transgenic and NT tomato plants per line were computed using infrared gas analyzer (LC pro+, BioScientific Ltd, Germany) as detailed by Long and Bernacchi (2003). During these analyses, the actual temperature of leaf chamber, relative air humidity, CO_2 concentration and saturation light were kept at 25 ± 2 ºC, 80%, 380 µl l^{-1} and 800 µmol $m^{-2}s^{-1}$, respectively and the instrument was calibrated after every 30 min to get more precocious results. All the analyses about photosynthetic parameters were performed on sunny days (10:00 a.m. to 2:00 p.m). The measurements persisted about 10 min and no distinct recovery was noticed during this measurement duration. Each representative leaf sample was enclosed in the instrument chamber for about 60 s and the measurements were scored 3 times for each treatment.

3.5.2 Relative water contents (%)

The relative water contents (RWC) of tomato leaves were computed according to the method recommended by Barrs and Weatherly (1962) for the assessment of water status

of transgenic and NT tomato lines. The fresh weight (FW) of leaves was taken immediately after removing from the plants, saturated in dH_2O for four hours at room temperature, and then turgid weight (TW) was determined. Subsequently, dry weight (DW) was estimated after drying the samples at 80 °C for twenty-four hours. Finally, RWC was determined employing following formula:

$$\text{RWC}\ (\%)\ = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times\ 100$$

3.5.3 Leaf osmotic potential (MPa)

The leaf osmotic potential was computed following the method devised by Capell and Doerffling (1993) using freezing-point osmometer (Osmomat 010, Model Gonatec GMBH, D-10823 Berlin Germany). In order to obtain cell sap, the frozen-thawed tomato leaf samples were put in two milliliters of plastic syringe and pressed it to exude out the sap (60 µl) and the readings were taken in milliosmol. These readings were converted into MPa from osmometer.

3.6 Techniques employed in analyzing biochemical characteristics

The 3-week old T_2 transgenic and NT plants (obtained from normal seeds) were exposed to different low temperature stresses (4, 6 and 8 °C) under controlled environment conditions (16/8 hrs photoperiod and 50 µmole $m^{-2}s^{-1}$ fluorescence light) for seven days and then returned to 25 °C for recovery as described by Hsieh *et al.,* 2002; Singh *et al.,* 2011; Zhang *et al.,* 2011. Successively, biochemical characteristics such as membrane leakage, proline contents, total soluble sugar contents, chlorophyll contents, carotenoid contents, ascorbic acid contents and malondialdehyde contents were assessed in transgenic and NT lines under cold stressed as well as normal conditions.

3.6.1 Membrane leakage (%)

The membrane leakage was measured by following the method devised by Campos and Thi (1997). The leaf samples (0.2 g fresh weight) were detached from three weeks old seedlings (T_2) and rinsed in ddH_2O for 3 times to eliminate the ions from damaged cell surfaces and then measured the initial/blank conductivity (EC_0) of the samples immediately after immersion in 20 ml distilled water by a conductivity meter

(inoLab® Germany). The material was kept at 25 °C for 2-3 hours and subsequently electrical conductivity (EC_1) was assessed. Subsequently, the material was autoclaved at 121 ºC for thirty minutes and total ionic strength (EC_2) from the tissue after heat-killing was measured when the solution was cooled down to room temperature.

The electrolyte leakage was calculated by using the following formula:

$$\text{Membrane ion leakage (\%)} = \frac{\text{EC1} - \text{EC0}}{\text{EC2} - \text{EC0}} \times 100$$

3.6.2 Total soluble sugar contents (mg/g FW)

The total soluble sugar content was measured according to the method specified by Dubois *et al.* (1956). The sugar stock solution was made dissolving 0.2 g of glucose (anhydrous) in 100 ml of ddH_2O. From this stock solution, six dilutions (10, 15, 20, 25, 30 and 35 ppm) of glucose were prepared and total volume of each dilution was made up to 50 ml. For dilution-I (10 ppm) 10: 40 (v/v), for dilution-II (15 ppm) 15: 35 (v/v), for dilution-III (20 ppm) 20: 30 (v/v), for dilution-IV (25 ppm) 25: 25 (v/v), for dilution-V (30 ppm) 30: 20 (v/v) and for dilution-VI (35 ppm) 35: 15 (v/v) (dilution: distilled water) were transferred in sterilized centrifuge tubes. From these dilutions, the sugar standards were prepared by pipetting one ml of respective dilutions into 10 ml of concentrated sulphuric acid for constructing a standard curve and to check the proper functioning of spectrophotometer. The colour of sugar standards was changed to yellow-orange by the reaction of glucose and sulphuric acid and its intensity was directly related to concentration of glucose available in the sample at 420 nm.

The fresh leaf samples (0.5 g) were ground in autoclaved pestle and mortar with ten milliliter distilled water. The homogenized mixture was transferred into centrifuge tubes and was spun at 3000 rpm for five minutes. The upper liquid phase (0.1 ml) of the mixture was transferred into fresh centrifuge tubes and 1 ml of 80% (v/v) phenol was supplemented. Subsequently, the tubes were incubated at room temperature for one hour. After incubation, the concentrated sulphuric acid (10 ml) was added to the suspension. The blank solution having 1 ml of ddH_2O and 10 ml of concentrated H_2SO_4 was also run for calibrating the instrument. The colour of blank solution didn't change. The aliquot of 3 ml was taken from each sample and absorbance was assessed at 420 nm wavelength by

spectrophotometer. The quantification of unknown samples was recorded with reference to the standard curve by following the linear regression equation produced from calibration curve.

3.6.3 Proline contents (µg/g FW)

The proline contents were assessed using optimized protocol of Bates *et al.* (1973). The plant material (0.5 g) was harmonized in 10 ml of 3% sulphosalicylic acid. The homogenized material was sieved through a filter paper; Whatman No. 2 (Thomas Scientific). The filtrate (2 ml) was taken in test tube and 2 ml of glacial acid as well as 2 ml of acid ninhydrin was mixed with the filtrate and the mixture was heated in water bath at 100 ºC for 1 h. The reaction was ceased by placing the material in an ice-bath. Four milliliters of toluene was supplemented with reaction mixture, stirred for thirty seconds and phase separation was done separating the toluene layer using a pipette and then red colour intensity was calculated at 520 nm absorbance of proline-ninhydrin complex. Toluene was run as an empty sample.

The preparation of acid ninhydrin was carried out by dissolving 1.25 grams of ninhydrin in 30 milliliters of glacial acetic-acid and 20 milliliters of 6 molar phosphoric acid and continuous agitation was done to homogenize mixture and it was stored at 4 ºC and used within 24 h.

A standard curve was prepared by running a series of standards of pure proline which were prepared as follows: The proline stock solution was synthesized dissolving 0.1 g of proline in 100 ml of ddH_2O. From this proline solution, 10 concentrations were made by measuring 1 – 10 ml and then volume of each concentration was raised up to 100 ml adding ddH_2O. Now 1 ml from each solution was taken and supplemented with 1 ml of sulphosalicylic acid, 2 ml of glacial acetic acid and two milliliters of acid ninhydrin. The material was heated in boiling water-bath for 1 h and then placed in ice-bath to cease the reaction. Four milliliters of toluene was supplemented with reaction mixture, stirred it well for 30 sec and the toluene layer was separated by a pipette after warming the mixture for 10 minutes at 25 °C. The absorbance of each standard solution was calculated at 520 nm using Optima (SP 3000 plus) and distilled water was run as a

blank solution. The amount of proline from each plant sample was measured from the curve after calculating the values of proline from the following equation:

$$\text{Proline (µg per g tissue)} = \frac{\text{µg proline/ml} \times \text{ml toluene}}{115.5} \times \frac{5}{\text{g sample}}$$

Where 115.5 denotes molecular weight of proline

3.6.4 Chlorophyll contents (mg/g FW)

Chlorophyll contents (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were measured according to the protocol of Arnon (1949). The leaf samples were cut into small pieces by removing the veins and any tough (fibrous) tissue and weighed 0.1 g of each sample. The tissues were ground to a homogeneous suspension into a sterilized pestle and mortar by the addition of 2.0 ml pre-chilled 80% acetone. The homogenate was transferred in large centrifugation tubes and spun at 12000 rpm for ten min. The filtrate was stored and pellet was extracted with more two milliliter of 80% acetone (pre-chilled) and again centrifuged at 12000 rpm for ten minutes. This procedure was repeated until there was no green colour in pellet. A clean cuvette was filled with 3 ml of 80% acetone; it was the blank. The cuvette was wiped out with tissue paper and placed into the spectrophotometer [UV-VIS SP-3000 plus Optima, USA] and wavelength was set at 663, 645 and 450 nm, while spectrophotometer (UV visible) was adjusted to zero absorbance for the blank. The sample filtrate (3 ml each) was put in clean cuvettes, inserted in spectrophotometer and absorbance of the solutions at 663, 645 and 450 nm was recorded against eighty percent acetone (solvent). Chlorophyll contents (present in mg extract per gram tissue) were calculated by the following formulae:

Chlorophyll a (mg/g FW) = [12.7 (OD 663) – 2.69 (OD 645)] × V/1000 × W

Chlorophyll b (mg/g FW) = [22.9 (OD 645) – 4.69 (OD 663)] × V/1000 × W

Total chlorophyll (mg/g FW) = [20.2 (OD 645) + 8.02 (OD 663)] × V/1000 × W

Carotenoids (mg/g FW) = 4.07 × (OD 450) – (0.0435 × chlorophyll a + 0.367 × chlorophyll b)

Where OD = Optical density at particular wavelength, V = Final volume of extract in eighty percent acetone (ml), W = Fresh weight of leaf tissue (g)

3.6.5 Ascorbic acid (AsA) contents (mg/100 g FW)

The ascorbic acid contents were measured following the optimized method of Hans (1992). In brief, one gram leaf samples were ground thoroughly with clean mortar and pestle adding 3 ml of 1% HCl. The homogenate was poured in centrifuge tubes and spun at 10,000 rpm for ten min. The optical density of leaf samples was assessed at 243 nm absorption by spectrophotometer (Optima, SP 3000 plus).

3.6.6 Malondialdehyde contents (nmol/g FW)

The malondialdehyde (MDA) contents were calculated following the protocol devised by Zhao *et al.* (1994) with some modification. Two hundred gm of tomato leaf sample was homogenized in 10 milliliters of ten percent trichloroacetic acid (TCA) and spun at 12000 rpm at 4 ºC for ten minutes. The clear supernatant (2 ml) was mixed with two milliliters of 0.5% thiobarbituric acid (TBA) (dissolved in ten percent TCA) and heated in water bath at 95 ºC for twenty minutes. The material was put immediately on ice to terminate the reaction and then it was spun at 12000 rpm for fifteen minutes and absorbance of supernatant solution was measured at 450, 532 and 600 nm. The MDA contents were determined using the following equation:

$$\text{MDA (nmol/g FW)} = 6.45 \times (OD_{532\ nm} - OD_{600\ nm}) - 0.56 \times OD_{450\ nm}$$

3.7 Statistical analysis

All the experiments were performed in completely randomized design (CRD) in a factorial system. The values indicated mean ± standard deviation. Each experiment was repeated three times and data was analyzed by ANOVA at $p \leq 0.05$. The least significant difference test (LSD) was employed to compare the statistical differences between means (Steel *et al.*, 1997) using Statistical Software; The Statistix v. 8.1 (Analytical Software, 2005). The mean values indicated by the different letters within a column were statistically different at 5% level of significance.

IV. RESULTS

The genotypic responses of three tomato cultivars to various factors affecting the tissue culture and genetic transformation were studied. Subsequently, responses to cold stress of transgenic and NT tomato lines were evaluated through analyses of various physiological and biochemical parameters. The results of these studies are given below:

4.1 Bulking of explants after decontamination

Surface sterilization is an important step that inhibits the growth of microorganisms. The effect of different concentrations of clorox (5.25% sodium hypochlorite) was evaluated for contamination frequency and *in vitro* seed germination of three tomato genotypes (Rio Grande, Moneymaker and Roma). Seeds were surface sterilized in an autoclaved falcon tube in 70% ethanol for one minute. After removal of ethanol, these seeds were treated with five different concentrations of clorox (10, 20, 30, 40 and 50%) (v/v) for twenty minutes and then cultured on simple MS medium. Results indicated that the contamination frequency was maximum (28.12, 22.61 and 21.87%) in Moneymaker, Roma and Rio Grande respectively at low level of clorox (10%), while it was gradually decreased on further increase of clorox (Table 4.1). The most effective clorox concentration was 50%, giving the lowest contamination frequency (1.04, 2.08 and 0%) in Rio Grande, Moneymaker and Roma but the germination frequency was lower (82.93, 71.62 and 64.48%) as compared to 40% clorox which yielded the highest germination frequency (88.09, 83.72 and 79.75%) in Rio Grande, Moneymaker and Roma (Table 4.2). The seeds of all the genotypes germinated within one week and 2-3 weeks old *in vitro* seedlings were used for callus induction and *in vitro* shoot regeneration.

4.2 Comparison of various explants for efficient shoot regeneration and number of primordial shoots per explant

Five different types of explants namely cotyledons, epicotyls, leaf discs, internodes and hypocotyls from fifteen days old *in vitro* seedlings were appraised in three tomato genotypes on the basis of their regeneration frequency and potential of producing the mean number of shoots primordial per explant. All the explants were cultured on

Murashige and Skoog (1962) medium supplemented with 6-benzylaminopurine (BAP) (2.0 mg/l) and indole-3-acetic acid (IAA) (0.2 mg/l). All the explants responded positively from their cutting edges yielding green calli within fifteen days after culturing and then produced primordial shoots.

In case of cv. Rio Grande, the highest regeneration frequency (72.66 and 66.66%) was recorded culturing leaf discs and hypocotyls followed by cotyledons and epicotyls which exhibited 45.0 and 58.33% regeneration frequency, respectively (Table 4.3). The lowest regeneration frequency (24.33%) was noticed in internodes explants. In case of Roma, the best regeneration frequency (67.66 and 59%) was secured culturing leaf discs and hypocotyls followed by epicotyls (52%) and cotyledons (39.66%). The lowest regeneration frequency (18.33%) was recorded in internodal explants. The similar trend was noticed in cv. Moneymaker (Table 4.3). The overall mean for *in vitro* shoot regeneration frequency was in the order of leaf discs > hypocotyls > epicotyls > cotyledons > internodes.

The analysis of variance revealed significant differences among explant types and genotypes for regeneration frequency. Statistical genotype x explant interactions was also significant. On the basis of number of shoots primordial, significant differences were noticed among explants, genotypes and explant x genotype interactions (Appendices 3 & 4). The number of shoots primordial was versatile for different explants in three tomato genotypes. The highest number of shoots primordial (9.0 and 7.67) was secured culturing leaf discs and hypocotyls (Table 4.3). The lower number of primordial shoots (6.33, 5.67 and 2.33) was recorded culturing epicotyls, cotyledons and internodes explants than that of hypocotyls and leaf discs.

Table 4.1 Assessment of different concentrations of clorox (v/v) on contamination frequency (%) in three cultivars of tomato

Percentage of clorox (v/v) used	No. of contaminated seeds			No. of non-contaminated seeds			Contamination frequency (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
10	21.00	27.00	38.00	75.00	69.00	130.00	$21.87^{bc} \pm 2.76$	$28.12^{a} \pm 2.09$	$22.61^{b} \pm 1.58$
20	10.00	18.66	23.00	86.00	77.34	145.00	$10.41^{ef} \pm 2.09$	$19.44^{c} \pm 4.21$	$13.69^{d} \pm 1.19$
30	8.00	12.66	16.00	88.00	83.33	152.00	$8.33^{f} \pm 2.08$	$13.19^{de} \pm 2.62$	$9.52^{f} \pm 1.58$
40	2.00	4.66	5.00	94.00	91.34	163.00	$2.08^{gh} \pm 1.04$	$4.85^{g} \pm 1.21$	$2.97^{gh} \pm 0.60$
50	1.00	2.00	0.00	95.00	94.00	168.00	$1.04^{h} \pm 1.04$	$2.08^{gh} \pm 1.04$	$0.00^{h} \pm 0.00$

Each data is the average of three replicates. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). The total number of seeds inoculated for cv. Rio Grande and Moneymaker was ninety-six while the total number of seeds inoculated for Roma was one hundred and sixty-eight. LSD value was 3.08 at $p \leq 0.05$.

Table 4.2 Assessment of clorox (40 and 50%) on germination frequency in three cultivars of tomato

Percentage of clorox (v/v) used	No. of germinated seeds			No. of contaminated seeds			Germination percentage (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
40	148.0	140.66	134.0	5.00	4.00	6.00	$88.09^{a} \pm 3.31$	$83.72^{ab} \pm 4.8$	$79.75^{b} \pm 4.49$
50	139.3	120.3	108.3	3.00	2.00	4.00	$82.93^{ab} \pm 3.95$	$71.62^{c} \pm 2.08$	$64.48^{d} \pm 3.38$

Each data is the average of three replicates. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). The total number of seeds inoculated for all the cultivars was one hundred and sixty-eight. LSD value was 5.74 at $p \leq 0.05$.

Table 4.3 Assessment of various explants on the basis of their regeneration frequency and number of primordial shoots per explant culturing on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA in tomato

Genotypes	Epicotyls	Leaf discs	Internodes	Hypocotyls	Cotyledons
Rio Grande	**58.33 ± 1.53**	**72.66 ± 0.58**	**24.33 ± 0.76**	**66.66 ± 0.58**	**45.00 ± 0.50**
	6.00 ± 0.64	8.33 ± 0.57	2.33 ± 0.91	7.00 ± 0.76	4.67 ± 0.77
Moneymaker	**47.33 ± 2.08**	**61.00 ± 1.00**	**13.66 ± 0.58**	**53.00 ± 0.00**	**32.33 ± 1.04**
	4.33 ± 1.01	7.66 ± 0.8	2.00 ± 0.76	6.33 ± 0.88	5.67 ± 0.86
Roma	**52.00 ± 0.50**	**67.66 ± 0.76**	**18.33 ± 0.76**	**59.00 ± 0.50**	**39.66 ± 0.76**
	6.33 ± 0.39	9.00 ± 0.41	2.00 ± 0.39	7.67 ± 0.68	5.00 ± 0.3
Overall mean	**52.55**	**67.11**	**18.77**	**59.55**	**38.99**

All values are means of three replicates and the values after ± sign indicate standard deviation (n = 3). The upper bold values represent the *in vitro* shoot regeneration frequency and lower values demonstrate the mean number of primordial shoots per explant. LSD value for regeneration frequency was 1.53 at $p \leq 0.05$. LSD value for number of primordial shoots per explant was 0.94 at $p \leq 0.05$.

4.3 Evaluation of media composition and explants type on callus induction

Various composition of callus induction media (CIM_1 – CIM_8; Table 3.1) were evaluated for efficient callus induction culturing hypocotyls and leaf discs as explants sources. Both type of explants produced callus induction within thirty days of inoculation on MS medium supplemented with diverse hormonal regimes. Analysis of variance revealed that the effect of explant, genotype and media were highly significant for callus induction ability at $p \leq 0.05$. Similarly, the interactions between explant × media and media × genotype were also highly significant. Statistically, significant differences were found among explant × genotype and explant × media × genotype interactions (Appendix 5).

The highest callus induction frequency (CIF) (67.48%) was recorded in Rio Grande culturing hypocotyls on CIM_6 (MS + IAA-BAP; 2.0 - 2.5 mg/l) as shown in table 4.4. Similarly, the highest CIF (62%) was secured in Roma on CIM_5 (MS + NAA-BAP; 1.0 - 2.5 mg/l) followed by Moneymaker (58.23%) on CIM_4 (MS + NAA-BAP; 2.0 - 2.0 mg/l). There was no callus induction when explants were cultured on MS medium supplemented with 2,4-D (3.0 and 4.0 mg/l). During this experiment it was found that 2.5 mg/l BAP in combination with 1.0 mg/l NAA frequently produced callus in a shorter period of time than that of other hormonal combinations. Likewise, the highest CIF (63.69%) was recorded in Rio Grande on CIM_6 followed by Roma (60%) on CIM_5 and Moneymaker (53%) on CIM_4 culturing leaf discs (Table 4.4). As far as callus morphology is concerned, it was embryogenic calli from hypocotyls showing shoot regeneration in Rio Grande on CIM_{6A} (Fig. 4.1C), while hard, compact and dark green callus from leaf discs were observed in Moneymaker on CIM_4 (Fig. 4.1A). In case of Roma, the embryogenic calli with many embryoids were recorded from leaf discs on CIM_5 (Fig. 4.1B).

4.4 Assessment of silver nitrate response on callus induction in tomato

The capability of silver nitrate ($AgNO_3$) to induce embryogenic calli was assessed in three tomato genotypes namely Rio Grande, Moneymaker and Roma. The hypocotyls and leaf discs were cultured for fifteen days on diverse callus induction media; MS with

varying levels of $AgNO_3$ (2.5 – 20 mg/l). The supplementation of $AgNO_3$ in callus induction media enhanced significantly CIF in all the genotypes (Table 4.5). The callus formation was initiated after 5-8 days culturing of explants on $AgNO_3$ fortified media. All the treatments of $AgNO_3$ increased the volume of tissues by swelling and showed healthy green appearance. The explants orientation also had great influence on callus induction. The abaxial side of leaf discs and horizontal surface of hypocotyls on culture media produced the best callogenesis compared to adaxial and vertical sides. The analysis of variance showed highly significant differences among explants, genotypes, treatments and treatment × genotype interaction for callus induction frequency. The interaction effects of explant × treatment, explant × genotype and explant × treatment × genotype were also significant (Appendix 6). The response of $AgNO_3$ on CIF was also compared with $AgNO_3$ – free media. All the genotypes responded differently to $AgNO_3$ concentrations. In Rio Grande, the highest CIF (88.66 and 91.83%) was recorded on CIM_{6A} (MS media supplemented with IAA (2.0 mg/l) and BAP (2.5 mg/l) along with $AgNO_3$ (15.0 mg/l) culturing leaf discs and hypocotyls, respectively which indicated 39.2 and 36.08% increase in CIF compared to the same culture media devoid of $AgNO_3$ as control (Table 4.5). The best callogenesis in Moneymaker (80 and 82.66%) was secured on CIM_{4A} (MS + 10.0 mg/l $AgNO_3$ + NAA; 2.0 mg/l and BAP; 2.0 mg/l) using leaf discs and hypocotyls which exhibited 50.94 and 41.95% increase in CIF compared to control. Similarly, the efficient CIF (85.66 and 88.33%) was obtained in Roma on CIM_{5A} (MS medium supplemented with NAA; 1.0 mg/l and BAP; 2.5 mg/l along with $AgNO_3$; 12.5 mg/l) culturing leaf discs and hypocotyls which demonstrated 42.76 and 42.46% increase in CIF using leaf discs and hypocotyls as compared to control (Table 4.5). The CIF started to decline rapidly by increasing $AgNO_3$ concentrations more than 15 mg/l. $AgNO_3$ at 20 mg/l was found to be restrictive for embryogenic callus induction and yielded the lowest CIF. The browning was too much evident in hypocotyls and leaf discs derived callus induction on culture media with only hormonal combinations in all the genotypes. Therefore, plant regeneration from calli clumps was reduced due to browning effect. It was also found that appropriate concentrations of $AgNO_3$ reduced the browning of calli.

Table 4.4 Assessment of various combinations of PGRs on callus induction in tomato

Callus induction media	Hypocotyls-derived callus induction frequency (%)			Leaf discs-derived callus induction frequency (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
CIM_1	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$
CIM_2	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$
CIM_3	$32.68^{r} \pm 1.35$	$23.63^{u} \pm 0.79$	$39.43^{no} \pm 1.25$	$28.39^{s} \pm 1.06$	$20.82^{v} \pm 1.38$	$36.16^{pq} \pm 0.28$
CIM_4	$47.88^{i} \pm 5.17$	**$58.23^{cd} \pm 1.08$**	$42.77^{kl} \pm 0.8$	$41.49^{lm} \pm 0.9$	**$53.00^{f} \pm 1.08$**	$40.00^{mno} \pm 0.81$
CIM_5	$57.64^{d} \pm 1.02$	$39.00^{o} \pm 1.66$	**$62.00^{b} \pm 0.56$**	$54.00^{ef} \pm 1.36$	$39.94^{q} \pm 0.63$	**$60.00^{c} \pm 1.2$**
CIM_6	**$67.48^{a} \pm 0.7$**	$50.15^{g} \pm 1.39$	$55.32^{e} \pm 1.12$	**$63.69^{b} \pm 1.08$**	$47.71^{i} \pm 1.08$	$52.68^{f} \pm 0.83$
CIM_7	$49.82^{gh} \pm 1.05$	$45.66^{j} \pm 0.88$	$48.21^{hi} \pm 1.09$	$40.94^{lmn} \pm 0.23$	$43.91^{jk} \pm 1.66$	$42.00^{l} \pm 0.23$
CIM_8	$37.00^{p} \pm 0.89$	$18.56^{w} \pm 0.58$	$25.78^{t} \pm 0.97$	$30.84^{r} \pm 1.4$	$14.83^{x} \pm 0.54$	$21.53^{v} \pm 1.11$

The concentrations of plant growth regulators were taken in (mg/l). After six weeks of culture, data were collected. According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. Each data is the average of three replicates. The values after ± sign indicate standard deviation (n = 3). The bold letters demonstrate the best results. The number of explants cultured per treatment for each genotype was eighty-five and each experiment was repeated three times. LSD value was 1.89 at $p \leq 0.05$.

Table 4.5 Assessment of $AgNO_3$ in combination with various PGRs on callus induction in tomato

Callus induction media	Hypocotyls-derived callus induction frequency (%)			Leaf discs-derived callus induction frequency (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
CIM_{1A}	$34.32^{s} \pm 2.58$	$32.66^{st} \pm 4.14$	$30.00^{tuv} \pm 1.88$	$25.56^{w} \pm 1.63$	$30.00^{tuv} \pm 3.52$	$27.6^{vw} \pm 1.78$
CIM_{2A}	$42.33^{q} \pm 3.83$	$40.33^{q} \pm 4.79$	$38.66^{qr} \pm 2.16$	$38.66^{qr} \pm 0.61$	$38.66^{qr} \pm 2.96$	$35.33^{rs} \pm 2.44$
CIM_{3A}	$70.33^{hi} \pm 2.82$	$67.33^{ij} \pm 4.06$	$65.33^{jk} \pm 4.29$	$66.66^{ij} \pm 5.26$	$66.00^{j} \pm 3.94$	$62.00^{kl} \pm 4.64$
CIM_{4A}	$60.68^{l} \pm 1.41$	**$82.66^{cd} \pm 5.53$**	$55.33^{nop} \pm 3.53$	$56.33^{mn} \pm 4.88$	**$80.00^{de} \pm 6.02$**	$52.00^{p} \pm 3.49$
CIM_{5A}	$84.89^{bc} \pm 2.07$	$67.33^{ij} \pm 4.48$	**$88.33^{ab} \pm 6.91$**	$82.00^{cd} \pm 5.74$	$64.66^{jk} \pm 4.41$	**$85.66^{bc} \pm 6.52$**
CIM_{6A}	**$91.83^{a} \pm 2.92$**	$74.66^{fg} \pm 4.43$	$77.33^{ef} \pm 5.17$	**$88.66^{ab} \pm 6.69$**	$72.33^{gh} \pm 4.58$	$75.66^{fg} \pm 5.31$
CIM_{7A}	$59.76^{lm} \pm 1.75$	$58.66^{lmn} \pm 3.93$	$56.00^{mno} \pm 4.11$	$52.33^{op} \pm 4.66$	$55.00^{nop} \pm 3.63$	$51.66^{p} \pm 3.92$
CIM_{8A}	$31.66^{stu} \pm 3.3$	$31.66^{stu} \pm 2.67$	$29.00^{t-w} \pm 2.17$	$27.43^{vw} \pm 0.66$	$28.00^{uvw} \pm 2.94$	$26.3^{vw} \pm 2.43$

According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. Each data is the average of three replicates. The values after ± sign indicate standard deviation (n = 3). The bold letters demonstrate the best results. The number of explants cultured per treatment for each genotype was one hundred. LSD value was 3.96 at $p \leq 0.05$.

4.5 Assessment of cobalt chloride ($CoCl_2$) response on callus induction in tomato

The callus induction was examined using eight different concentrations (0.5 – 10.0 mg/l) of $CoCl_2$ on MS media with various hormonal regimes and initiation of callus was noticed after 4-5 days of culture in this culture. It was found that increasing $CoCl_2$ concentration in culture media considerably enhanced the callus induction in all the genotypes. The enhancement in callus fresh weight and percent response was recorded up to 5.5 mg/l $CoCl_2$, but there was a rapid decrease in callogenesis on further increase of $CoCl_2$ (Table 4.6). The influence of $CoCl_2$ to induce callus *in vitro* was prominent and the genotypes showed different response to varying levels of $CoCl_2$. The compact and embryogenic calli were obtained in all the genotypes after sub-culturing up to six weeks.

The analysis of variance unfolded that there were highly significant differences among the effects of genotypes, explants and treatments on callus induction frequency. Similarly, the interaction effects of treatment × genotype and explant × treatment × genotype had significant differences for CIF. While the interaction effects of explant × treatment and explant × genotype had no significant differences (Appendix 7).

In Rio Grande, the highest CIF (72 and 75.65%) was recorded on CIM_{6B} (MS medium supplemented with $CoCl_2$; 5.5 mg/l, IAA; 2.0 mg/l and BAP; 2.5 mg/l) culturing leaf discs and hypocotyls, respectively which showed 13.04 and 12.1% increase compared to the same culture media devoid of $CoCl_2$ as control (Table 4.6). In Moneymaker, the best callogenesis frequency (65 and 68%) using leaf discs and hypocotyls was secured on CIM_{4B} (MS media + $CoCl_2$; 3.5 mg/l and hormonal permutation NAA – BAP (2.0 – 2.0 mg/l) which depicted 22.64 and 16.77% increase over control. It was followed by Roma in which the efficient CIF (69 and 73.66%) was obtained applying 4.5 mg/l $CoCl_2$ in MS medium supplemented with hormonal amalgamation; NAA – BAP (1.0 – 2.5 mg/l) (CIM_{5B}) which represented 15 and 18.8% increase over control.

4.6 Assessment of media composition and explants type on *in vitro* shoot regeneration

Analysis of variance showed that impact of explant, genotype and media on regeneration frequency was highly significant. The interactions effect between media × explants, media × genotype and media × explants × genotype were also significant. While the interaction impact between explant × genotype was non-significant at $p \leq 0.05$ (Appendix 8).

The various permutations and concentrations of plant growth regulators (PGRs) (auxins, cytokinins and gibberellins) were scrutinized for their effects on *in vitro* shoot regeneration and number of primordial shoots per calli clump in three tomato genotypes. Genotypes and explant types were also assessed on *in vitro* shoot regeneration. Two – three weeks old *in vitro* seedlings-derived hypocotyls and leaf discs were used as explant sources. These explants were first cultured on CIM and after six weeks, the hypocotyls and leaf discs – derived calli were transferred to various shoot induction media (SIM_1 – SIM_{11}). No regeneration response was noticed from calli clumps cultured on MS plain medium (devoid of PGRs). Among all the media investigated, the optimal medium for efficient *in vitro* shoot regeneration was SIM_7 (MS medium supplemented with 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP) which produced maximum shoot regeneration in Rio Grande and Roma from both type of explants. While in Moneymaker, the efficient shoot regeneration was recorded on SIM_6 (MS medium fortified with 0.1 mg/l IAA and 3.0 mg/l BAP). During this experiment, it was noticed that sub-culturing of calli clumps on recovery medium after every two week was too necessary for securing maximum shoot regeneration frequency; otherwise calli clumps limited the shoot regeneration. The highest shoot regeneration frequency (66.65 and 60.66%) and the highest mean number of primordial shoots (7.0) per explants culturing hypocotyls – derived calli were recorded in Rio Grande and Roma on SIM_7 (Table 4.7; Figs. 4.1E & 4.2A). It was followed by Moneymaker in which the best shoot regeneration frequency (62.66%) and mean number of shoots primordial (6.0) from hypocotyls – derived calli was obtained on SIM_6. Similarly, the highest shoot regeneration frequency (69.65 and 65.32%) and the highest mean number of primordial shoots (7.66) culturing leaf discs – derived calli were

recorded in Rio Grande and Roma on SIM_7 followed by Moneymaker whose highest *in vitro* shoot regeneration frequency (67.32%) and the efficient average number of shoots primordial (6.66) from leaf discs – derived calli was secured on SIM_6 (Table 4.7; Figs. 4.1D & 4.2B).

4.7 Effect of $AgNO_3$ on *in vitro* shoot regeneration in tomato

In current study, influence of $AgNO_3$ was investigated on indirect *in vitro* shoot regeneration culturing hypocotyls and leaf discs-derived calli clumps in three tomato genotypes. The culture media, devoid of plant growth regulators neither initiated callus induction nor *in vitro* shoot regeneration in all the genotypes. Different concentrations of $AgNO_3$ (2.0 – 15.0 mg/l) were examined in combination with different plant growth regulators for the enhancement of shoot regeneration. It was found that calli clumps responded to initiate the shoot regeneration after fifteen days of culturing on SIM supplemented with different levels of $AgNO_3$ with outgrowth appearance.

The prolonged culturing of calli on the same media failed to differentiate into shoots, but sub-culturing of calli on respective fresh media developed the shoots considerably. The organogenesis frequency was increased gradually by increasing the concentration of $AgNO_3$ up to 10 mg/l but decreased rapidly by further increase of $AgNO_3$. All the genotypes and explants responded in a different way to diverse concentrations of $AgNO_3$. Analysis of variance depicted that influence of media, explants, genotypes on *in vitro* shoot regeneration frequency and interaction effect between media × genotype were highly significant. The interactions between explants × genotype had no significant effect on shoot regeneration frequency. While a significant difference was found between the interaction effects of media × explants and media × explants × genotype at $p \leq 0.05$ (Appendix 10). The highest *in vitro* shoot regeneration frequency (93 and 96.65%) and mean number of shoots primordial (10.0, 10.66) from hypocotyls and leaf discs-derived calli, respectively were recorded in Rio Grande on SIM_{7A} (MS medium enriched with 10.0 mg/l $AgNO_3$ along with hormonal regime, IAA – ZEA – BAP; 0.1 – 1.0 – 2.0 mg/l) which depicted 39.53 and 38.76% increase over control (Table 4.8; Figs. 4.1F & 4.2C and D).

Table 4.6 Assessment of $CoCl_2$ in combination with various PGRs on callus induction in tomato

Callus induction media	Hypocotyls-derived callus induction frequency (%)			Leaf discs-derived callus induction frequency (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
CIM_{1B}	$25.69^{za} \pm 0.97$	$18.66^{cd} \pm 1.24$	$20.66^{c} \pm 0.93$	$24.00^{ab} \pm 0.89$	$12.96^{e} \pm 1.23$	$17.00^{d} \pm 0.79$
CIM_{2B}	$33.33^{vw} \pm 1.55$	$26.00^{za} \pm 1.57$	$30.66^{xy} \pm 2.09$	$29.69^{y} \pm 1.76$	$22.86^{b} \pm 0.91$	$27.00^{z} \pm 0.65$
CIM_{3B}	$55.99^{hi} \pm 1.8$	$48.00^{no} \pm 1.3$	$53.65^{jk} \pm 0.69$	$52.00^{kl} \pm 2.14$	$39.54^{rs} \pm 0.88$	$51.00^{lm} \pm 1.31$
CIM_{4B}	$48.66^{n} \pm 1.98$	**$68.00^{c} \pm 1.48$**	$51.33^{l} \pm 4.16$	$46.00^{op} \pm 2.11$	**$65.00^{de} \pm 1.79$**	$49.00^{mn} \pm 1.55$
CIM_{5B}	$67.00^{cd} \pm 1.75$	$32.33^{wx} \pm 1.22$	**$73.66^{ab} \pm 2.23$**	$64.00^{ef} \pm 1.15$	$30.00^{y} \pm 1.34$	**$69.00^{c} \pm 1.36$**
CIM_{6B}	**$75.65^{a} \pm 1.15$**	$60.66^{g} \pm 0.88$	$62.99^{ef} \pm 3.1$	**$72.00^{b} \pm 2.95$**	$57.00^{h} \pm 1.42$	$62.00^{fg} \pm 1.86$
CIM_{7B}	$45.66^{p} \pm 1.45$	$54.64^{ij} \pm 0.89$	$52.33^{kl} \pm 2.02$	$43.00^{q} \pm 2.39$	$52.00^{kl} \pm 2.48$	$46.00^{op} \pm 1.3$
CIM_{8B}	$40.00^{qr} \pm 1.28$	$39.66^{rs} \pm 1.49$	$35.00^{uv} \pm 0.77$	$38.00^{st} \pm 1.5$	$36.00^{tu} \pm 1.36$	$33.00^{vw} \pm 1.56$

According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. Each data is the average of three replicates. The values after ± sign indicate standard deviation (n = 3). LSD value was 2.12 at $p \leq 0.05$.

Table 4.7 Assessment of various combinations of PGRs on *in vitro* shoot regeneration in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls-derived calli clumps (%)			*In vitro* shoot regeneration frequency culturing leaf discs-derived calli clumps (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
SIM_1	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$
SIM_2	$11.46^{g-j} \pm 0.52$	$10.00^{i-l} \pm 0.62$	$14.66^{e} \pm 0.74$	$14.26^{e} \pm 0.75$	$13.33^{efg} \pm 0.82$	$12.00^{f-i} \pm 0.66$
SIM_3	$34.00^{vwx} \pm 0.97$	$31.00^{yz} \pm 1.24$	$32.33^{xyz} \pm 0.76$	$36.00^{tuv} \pm 0.78$	$33.06^{wxy} \pm 1.79$	$35.00^{uvw} \pm 1.87$
SIM_4	$54.00^{l} \pm 1.92$	$50.00^{no} \pm 1.24$	$48.33^{op} \pm 0.89$	$56.33^{k} \pm 1.38$	$52.65^{lm} \pm 1.84$	$51.66^{mn} \pm 1.54$
SIM_5	$59.00^{ij} \pm 1.22$	$57.00^{jk} \pm 1.16$	$58.00^{jk} \pm 1.04$	$61.66^{gh} \pm 1.66$	$58.66^{ij} \pm 1.14$	$64.00^{def} \pm 2.11$
SIM_6	$63.66^{d-g} \pm 1.31$	**$62.66^{fgh} \pm 2.12$**	$59.00^{ij} \pm 1.05$	$65.00^{cde} \pm 1.44$	**$67.32^{b} \pm 1.74$**	$63.00^{efg} \pm 3.35$
SIM_7	**$66.65^{bc} \pm 1.85$**	$47.32^{p} \pm 0.76$	**$60.66^{hi} \pm 1.36$**	**$69.65^{a} \pm 2.17$**	$50.66^{mn} \pm 0.79$	**$65.32^{bcd} \pm 4.26$**
SIM_8	$38.00^{st} \pm 1.13$	$35.33^{uv} \pm 1.18$	$30.65^{z} \pm 0.85$	$42.00^{q} \pm 1.81$	$39.65^{rs} \pm 1.42$	$32.32^{xyz} \pm 3.64$
SIM_9	$18.00^{d} \pm 1.11$	$22.66^{b} \pm 1.21$	$36.33^{tu} \pm 1.52$	$21.00^{bc} \pm 1.33$	$25.00^{a} \pm 0.49$	$41.65^{qr} \pm 3.48$
SIM_{10}	$10.00^{i-l} \pm 0.37$	$7.00^{no} \pm 0.57$	$15.00^{e} \pm 1.55$	$13.66^{ef} \pm 1.67$	$9.00^{k-n} \pm 0.64$	$20.00^{cd} \pm 1.25$
SIM_{11}	$7.65^{mno} \pm 0.77$	$6.00^{o} \pm 0.28$	$9.66^{j-m} \pm 0.39$	$11.00^{h-k} \pm 0.52$	$8.66^{lmn} \pm 0.82$	$13.00^{e-h} \pm 0.84$

According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. Each data is the average of three replicates. The values after ± sign indicate standard deviation (n = 3). The bold letters demonstrate the best results. LSD value was 2.12 at $p \leq 0.05$.

Table 4.8 Assessment of $AgNO_3$ in combination with various PGRs on *in vitro* shoot regeneration in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls-derived calli clumps (%)			*In vitro* shoot regeneration frequency culturing leaf discs-derived calli clumps (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
SIM_{1A}	$20.00^{gh} \pm 1.27$	$18.32^{hi} \pm 0.85$	$10.66^{j} \pm 0.87$	$23.66^{ef} \pm 1.26$	$21.66^{fg} \pm 0.94$	$17.00^{i} \pm 0.13$
SIM_{2A}	$40.33^{wx} \pm 1.59$	$38.33^{xy} \pm 1.12$	$44.00^{uv} \pm 0.21$	$44.00^{uv} \pm 1.66$	$42.00^{vw} \pm 0.75$	$46.65^{st} \pm 0.7$
SIM_{3A}	$60.00^{op} \pm 2.93$	$61.00^{o} \pm 1.77$	$57.00^{q} \pm 0.9$	$63.65^{n} \pm 0.88$	$65.00^{mn} \pm 1.44$	$59.66^{op} \pm 0.75$
SIM_{4A}	$78.00^{i} \pm 2.99$	$75.00^{j} \pm 3.04$	$71.66^{kl} \pm 1.88$	$80.00^{hi} \pm 2.88$	$78.33^{i} \pm 2.95$	$73.65^{jk} \pm 1.66$
SIM_{5A}	$83.66^{fg} \pm 4.00$	$77.66^{i} \pm 1.31$	$78.66^{i} \pm 1.35$	$86.32^{de} \pm 2.25$	$82.00^{gh} \pm 1.92$	$83.66^{fg} \pm 2.76$
SIM_{6A}	$87.66^{cd} \pm 3.06$	**$84.73^{ef} \pm 3.69$**	$70.00^{l} \pm 1.15$	$84.33^{efg} \pm 3.32$	**$90.00^{c} \pm 3.67$**	$74.66^{j} \pm 2.4$
SIM_{7A}	**$93.00^{b} \pm 3.67$**	$73.00^{jk} \pm 2.84$	**$88.00^{cd} \pm 2.76$**	**$96.65^{a} \pm 2.8$**	$77.65^{i} \pm 2.91$	**$92.66^{b} \pm 3.83$**
SIM_{8A}	$66.65^{m} \pm 1.78$	$58.33^{pq} \pm 1.86$	$79.00^{i} \pm 1.83$	$69.32^{l} \pm 2.78$	$61.00^{o} \pm 1.37$	$82.00^{gh} \pm 2.79$
SIM_{9A}	$44.66^{tu} \pm 1.87$	$48.66^{s} \pm 1.37$	$46.65^{st} \pm 1.03$	$47.33^{s} \pm 2.07$	$52.00^{r} \pm 1.56$	$47.66^{s} \pm 0.95$
SIM_{10A}	$38.66^{x} \pm 1.95$	$34.00^{zab} \pm 1.3$	$44.00^{uv} \pm 0.93$	$41.66^{vw} \pm 2.81$	$35.33^{za} \pm 0.97$	$43.66^{uv} \pm 1.18$
SIM_{11A}	$32.66^{b} \pm 2.76$	$28.66^{c} \pm 1.1$	$25.66^{de} \pm 1.24$	$36.00^{yz} \pm 2.28$	$33.00^{ab} \pm 0.6$	$27.32^{cd} \pm 1.07$

Different concentrations of plant growth regulators and ethylene inhibitor were taken in (mg/l). After six weeks of culture, data were collected about *in vitro* shoot regeneration frequency. According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. The number of calli clumps cultured per treatment for each genotype was ninety-five and each experiment was repeated three times. The values after ± sign indicate standard deviation (n = 3). The bold letters demonstrate the best results. LSD value was 2.39 at $p \leq 0.05$.

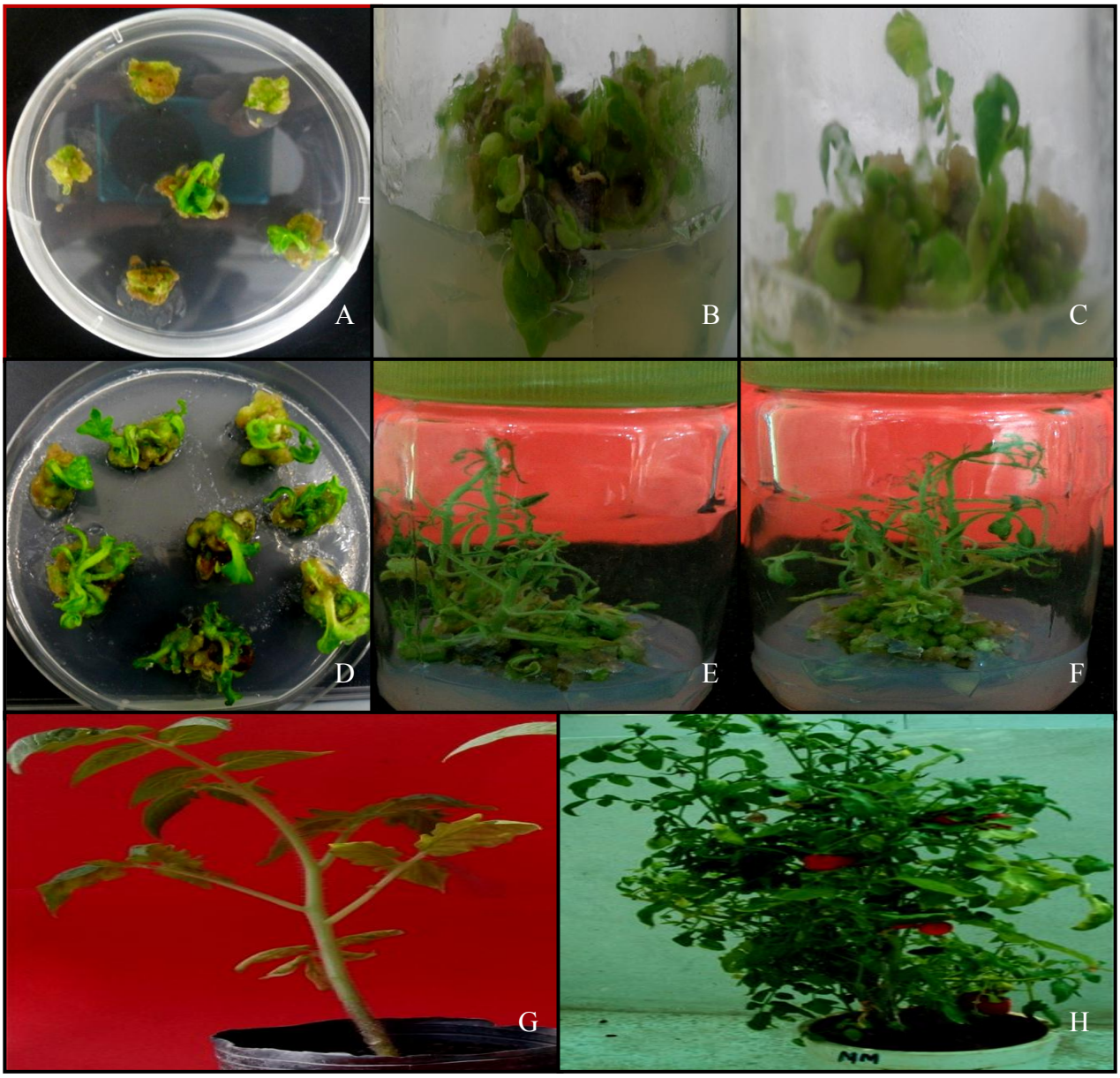

Fig. 4.1 Establishment of tomato plants through callus culture and *in vitro* shoot regeneration (A) Initiation of calli on CIM_5 (B) *In vitro* embryogenic calli produced on CIM_6 (C) *In vitro* calli induction on CIM_{6A} (D) Regeneration of calli clumps on SIM_6 (E) Multiple shoots primordia from regenerating calli produced on SIM_7 (F) Multiple shoots from regenerating calli clumps on SIM_{7A} (G) Acclimatization of regenerating plantlets after culturing on RIM for 4 weeks in growth room (H) Mature tomato (T_0) plants under controlled conditions bearing normal fruits

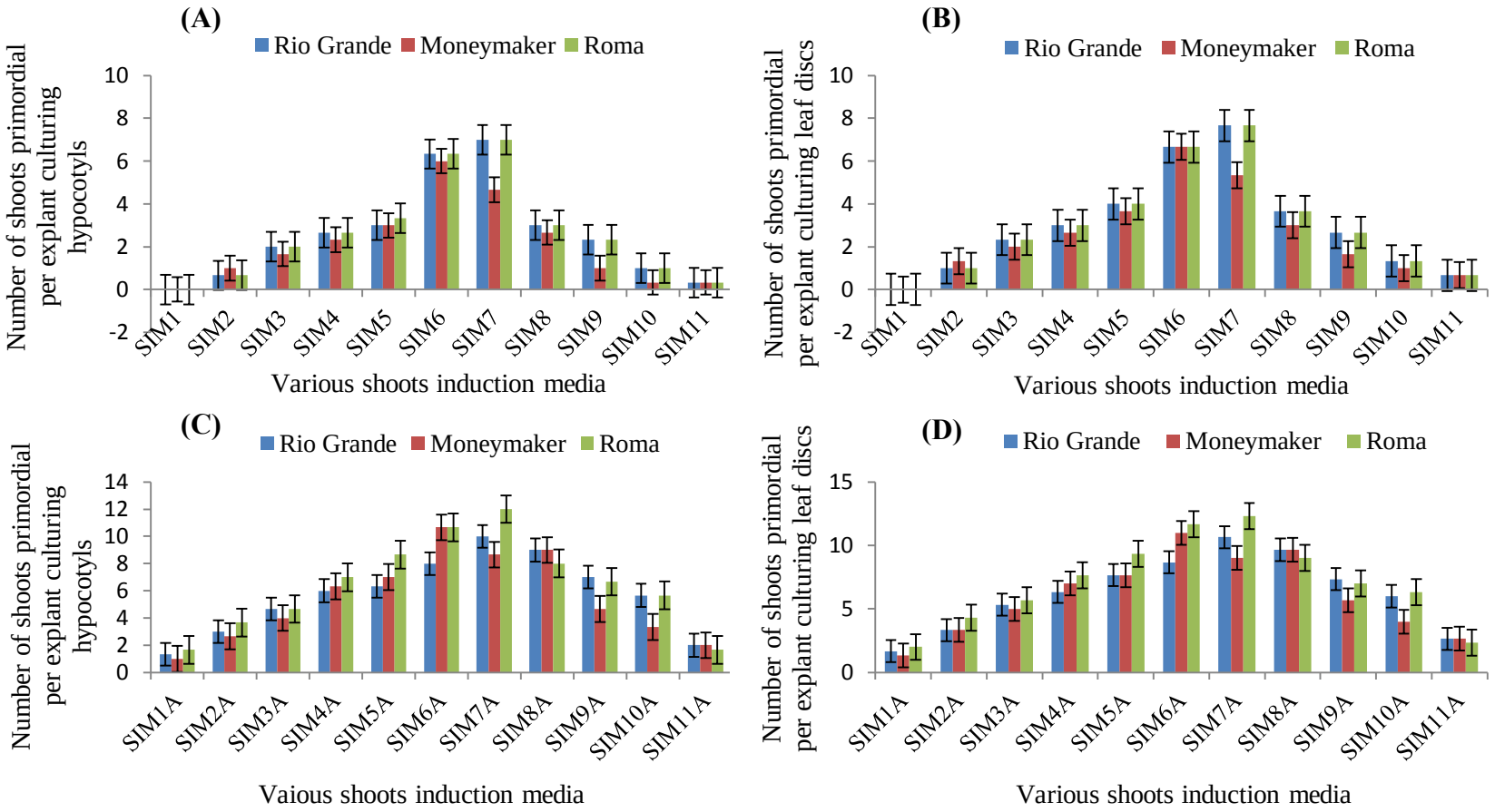

Fig. 4.2 Effect of various shoots induction media on the number of shoots primordial per explant in three tomato genotypes (A) Number of shoots primordial per explant on various concentrations of PGRs culturing hypocotyls (B) Number of shoots primordial per explant on various concentrations of PGRs culturing leaf discs (C) Number of shoots primordial per explant on $AgNO_3$-supplemented shoots induction media culturing hypocotyls (D) Number of shoots primordial per explant on $AgNO_3$-supplemented shoots induction media culturing leaf discs. Vertical bars depict the standard error of the means (n = 3)

It was followed by Roma whose best shoot regeneration frequency (88 and 92.66%) and average number of primordial shoots (12.0, 12.33) culturing hypocotyls and leaf discs-derived calli were secured on SIM_{7A} which represented 45.07 and 41.85% increase over control (Table 4.8; Figs. 4.1F & 4.2C and D). The same trend was noticed in Moneymaker whose efficient shoot regeneration and mean number of shoots primordial was obtained on SIM_{6A} (MS + 8.5 mg/l $AgNO_3$; 0.1 mg/l IAA and 3.0 mg/l BAP) which exhibited 35.22 and 33.68% increase in shoot regeneration frequency over control culturing hypocotyls and leaf discs – derived calli (Table 4.8).

4.8 Effect of $CoCl_2$ on *in vitro* shoot regeneration

The effect of $CoCl_2$ on *in vitro* shoot regeneration of three tomato genotypes was assessed indirectly from hypocotyls and leaf discs-derived calli. The regeneration frequency varied according to the amount of $CoCl_2$ used in shoot induction media.

The organogenesis response adding $CoCl_2$ was comparable to the response obtained on $CoCl_2$ – free SIM. During this experiment, it was found that $CoCl_2$ significantly reduced the loss of *in vitro* regeneration ability in embryogenic cultures of *Solanum lycopersicum* minimizing the browning of hypocotyls and leaf discs-derived calli. Incorporation of $CoCl_2$ into SIM significantly increased the development of plantlets from the embryogenic calli and the regeneration response increased linearly from lower levels to an optimum higher level. During this experiment, $CoCl_2$ in different concentrations (0.5 – 10.0 mg/l) was amalgamated in various culture media for scrutinizing their effects on *in vitro* shoot regeneration in three tomato genotypes.

Analysis of variance demonstrated that effect of media, explants and genotypes on *in vitro* shoot regeneration frequency were highly significant. The interactions between explants × media, media × genotype and explants × media × genotype had significant effects on *in vitro* shoot regeneration frequency. On the contrary, there was no significant difference between the interaction effect of explant × genotype at $p \leq 0.05$ (Appendix 12). All the genotypes exhibited different response to various levels of $CoCl_2$. The highest *in vitro* shoot regeneration was recorded on SIM_{7B} (MS medium fortified with 5.0 mg/l $CoCl_2$ and hormonal regime IAA – ZEA – BAP; 0.1 – 1.0 – 2.0 mg/l).

In case of Rio Grande, the best shoot regeneration frequency (81.32 and 85%) and the average number of primordial shoots (6.0, 6.66) from hypocotyls and leaf discs – derived calli, were recorded on SIM_{7B} which showed 22.01 and 22.03% increase over control (same SIM lacking $CoCl_2$) (Table 4.9; Figs. 4.3A, B & 4.4E). It was followed by Moneymaker whose the highest organogenesis frequency (77 and 81%) and mean number of shoots primordial (5.33, 6.0) culturing hypocotyls and leaf discs – derived calli were recorded on SIM_{6B} (MS + 4.25 mg/l $CoCl_2$, 0.1 mg/l IAA and 3.0 mg/l BAP. This *in vitro* shoot regeneration frequency indicated 22.88 and 20.32% increase over control (Table 4.9; Figs. 4.3A, B & 4.4D). In Roma, the maximum shoot regeneration frequency (73 and 78%) and average number of primordial shoots (6.66, 7.0) was secured on SIM_{7B} culturing hypocotyls and leaf discs, respectively which demonstrated 20.34 and 19.41% increase over control (Table 4.9; Figs. 4.3A, B & 4.4E). The least responsive media was SIM_{11B} (MS medium enriched with 10.0 mg/l AgNO3 and 2.0 mg/l BAP which produced the minimum *in vitro* shoot regeneration frequency in all the genotypes.

4.9 Individual effect of sucrose and sorbitol on *in vitro* shoot regeneration in tomato

The individual effect of different levels of carbon sources (sucrose and sorbitol) supplemented with MS and N6 basal media were investigated. The hypocotyls and leaf discs were excised from fifteen days old *in vitro* seedlings and used as explant sources. These explants were cultured on MS medium supplemented with various levels of sorbitol and sucrose in separate experiments (Table 3.7). Similarly, the explants were cultured on N6 medium having the same concentrations of sucrose and sorbitol individually (Table 3.8). No regeneration response was noticed in all the genotypes from both types of explants even after thirty days of culturing.

Table 4.9 Assessment of $CoCl_2$ in combination with various PGRs on *in vitro* shoot regeneration in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls-derived calli clumps (%)			*In vitro* shoot regeneration frequency culturing leaf discs-derived calli clumps (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
SIM_{1B}	$15.00^{fgh} \pm 0.84$	$10.00^{i} \pm 0.42$	$12.65^{hi} \pm 0.77$	$17.00^{f} \pm 0.93$	$12.33^{hi} \pm 0.65$	$14.00^{gh} \pm 1.41$
SIM_{2B}	$24.66^{bc} \pm 0.79$	$21.33^{de} \pm 0.88$	$27.00^{ab} \pm 2.2$	$27.00^{ab} \pm 1.35$	$24.00^{cd} \pm 0.74$	$30.00^{xyz} \pm 2.67$
SIM_{3B}	$46.66^{rs} \pm 2.46$	$50.66^{op} \pm 1.22$	$49.00^{pqr} \pm 2.93$	$51.00^{nop} \pm 1.69$	$46.00^{s} \pm 1.73$	$47.33^{qrs} \pm 3.46$
SIM_{4B}	$55.65^{lm} \pm 1.58$	$53.65^{mn} \pm 2.03$	$58.00^{jkl} \pm 3.74$	$58.00^{jkl} \pm 0.62$	$57.00^{kl} \pm 1.73$	$60.00^{ij} \pm 4.75$
SIM_{5B}	$67.00^{fg} \pm 1.22$	$65.33^{gh} \pm 0.92$	$62.66^{hi} \pm 5.26$	$70.00^{e} \pm 1.82$	$67.00^{fg} \pm 1.62$	$64.00^{h} \pm 4.61$
SIM_{6B}	$74.00^{d} \pm 1.63$	**$77.00^{c} \pm 1.1$**	$69.00^{ef} \pm 4.65$	$77.00^{c} \pm 1.76$	**$81.00^{b} \pm 1.69$**	$71.33^{de} \pm 4.5$
SIM_{7B}	**$81.33^{b} \pm 2.95$**	$70.00^{e} \pm 1.81$	**$73.00^{d} \pm 5.85$**	**$85.00^{a} \pm 2.73$**	$71.66^{de} \pm 2.83$	**$78.00^{c} \pm 6.27$**
SIM_{8B}	$52.66^{no} \pm 1.46$	$49.66^{pq} \pm 0.79$	$56.65^{kl} \pm 3.67$	$55.66^{lm} \pm 2.46$	$53.00^{mno} \pm 0.98$	$59.00^{jk} \pm 2.88$
SIM_{9B}	$43.00^{tuv} \pm 1.36$	$44.65^{stu} \pm 1.11$	$40.65^{v} \pm 2.37$	$45.32^{st} \pm 1.26$	$42.33^{uv} \pm 1.46$	$43.00^{tuv} \pm 4.43$
SIM_{10B}	$29.66^{yza} \pm 0.93$	$28.00^{za} \pm 1.43$	$32.65^{wx} \pm 2.11$	$32.00^{xy} \pm 1.42$	$30.00^{xyz} \pm 1.66$	$35.00^{w} \pm 1.72$
SIM_{11B}	$20.00^{e} \pm 1.28$	$16.00^{fg} \pm 0.26$	$22.66^{cde} \pm 1.95$	$22.00^{cde} \pm 1.24$	$21.00^{e} \pm 0.94$	$24.00^{cd} \pm 1.82$

In vitro shoot regeneration was obtained on MS (1962) basal media supplemented with $CoCl_2$ in combination with auxins (IAA, NAA), cytokinins (BAP, Zeatin and Kinetin) and Gibberellins (GA_3). Different concentrations of plant growth regulators and ethylene inhibitor were taken in (mg/l). After six weeks of culture, data were collected about *in vitro* shoot regeneration frequency. According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. The number of calli clumps (n) cultured per treatment for each genotype was one hundred and ten, and each experiment was repeated three times. The values after ± sign indicate standard deviation (n = 3). The bold letters demonstrate the best results. LSD value was 2.82 at $p \leq 0.05$.

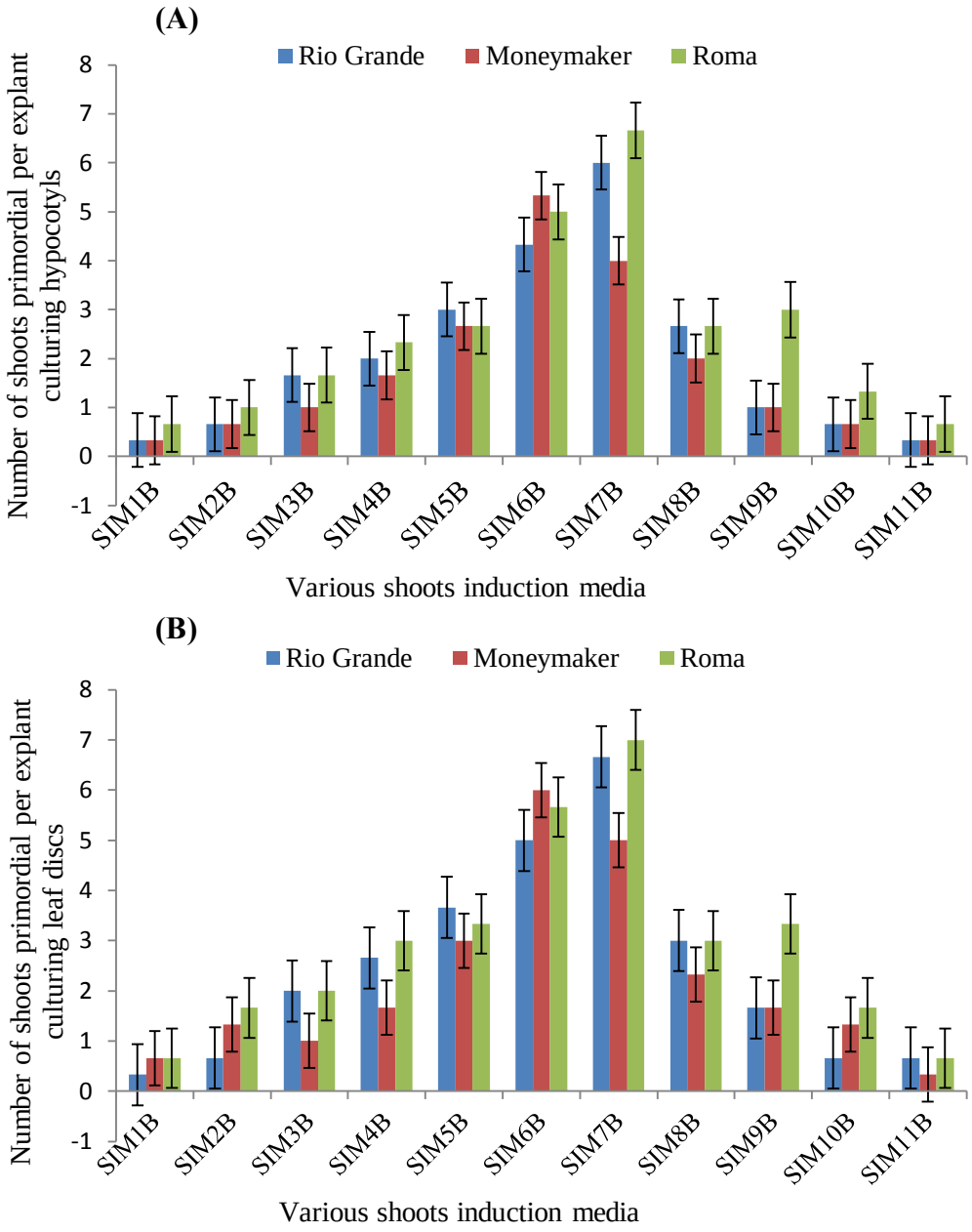

Fig. 4.3 Effect of various shoots induction media on the number of shoots primordial per explant in three tomato genotypes (A) Number of shoots primordial per explant on $CoCl_2$-supplemented shoots induction media culturing hypocotyls (B) Number of shoots primordial per explant on $CoCl_2$-supplemented shoots induction media culturing leaf discs. Vertical bars indicate standard error of the means (n = 3)

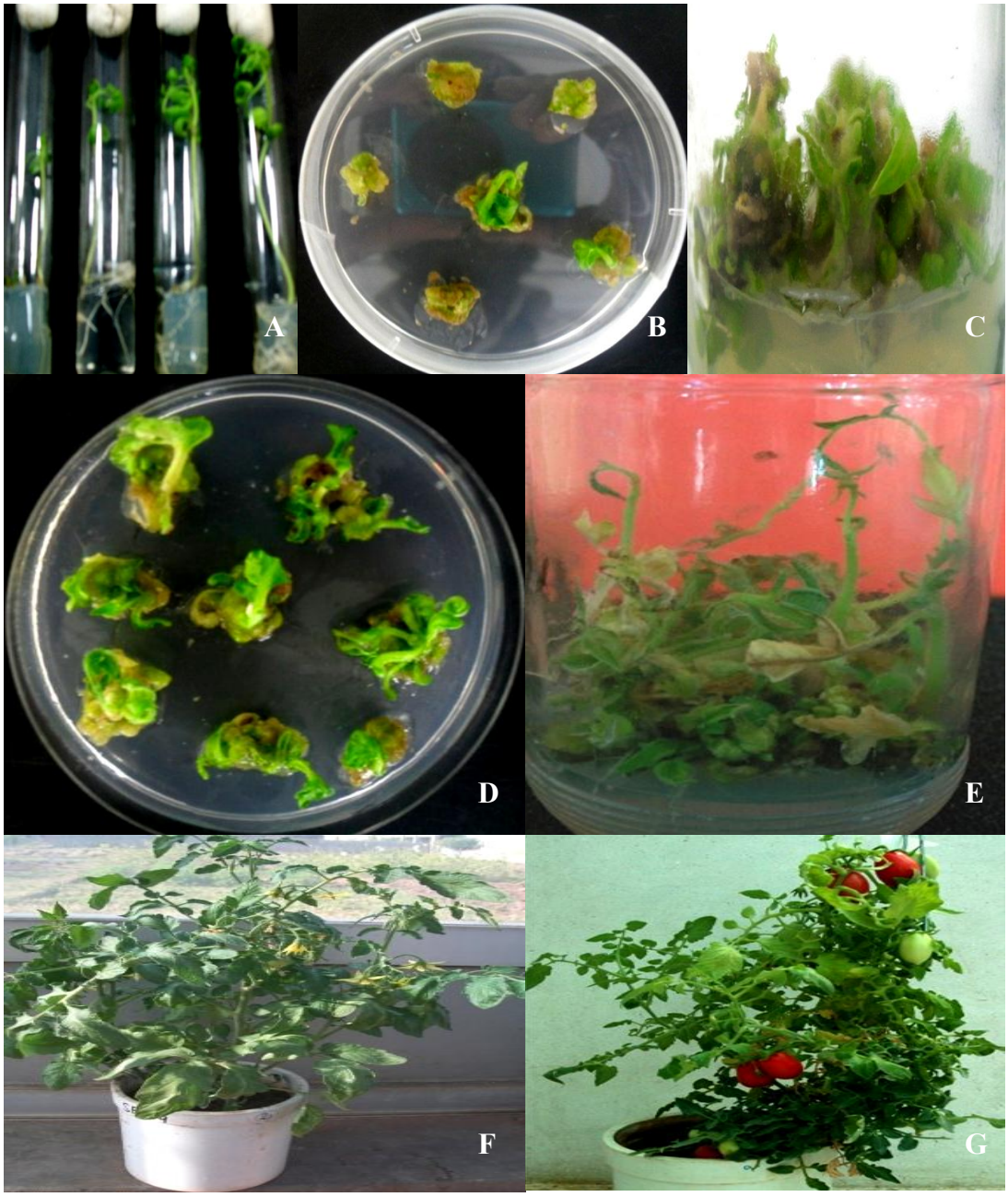

Fig. 4.4 Various tissue culture steps in establishment of tomato plants (A) Fifteen days old *in vitro* seedlings as a source for hypocotyls and leaf discs explants on MS basal medium (B) Callus induction on CIM_6 (C) Embryogenic calli emerging shoots on CIM_{5B} i.e. $CoCl_2$ (4.5 mg/l), NAA (1.0 mg/l) and BAP (2.5 mg/l) (D) Calli clumps regenerating *in vitro* shoots on shoot induction medium supplemented with $CoCl_2$ (4.25 mg/l) (SIM_{6B}) (E) Multiple primordial shoots produced on SIM_{7B} having hormonal regime IAA – ZEA – BAP (0.1 – 1.0 – 2.0 mg/l) and $CoCl_2$ (5.0 mg/l) (F) Tissue culture plants at flowering stage in glasshouse after four weeks of acclimatization in growth room (G) Mature T_0 *Solanum lycopersicum* plants produced in controlled conditions yielding normal fruits

4.10 Sucrose and sorbitol in MS basal media in absence of PGRs rapidly enhances *in vitro* shoot regeneration in tomato

The synergistic effect of sucrose and sorbitol was assessed in three cultivars of tomato using hypocotyls and leaf discs. These explants were cultured on diverse regeneration media i.e. MS media with various concentrations of carbon sources in equal ratio but devoid of any exogenously applied PGRs (Table 3.7). The synergism of carbon sources showed significant effects on *in vitro* shoot regeneration frequency and shoot length. Both the shoot regeneration frequency as well as shoot length was enhanced gradually with the increase in application of sucrose and sorbitol up to 30: 30 g/l and decreased on higher concentration. The highest regeneration frequency (100%) was recorded in Rio Grande followed by Roma (99%) and Moneymaker (97.69%) on RMS_6 culturing hypocotyls. Similarly, the leaf discs-derived the highest *in vitro* shoot regeneration frequency (100, 95.86 and 93.48%) was recorded in Rio Grande, Roma and Moneymaker on RMS_6 (Table 4.10; Fig. 4.7A, B & C). The highest *in vitro* shoot length (9.66 and 8.63 cm) was secured in Rio Grande culturing hypocotyls and leaf discs, followed by Roma where the best shoot length (8.79 and 8.17 cm) was recorded using hypocotyls and leaf discs on RMS_6 (Fig. 4.5A & B). Similarly, the maximum shoot length (7.48 and 7.25 cm) was achieved in Moneymaker culturing hypocotyls and leaf discs (Fig. 4.5A & B). The rooting frequency increased by increasing the ratio of these carbon sources. The highest rooting response was observed on regeneration medium having sucrose: sorbitol (30: 30 g/l) in all the genotypes tested and reduction in roots formation was recorded when explants were cultured to sucrose: sorbitol other than the optimal level (Data not shown). In this experiment, no PGRs were applied for rooting in all the genotypes.

4.11 Synergistic effect of carbon sources in N6 basal medium on *in vitro* shoot regeneration in absence of PGRs

During this study, various concentrations of sucrose and sorbitol incorporated into N6 basal media were examined on *in vitro* shoot regeneration frequency and shoot length. Significant differences ($p \leq 0.05$) were recorded in genotypes, explants and synergistic effects of carbon sources. The marked differences were also observed in interaction

between genotypes and carbon sources (Appendix 16). The growth of tomato explants was directly related to type of basal media, ratio of carbon sources and type of genotype. The explants showed swelling within three to four days and initiation of shoots occurred after seven days of culturing by using optimum level of carbon sources in regeneration medium. The highest *in vitro* shoot regeneration frequency (96.33 and 93.69%) was recorded in Rio Grande culturing hypocotyls and leaf discs, respectively on RMN_6 (N6 basal medium fortified with sucrose: sorbitol; 30: 30 g/l) followed by Roma (94.45 and 88.66%) and Moneymaker (92.75 and 86.56%) (Table 4.11; Fig. 4.7D, E & F). Similarly, the highest *in vitro* shoot length (11.12 and 10.89 cm) was secured in Rio Grande followed by Roma (10.49 and 9.76 cm) and Moneymaker (9.93 and 9.19 cm) culturing hypocotyls and leaf discs on RMN_6 (Fig. 4.5C & D). The concentration of carbohydrate sources higher than 30 g/l had inhibitory role to both shoot regeneration frequency as well as shoot length.

4.12 Influence of carbon sources in MS medium along with various PGRs on shoots organogenesis and multiple shoots formation

During this experiment, different ratio of sucrose and sorbitol were amalgamated with various PGRs to compare the efficiencies of carbon sources supplemented with MS basal media only, as well as with PGRs. The different treatments having sucrose and sorbitol along with PGRs produced multiple shoots in all the cultivars. Due to massive multiplication from both types of explants, it was difficult to measure the *in vitro* shoot length variations and therefore this factor was not considered. Rather than, the parameter about number of shoot primordial was undertaken during this study. Analysis of variance demonstrated that *in vitro* shoot regeneration frequency and number of shoot primordial were influenced significantly ($p \leq 0.05$) by various parameters studied (Appendices 18 & 19).

Table 4.10 Assessment of synergistic effect of sucrose and sorbitol in MS basal media without PGRs on *in vitro* shoot regeneration in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls (%)			*In vitro* shoot regeneration frequency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
RMS_1	$16.00^{xyz} \pm 2.65$	$10.26^{z} \pm 1.13$	$20.00^{wx} \pm 1.33$	$10.39^{z} \pm 2.35$	$13.00^{yz} \pm 1.66$	$29.52^{uv} \pm 2.56$
RMS_2	$25.45^{vw} \pm 2.44$	$32.46^{tu} \pm 3.61$	$40.82^{rs} \pm 2.72$	$17.75^{xy} \pm 3.08$	$37.52^{rst} \pm 4.31$	$36.59^{st} \pm 2.77$
RMS_3	$42.65^{r} \pm 4.41$	$49.54^{q} \pm 3.04$	$51.91^{pq} \pm 2.99$	$32.89^{tu} \pm 3.23$	$40.66^{rs} \pm 2.9$	$56.26^{p} \pm 3.76$
RMS_4	$73.59^{lmn} \pm 5.00$	$62.92^{o} \pm 5.17$	$78.35^{jkl} \pm 4.49$	$68.00^{no} \pm 5.33$	$54.83^{pq} \pm 2.91$	$70.79^{mn} \pm 4.23$
RMS_5	$86.25^{f-i} \pm 6.2$	$77.25^{kl} \pm 3.5$	$83.44^{g-j} \pm 4.2$	$79.63^{jk} \pm 5.43$	$70.18^{n} \pm 2.5$	$86.44^{fgh} \pm 4.08$
RMS_6	**$100.00^{a} \pm 0.00$**	**$97.69^{abc} \pm 3.98$**	**$99.00^{ab} \pm 1.72$**	**$100.00^{a} \pm 0.00$**	**$93.48^{b-e} \pm 4.54$**	**$95.86^{a-d} \pm 3.95$**
RMS_7	$95.72^{a-d} \pm 4.01$	$90.15^{def} \pm 2.17$	$92.56^{cde} \pm 4.46$	$88.49^{efg} \pm 5.31$	$81.25^{h-k} \pm 3.77$	$89.00^{efg} \pm 4.19$
RMS_8	$89.32^{efg} \pm 5.71$	$84.00^{g-j} \pm 4.01$	$80.35^{ijk} \pm 3.32$	$83.73^{g-j} \pm 5.1$	$76.39^{klm} \pm 2.7$	$73.42^{lmn} \pm 3.71$

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences ($p \leq 0.05$) by ANOVA (Statistix v. 8.1). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 5.95 at $p \leq 0.05$.

Table 4.11 Assessment of synergistic effect of sucrose and sorbitol in N6 basal media without PGRs on *in vitro* shoot regeneration frequency in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls (%)			*In vitro* shoot regeneration frequency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
RMN_1	$22.75^{zab} \pm 2.30$	$12.54^{c} \pm 2.39$	$18.00^{abc} \pm 2.00$	$16.00^{bc} \pm 3.38$	$21.00^{z-c} \pm 2.09$	$14.33^{bc} \pm 2.05$
RMN_2	$35.85^{u-x} \pm 3.70$	$25.63^{yza} \pm 1.84$	$29.39^{w-z} \pm 1.66$	$27.33^{xyz} \pm 1.83$	$17.33^{abc} \pm 2.32$	$24.66^{stu} \pm 1.68$
RMN_3	$48.66^{qrs} \pm 4.16$	$32.92^{v-y} \pm 3.68$	$38.42^{t-w} \pm 3.82$	$57.49^{n-q} \pm 3.93$	$41.75^{s-v} \pm 4.17$	$45.58^{rst} \pm 3.65$
RMN_4	$69.45^{j-m} \pm 4.87$	$54.69^{o-r} \pm 3.93$	$60.34^{mno} \pm 3.87$	$73.82^{h-k} \pm 5.94$	$50.00^{p-s} \pm 4.00$	$65.42^{k-n} \pm 3.85$
RMN_5	$91.00^{a-d} \pm 4.33$	$77.62^{f-j} \pm 4.49$	$84.00^{c-g} \pm 4.00$	$89.75^{a-e} \pm 4.17$	$73.00^{i-l} \pm 5.00$	$78.25^{f-j} \pm 4.05$
RMN_6	**$96.33^{a} \pm 3.68$**	**$92.75^{abc} \pm 4.27$**	**$94.45^{ab} \pm 5.76$**	**$93.69^{ab} \pm 4.85$**	**$86.42^{b-f} \pm 4.54$**	**$88.66^{a-e} \pm 5.61$**
RMN_7	$88.76^{a-e} \pm 5.25$	$82.54^{d-h} \pm 5.79$	$90.12^{a-e} \pm 5.49$	$78.33^{f-j} \pm 4.80$	$75.85^{g-j} \pm 4.34$	$81.00^{e-i} \pm 5.65$
RMN_8	$66.51^{k-n} \pm 3.43$	$70.63^{jkl} \pm 3.49$	$80.85^{e-i} \pm 4.67$	$58.00^{nop} \pm 4.00$	$63.93^{l-o} \pm 3.77$	$73.85^{h-k} \pm 3.29$

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences ($p \leq 0.05$) by ANOVA (Statistix v. 8.1). The values after ± sign depict standard deviation (n = 3). LSD value was 9.3 at $p \leq 0.05$.

The highest shoot regeneration frequency and more number of primordial shoots were recorded on RM_{6A} (MS + Sucrose: Sorbitol (30: 30 g/l) + IAA (0.1 mg/l) + ZEA (1.0 mg/l) + BAP (2.0 mg/l) in all genotypes from both types of explants. During this study of indirect shoot regeneration, leaf discs were found to be more responsive giving better results than that of hypocotyls. The highest shoot regeneration frequency (90.72, 86.85 and 82.55%) was recorded in Roma, Rio Grande and Moneymaker, respectively culturing leaf discs on RM_{6A}. Similarly, the best shoot regeneration frequency (88, 80.35 and 79%) was recorded in Roma, Rio Grande and Moneymaker from hypocotyls-derived explants on RM_{6A} (Table 4.12; Fig. 4.8G, H & I). The highest number of shoots primordial per explant (14, 12) was recorded in Rio Grande followed by Roma (13, 11) and Moneymaker (12, 10) culturing leaf discs and hypocotyls on RM_{6A} (Fig. 4.6A & B). The *in vitro* shoot regeneration frequency and primordial shoots number decreased applying sucrose: sorbitol more or less than (30: 30 g/l). Hence sucrose and sorbitol in 30: 30 g/l was found to be optimal for all the genotypes.

4.13 Synergistic effect of sucrose and sorbitol in N6 medium along with various PGRs on *in vitro* shoot regeneration and multiple shoots formation

To investigate the mutual effect of sucrose and sorbitol on *in vitro* shoot regeneration frequency and multiple shoot formation, N6 basal medium was fortified with different concentrations of sucrose and sorbitol along with various levels of PGRs. *In vitro* shoot induction frequency and multiple shoot formation in Rio Grande, Moneymaker and Roma were noticed to be statistically different using different concentrations of sucrose and sobitol. In this experiment, calli formation occurred from the cut ends of explants and then these calli were maintained after every twelve to fifteen days on the same culture medium for successful shoots regeneration.

Table 4.12 Assessment of synergistic effect of sucrose and sorbitol in MS basal media enriched with various PGRs on *in vitro* shoot regeneration in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls (%)			*In vitro* shoot regeneration frequency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
RM_{1A}	$25.00^{c} \pm 1.00$	$34.00^{ab} \pm 1.96$	$42.57^{v-y} \pm 3.07$	$28.25^{bc} \pm 1.87$	$34.72^{za} \pm 1.85$	$38.24^{yza} \pm 2.79$
RM_{2A}	$34.56^{a} \pm 1.89$	$40.35^{xyz} \pm 2.43$	$45.00^{u-x} \pm 4.86$	$41.73^{wxy} \pm 3.38$	$44.84^{vwx} \pm 2.85$	$47.37^{t-w} \pm 3.26$
RM_{3A}	$50.69^{stu} \pm 2.31$	$47.82^{tuv} \pm 2.84$	$53.69^{qrs} \pm 3.73$	$58.44^{n-q} \pm 3.90$	$52.34^{rst} \pm 3.17$	$62.82^{lmn} \pm 3.83$
RM_{4A}	$58.85^{n-q} \pm 2.37$	$55.62^{p-s} \pm 4.14$	$57.00^{o-r} \pm 4.12$	$66.00^{j-m} \pm 4.23$	$59.49^{nop} \pm 2.95$	$61.63^{l-o} \pm 5.00$
RM_{5A}	$76.00^{d-h} \pm 3.74$	$76.42^{d-g} \pm 6.36$	$81.00^{cd} \pm 5.23$	$79.52^{cde} \pm 5.11$	$73.23^{f-i} \pm 3.73$	$84.00^{bc} \pm 5.97$
RM_{6A}	**$80.35^{cd} \pm 4.34$**	**$79.00^{c-f} \pm 5.82$**	**$88.00^{ab} \pm 5.6$**	**$86.85^{ab} \pm 3.83$**	**$82.55^{bc} \pm 5.94$**	**$90.72^{a} \pm 5.15$**
RM_{7A}	$63.92^{k-n} \pm 3.32$	$71.65^{g-j} \pm 5.27$	$66.33^{jkl} \pm 3.24$	$72.68^{ghi} \pm 3.46$	$68.78^{ijk} \pm 7.79$	$75.45^{d-h} \pm 5.11$
RM_{8A}	$60.00^{nop} \pm 3.58$	$60.33^{m-p} \pm 4.58$	$74.00^{e-i} \pm 4.14$	$68.79^{ijk} \pm 4.00$	$64.00^{k-n} \pm 4.55$	$70.41^{hij} \pm 4.83$

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences ($p \leq 0.05$) on the basis of ANOVA (Statistix v. 8.1). The values after ± sign indicate standard deviation (n = 3). LSD value was 5.77 at $p \leq 0.05$.

Table 4.13 Assessment of synergistic effect of sucrose and sorbitol in N6 basal media augmented with various PGRs on *in vitro* shoot regeneration in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls (%)			*In vitro* shoot regeneration frequency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
RM_{1B}	$18.00^{d} \pm 4.45$	$22.65^{bc} \pm 3.11$	$20.00^{bcd} \pm 3.31$	$22.95^{b} \pm 2.93$	$18.29^{cd} \pm 4.29$	$28.39^{h} \pm 2.75$
RM_{2B}	$30.31^{za} \pm 4.46$	$27.75^{a} \pm 5.65$	$34.65^{yz} \pm 3.55$	$36.66^{xy} \pm 5.65$	$31.54^{za} \pm 4.12$	$40.63^{wx} \pm 3.87$
RM_{3B}	$52.72^{v} \pm 6.66$	$40.00^{wx} \pm 4.70$	$43.74^{w} \pm 4.14$	$57.43^{u} \pm 5.80$	$48.69^{v} \pm 4.84$	$52.75^{v} \pm 3.51$
RM_{4B}	$63.92^{rst} \pm 5.99$	$65.43^{qr} \pm 5.20$	$76.49^{i-l} \pm 4.36$	$70.25^{m-p} \pm 4.79$	$73.58^{lmn} \pm 3.66$	$78.25^{ijk} \pm 4.17$
RM_{5B}	$77.64^{i-l} \pm 5.14$	$70.66^{mno} \pm 5.19$	$81.00^{ghi} \pm 3.77$	$83.56^{e-h} \pm 3.74$	$79.45^{hij} \pm 4.80$	$86.41^{def} \pm 5.61$
RM_{6B}	**$87.79^{cde} \pm 4.85$**	**$83.00^{fgh} \pm 6.07$**	**$92.25^{abc} \pm 5.40$**	**$92.69^{ab} \pm 5.34$**	**$88.74^{bcd} \pm 4.21$**	**$96.33^{a} \pm 3.85$**
RM_{7B}	$74.45^{klm} \pm 3.82$	$69.0^{n-q} \pm 3.81$	$78.36^{ijk} \pm 4.57$	$80.37^{g-j} \pm 5.77$	$75.86^{jkl} \pm 4.04$	$84.66^{d-g} \pm 3.11$
RM_{8B}	$66.00^{pqr} \pm 5.74$	$63.59^{rst} \pm 4.53$	$59.33^{tu} \pm 3.73$	$64.82^{qrs} \pm 4.83$	$60.66^{stu} \pm 4.19$	$67.84^{o-r} \pm 4.64$

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences ($p \leq 0.05$) on the basis of ANOVA (Statistix v. 8.1). The values after ± sign represent standard deviation (n = 3). LSD value was 4.63 at $p \leq 0.05$.

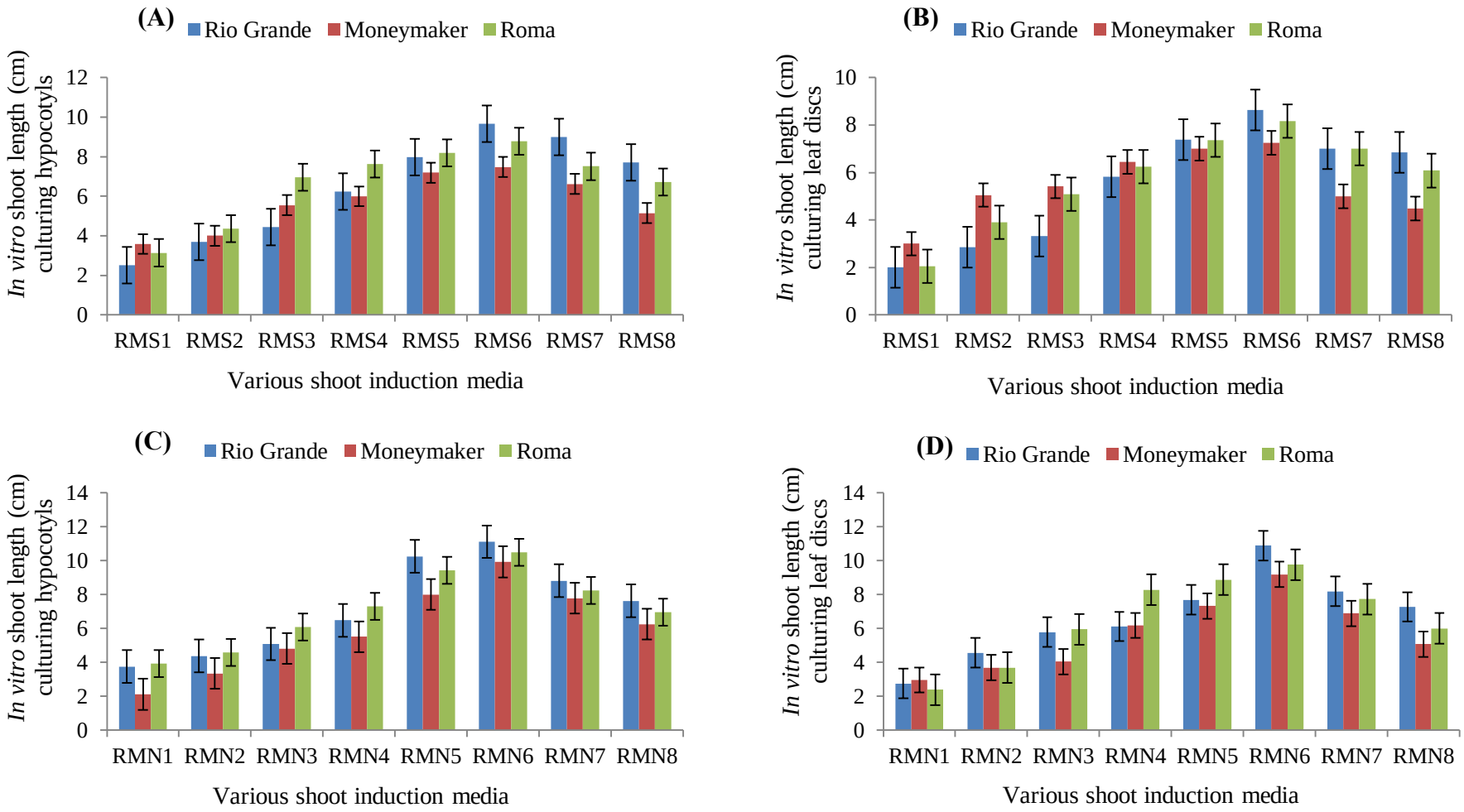

Fig. 4.5 Effect of various shoot induction media on *in vitro* shoot length (cm) in three tomato genotypes (A) *In vitro* shoot length by synergistic effect of sucrose and sorbitol in MS basal media without PGRs culturing hypocotyls (B) *In vitro* shoot length by synergistic effect of sucrose and sorbitol in MS basal media without PGRs culturing leaf discs (C) *In vitro* shoot length by synergistic effect of sucrose and sorbitol in N6 basal media without PGRs culturing hypocotyls (D) *In vitro* shoot length by synergistic effect of sucrose and sorbitol in N6 basal media without PGRs culturing leaf discs. Vertical bars indicate standard error of the means (n = 3)

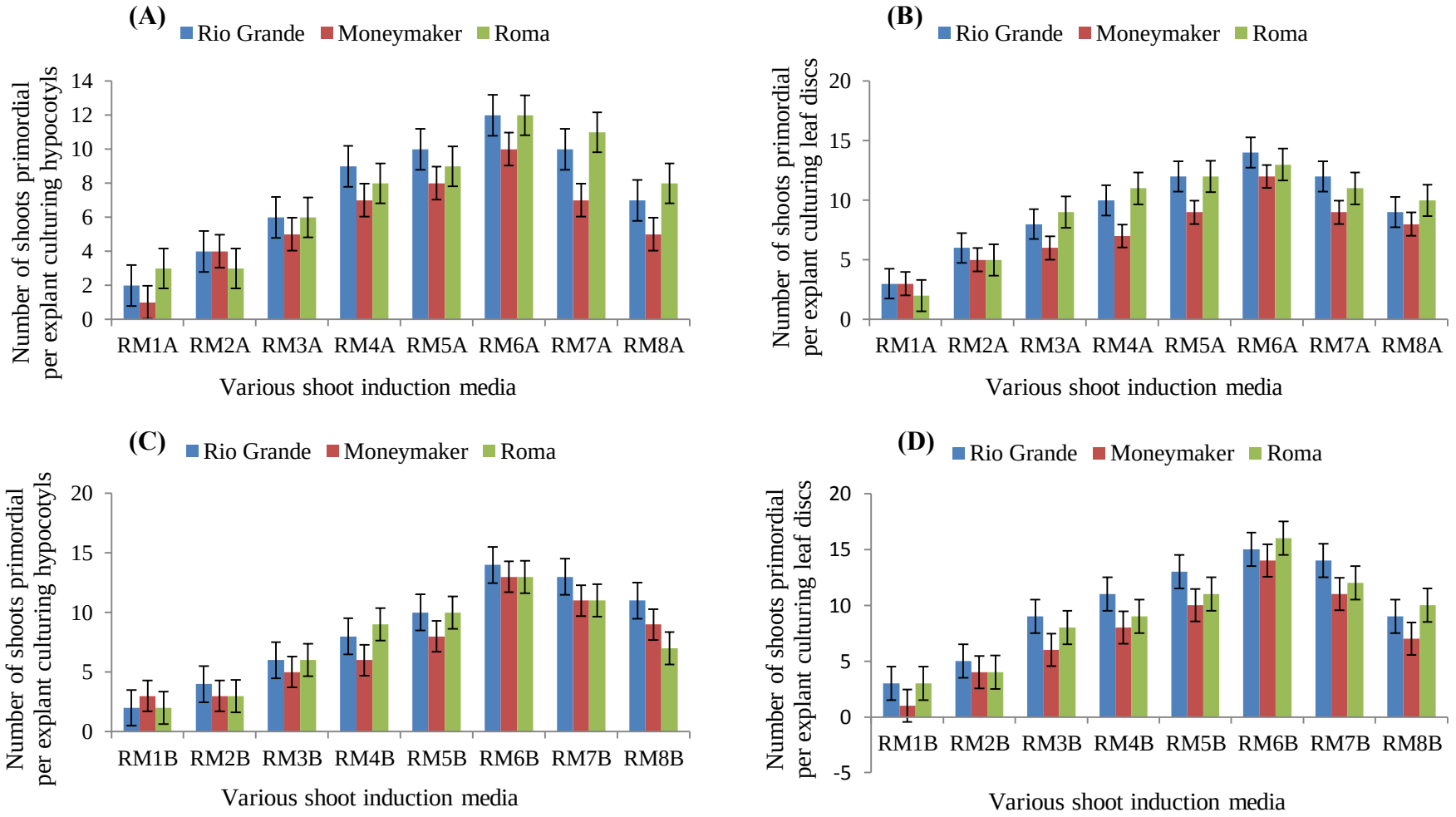

Fig. 4.6 Effect of various shoot induction media on the number of shoots primordial per explant in three tomato genotypes (A) Number of shoots primordial per explant by the synergistic effect of sucrose and sorbitol in MS basal media supplemented with various PGRs culturing hypocotyls (B) Number of shoots primordial per explant by the synergistic effect of sucrose and sorbitol in MS basal media supplemented with various PGRs culturing leaf discs (C) Number of shoots primordial per explant by the synergistic effect of sucrose and sorbitol in N6 basal media supplemented with various PGRs culturing hypocotyls (D) Number of shoots primordial per explant by the synergistic effect of sucrose and sorbitol in N6 basal media supplemented with various PGRs culturing leaf discs. Vertical bars indicate standard error of the means (n = 3)

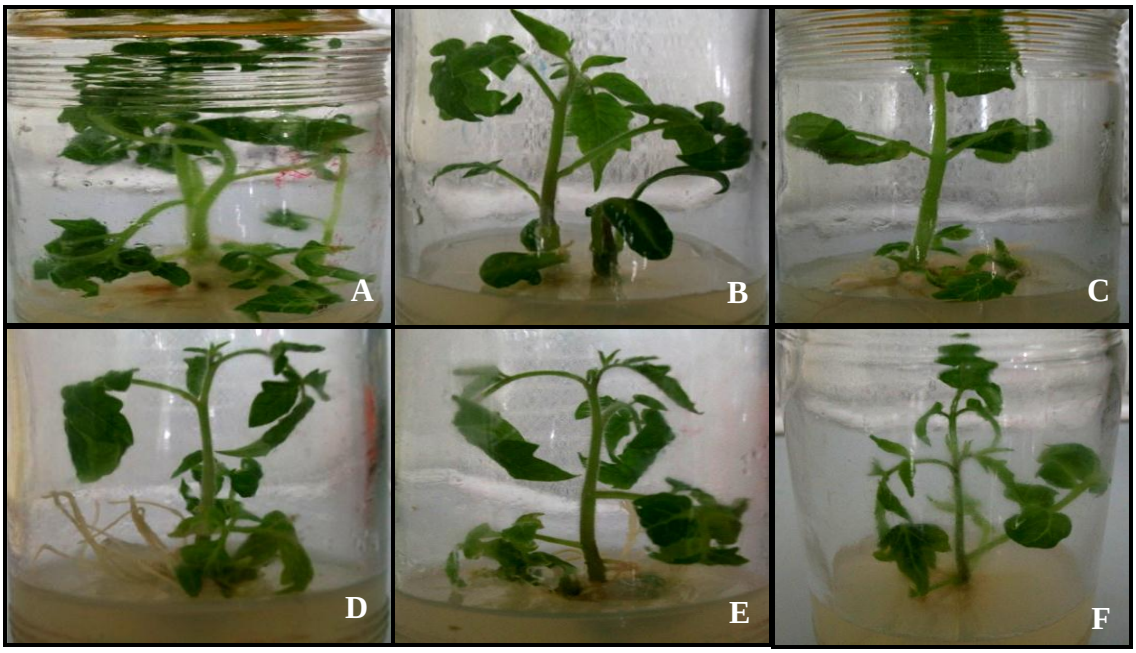

Fig. 4.7 Direct shoot organogenesis in tomato on MS and N6 basal media fortified with only sucrose and sorbitol (A, B and C): *In vitro* shoot regeneration in Rio Grande, Moneymaker and Roma, respectively on MS basal medium supplemented with sucrose: sorbitol (30: 30 g/l) **(D, E and F):** Shoot organogenesis in Rio Grande, Moneymaker and Roma on N6 basal medium augmented with sucrose: sorbitol (30: 30 g/l)

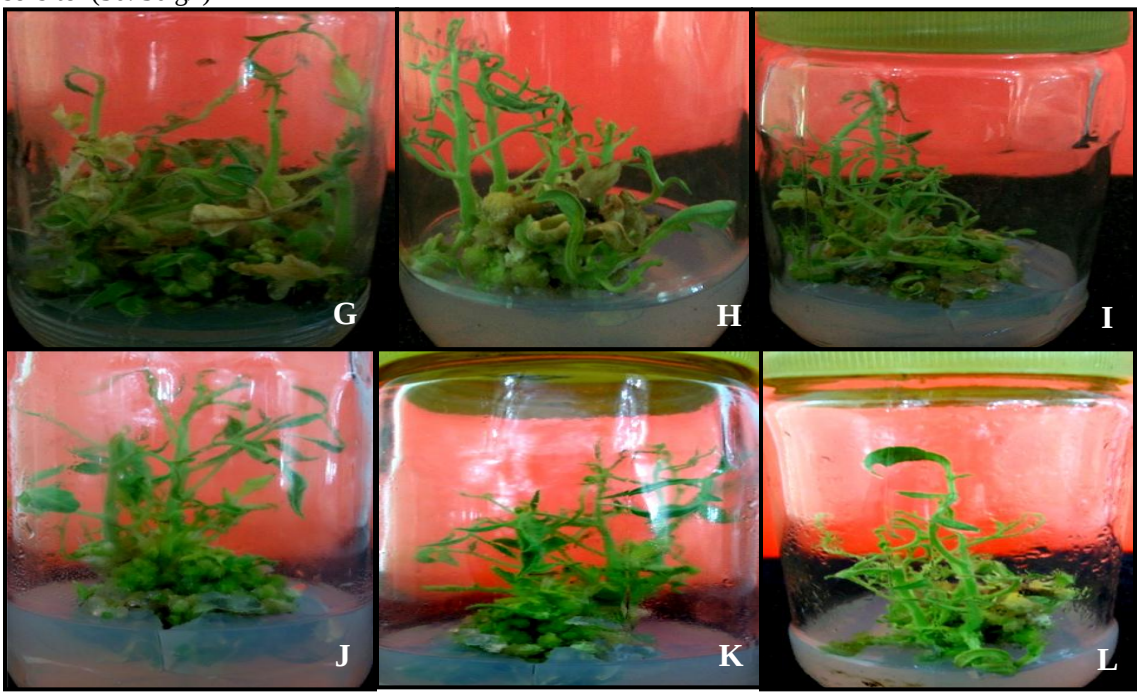

Fig. 4.8 Indirect shoot regeneration in tomato on MS and N6 basal media having sucrose and sorbitol and various phytohormones (G, H and I): Shoot organogenesis in Rio Grande, Moneymaker and Roma, respectively on MS basal medium + sucrose: sorbitol (30: 30 g/l) + (0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP) **(J, K and L):** *In vitro* shoot regeneration in Rio Grande, Moneymaker and Roma on N6 basal medium fortified with sucrose: sorbitol (30: 30 g/l) and IAA (0.1 mg/l) + ZEA (1.0 mg/l) + BAP (2.0 mg/l)

The highest *in vitro* shoot regeneration frequency (96.33 and 92.25%) was recorded in Roma followed by Rio Grande (92.69 and 87.79%) and Moneymaker (88.74 and 83%) culturing leaf discs and hypocotyls on RM_{6B} (N6 medium supplemented with sucrose: sorbitol; 30: 30 g/l along with 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP (Table 4.13; Fig. 4.8J, K & L). Similarly, the highest number of primordial shoots (16 and 13) was obtained in Roma followed by Rio Grande (15 and 14) and Moneymaker (14 and 11) culturing leaf discs and hypocotyls (Fig. 4.6C & D). The gross comparison of *in vitro* shoot regeneration and number of primordial shoots on various concentrations of sucrose and sorbitol clearly demonstrated that sucrose: sorbitol (30: 30 g/l) was proved to be the best concentration supporting a significant increase in frequency and number of *in vitro* shoots. In all genotypes tested, the higher concentrations of sucrose and sorbitol (more than 30 g/l) inhibited the *in vitro* shoot regeneration frequency and number of primordial shoots (Table 4.13; Fig. 4.6). As far as calli morphology is concerned, it didn't change appreciably from lower to higher concentrations of carbon sources but necrosis was evident in some portion of calli at higher levels of sucrose and sorbitol.

4.14 Factors affecting transformation efficiency

Several factors have been reported to affect transformation efficiency in tomato. The most critical factors of transformation via *Agrobacterium* include bacterial cell density, infection duration, antibiotic concentration and co-cultivation period. The results obtained for various parameters have been summarized below.

4.14.1 Effect of *in vitro* seedling age on transformation efficiency

Effect of *in vitro* seedling age on transformation efficiency (TE) was investigated in three tomato genotypes (Rio Grande, Moneymaker and Roma). A significant difference of seedling age was noticed for both hypocotyls and leaf discs in all the genotypes. The TE was increased with the increase in seedling age up to fifteen days. It was observed that TE was higher in 15-days old *in vitro* seedlings as compared to 5 and 10-days old seedlings in both types of explants in all the genotypes (Table 4.14). Similarly, 20 and 25 days old seedlings showed lower transformation efficiencies than that of fifteen days old seedlings. The highest TE was recorded in Roma (15.71%) followed by Rio Grande (14.61%) and Moneymaker (13.74%) respectively. Leaf discs derived maximum transformation efficiency which was 11.62, 10.52 and 9.5% in Roma, Rio Grande and Moneymaker at fifteen days old seedlings (Table 4.14). These results indicated that fifteen days old *in vitro* seedling was ideal for maximum TE in tomato.

4.14.2 Effect of pre-culture period on transformation efficiency

To investigate the effect of different pre-culture periods on TE in tomato, hypocotyls and leaf discs were pre-cultured on MS medium supplemented with different plant growth regulators. Analysis of variance elucidated that there were highly significant differences among different treatments, genotypes and explants and also for genotype x treatment, while it was non-significant for explant x treatment and explant x genotype interactions (Appendix 23). During this study, continual pre-culture experiments were conducted at regular intervals of 0, 24, 48, 72 and 96 hours. During pre-culture period, most of the explants increased significantly in size. The lowest TE (0.57 and 1.27%) was secured using leaf discs and hypocotyls without pre-culturing. The highest TE (8.25, 9.88 and 10.69%) was recorded in Moneymaker, Roma and Rio Grande when hypocotyls were

pre-cultured for forty-eight hours (Table 4.15). Similarly, the highest TE (6.4, 9.18 and 9.88%) was recorded in Moneymaker, Rio Grande and Roma culturing leaf discs for 48 hours. The pre-culture period beyond forty-eight hours significantly decreased TE in all the genotypes (Table 4.15).

4.14.3 Effect of *Agrobacterium* densities on transformation efficiency

Bacterial densities significantly affected TE in all the genotypes. Different optical densities (0.1, 0.2, 0.3, 0.4 and 0.5) at 600 nm were scrutinized their influence on TE giving infection to explants. Statistically, significant results for genotype, explants type and OD were recorded in all the cultivars (Appendix 24). All the genotypes showed maximum TE at $OD_{600\ nm}$ = 0.2. The highest TE (8.83%) at $OD_{600\ nm}$ = 0.2 was secured in Rio Grande culturing hypocotyls as a source of explants, followed by Moneymaker (7.33%) and Roma (6.66%). When OD was increased from 0.2, TE gradually decreased due to excessive growth of bacteria causing necrosis in both types of explants in all genotypes tested (Table 4.16). There was a significant linear relationship between bacterial concentration and percentage of necrotic explants and there was also a significant interaction of cultivar with bacterial concentration. On average, irrespective of genotype and explants, $OD_{600\ nm}$ = 0.2 was found to be optimal for further tissue-culture based transformation experiments.

4.14.4 Effect of acetosyringone on transformation efficiency

Different concentrations of acetosyringone (0, 20, 40, 60, 80 and 100 µM) were added in two experiments for the improvement of TE. In first experiment, 0-100 µM acetosyringone was supplemented with inoculation medium (Table 3.11; IM), while in second experiment, the hypocotyls and leaf discs were co-cultivated for two days on co-cultivation media (Table 3.11; CCM) supplemented with the same concentrations of acetosyringone. The TE was compared after four weeks of culture on selection medium and ANOVA results exhibited significant acetosyringone concentration effect on plantlet

Table 4.14 Assessment of *in vitro* seedling age on transformation efficiency in tomato

In vitro seedling age (days)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
5	$2.69^{qr} \pm 0.23$	$3.33^{pq} \pm 0.17$	$2.14^{rs} \pm 0.16$	$1.57^{st} \pm 0.48$	$0.53^{u} \pm 0.24$	$1.00^{tu} \pm 0.46$
10	$5.97^{lm} \pm 0.35$	$6.66^{kl} \pm 0.36$	$5.00^{n} \pm 0.32$	$3.1^{q} \pm 0.12$	$3.92^{op} \pm 0.3$	$2.89^{qr} \pm 0.26$
15	$\mathbf{14.61^{b} \pm 0.67}$	$\mathbf{13.74^{c} \pm 0.72}$	$\mathbf{15.71^{a} \pm 0.33}$	$\mathbf{10.52^{fg} \pm 0.58}$	$\mathbf{9.5^{hi} \pm 1.02}$	$\mathbf{11.62^{de} \pm 0.65}$
20	$10.99^{ef} \pm 0.33$	$9.93^{gh} \pm 0.36$	$12.13^{d} \pm 0.32$	$7.11^{k} \pm 0.5$	$6.50^{kl} \pm 0.43$	$7.90^{j} \pm 0.43$
25	$8.07^{j} \pm 0.92$	$8.05^{j} \pm 0.43$	$8.92^{i} \pm 0.15$	$5.25^{mn} \pm 0.32$	$4.09^{o} \pm 0.2$	$4.65^{no} \pm 0.07$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.75 at $p \leq 0.05$.

Table 4.15 Assessment of various pre-culture periods on transformation efficiency in tomato

Pre-culture period (hrs)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
0	$1.39^{o-r} \pm 0.36$	$1.83^{nop} \pm 0.51$	$1.27^{o-s} \pm 0.29$	$0.91^{qrs} \pm 0.18$	$0.74^{rs} \pm 0.14$	$0.57^{s} \pm 0.38$
24	$4.38^{hi} \pm 0.43$	$5.12^{gh} \pm 0.53$	$4.05^{ij} \pm 0.18$	$3.02^{kl} \pm 0.5$	$3.20^{k} \pm 0.05$	$3.42^{jk} \pm 0.42$
48	$\mathbf{10.69^{a} \pm 0.63}$	$\mathbf{8.25^{c} \pm 0.31}$	$\mathbf{9.95^{ab} \pm 0.24}$	$\mathbf{9.18^{b} \pm 0.74}$	$\mathbf{6.40^{ef} \pm 0.5}$	$\mathbf{9.88^{b} \pm 0.85}$
72	$7.44^{d} \pm 0.65$	$6.36^{ef} \pm 0.47$	$7.00^{de} \pm 0.73$	$5.83^{fg} \pm 0.34$	$4.80^{hi} \pm 0.6$	$5.81^{fg} \pm 0.13$
96	$2.32^{lmn} \pm 0.83$	$2.04^{mno} \pm 0.41$	$2.82^{klm} \pm 0.52$	$1.42^{o-r} \pm 0.6$	$1.60^{n-q} \pm 0.43$	$1.14^{p-s} \pm 0.17$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.79 at $p \leq 0.05$.

Table 4.16 Assessment of various *Agrobacterium* (EHA105) cell densities on transformation efficiency in tomato

Cell densities of *Agrobacterium* ($OD_{600\ nm}$)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
0.1	$5.66^{c} \pm 0.68$	$4.00^{ef} \pm 0.53$	$4.00^{ef} \pm 0.5$	$3.87^{ef} \pm 0.36$	$2.77^{ghi} \pm 0.19$	$2.21^{hij} \pm 0.5$
0.2	$\mathbf{8.83^{a} \pm 0.57}$	$\mathbf{7.33^{b} \pm 0.33}$	$\mathbf{6.66^{b} \pm 0.33}$	$\mathbf{5.55^{c} \pm 0.69}$	$\mathbf{5.00^{cd} \pm 0.33}$	$\mathbf{4.44^{de} \pm 0.69}$
0.3	$6.58^{b} \pm 0.77$	$5.33^{c} \pm 0.66$	$4.00^{ef} \pm 0.8$	$3.06^{g} \pm 0.34$	$3.88^{ef} \pm 0.19$	$3.33^{fg} \pm 0.33$
0.4	$2.89^{gh} \pm 0.4$	$2.66^{ghi} \pm 0.57$	$2.00^{ij} \pm 0.41$	$1.10^{k} \pm 0.95$	$1.66^{jk} \pm 0.55$	$1.10^{k} \pm 0.18$
0.5	$0.00^{l} \pm 0.00$	$0.00^{l} \pm 0.00$	$0.00^{l} \pm 0.00$	$0.00^{l} \pm 0.00$	$0.00^{l} \pm 0.00$	$0.00^{l} \pm 0.00$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.79 at $p \leq 0.05$.

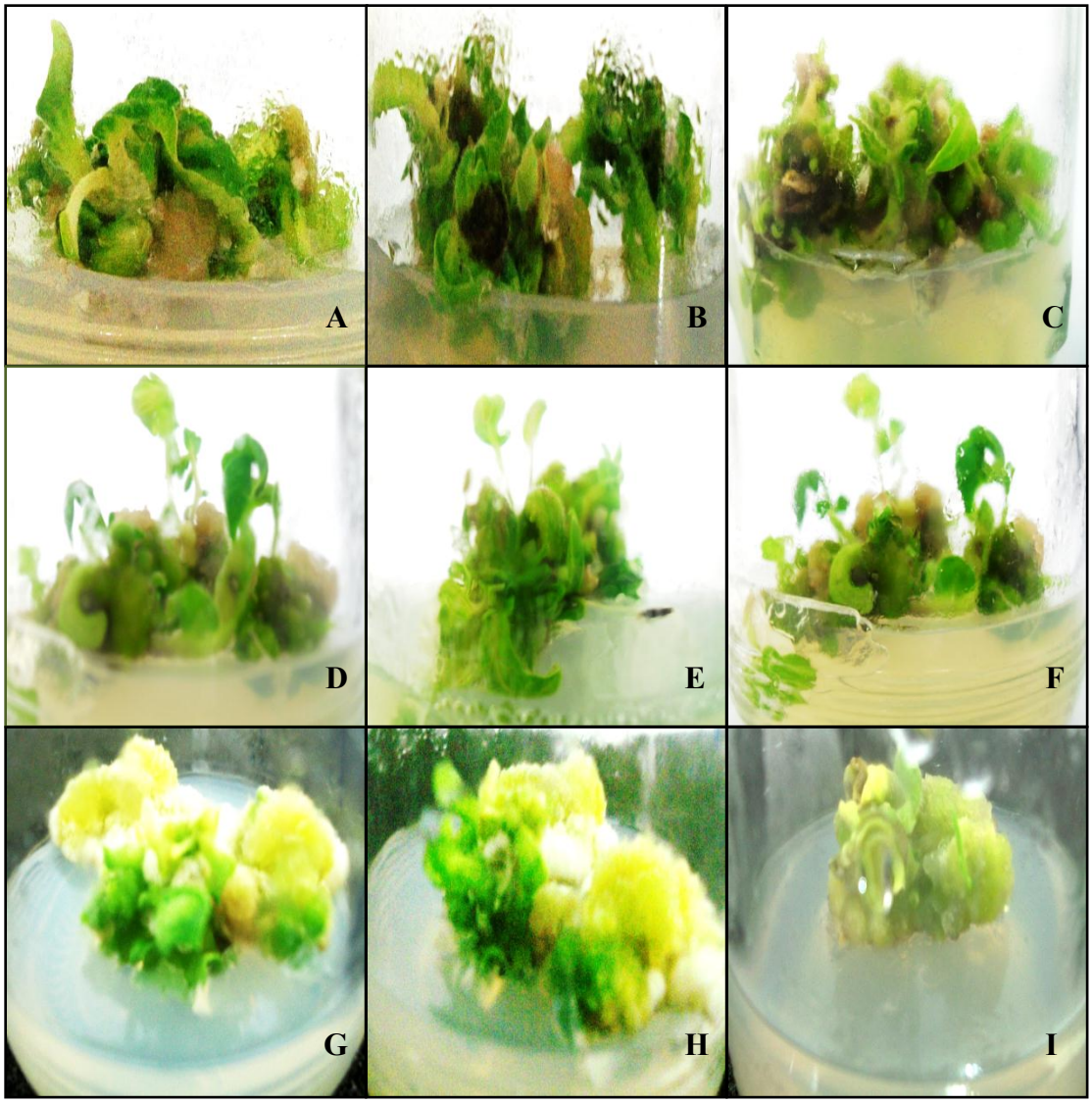

Fig. 4.9 Effect of various levels of acetsyringone on differentiation and morphology of hypocotyls-derived calli in three genotypes of tomato (*Solanum lycopersicum* Mill.) (A, B, C): Differentiation of embryogenic calli with whitish green, dry and relatively compact in appearance produced at 40 µM acetosyringone in Rio Grande, Moneymaker and Roma (D, E and F): Differentiation of embryogenic calli with compact and nodular in appearance produced at 60 µM acetosyringone in Rio Grande, Moneymaker and Roma (G, H and I): Differentiation of non-embryogenic calli with yellow, soft and granular in appearance produced at 80 µM acetosyringone in Rio Grande, Moneymaker and Roma

regeneration and subsequent TE, while the interaction of explant and genotype illustrated non-significant effect (ANOVA; $p \leq 0.05$) (Appendix 25). The minimum TE (0.57%) was recorded in an experiment with no acetosyringone (Table 4.17). It was found that TE increased up to 60 µM acetosyringone concentration, while further increase of acetosyringone gradually decreased TE. The TE increased considerably in explants cultured on co-cultivation media supplemented with 60 µM acetosyringone (Fig. 4.9). The highest TE (18.32%) was noticed in cv. Rio Grande followed by Moneymaker (16.13%) and Roma (15.73%) culturing hypocotyls using 60 µM acetosyringone. Similarly, leaf discs derived maximum TE was 15 and 13.77% in Rio Grande and Moneymaker followed by Roma (12.39%) at 60 µM acetosyringone (Table 4.17).

4.14.5 Effect of infection durations on transformation efficiency

Effect of various infection durations (the time for which explants were submerged in the co-cultivation liquid media containing the bacterial suspension) (2, 3, 5, 8 and 10 min) was investigated on TE. The bacterial $OD_{600\ nm}$ = 0.2 was maintained in all of the experiments. No transformant was obtained at 1 min infection duration in both types of explants in all the genotypes, indicating that bacterial culture did not properly associate with explants. Among various infection durations examined, 3 min at $OD_{600\ nm}$ = 0.2 was found to be optimal for explants survival and efficient transformation for all the cultivars. When the infection duration was increased beyond 3 min, TE was gradually decreased and necrosis occurred in the same manner due to excessive amount of bacteria (Table 4.18). To prevent the explants from necrosis, they were sub-cultured on fresh MS media supplemented with 300 mg/l claforan® (cefotaxime sodium). The highest TE (10.66%) was achieved in Roma followed by Rio Grande (9.21%) and Moneymaker (8.5%) at 3 min infection period culturing hypocotyls explants. Similarly from leaf discs, the best TE (8.94%) was recorded in Roma followed by Moneymaker (7.64%) and Rio Grande (7.44%) at 3 min infection period. From the present investigation, we concluded that infection period more than three minutes caused necrosis in explant tissues and reduced TE (Table 4.18).

Table 4.17 Assessment of various concentrations of acetosyringone on transformation efficiency in tomato

Acetosyringone concentration (µM)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
0	$2.41^{qr} \pm 0.52$	$1.87^{rs} \pm 0.23$	$3.05^{pq} \pm 0.25$	$1.13^{st} \pm 0.36$	$0.57^{t} \pm 0.2$	$0.92^{st} \pm 0.46$
20	$5.55^{lm} \pm 0.11$	$4.28^{no} \pm 0.75$	$4.90^{mn} \pm 0.99$	$4.07^{nop} \pm 0.08$	$3.63^{op} \pm 0.09$	$3.72^{op} \pm 0.93$
40	$9.00^{hi} \pm 0.6$	$10.35^{g} \pm 0.28$	$10.18^{g} \pm 0.75$	$7.13^{j} \pm 0.41$	$6.36^{jkl} \pm 0.09$	$6.86^{jk} \pm 1.18$
60	**$18.32^{a} \pm 0.61$**	**$16.13^{b} \pm 0.94$**	**$15.73^{bc} \pm 0.85$**	**$15.00^{c} \pm 0.69$**	**$13.77^{d} \pm 0.27$**	**$12.39^{e} \pm 0.43$**
80	$12.22^{e} \pm 0.61$	$11.42^{ef} \pm 0.35$	$11.69^{ef} \pm 0.75$	$10.71^{fg} \pm 0.56$	$8.63^{i} \pm 0.31$	$9.76^{gh} \pm 1.23$
100	$10.00^{gh} \pm 0.39$	$6.78^{jk} \pm 0.36$	$7.16^{j} \pm 0.65$	$6.42^{jkl} \pm 0.42$	$5.45^{lm} \pm 0.83$	$6.04^{kl} \pm 0.93$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.02 at $p \leq 0.05$.

Table 4.18 Assessment of different time periods of transfection on transformation efficiency in tomato

Infection time (min)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
2	$6.31^{e} \pm 0.55$	$5.00^{f} \pm 0.25$	$6.22^{e} \pm 0.89$	$4.22^{gh} \pm 0.34$	$2.79^{j} \pm 0.3$	$3.68^{hi} \pm 0.05$
3	**$9.21^{b} \pm 0.9$**	**$8.50^{c} \pm 0.5$**	**$10.66^{a} \pm 0.4$**	**$7.44^{d} \pm 0.18$**	**$7.64^{d} \pm 0.51$**	**$8.94^{bc} \pm 0.05$**
5	$3.67^{hi} \pm 0.46$	$4.50^{fg} \pm 0.5$	$4.85^{fg} \pm 0.16$	$2.66^{jk} \pm 0.33$	$3.05^{ij} \pm 0.08$	$2.10^{kl} \pm 0.13$
8	$2.57^{jk} \pm 0.52$	$1.85^{lm} \pm 0.5$	$1.35^{mno} \pm 0.25$	$1.33^{m-p} \pm 0.33$	$1.46^{lmn} \pm 0.2$	$1.04^{n-q} \pm 0.12$
10	$0.52^{q} \pm 0.04$	$1.00^{n-q} \pm 0.5$	$1.47^{lmn} \pm 0.46$	$0.66^{pq} \pm 0.27$	$0.58^{q} \pm 0.11$	$0.70^{opq} \pm 0.4$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.68 at $p \leq 0.05$.

4.14.6 Effect of co-cultivation media pH on transformation efficiency

In order to determine the influence of pH on transformation efficiency, leaf discs and hypocotyls were co-cultured with *Agrobacterium* for two days on co-cultivation media with varying pH values (5.3, 5.4, 5.5, 5.6, 5.7 and 5.8) as shown in Table 4.19. Analysis of variance revealed highly significant differences for explants, pH values and genotypes and also for their interactions i.e. explant x treatment, explant x genotype, treatment x genotype and explant x treatment x genotype (Appendix 27). In current study, the highest TE (16.18%) was recorded in Rio Grande from hypocotyls, followed by Moneymaker (15.94%) and Roma (15.89%) at pH 5.6. While in case of leaf discs, maximum efficiency of gene transfer (11.92%) was obtained in Roma followed by Moneymaker (10.97%) and Rio Grande (9.86%) on co-cultivation medium with pH 5.7 (Table 4.19). The minimum efficiency of gene transfer was recorded on pH 5.3 from both types of explants in all the genotypes. The pH values above or below 5.6-5.7 reduced the TE significantly ($p \leq 0.05$) in all the genotypes (Table 4.19).

4.14.7 Effect of claforan® (cefotaxime sodium) on transformation efficiency

In order to remove *Agrobacterium* completely from infected explants, six different concentrations of claforan® (200, 300, 400, 500, 600 and 700 mg/l) were added in the regeneration media after two days of co-cultivation. Data recorded after one month clearly indicated that increasing levels of claforan® significantly enhanced the regeneration and subsequent TE in all three genotypes (Table 4.20; Fig. 4.10). It was found that increase in claforan® concentration up to 500 mg/l increased TE gradually but further increase in claforan® level suppressed the TE in all the genotypes (Table 4.20). Statistically, there were significant differences among different treatments of claforan® and also between two types of explants and genotypes, but non-significant differences were found between explant x treatment (Appendix 28). The highest TE (16.45%) was recorded in cv. Roma followed by Rio Grande (16.22%) and Moneymaker (14.32%) culturing hypocotyls as explants at 500 mg/l claforan®. Similarly, maximum leaf discs derived TE was recorded in Roma (14.16%) followed by Moneymaker (14%) and Rio Grande (12%) as shown in table 4.20.

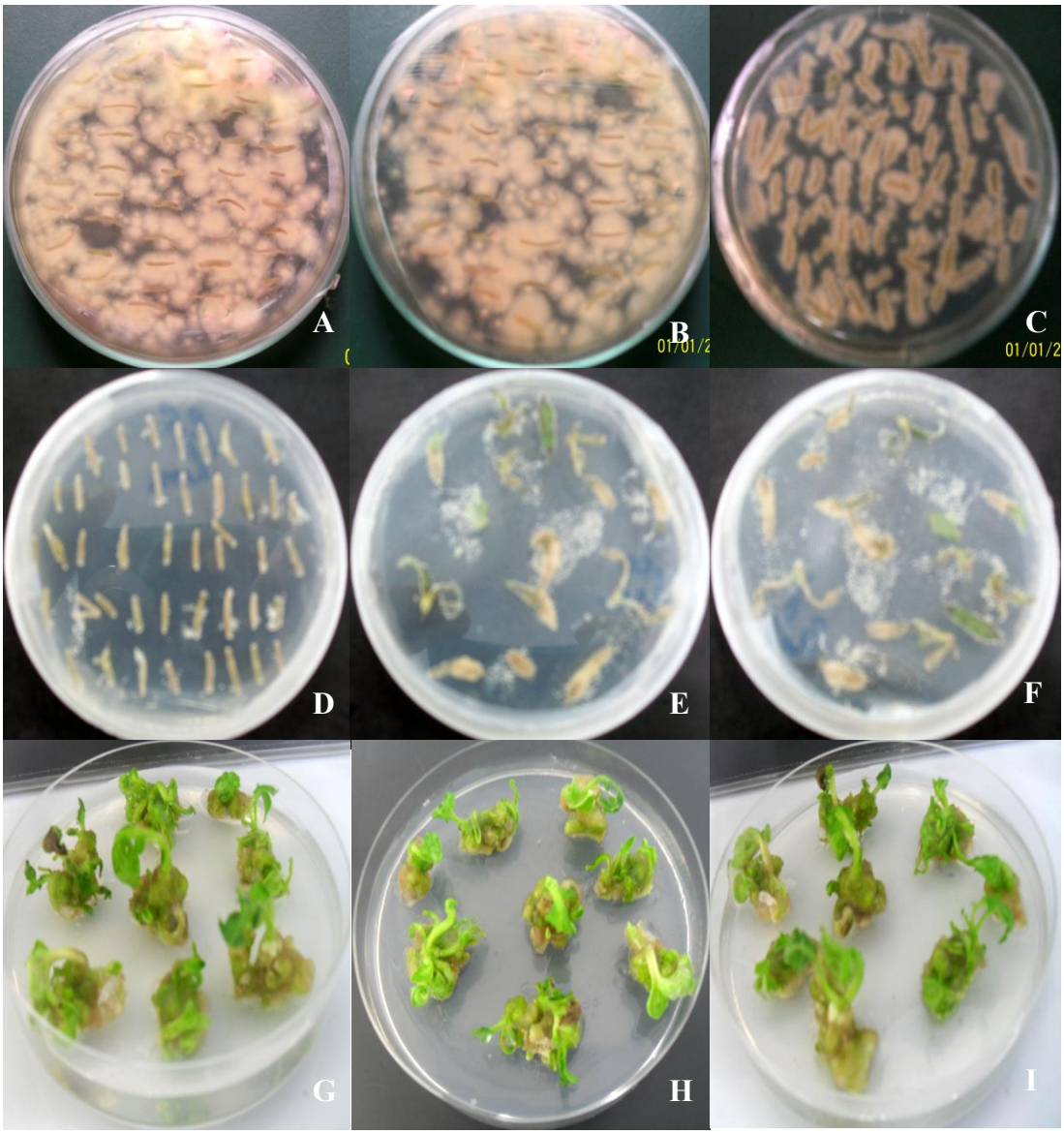

Fig. 4.10 Effect of different concentrations of claforan® on bacterial overgrowth and regeneration potential in three genotypes of tomato (*Solanum lycopersicum* Mill.) after 3-4 weeks, keeping $OD_{600\ nm}$ = 0.2 and acetosyringone 60 µM (A, B and C): The highest degree of *Agrobacterium tumefaciens* overgrowth at 300 mg/l claforan® with no regeneration in cvs. Rio Grande, Moneymaker and Roma culturing hypocotyls (D, E and F): The reduced overgrowth of *Agrobacterium tumefaciens* at 400 mg/l claforan® with no regeneration in Rio Grande culturing hypocotyls while in cvs. Moneymaker and Roma, overgrowth was obtained culturing leaf discs as explants (G, H and I): The efficient regeneration at 500 mg/l claforan® after 48-h co-cultivation period with no bacterial growth in Rio Grande, Moneymaker and Roma culturing leaf discs explants

At 500 mg/l concentration of claforan®, no bacterial growth was observed and hence it was found to be optimal level for tomato transformation (Fig. 4.10).

4.14.8 Effect of co-cultivation duration on transformation efficiency

Leaf discs and hypocotyls from fifteen days old seedlings of three tomato cultivars were cultured on co-cultivation media (Table 3.11; CCM) at 28°C for different time intervals (24, 48, 72 and 96 hours). Analysis of variance revealed highly significant differences among treatments, genotypes and explants, while no significant differences were found between explant x genotype interaction (Appendix 29). Maximum TE was recorded at 48 hours co-cultivation period for all cultivars from both types of explants (Fig. 4.12D & E). The highest TE (14.83%) was recorded in Roma followed by Rio Grande (13.64%) and Moneymaker (10.54%) culturing hypocotyls, while maximum leaf discs derived TE was also secured in Roma (11.45%) followed by Rio Grande (9.89%) and Moneymaker (8.26%) at 48 hours co-cultivation period (Table 4.21). Minimum transformant was obtained in cultures co-cultivated for 96 hours. The prolonged co-cultivation period adversely affected the plant tissues due to bacterial overgrowth on the infected explants and explants showed poor health and browning at cut surfaces resulting in no regeneration response.

4.14.9 Effect of pre-selection periods on transformation efficiency

After forty-eight hours co-cultivation period, the hypocotyls and leaf discs were shifted on pre-selection medium (Table 3.11; PSM) for 0, 2, 4, 6, and 8 days in an attempt to investigate the effect of pre-selection period on TE. The results of ANOVA for TE in tomato genotypes clearly demonstrated the highly significant differences for explants, pre-selection period and genotypes, and also their various interactions i.e. explant x treatment, explant x genotype, treatment x genotype and explants x pre-selection period x genotype interactions indicating that genotypes interacted significantly with the pre-selection periods at 5% level of probability (Appendix 30).

Table 4.19 Assessment of co-cultivation media pH on transformation efficiency in tomato

pH	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
5.3	$5.39^{mno} \pm 0.39$	$3.47^{q} \pm 0.22$	$4.35^{opq} \pm 0.69$	$1.08^{r} \pm 0.13$	$1.17^{r} \pm 0.2$	$1.75^{r} \pm 0.6$
5.4	$8.93^{fgh} \pm 0.55$	$6.66^{j-m} \pm 0.43$	$7.69^{h-k} \pm 0.6$	$3.98^{pq} \pm 0.7$	$3.91^{pq} \pm 0.41$	$3.85^{pq} \pm 1.21$
5.5	$11.06^{cd} \pm 0.47$	$8.98^{fgh} \pm 0.64$	$10.51^{de} \pm 0.53$	$5.07^{nop} \pm 0.7$	$6.66^{j-m} \pm 0.59$	$5.61^{mno} \pm 1.6$
5.6	**$16.18^{a} \pm 0.9$**	**$15.94^{a} \pm 0.39$**	**$15.89^{a} \pm 0.23$**	$7.96^{g-j} \pm 0.65$	$8.93^{fgh} \pm 0.35$	$6.31^{k-n} \pm 2.1$
5.7	$13.01^{b} \pm 0.51$	$11.01^{cd} \pm 0.56$	$12.05^{bc} \pm 0.76$	**$9.86^{def} \pm 0.23$**	**$10.97^{cd} \pm 0.41$**	**11.92 ± 1.6**
5.8	$9.20^{efg} \pm 0.55$	$7.11^{i-l} \pm 0.3$	$8.46^{ghi} \pm 2.03$	$6.15^{lmn} \pm 0.56$	$5.18^{nop} \pm 0.68$	$5.96^{lmn} \pm 1.6$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.38 at $p \leq 0.05$.

Table 4.20 Assessment of different concentrations of cefotaxime on transformation efficiency in tomato

Cefotaxime concentration (mg/l)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
200	$8.22^{nop} \pm 0.69$	$9.26^{k-n} \pm 0.86$	$8.62^{mn} \pm 0.50$	$6.66^{o-r} \pm 0.75$	$6.17^{qr} \pm 0.64$	$6.38^{pqr} \pm 0.66$
300	$10.88^{ijk} \pm 1.26$	$11.66^{g-j} \pm 1.13$	$11.17^{hij} \pm 0.76$	$8.48^{mno} \pm 1.00$	$8.63^{mn} \pm 0.88$	$8.61^{mn} \pm 0.74$
400	$13.78^{c-f} \pm 1.41$	$13.00^{d-h} \pm 1.58$	$14.5^{bcd} \pm 0.92$	$10.60^{i-l} \pm 1.16$	$10.28^{j-m} \pm 1.63$	$11.66^{g-j} \pm 0.86$
500	**$16.22^{ab} \pm 1.52$**	**$15.32^{abc} \pm 1.68$**	**$16.45^{a} \pm 1.11$**	**$13.32^{d-g} \pm 1.34$**	**$14.00^{cde} \pm 1.82$**	**$14.16^{cde} \pm 1.66$**
600	$11.33^{hij} \pm 1.42$	$12.4^{e-i} \pm 1.39$	$11.95^{f-j} \pm 1.05$	$7.57^{n-q} \pm 0.54$	$10.61^{i-l} \pm 1.29$	$8.88^{lmn} \pm 2.09$
700	$5.86^{qr} \pm 0.98$	$4.81^{rst} \pm 0.56$	$5.48^{rs} \pm 0.51$	$3.93^{st} \pm 0.44$	$2.96^{t} \pm 0.48$	$3.33^{t} \pm 0.83$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.47 at $p \leq 0.05$.

The pre-selection period of six days gave the highest TE in all the genotypes (Table 4.22). In six days pre-selection period, the maximum efficiency of gene transfer from hypocotyls was 14.44, 10.58 and 10% in Rio Grande, Moneymaker and Roma, respectively. Similarly, the highest leaf discs derived TE was 9.15, 7.49 and 6.86% in Rio Grande, Roma and Moneymaker (Table 4.22). The transformation frequency for four days pre-selection period ranked second from both hypocotyls and leaf discs in all the genotypes. No TE was recorded in explants that were directly transferred to selection medium after two days co-cultivation. Hence pre-selection period of six days was found to be optimal for tomato transformation (Fig. 4.12F & G).

4.14.10 Investigation of hygromycin optimum dose for transformation experiments

In order to assess the effect of hygromycin on *in vitro* shoot regeneration and to screen out the optimum level of hygromycin for selecting transformants, various levels of hygromycin B water solution (0, 10, 25, 35 and 45 mg/l) were investigated culturing hypocotyls and leaf discs as explants. It was found that plant regeneration was inversely related to concentration of hygromycin (Table 4.23). *In vitro* shoot regeneration from both types of explants was completely blocked within seven days on regeneration media supplementing with 35 mg/l hygromycin and no shoot regeneration was recorded even after one month. With increasing concentration of hygromycin beyond 35 mg/l, all the explants turned brown and ultimately died. From these trials, it was concluded that hygromycin (35 mg/l) was optimum dose for selecting transformants in further *Agrobacterium*-mediated transformation experiments and also response of hygromycin was tissue as well as genotype dependent (Fig. 4.11).

Table 4.21 Assessment of different time periods of co-cultivation on transformation efficiency in tomato

Co-cultivation period (hrs)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
24	$7.48^{hi} \pm 0.32$	$6.11^{j} \pm 0.14$	$8.19^{g} \pm 0.32$	$5.28^{k} \pm 0.29$	$4.00^{l} \pm 0.06$	$6.16^{j} \pm 0.2$
48	$\mathbf{13.64^{b} \pm 0.49}$	$\mathbf{10.54^{d} \pm 0.44}$	$\mathbf{14.83^{a} \pm 0.72}$	$\mathbf{9.89^{e} \pm 0.18}$	$\mathbf{8.26^{g} \pm 0.3}$	$\mathbf{11.45^{c} \pm 0.29}$
72	$9.11^{f} \pm 0.46$	$5.75^{jk} \pm 0.28$	$7.54^{h} \pm 0.56$	$6.91^{i} \pm 0.24$	$3.07^{mn} \pm 0.51$	$5.44^{k} \pm 0.23$
96	$2.66^{n} \pm 0.00$	$1.93^{o} \pm 0.41$	$3.65^{lm} \pm 0.37$	$1.12^{p} \pm 0.25$	$0.96^{p} \pm 0.23$	$2.00^{o} \pm 0.21$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.6 at $p \leq 0.05$.

Table 4.22 Assessment of various pre-selection periods on transformation efficiency in tomato

Pre-selection period (days)	Transformation efficiency culturing hypocotyls (%)			Transformation efficiency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
0	$0.00^{o} \pm 0.00$	$0.00^{o} \pm 0.00$	$0.00^{o} \pm 0.00$	$0.00^{o} \pm 0.00$	$0.00^{o} \pm 0.00$	$0.00^{h} \pm 0.00$
2	$1.96^{klm} \pm 0.34$	$1.45^{lmn} \pm 0.27$	$0.9^{no} \pm 0.45$	$1.06^{mn} \pm 0.29$	$0.89^{no} \pm 0.19$	$0.62^{no} \pm 0.17$
4	$8.95^{d} \pm 0.31$	$6.13^{fg} \pm 0.37$	$6.36^{f} \pm 0.45$	$5.32^{gh} \pm 0.55$	$4.42^{hi} \pm 0.53$	$3.75^{ij} \pm 1.25$
6	$\mathbf{14.44^{a} \pm 0.5}$	$\mathbf{10.58^{b} \pm 0.21}$	$\mathbf{10.00^{bc} \pm 1.2}$	$\mathbf{9.15^{cd} \pm 0.44}$	$\mathbf{6.86^{ef} \pm 0.22}$	$\mathbf{7.49^{e} \pm 1.65}$
8	$6.91^{ef} \pm 0.37$	$4.16^{i} \pm 0.29$	$3.63^{ij} \pm 0.45$	$2.94^{jk} \pm 0.58$	$2.28^{kl} \pm 0.32$	$2.50^{k} \pm 1.25$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.98 at $p \leq 0.05$.

Table 4.23 Assessment of optimum level of hygromycin for transformation experiments in tomato

Hygromycin concentration (mg/l)	Regeneration frequency culturing hypocotyls (%)			Regeneration frequency culturing leaf discs (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
0	$79.66^{b} \pm 2.00$	$68.74^{d} \pm 0.84$	$69.20^{d} \pm 1.05$	$83.67^{a} \pm 1.61$	$71.48^{c} \pm 2.03$	$72.37^{bc} \pm 0.95$
10	$55.33^{e} \pm 1.46$	$37.62^{h} \pm 1.27$	$40.80^{g} \pm 1.05$	$51.93^{f} \pm 1.3$	$34.36^{i} \pm 2.42$	$33.27^{i} \pm 1.64$
25	$24.62^{j} \pm 1.04$	$18.66^{l} \pm 1.75$	$14.00^{m} \pm 1.05$	$22.00^{k} \pm 1.24$	$11.63^{n} \pm 0.73$	$8.00^{o} \pm 0.25$
35	$\mathbf{0.00^{p} \pm 0.00}$	$\mathbf{0.00^{p} \pm 0.00}$	$\mathbf{0.00^{p} \pm 0.00}$	$\mathbf{0.00^{p} \pm 0.00}$	$\mathbf{0.00^{p} \pm 0.00}$	$\mathbf{0.00^{p} \pm 0.00}$
45	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$	$0.00^{p} \pm 0.00$

The values are means and percentages of three separate experiments. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.78 at $p \leq 0.05$.

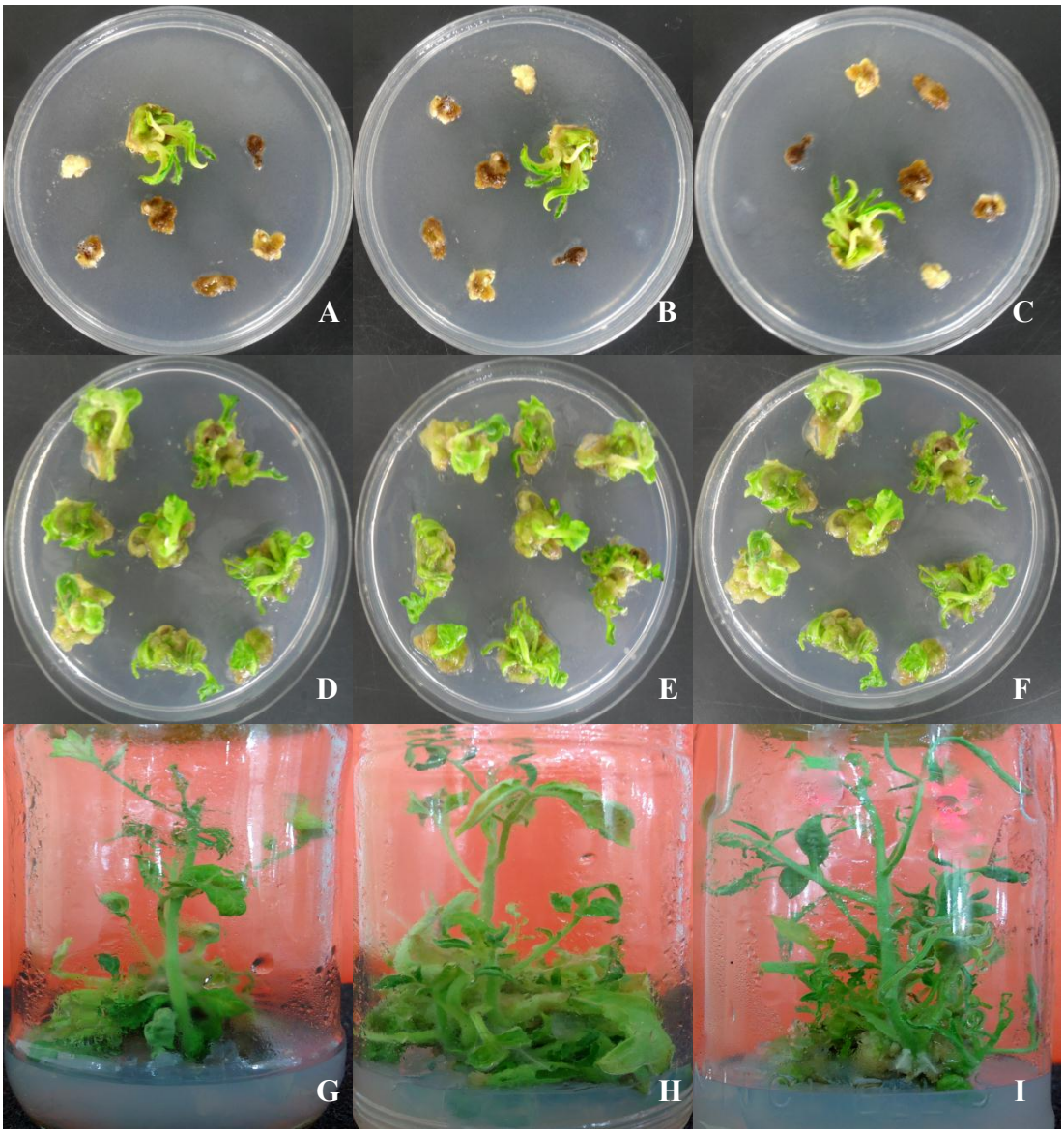

Fig. 4.11 Development of hygromycin resistant plantlets in three genotypes of tomato (*Solanum lycopersicum* Mill.) (A, B and C): Selection of hygromycin resistant calli derived from leaf discs at 35 mg/l hygromycin in cvs. Rio Grande, Moneymaker and Roma (D, E and F): *In vitro* morphogenesis from hygromycin resistant hypocotyls derived calli at 35 mg/l hygromycin 3-4 weeks after transformation in Rio Grande, Moneymaker and Roma (G, H and I): Hygromcin resistant plants obtained after culturing hypocotyls before acclimatization in Rio Grande, Moneymaker and Roma

Fig. 4.12 Development of tomato transgenic plants in a sequential manner via *Agrobacterium*-mediated transformation method (A) Two-three week old *in vitro* seedlings for hypocotyls and leaf discs explants (B) Pre-culturing of hypocotyls for 48-h (C) Pre-culturing of leaf discs for 48-h (D) Co-cultivation of hypocotyls for 48-h (E) Co-cultivation of leaf discs for 48-h (F) Pre-selection of hypocotyls for 6-d (G) Pre-selection of leaf discs for 6-d (H) Hygromycin resistant calli showing root and shoot development (I) Hygromycin resistant plants produced on MS medium fortified with 10 mg/l AgNO3, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP and 35 mg/l hygromycin (J) Hygromycin resistant plants produced on MS medium enriched with sucrose: sorbitol (30: 30 g/l) and 35 mg/l hygromycin (K) Acclimatization of hygromycin resistant plants on soil: vermiculite (1: 1) growth medium for 4-week in growth room (L) Transgenic tomato plants at flowering stage (M) Transgenic tomato plants at fruiting stage (N) Transgenic tomato plants at maturity stage bearing normal fruit

4.15 Factors affecting *in planta* transformation efficiency

4.15.1 Assessment of optical densities on *in planta* transformation

Optical density of *Agrobacterium* suspension denotes the cell number of bacteria using in transformation experiments. During this study, various *Agrobacterium* culture densities (0.5, 1.0, 1.5, and 2.0) at 600 nm absorption were evaluated to enhance the possibility of stable *in planta* TE in tomato and $OD_{600\ nm}$ = 1.0 was found to be optimal level in all the transgenic lines (Fig. 4.13). The highest TE (6.35%) was recorded in Rio Grande followed by Roma (5.25%) and Moneymaker (4.36%) at $OD_{600\ nm}$ = 1.0 with no bacterial growth (Figs. 4.13 & 4.17B). In case of higher density ($OD_{600\ nm}$ = 1.5), higher bacterial growth was noticed which was lethal to cell feasibility (Fig. 4.17C), resulting in lower TE in Rio Grande (4.65%) followed by Roma (3.85%) and Moneymaker (2.39%). Similarly, TE decreased drastically in all the genotypes when *Agrobacterium* culture density ($OD_{600\ nm}$) was 2.0 (Fig. 4.13 & Fig. 4.17D).

4.15.2 Assessment of acetosyringone concentration on *in planta* transformation

To investigate the effect of acetosyringone on *in planta* transformation efficiency in tomato, five different concentrations of acetosyringone (0, 20, 40, 60, 80 and 100 µM) were added in co-cultivation media. The co-cultivation media devoid of acetosyringone did not give any transformant. On the contrary, a gradual increase in TE was recorded with the increase in acetosyringone concentration up to 60 µM for all the genotypes (Fig. 4.14). The highest TE (6.72 %) in Rio Grande was recorded at 60 µM acetosyringone followed by Roma (5.49 %) and Moneymaker (3.82 %). Beyond the concentration of 60 µM, a sudden decrease in TE was noticed for all the genotypes due to bacterial overgrowth and explants browning. Hence, 60 µM acetosyringone was confirmed as the paramount level for further *in planta* transformation experiments.

Table 4.24 Impact of different growth media on germination and seedling establishment in tomato

Genotypes	Media used	No. of seeds inoculated	No. of embryos germinated	Germination frequency	No. of seedlings developed	Seedling establishment frequency
Rio Grande	Soil	92	63.33	$68.76^{e} \pm 3.43$	41.66	$65.67^{f} \pm 3.51$
	Vermiculite	92	76.33	$82.96^{b} \pm 4.11$	57.66	$75.44^{c} \pm 3.13$
	Soil: Vermiculite (1: 1)	**92**	**82.00**	**$89.12^{a} \pm 4.34$**	**71.66**	**$87.23^{a} \pm 4.92$**
Moneymaker	Soil	92	58.00	$62.7^{g} \pm 2.73$	34.66	$59.67^{g} \pm 2.89$
	Vermiculite	92	67.66	$73.54^{d} \pm 1.66$	47.33	$69.9^{e} \pm 2.95$
	Soil: Vermiculite (1: 1)	**92**	**70.66**	**$76.8^{c} \pm 3.81$**	**56.33**	**$79.56^{b} \pm 4.62$**
Roma	Soil	92	60.66	$65.93^{f} \pm 2.73$	39.33	$64.72^{f} \pm 4.02$
	Vermiculite	92	68.66	$74.63^{cd} \pm 5.13$	50.33	$73.12^{d} \pm 3.89$
	Soil: Vermiculite (1: 1)	**92**	**75.33**	**$81.88^{b} \pm 6.17$**	**61.33**	**$81.17^{b} \pm 5.6$**

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences ($p \leq 0.05$) by ANOVA (Statistix v. 8.1). The values after ± sign demonstrate standard deviation (n = 3). Data was recorded about germination and seedling establishment frequency in three tomato genotypes. For germination frequency, LSD value was 2.75 at $p \leq 0.05$. Similarly for seedling establishment frequency, LSD value was 2.23 at $p \leq 0.05$.

Table 4.25 Investigation of hygromycin sensitivity level on 3-weeks old seedlings developed from shoot apical meristem

Hygromycin concentration (mg/l)	No. of seedlings treated	No. of seedlings survived	Response of plantlets	Plantlets survival percentage
0	72	54	Normal growth	$74.83^{a} \pm 5.08$
10	72	35	Normal growth but retarded root development	$48.61^{b} \pm 4.17$
25	72	15	Albino growth with no appropriate root development	$20.83^{c} \pm 2.78$
35	72	7	Nil	$0.00^{d} \pm 0.00$
45	72	Nil	Nil	$0.00^{d} \pm 0.00$

Mean values superscripted with different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). Data was recorded about plantlet survival percentage. LSD value was 6.65 at $p \leq 0.05$.

4.15.3 Assessment of incubation period on *in planta* transformation

The time for incubating the explants in *Agrobacteium* culture is the most critical step during *Agrobacterium*-mediated *in planta* transformation. In current investigation, four different incubation regimes (10, 20, 30 and 40 min) were applied for producing transgenic plants in three tomato genotypes. The highest TE (7.63%) was secured in Rio Grande with the incubation period of 20 min on co-cultivation medium for two days followed by Roma (6.28%) and Moneymaker (4.43%) (Fig. 4.15). Consequently, twenty minutes of incubation period was found to be optimal time of infection for efficient explants regeneration and TE. On the contrary, infection period more than twenty minutes negatively affected the TE by limiting the explants survival rate due to bacterial overgrowth (Fig. 4.15).

4.15.4 Assessment of co-cultivation period on *in planta* transformation

A range of co-cultivation periods (24–96 h) were evaluated for securing maximum TE in tomato. The shoot apical meristem were infiltered with *Agrobacterium* ($OD_{600\ nm}$ = 1.00) and none of the explants was transformed when they were directly transferred to sterilized growth medium (soil: vermiculite) without incubating on co-cultivation medium. The highest TE (8.28%) was obtained at 48 h co-cultivation period in Rio Grande followed by Roma (7%) and Moneymaker (5.49%) keeping OD and incubation period constant (Fig. 4.16). In case of prolonged co-cultivation periods (72 and 96 h), lower transformation efficiencies were recorded due to the excessive growth of *Agrobacterium* resulting in the explants death in all the genotypes (Fig. 4.16).

4.15.5 Assessment of growth media on seedlings establishment

During present study, three different types of growth media were investigated on germination and seedling establishment of agro-infected shoot apical meristem of tomato in separate experiments. For successful acclimatization and seedling establishment, Agro-infected shoot apical meristem were transferred in three types of media; soil, vermiculite and soil: vermiculite (1: 1). The significant differences at $p \leq 0.05$ were noticed for both germination and seedling establishment as mentioned in Table 4.24. The most efficient response of these meristems was pronounced in the soil: vermiculite (1: 1) growth media

giving the highest germination frequency (89.12, 81.88 and 76.8%) in Rio Grande, Roma and Moneymaker, respectively. Similarly, the highest seedling establishment frequency (87.23, 81.17 and 79.56%) was recorded on soil: vermiculite (1: 1) growth media in Rio Grande, Roma and Moneymaker (Table 4.24; Fig. 4.17E). Moreover, the plants were taller in length and more in weight grown on soil: vermiculite medium as compared to plants grown on vermiculite growing medium (Data not shown). These results suggest that soil: vermiculite (1: 1) served as an efficient germination medium for better seedling establishment from *Agrobacterium*-treated shoot apical meristem.

4.15.6 Optimization of hygromycin for *in planta* transformation

In order to find suitable selection dose for *in planta* tomato transformation, various concentrations of hygromycin (0, 10, 25, 35, 45 and 60 mg/l) were directly applied on 3-week old seedlings of tomato. During the experiment, it was noticed that gradual increase of hygromycin adversely affected the plantlets survival percentage with retarded growth, showing the diverse responses at various levels of hygromycin (Table 4.25). It was observed that the seedlings were highly sensitive to 25 mg/l hygromycin concentration exhibiting the albino growth with no proper rooting, while complete necrosis occurred at 35 mg/l hygromycin resulting in the lethality of seedlings (Fig. 4.17F). Consequently, 35 mg/l hygromycin was found to be the optimum sensitivity level for further *in planta* transformation experiments.

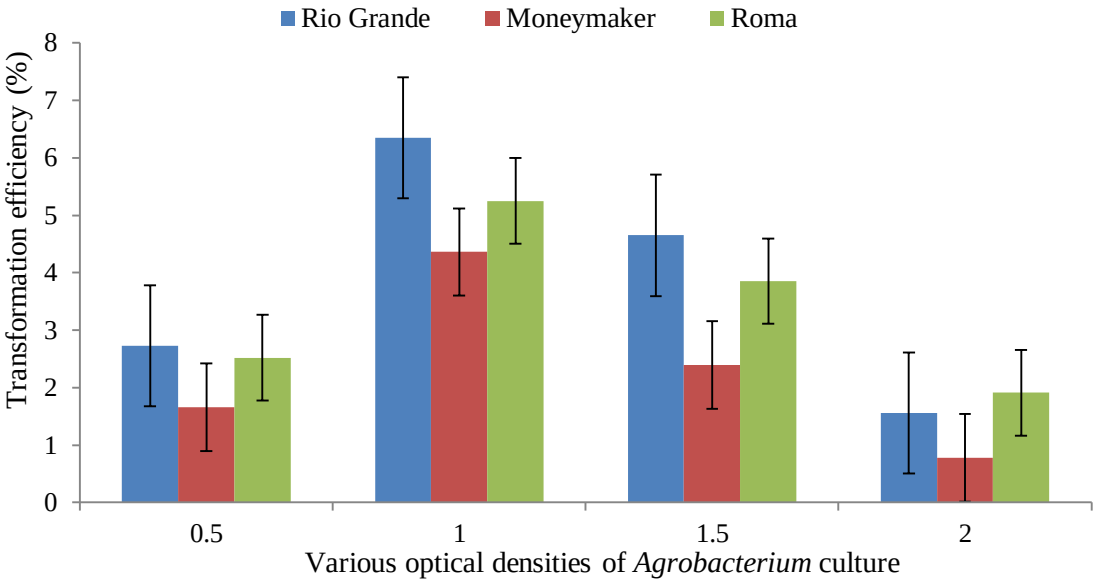

Fig. 4.13 Assessment of various optical densities of bacterial culture on *in planta* transformation efficiency in tomato. Vertical bars indicate standard error of the means (n = 3)

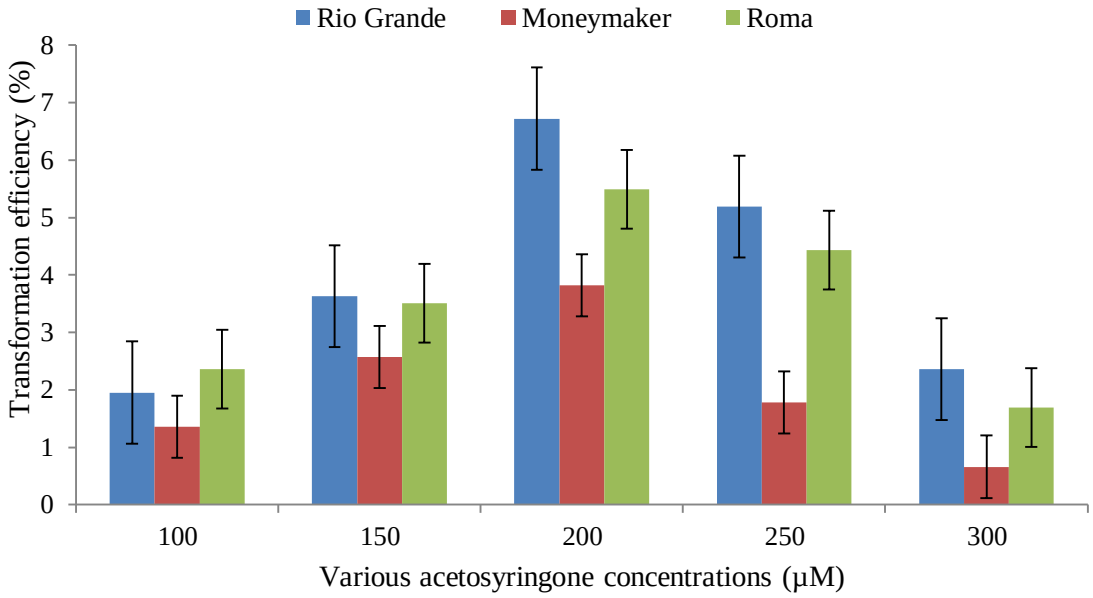

Fig. 4.14 Assessment of varying levels of acetosyringone on *in planta* transformation efficiency in tomato. Vertical bars indicate standard error of the means (n = 3)

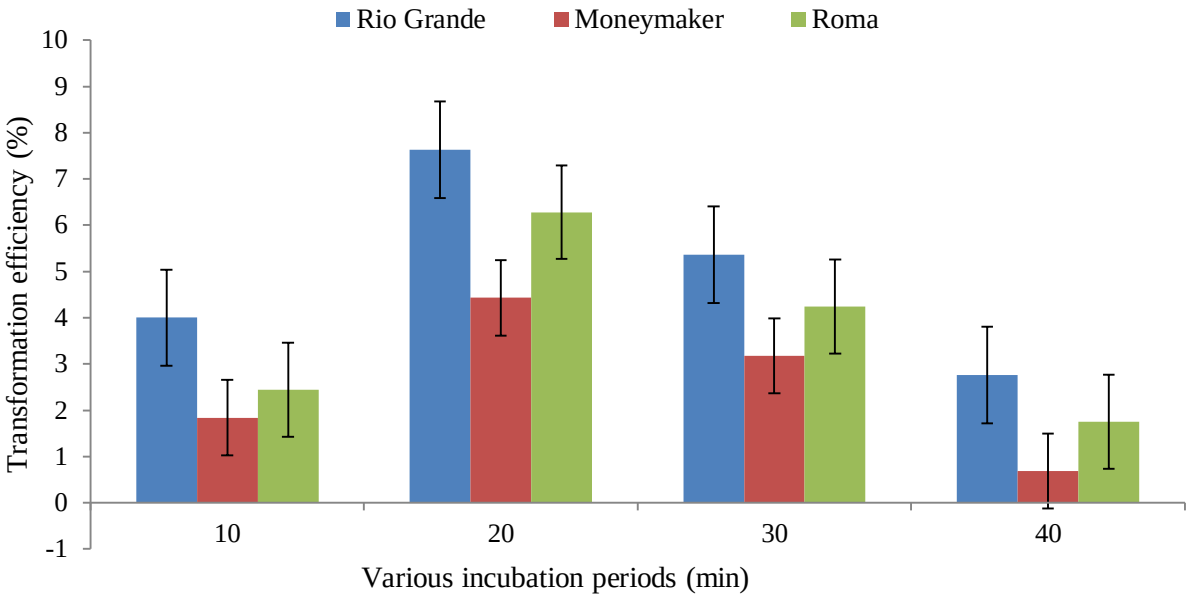

Fig. 4.15 Assessment of various incubation periods on *in planta* transformation efficiency in tomato. Vertical bars denote standard error of the means (n = 3)

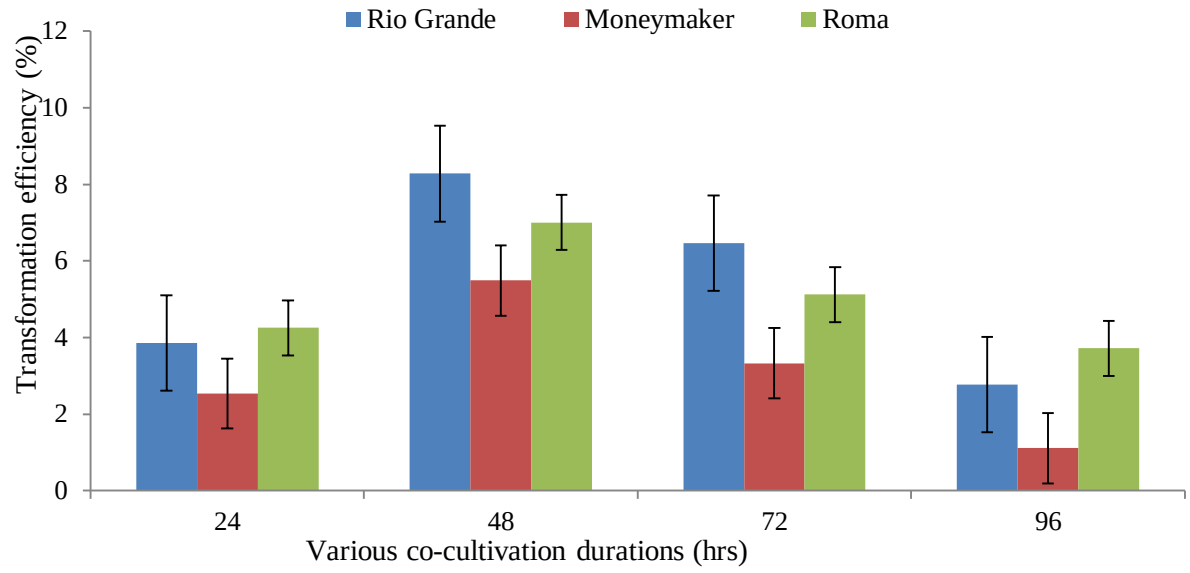

Fig. 4.16 Assessment of various co-cultivation durations on *in planta* transformation efficiency in tomato. Vertical bars denote standard error of the means (n = 3)

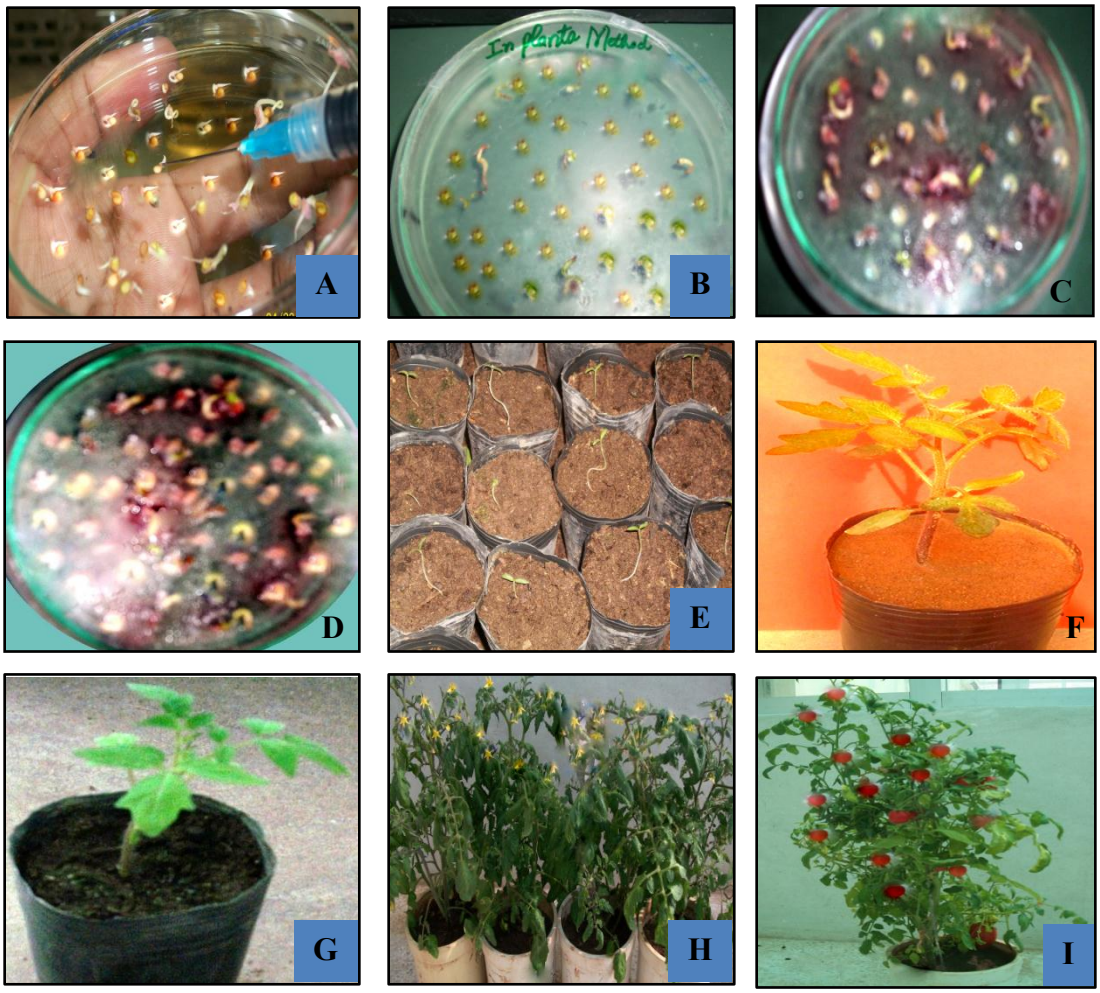

Fig. 4.17 Development of tomato transgenic plants via *Agrobacterium*-mediated tissue culture independent transformation method (A) Piercing and inserting a drop of bacterial suspension in 3-d-old shoot apical meristem through sterilized needle of syringe (3cc) (B) Co-cultivation stage of infected shoot apical meristem with *Agrobacterium* culture ($OD_{600\ nm}$ = 1.0) indicating no bacterial growth on sterilized wet filter paper (C) Co-cultivation stage of infected shoot apical meristem with *Agrobacterium* culture ($OD_{600\ nm}$ = 1.5) indicating bacterial growth on sterilized wet filter paper (D) Co-cultivation stage of infected shoot apical meristem with *Agrobacterium* culture ($OD_{600\ nm}$ = 2.0) with excessive bacterial growth on sterilized wet filter paper (E) Establishment of seedling from agro-infected shoot apical meristem on soil: vermiculite (1: 1) growth medium (F) Phenotypes of non-transformants after the application of 35 mg/l hygromycin (G) Hygromycin (35 mg/l) resistant tomato transgenic plants developed by following various sequenctial steps of *in planta* transformation technique (H) Transgenic plants at flowering stage in glasshouse conditions (I) Transgenic plants at ripening stage in glasshouse

4.16 Molecular analysis of tomato transgenes

4.16.1 Polymerase chain reaction

For the confirmation of *CBF3* and *HPT* genes in three putative tomato transgenic lines, Polymerase chain reaction (PCR) was performed. The genomic DNA was isolated from transgenic as well as NT plants. The DNA from NT plants was served as negative control. From *Agrobacterium tumefaciens*, plasmid DNA was also extracted and used as positive control. The genes - specified primers for *CBF3* and *HPT* were used for PCR scrutiny. The desired 649 bp of *CBF3* and 399 bp of *HPT* specified fragments were amplified from transgenic plants, while no fragment was recorded from NT plants (Figs. 4.18 & 4.19).

4.16.2 Segregation analysis by gel electrophoresis

In order to examine the stable integration of *CBF3* gene, segregation analysis of three tomato transgenic lines was done by gel electrophoresis. All T_1 transgenic lines produced by self-fertilized T_0 progeny followed by Mendelian inheritance ratio (3: 1) as shown in Fig. 4.20.

4.16.3 Multiplex polymerase chain reaction

In order to confirm the stable integration of desired genes, Multiplex PCR was carried out for simultaneous amplification of *CBF3* gene and plant selectable marker gene i.e. *HPT*. The genomic DNA was extracted from transgenic as well as NT plants of three tomato genotypes. The DNA from NT plants acted as negative control and plasmid DNA (pBIH) was used as positive control. The specific primer pairs for *CBF3* and *HPT* were designed to carry out the desired PCR assays. The identified fragments i.e. 649 bp of *CBF3* and 399 bp of *HPT* were amplified from transgenic plants in a single reaction, while no fragment was found from NT plants (Fig. 4.21). This standardized protocol for Multiplex PCR is simple, economical, time-saving and could be employed as an efficient tool for qualitative screening of tomato as well as other economical GM crops.

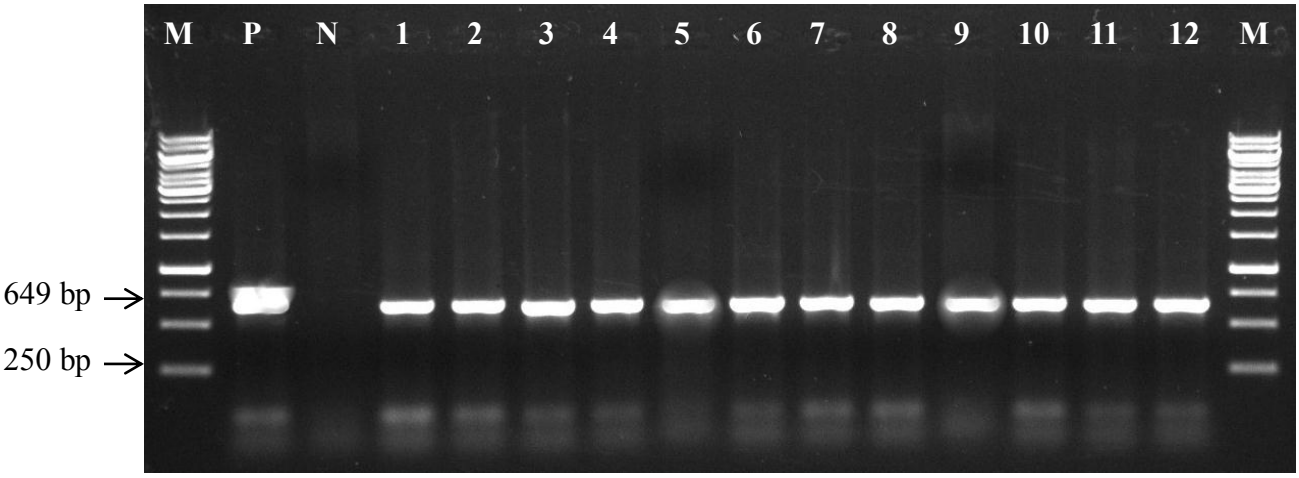

Fig. 4.18 PCR analysis of putative transgenic tomato plants carrying *CBF3* gene fragment (649 bp) using *Agrobacterium* strain EHA105 (pBIH): Lanes M 1 kb DNA Ladder (Fermentas), Lane P positive control (plasmid pBIH), Lane N negative control (non-transgenic tomato), Lanes 1–4 transgenic plants of Rio Grande, Lanes 5–8 transgenic plants of Moneymaker, Lanes 9–12 transgenic plants of Roma.

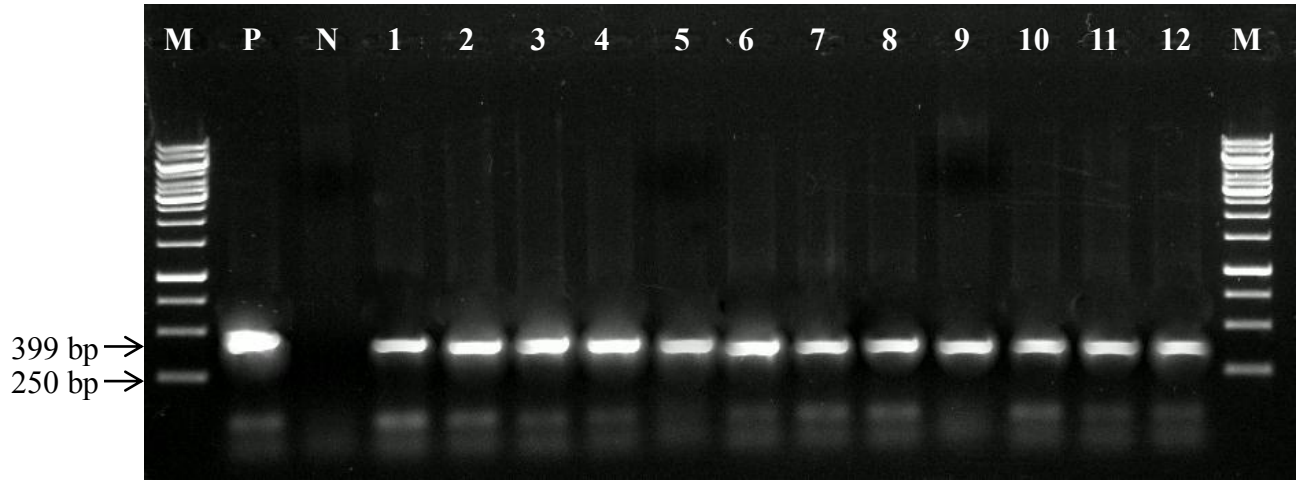

Fig. 4.19 PCR analysis for presence of *hpt* gene fragment (399 bp) in putative tomato transgenics: Lanes M 1 kb DNA Ladder (Fermentas), Lane P positive control (plasmid pBIH), Lane N negative control (non-transgenic tomato), Lanes 1–4 transgenic plants of Rio Grande, Lanes 5–8 transgenic plants of Moneymaker, Lanes 9–12 transgenic plants of Roma.

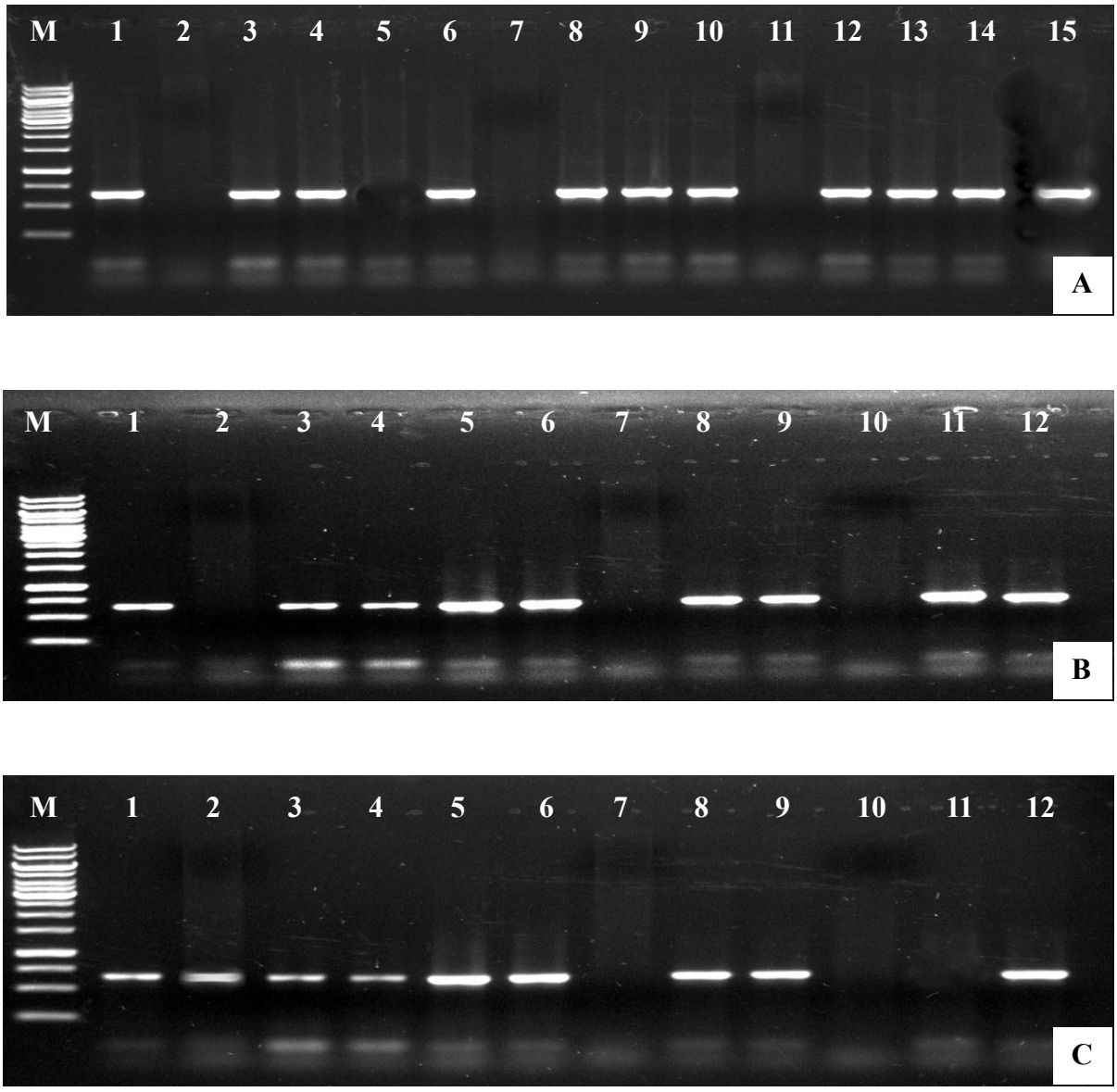

Fig. 4.20 Segregation analysis of tomato independent transgenic lines produced by self-fertilized T0 progeny followed by Mendelian inheritance ratio (3: 1). (A) Segregation analysis of Rio Grande transgenic line (B) Segregation analysis of Moneymaker transgenic line (C) Segregation analysis of Roma transgenic line

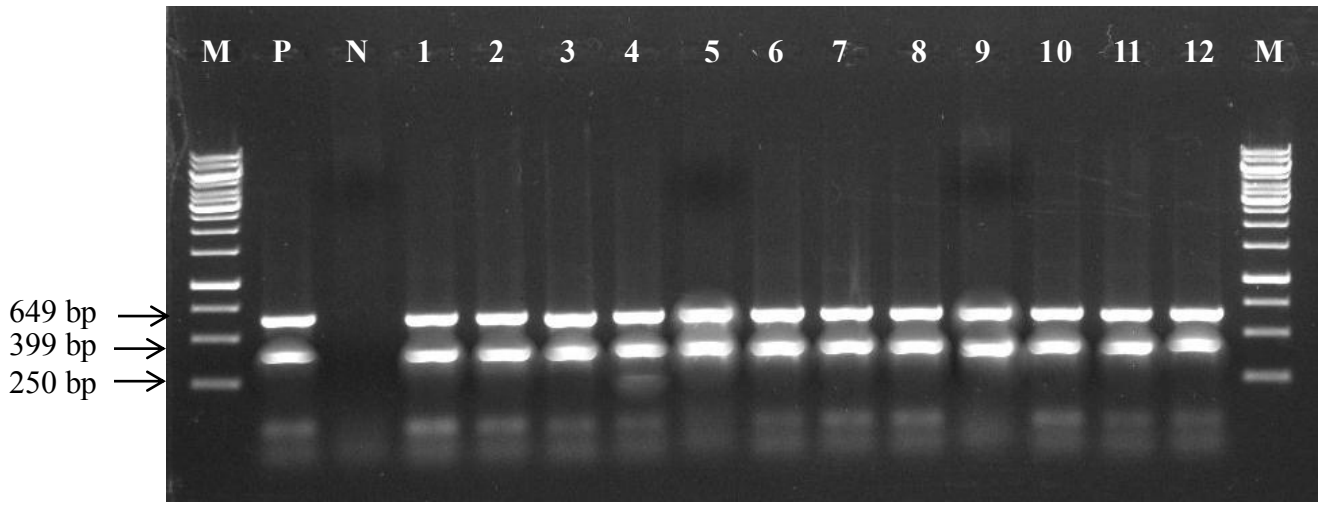

Fig. 4.21 Multiplex PCR screening for *CBF3* and *hpt* genes in putative tomato transgenic plants: Lanes M 1 kb DNA Ladder (Fermentas), Lane P positive control (plasmid pBIH), Lane N negative control (non-transgenic tomato), Lanes 1–4 transgenic plants of Rio Grande, Lanes 5–8 transgenic plants of Moneymaker, Lanes 9–12 transgenic plants of Roma.

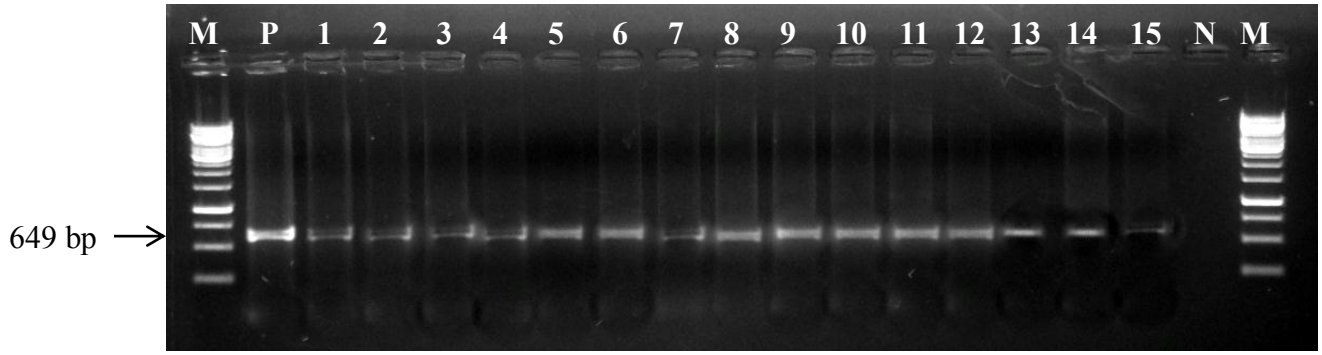

Fig. 4.22 RT PCR analysis of putative transgenic tomato plants. Lanes M 1 kb DNA Ladder (Fermentas), Lane P positive control (pBIH-*CBF3* as template), Lane N negative control (non-transgenic tomato), Lanes 1–5 transgenic plants of Rio Grande, Lanes 6–10 transgenic plants of Moneymaker, Lanes 11–15 transgenic plants of Roma.

4.16.4 Expression analysis of *CBF3* gene by Reverse-transcriptase (RT) PCR

In order to assess whether the gene has been stably integrated only, or it has been expressed to various mRNA transcript levels, RT-PCR analysis was performed in three tomato transgenic lines. Total RNA extracted from cold treated transgenic as well as NT plants employed as template for cDNA synthesis. The transcript expression of *AtCBF3* gene detected using gene specific primers. The RT-PCR assay showed that relative expression of *AtCBF3* gene induced by chilling stress and yielded differential gene expression in target leaf tissues. In case of Rio Grande transgenic line, the transcript signal was easily detectable in all five transgenic plants, while in case of Moneymaker transgenic line, the transcription signal was strongly detectable in four transgenic plants and weakly detectable in one transgenic plant. Similarly, in case of Roma transgenic line, the transcript signal was highly detectable in four transgenic plants and barely detectable in one transgenic plant. The expected size (649 bp) of *AtCBF3* was visualized in transgenic lines only having cold stress, but no band was observed in NT plants, showing that the introduced gene has also been expressed in transgenic lines (Fig. 4.22). Hence, RT-PCR is an efficient technique for detection and quantification of mRNA, and it is also more sensitive because it may give mRNA quantification in a single cell.

4.16.5 Southern blot analysis

In order to confirm the stable integration pattern of foreign gene in nuclear genome of putative tomato transgenic lines, Southern blot analysis was done. The genomic DNA of eight PCR-positive transgenic plants (T_1) of Rio Grande, Moneymaker and Roma were digested with BamH1, which yielded hybridization signals at different locations when hybridized with *AtCBF3* gene specific probe. The results of DNA blot analysis revealed the occurrence of 1-2 copies of introduced gene per individual tomato genome (Fig. 4.23).

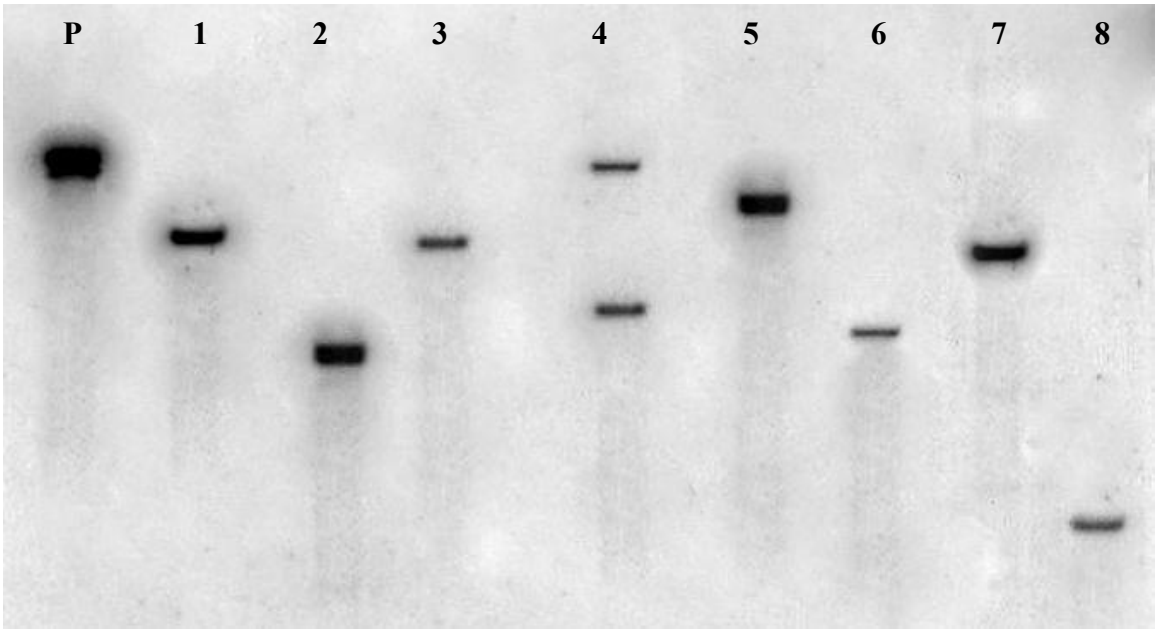

Fig. 4.23 Southern blot analysis of tomato transgenic lines: P positive control (pBIH-*DREB1A* plasmid) digested with *BamHI* and hybridized with *DREB1A* gene specific probe, 1-3 transgenic plants of Rio Grande, 4-5 transgenic plants of Moneymaker, 6-8 transgenic plants of Roma

4.17 Morphological comparison of transgenic and NT plants of tomato (*Solanum lycopersicum* Mill.)

Four week old transgenic and NT plants of three tomato lines were compared on the basis of following morphological characteristics:

4.17.1 Plant height (cm)

ANOVA for plant height recorded in transgenic and NT plants showed non-significant differences among transgenic and NT plants and also their interaction with genotypes i.e. plants × genotypes, while highly significant differences were found only among genotypes (Appendix 36). Plant height of transgenic plants was non-significantly less than that of NT plants (Fig. 4.24A & B). The maximum plant height (71.35 cm) was recorded in transgenic Rio Grande followed by Roma (65.95 cm) and Moneymaker (57 cm) as compared to their NT counterparts that exhibited 72.55, 67.26 and 59.63 cm plant height (Table 4.26).

4.17.2 Number of leaves per plant

Data regarding the number of leaves per plant given in table 4.32 demonstrated that no variations were found in number of leaves among transgenic and NT plants. Statistically, no significant differences were recorded among transgenic and NT plants and also their interaction i.e. plants × genotypes, while highly significant differences were noticed among genotypes (Appendix 37). The maximum no. of leaves/plant (46.75) was recorded in transgenic Rio Grande followed by Roma (33.57) and Moneymaker (24.69) as compared to their NT counterparts of Rio Grande (45.26), Roma (30.93) and Moneymaker (22.92) (Table 4.27).

4.17.3 Fresh weight (g/plant)

Data about plant fresh weight given in table 4.28 showed minute differences in fresh weight of transgenic and NT plants. Fresh weight per plant obtained for transgenic and NT plants demonstrated no significant variations among transgenic and NT plants and also their interaction with genotypes, while highly significant differences were recorded among genotypes (Appendix 38).

Table 4.26 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of plant height (cm) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	71.35 ± 3.87	57.00 ± 2.01	65.95 ± 3.81	**64.76**
NT plants	72.55 ± 3.15	59.63 ± 2.91	67.26 ± 2.74	**66.48**
Mean	**71.95**	**58.00**	**66.60**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 4.75 at $p \leq 0.05$.

Table 4.27 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of leaves per plant in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	46.75 ± 4.53	24.69 ± 2.36	33.57 ± 3.36	**35.00**
NT plants	45.26 ± 3.28	22.92 ± 3.16	30.93 ± 3.71	**33.03**
Mean	**46.00**	**23.80**	**32.25**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 4.59 at $p \leq 0.05$.

Table 4.28 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of fresh weight (g/plant) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	64.45 ± 5.46	45.77 ± 4.07	56.50 ± 3.89	**55.57**
NT plants	63.17 ± 7.56	43.71 ± 4.78	53.82 ± 6.11	**53.56**
Mean	**63.81**	**44.74**	**55.16**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 5.1 at $p \leq 0.05$.

Table 4.29 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of dry weight (g/plant) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	6.95 ± 1.47	5.26 ± 1.15	6.09 ± 1.44	**6.1**
NT plants	6.21 ± 1.44	4.52 ± 1.32	5.49 ± 0.76	**5.4**
Mean	**6.58**	**4.89**	**5.79**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.42 at $p \leq 0.05$.

The highest fresh weight per plant (64.45, 56.5 and 45.77 g) was recorded in transgenic plants of Rio Grande, Roma and Moneymaker as compared to their NT plants that exhibited 63.17, 53.82 and 43.71 g, respectively (Table 4.28).

4.17.4 Dry weight (g/plant)

Dry weight per plant varied non-significantly among transgenic and NT plants and also their interaction with genotypes, while it was significant for genotypes (Appendix 39). The highest dry weight per plant (6.95 g) was recorded in transgenic Rio Grande followed by Roma (6.09 g) and Moneymaker (5.26 g) as compared to their isogenic NT plants that exhibited 6.21, 5.49 and 4.52 g (Table 4.29).

4.17.5 Root length (cm)

Twelve uniform plants from each line were chosen randomly for calculating root length at 30 days after germination. The growth rate of root length showed non-significant differences among transgenic and NT plants, and interaction i.e. plants × genotypes at 25 °C, whereas significant differences were noticed among genotypes (Appendix 40). Data in table 4.30 indicated that the highest length of roots (26.65 cm) was obtained in transgenic Rio Grande followed by Roma (22.15 cm) and Moneymaker (19.23 cm), while NT plants of Rio Grande yielded 24.99 cm root length followed by NT Roma (20.38 cm) and NT Moneymaker (16.95 cm) as shown in table 4.30.

4.17.6 Days to flowering

The critical growth attribute like time needed for flowering was scored in transgenic and NT tomato genotypes, which demonstrated non-significant differences among transgenic and NT plants and interaction of plants × genotypes, while it was significant for genotypes at normal temperature (Appendix 41). The transgenic plants non-significantly took longer time to flower than that of NT plants in all the lines (Table 4.31). The longest time to flower (67 days) was recorded in transgenic Moneymaker followed by Roma (62 days) and Rio Grande (55 days) as compared to NT plants of Moneymaker (65 days), Roma (61 days) and Rio Grande (52 days).

Fig. 4.24 Morphological comparison of transgenic and NT plants of three tomato genotypes under normal growth environment in glasshouse (A) Plant height of NT plants (B) Plant height of transgenic plants (C) Transgenic plants at flowering stage (D) NT plants at flowering stage

Table 4.30 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of root length (cm) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	26.65 ± 3.62	19.23 ± 3.24	22.15 ± 3.92	**22.67**
NT plants	24.99 ± 1.71	16.95 ± 3.27	20.38 ± 4.54	**20.77**
Mean	**25.82**	**18.09**	**21.26**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 6.35 at $p \leq 0.05$.

Table 4.31 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of days to flowering in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	55.00 ± 5.57	67.00 ± 7.94	62.00 ± 5.29	**61.33**
NT plants	52.00 ± 5.29	65.00 ± 4.58	61.00 ± 4.58	**59.33**
Mean	**53.5**	**66.00**	**61.5**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 10.02 at $p \leq 0.05$.

Table 4.32 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of flowers per plant in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	35.65 ± 4.82	21.98 ± 2.99	30.84 ± 3.71	**29.49**
NT plants	37.29 ± 5.22	24.93 ± 4.84	32.91 ± 5.21	**31.71**
Mean	**36.47**	**23.45**	**31.87**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 8.87 at $p \leq 0.05$.

Table 4.33 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of first fruit set (days) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	65.00 ± 5.20	76.00 ± 6.08	70.00 ± 5.00	**70.33**
NT plants	62.00 ± 4.36	74.00 ± 6.24	69.00 ± 5.29	**68.33**
Mean	**63.5**	**75.00**	**69.5**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 10.44 at $p \leq 0.05$.

4.17.7 Number of flowers per plant

The number of flowers per plant in all the transgenic lines was non-significantly different from NT plants at 25 °C (Table 4.32). ANOVA for number of flowers per plant showed no significant variations for transgenic and NT plants and interaction of genotypes × plants. However, genotypes differed significantly from each other with respect to number of leaves per plant (Appendix 42). Data given in table 4.32 exhibited non-significantly less number of flowers per transgenic plants than that of NT plants in all the genotypes tested (Fig. 4.24C & D). The maximum number of flowers (46.62) was secured in transgenic Rio Grande followed by Roma (40.78) and Moneymaker (32.59) as compared to their isogenic NT plants that produced 37.29, 32.91 and 24.93 number of flowers per plant, respectively (Table 4.32).

4.17.8 First fruit set (days)

The agronomic performance of *lip9-CBF3* and NT plants was investigated with respect to first fruit set at normal temperature. The results presented in table 4.33 clearly indicated that time required to the initiation of fruiting in transgenic lines was almost similar to that of NT plants under optimum growth conditions. Statistically, non-significant differences were noticed among transgenic and NT plants and also their interaction (genotypes × plants), while significant differences were found among genotypes (Appendix 43). The highest days to fruiting (76 days) were recorded in transgenic Moneymaker followed by Roma (70) and Rio Grande (65) as compared to their isogenic NT plants, where days to fruiting (74, 69 and 62) were recorded in Moneymaker, Roma and Rio Grande (Table 4.33).

4.17.9 Number of fruit per plant

ANOVA exhibited non-significant differences among transgenic and NT plants and also in the interaction of genotypes × plants, while it was significant for genotypes (Appendix 44). Results from investigation of transgenic lines (T_2 generation) revealed that the average maximum no. of fruit per plant (21.66) was obtained in transgenic line of Rio Grande followed by Roma (15.72) and Moneymaker (10.66) as compared to their NT

plants of Rio Grande (24.61), Roma (18.49) and Moneymaker (13.86) (Table 4.34; Fig. 4.25A & B).

4.17.10 Fruit diameter (cm)

ANOVA for fruit diameter showed non-significant variations among transgenic and NT plants and also interaction of genotypes × plants, while it showed significant differences among genotypes (Appendix 45). The fruit diameter was non-significantly lower in transgenic plants than that of NT plants (Fig. 4.25C & D). The maximum fruit diameter (5.46 cm) was recorded in transgenic Rio Grande followed by Moneymaker (4.18 cm) and Roma (3.34 cm) as compared to their isogenic NT plants that produced 6.15, 4.86 and 4.46 cm fruit diameter of Rio Grande, Moneymaker and Roma, respectively (Table 4.35).

4.17.11 Fruit mean weight (g)

The average fruit weight of *CBF3* expressing lines (*lip9:CBF3*) and NT tomato plants was analyzed at 25 °C. Data given in table 4.36 indicated non-significantly less fruit weight in transgenic lines as compared to their counterparts. The maximum fruit weight (39.59 g) was recorded in transgenic Rio Grande followed by Roma (35.65 g) and Moneymaker (30.84 g) as compared to NT plants that produced 42.72, 38.31 and 32.45 g fruit mean weight of Rio Grande, Roma and Moneymaker, respectively. ANOVA for fruit weight indicated significant variations among genotypes, while it exhibited non-significant differences among transgenic and NT plants (Appendix 46).

4.17.12 Number of seeds per fruit

ANOVA for number of seeds per fruit showed no significant variations for transgenic and NT plants and also their interaction with genotypes (Appendix 47). Data in table 4.37 indicated that the maximum number of seeds per fruit (120.78) was obtained in Rio Grande transgenic line followed by Roma (116.67) and Moneymaker (110.37) as compared to their NT counterparts that produced 123.87, 121.59 and 115.23 number of seeds per fruit of Rio Grande, Roma and Moneymaker (Table 4.37).

Fig. 4.25 Comparison of transgenic and NT plants of three tomato genotypes on the basis of morphological parameters under normal growth conditions in glasshouse (A) Transgenic plants at fruiting stage (B) NT plants at fruiting stage (C) Fruit size of transgenic plants (D) Fruit size of NT plants

Table 4.34 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of fruit per plant in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	21.66 ± 3.94	10.66 ± 2.67	15.72 ± 4.44	**16.01**
NT plants	24.61 ± 5.09	13.86 ± 2.72	18.49 ± 3.83	**18.98**
Mean	**23.13**	**12.26**	**17.1**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 6.74 at $p \leq 0.05$.

Table 4.35 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of fruit diameter (cm) at ripening in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	5.46 ± 0.84	4.18 ± 0.96	3.34 ± 0.63	**4.32**
NT plants	6.15 ± 1.14	4.86 ± 0.70	4.46 ± 1.07	**5.15**
Mean	**5.8**	**4.52**	**3.9**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.67 at $p \leq 0.05$.

Table 4.36 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of fruit mean weight (g) in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	39.59 ± 4.17	30.84 ± 3.71	35.65 ± 4.82	**35.36**
NT plants	42.72 ± 4.91	32.45 ± 4.35	38.31 ± 5.96	**37.82**
Mean	**41.15**	**31.64**	**36.98**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 8.19 at $p \leq 0.05$.

Table 4.37 Comparison of T_2 transgenic plants carrying *AtCBF3* gene and NT plants on the basis of number of seeds per fruit in tomato

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	120.78 ± 9.91	110.37 ± 6.91	116.67 ± 7.76	**115.94**
NT plants	123.87 ± 5.18	115.23 ± 5.87	121.59 ± 4.26	**120.23**
Mean	**122.32**	**112.8**	**119.13**	

Each data is the average of three replicates. The values after ± sign demonstrate standard deviation (n = 3). LSD value was 12.79 at $p \leq 0.05$.

4.18 Evaluation of cold tolerance in tomato on physiological basis

4.18.1 Stomatal conductance (mole/m^2/sec)

The transgenic and NT plants (T_2) of three tomato genotypes (Rio Grande, Moneymaker and Roma) were assessed under various low temperature stresses compared with normal temperature (Figs. 4.26, 4.27 & 4.28). Data given in table 4.38 indicated that different cold stresses markedly affected the stomatal conductance in all tested genotypes. At normal temperature (25 ºC), no apparent differences were found among transgenic and NT plants for stomatal conductance, which was around 0.29 – 0.34 mole/m^2/sec in transgenic lines and 0.26 – 0.33 mole/m^2/sec in respective NT plants. After cold treatments, the stomatal conductance enhanced significantly in all the transgenic lines. The highest stomatal conductance (0.78, 0.67 and 0.86 mole/m^2/sec) was recorded in transgenic plants of Rio Grande, Moneymaker and Roma at 8 ºC as compared to their counterparts whose maximum conductance (0.07, 0.08 and 0.09 mole/m^2/sec) was recorded at the same temperature (Table 4.38). A sudden change was noticed below 8 ºC by 0.68, 0.53 and 0.79 mole/m^2/sec conductances in transgenic Rio Grande, Moneymaker and Roma at 6 ºC. Similarly, the lowest stomatal conductance (0.35, 0.41 and 0.55 mole/m^2/sec) was secured in transgenic plants of Rio Grande, Moneymaker and Roma at 4 ºC.

4.18.2 Transpiration rate (mole/m^2/sec)

The transpiration rate of transgenic and NT tomato plants were calculated under normal and cold stress conditions. The statistical analysis revealed non-significant differences of transpiration rate in transgenic (3.17 – 4.41 mole/m^2/sec) and NT (3.12 – 4.45 mole/m^2/sec) tomato lines at normal temperature (Table 4.39). Our results indicated that under chilling stress, the transpiration rate decreased in NT plants, while it enhanced in transgenic lines about two-folds compared with NT ones (Table 4.39). The minimum transformation rate (0.65, 0.89 and 0.91 mole/m^2/sec) was recorded in transgenic lines of Moneymaker, Rio Grande and Roma upon exposure to chilling stress (4 °C). The highest transpiration rate (2.42, 1.96 and 1.51 mole/m^2/sec) was recorded at 8 °C followed by 1.95, 1.35 and 1.22 mole/m^2/sec in Roma, Rio Grande and Moneymaker transgenic lines,

upon exposure to 6 °C (Table 4.39). All the transgenic lines behaved in a same manner and Roma was proved to be the most efficient in transpiration rate among all the genotypes.

4.18.3 CO_2 concentration rate (µmole/mole)

The leaf CO_2 concentration rate is a critical physiological parameter that directly affects the photosynthetic systems in plants. In order to examine the effects of inducible *DREB1A* expression on CO_2 concentration rate, we analyzed the transgenic and NT plants of three tomato genotypes that were exposed to various low temperature stresses (4, 6 and 8 ºC) for seven days (Figs. 4.26, 4.27 & 4.28). Under normal growth temperature, T_2 transgenic and NT tomato plants gave almost similar CO_2 concentration rate, whereas it decreased in both types of plants after chilling treatments (Table 4.40). The highest CO_2 concentration rate (185.57, 209.82 and 238.28 µmole/mole) was recorded in transgenic Roma plants at 4, 6 and 8 ºC, respectively which was comparable to its NT plants that showed 109.94, 145.37 and 198.69 (µmole/mole) CO_2 concentration rate. It was followed by Rio Grande transgenic line whose maximum CO_2 concentration rate (150.78, 178.17 and 217.66 µmole/mole) was obtained at chilling stresses of 4, 6 and 8 ºC compared with its counterparts that exhibited 86.78, 105.17 and 145.66 µmole/mole CO_2 concentration (Table 4.40). Similarly, in Moneymaker, the maximum CO_2 concentration (132.46, 156.68 and 196.82 µmole/mole) was secured at 4, 6 and 8 ºC as compared to NT Moneymaker plants (92.46, 116.35 and 124.95 µmole/mole).

4.18.4 Photosynthetic rate (µmole/m^2/sec)

Recent study was performed to understand the phenomena of *DREB1A*/*CBF3* gene expression under cold stress in transgenic and NT tomato plants. Transcriptional factors especially *CBF3* attributed the cold tolerance in tomato transgenic plants that enhanced photosynthetic rate by overexpressing *CBF3* gene. The photosynthetic rate of transgenic plants significantly enhanced under cold stresses relative to NT plants. Although under low temperature, photosynthetic rates decreased in all the transgenic lines but Roma showed a significant increase in photosynthetic rates at low temperature compared to other transgenic lines. The highest photosynthetic rate (12.71, 11.68 and 10

µmole/m^2/sec) was recorded in transgenic plants of Roma, Rio Grande and Moneymaker at 8 ºC compared with their isogenic NT plants (7.11, 6.82 and 5.12 µmole/m^2/sec) at the same temperature (Table 4.41). At 25 ºC, all the transgenic and NT tomato plants showed almost similar photosynthetic rates. Photosynthetic rate dramatically lowered with lowering temperature in all the transgenic lines investigated. Minimum photosynthetic rate (2.36, 3.88 and 4.96 µmole/m^2/sec) was recorded at 4 ºC in transgenic plants of Moneymaker, Rio Grande and Roma compared with their NT counterparts that exhibited 1.81, 1.34 and 2.42 µmole/m^2/sec at 4 ºC. Relative to NT tomato plants, transgenic plants were dark green with higher chlorophyll contents and greater photosynthetic rates (Figs. 4.26, 4.27 & 4.28).

4.18.5 Relative water contents (%)

During this study, the chilling tolerance was investigated on the basis of increment of RWC of leaves in 3 week old transgenic lines upon exposure to various chilling stresses for seven days, as compared to their respective NT ones. The three tomato transgenic lines along with NT plants were exposed to three cold treatments (4, 6 and 8 ºC) for seven days and then were maintained for one day to measure RWC. The analysis of variance showed no statistical differences among transgenic and NT plants at normal growth conditions (25 ºC), whose RWC in leaves were ranged between 83.43 – 89.94%. Upon exposure to low temperature stresses, the RWC decreased in both transgenic and NT plants, but rate of decrease of RWC in NT plants was more than that of transgenic ones (Figs. 4.26, 4.27 & 4.28). The highest RWC (78.48, 71.86 and 65.05%) were recorded in transgenic plants of Roma, Rio Grande and Moneymaker at 8 ºC compared with their NT plants that exhibited 63.54, 60.59 and 49% RWC at the same temperature. It was followed by RWC (55.21, 48.62 and 43.34%) in Roma, Rio Grande and Moneymaker transgenic lines at 6 ºC as compared to their counterparts that gave 45.47, 38.78 and 32.36% RWC at the same temperature (Table 4.42). The minimum RWC (29.14, 34.87 and 39%) were recorded in transgenic plants of Moneymaker, Rio Grande and Roma at 4 ºC that were comparable to their respective NT plants (Table 4.42).

Fig. 4.26 Screening of cold tolerance in transgenic tomato line i.e. Rio Grande under various low temperature regimes on the basis of physiological parameters (A) Phenotype of six NT plants (left) which displayed altered responses to cold stress (4 ºC) in term of tissue damage in relation to the respective six transgenic plants (right) (B) Phenotypically, three *CBF3*-plants (left) are noticeably distinguishable from three NT plants (right) in the sense that leaves of *CBF3*-plants are green, more in diameter and erect as compared to NT plants (C) Phenotype of two transgenic plants (left) compared to two NT plants (right) showing wilting and white green leaves

Fig. 4.27 Screening of cold tolerance in transgenic tomato line i.e. Moneymaker under various low temperature regimes on the basis of physiological parameters. All conditions were similar to those specified for fig. 4.24 (A) Images of first six transgenic plants from right and six NT plants which demonstrate the clear differences after 7-d cold stress (4 ºC) (B) Morphological changes in first three NT plants from right and three transgenic plants after 7-d cold stress (6 ºC) (C) Images of first two transgenic plants from left and three NT plants after 7-d cold stress (8 ºC). Images are indicatives for three independent experiments. All the measurements were taken after various chilling stresses for seven days and then one day recovery period at normal temperature

Fig. 4.28 Screening of cold tolerance in transgenic tomato line i.e. Roma under various low temperature regimes on the basis of physiological parameters (A) Phenotype of first six NT plants from left showing necrosis compared to six transgenic plants which show normal growth at 4 ºC (B) Phenotype of first three NT plants from right and three transgenic plants which demonstrate the clear differences at 6 ºC (C) Phenotype of first three NT plants from left and three transgenic plants at 8 ºC

Table 4.38 Effect of various levels of chilling stress on stomatal conductance (mole/m^2/sec) in tomato

Chilling stress	Stomatal conductance in non-transgenic plants			Stomatal conductance in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$0.05^{f} \pm 0.01$	$0.02^{f} \pm 0.01$	$0.04^{f} \pm 0.03$	$0.35^{de} \pm 0.22$	$0.41^{d} \pm 0.03$	$0.54^{c} \pm 0.05$
6 ºC	$0.05^{f} \pm 0.01$	$0.03^{f} \pm 0.01$	$0.06^{f} \pm 0.01$	$0.66^{b} \pm 0.03$	$0.52^{c} \pm 0.04$	$0.79^{a} \pm 0.03$
8 ºC	$0.07^{f} \pm 0.01$	$0.08^{f} \pm 0.03$	$0.08^{f} \pm 0.07$	$0.78^{a} \pm 0.02$	$0.66^{b} \pm 0.07$	$0.85^{a} \pm 0.04$
25 ºC	$0.30^{e} \pm 0.05$	$0.25^{e} \pm 0.03$	$0.33^{de} \pm 0.02$	$0.32^{de} \pm 0.08$	$0.28^{e} \pm 0.02$	$0.34^{de} \pm 0.03$

Each value is the mean of stomatal conductance of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.09 at $p \leq 0.05$.

Table 4.39 Effect of various levels of chilling stress on transpiration rate (mole/m^2/sec) in tomato

Chilling stress	Transpiration rate in non-transgenic plants			Transpiration rate in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$0.58^{m} \pm 0.03$	$0.33^{n} \pm 0.06$	$0.25^{n} \pm 0.04$	$0.89^{k} \pm 0.06$	$0.65^{m} \pm 0.05$	$0.91^{k} \pm 0.06$
6 ºC	$0.85^{kl} \pm 0.13$	$0.72^{lm} \pm 0.11$	$1.46^{g} \pm 0.24$	$1.35^{gh} \pm 0.14$	$1.22^{hi} \pm 0.18$	$1.95^{e} \pm 0.05$
8 ºC	$1.10^{ij} \pm 0.10$	$0.96^{jk} \pm 0.2$	$1.65^{f} \pm 0.16$	$1.96^{e} \pm 0.05$	$1.51^{fg} \pm 0.08$	$2.42^{d} \pm 0.08$
25 ºC	$3.82^{b} \pm 0.19$	$3.12^{c} \pm 0.08$	$4.45^{a} \pm 0.09$	$3.78^{b} \pm 0.17$	$3.17^{c} \pm 0.19$	$4.41^{a} \pm 0.05$

Each value is the mean of transpiration rate of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.16 at $p \leq 0.05$.

Table 4.40 Effect of various levels of chilling stress on CO_2 concentration rate (µmole/mole) in tomato

Chilling stress	CO_2 concentration rate in non-transgenic plants			CO_2 concentration rate in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$86.78^{o} \pm 8.31$	$92.46^{no} \pm 10.63$	$109.94^{m} \pm 9.89$	$150.78^{i} \pm 16.77$	$132.46^{jk} \pm 12.05$	$185.57^{gh} \pm 19.76$
6 ºC	$105.17^{mn} \pm 12.77$	$116.35^{lm} \pm 11.8$	$145.37^{ij} \pm 15.84$	$178.17^{h} \pm 17.37$	$156.68^{i} \pm 16.93$	$209.82^{ef} \pm 18.52$
8 ºC	$145.66^{ij} \pm 19.3$	$124.95^{kl} \pm 13.28$	$198.69^{fg} \pm 19.48$	$217.66^{e} \pm 20.09$	$196.82^{fg} \pm 18.59$	$238.28^{d} \pm 21.93$
25 ºC	$264.63^{ab} \pm 23.29$	$255.38^{bc} \pm 24.59$	$278.21^{a} \pm 25.1$	$253.58^{bc} \pm 21.48$	$249.53^{cd} \pm 22.44$	$272.82^{a} \pm 23.52$

Each value is the mean of CO_2 concentration rate of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 13.73 at $p \leq 0.05$.

4.18.6 Leaf osmotic potential (MPa)

The three tomato transgenic lines were screened for their chilling tolerance on the basis of leaf osmotic potential (Ψs) before and after cold stress. The data given in table 4.43 indicated that the values of osmotic potential in leaf sap were almost alike (ranged from - 0.25 to – 0.44 MPa) between transgenic and NT plants of all the genotypes at normal growth conditions, while the osmotic potential in both types of plants decreased significantly with the increased chilling stress. The rate of decrease of Ψs was higher in transgenic lines than that of NT ones upon exposure to various low temperature stresses (Table 4.43). The respective values of Ψs in Rio Grande transgenic line were - 1.1, - 0.82 and - 0.64 MPa at 4, 6 and 8 ºC which exhibited 50.68, 26.15 and 20.75% decrease Ψs than that of NT plants. Similarly, the respective values of Ψs in Moneymaker transgenic line were 33.33, 32.2 and 17.02% lower than that of NT plants. The highest decrease in Ψs values were recorded in Roma transgenic line (Table 4.43). These results indicated the negative correlation between the overexpression of *DREB1A* under chilling stress and leaf osmotic potential, which is essential for maintaining higher relative water contents in transgenic tomato lines.

Table 4.41 Effect of various levels of chilling stress on photosynthetic rate (µmole/m^2/sec) in tomato

Chilling stress	Photosynthetic rate in non-transgenic plants			Photosynthetic rate in transgenic plants		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
4 ºC	$1.34^{o} \pm 0.08$	$1.81^{no} \pm 0.08$	$2.42^{mn} \pm 0.07$	$3.88^{k} \pm 0.18$	$2.36^{mn} \pm 0.12$	$4.96^{j} \pm 0.64$
6 ºC	$3.56^{kl} \pm 0.28$	$2.83^{lm} \pm 0.06$	$3.82^{k} \pm 0.05$	$8.26^{g} \pm 0.29$	$6.79^{hi} \pm 0.42$	$5.82^{ij} \pm 0.53$
8 ºC	$6.82^{h} \pm 0.15$	$5.12^{j} \pm 0.39$	$7.11^{h} \pm 0.46$	$11.68^{e} \pm 0.59$	$10.00^{f} \pm 0.22$	$12.71^{d} \pm 0.73$
25 ºC	$26.72^{b} \pm 0.89$	$19.67^{c} \pm 0.61$	$32.85^{a} \pm 1.36$	$26.47^{b} \pm 1.3$	$18.96^{c} \pm 0.59$	$32.32^{a} \pm 1.01$

Each value is the mean of photosynthetic rate of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.97 at $p \leq 0.05$.

Table 4.42 Effect of various levels of chilling stress on relative water contents (%) in tomato

Chilling stress	Relative water contents in non-transgenic plants			Relative water contents in transgenic plants		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
4 ºC	$23.77^{m} \pm 3.21$	$18.98^{m} \pm 2.91$	$13.82^{n} \pm 1.28$	$34.87^{jk} \pm 4.3$	$29.14^{l} \pm 2.47$	$39.00^{ij} \pm 1.53$
6 ºC	$38.78^{ij} \pm 4.94$	$32.36^{kl} \pm 4.23$	$45.47^{gh} \pm 5.95$	$48.62^{g} \pm 6.72$	$43.34^{hi} \pm 5.38$	$55.21^{f} \pm 6.3$
8 ºC	$60.59^{e} \pm 8.29$	$49.00^{g} \pm 7.3$	$63.54^{e} \pm 7.86$	$71.86^{d} \pm 7.67$	$65.05^{e} \pm 6.4$	$78.48^{c} \pm 4.64$
25 ºC	$86.24^{ab} \pm 9.16$	$83.43^{bc} \pm 8.95$	$89.49^{a} \pm 10.17$	$85.91^{ab} \pm 9.45$	$84.35^{b} \pm 8.04$	$89.94^{a} \pm 9.56$

Each value is the mean of relative water contents (RWC) of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 5.01 at $p \leq 0.05$.

Table 4.43 Effect of various levels of chilling stress on leaf osmotic potential (MPa) in tomato

Chilling stress	Leaf osmotic potential in non-transgenic plants			Leaf osmotic potential in transgenic plants		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
4 ºC	$-0.73^{def} \pm 0.02$	$-0.69^{efg} \pm 0.07$	$-0.81^{cd} \pm 0.08$	$-1.1^{a} \pm 0.11$	$-0.92^{bc} \pm 0.14$	$-1.17^{a} \pm 0.07$
6 ºC	$-0.65^{fgh} \pm 0.05$	$-0.59^{ghi} \pm 0.03$	$-0.69^{efg} \pm 0.05$	$-0.82^{cd} \pm 0.08$	$-0.78^{de} \pm 0.11$	$-0.96^{b} \pm 0.12$
8 ºC	$-0.53^{ijk} \pm 0.03$	$-0.47^{jkl} \pm 0.03$	$-0.33^{mn} \pm 0.07$	$-0.64^{fgh} \pm 0.09$	$-0.55^{hij} \pm 0.08$	$-0.71^{def} \pm 0.07$
25 ºC	$-0.43^{klm} \pm 0.04$	$-0.39^{lm} \pm 0.04$	$-0.27^{n} \pm 0.03$	$-0.44^{j-m} \pm 0.02$	$-0.41^{lm} \pm 0.04$	$-0.25^{n} \pm 0.05$

Each value is the mean of leaf osmotic potential of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.11 at $p \leq 0.05$.

4.19 Evaluation of cold tolerance in tomato on biochemical basis

4.19.1 Membrane leakage (%)

The stability of plasma membrane in transgenic plants was examined under various chilling treatments in term of electrolyte leakage (%) and compared with NT plants. Data about membrane leakage presented in Table 4.44 indicated that cold stress significantly affected ion leakage in transgenic and NT tomato lines. Under non-stressed conditions, no significant differences were recorded among transgenic and NT lines having values ranging between 16.78 – 24.87% (Table 4.44). Increase in chilling stress was accompanied by the increase in ion leakage in the leaves of all transgenic lines. The highest leakage was recorded in NT plants of Moneymaker (78.65%) followed by Rio Grande (68.72%) and Roma (58.88%) that was comparable to the isogenic counterparts of Moneymaker (35.75%), Rio Grande (29.08%) and Roma (18.78%) when plants were exposed to 4 ºC for seven days. The maximum reduction in membrane leakage was observed in transgenic Roma (10.85%) followed by Rio Grande (13.94%) and Moneymaker (22.24%) at 6 ºC as compared to their NT counterparts that exhibited 62.25, 60.26 and 50.89% membrane leakage, respectively (Table 4.44). Among various chilling stresses, the lowest leakage percentage was obtained at 8 ºC in transgenic plants of Roma (8.65%) followed by Rio Grande (10%) and Moneymaker (15.63%) that was too much lower than that of NT plants of Roma, Rio Grande and Moneymaker.

4.19.2 Proline contents (µg/g FW)

In current research study, three weeks old transgenic and NT plants of Rio Grande, Moneymaker and Roma were subjected to chilling stresses (4, 6 and 8 °C) for 7 days which exhibited pronounced wilting symptoms in all NT plants due to turgor injury (increase in osmotic stress). Chilling stress at 4 °C showed more wilting and necrosis symptoms as compared to 6 and 8 °C chilling stresses in NT plants, while all the transgenic plants regained their growth after one day recovery period (Figs. 4.29, 4.30 & 4.31). Chilling treatements enhanced proline contents in leaves of 3-week old tomato seedlings during recovery period. But the rate of increment of proline contents was significantly higher in transgenic lines than that of their isogenic NT plants. The

significant variation in proline contents was observed among transgenic and NT plants at 4, 6 and 8 ºC, while no significant variation was noticed among transgenic and NT plants at 25 ºC. The proline contents gradually decreased with the decrease in temperature in NT plants (cold sensitive), indicating the influence of low temperature on proline production. The transgenic plants of all the genotypes showed an increase in proline contents under low temperature (Table 4.45). Our results suggested that Roma transgenic line was the best adaptive to cold stress accumulating the highest proline contents (674.94 µg/g FW) triggered by *CBF3* at 8 ºC followed by Rio Grande (645.5 µg/g FW) and Moneymaker (586.52 µg/g FW) (Table 4.45). These results clearly demonstrated that chilling stresses could improve endogenous proline contents in transgenic lines due to inducible overexpression of *CBF3* gene.

4.19.3 Total soluble sugar contents (mg/g FW)

To investigate whether the expression of *CBF3* gene was correlated with the enhancement of total soluble sugars (TSS) contents in tomato under cold stress; transgenic and NT plants (T_2) were exposed to various low temperatures (4, 6 and 8 ºC) for seven days (Figs. 4.29, 4.30 & 4.31). At 25 ºC, no significant differences were found among transgenic and NT plants in total soluble sugar contents in three tomato genotypes which were around 3.62 to 10.7 mg/g FW (Table 4.46). After exposure to cold stress, the total soluble sugars contents enhanced in both transgenic and NT plants. The sugar levels decreased gradually by lowering the temperature. The highest TSS level accumulated in transgenic Roma (90.75 mg/g) followed by Rio Grande (85.63 mg/g) and Moneymaker (75.03 mg/g) at 8 ºC, compared to the NT counterparts of Roma (43.56 mg/g), Rio Grande (37.24 mg/g) and Moneymaker (32.07 mg/g). Similarly, the respective TSS contents in transgenic lines; Roma, Rio Grande and Moneymaker were 79.81, 69.32 and 63.57 (mg/g) that were significantly higher than that of NT plants of Roma (34.77 mg/g), Rio Grande (29.92 mg/g) and Moneymaker (26.09 mg/g) at 6 ºC (Table 4.46). The lowest TSS contents were recorded at 4 ºC in transgenic tomato plants which were significantly higher than that of their isogenic counterparts (Table 4.46).

Table 4.44 Comparison of membrane leakage percentage of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Membrane leakage (%) in non-transgenic plants			Membrane leakage (%) in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$68.72^{b} \pm 4.75$	$78.65^{a} \pm 5.77$	$58.88^{c} \pm 4.7$	$29.08^{g} \pm 3.17$	$35.75^{f} \pm 3.34$	$18.78^{kl} \pm 1.5$
6 ºC	$60.26^{c} \pm 3.21$	$62.25^{c} \pm 4.36$	$50.89^{d} \pm 3.12$	$13.94^{mn} \pm 0.71$	$22.24^{ij} \pm 1.63$	$10.85^{no} \pm 0.8$
8 ºC	$35.86^{f} \pm 2.67$	$43.68^{e} \pm 2.95$	$26.57^{gh} \pm 2.69$	$10.00^{o} \pm 0.85$	$15.63^{lm} \pm 0.42$	$8.65^{o} \pm 0.26$
25 ºC	$24.87^{hi} \pm 1.56$	$21.29^{jk} \pm 1.39$	$16.78^{lm} \pm 1.07$	$22.57^{ij} \pm 1.37$	$20.95^{jk} \pm 0.78$	$17.29^{lm} \pm 0.93$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 3.44 at $p \leq 0.05$.

Table 4.45 Comparison of proline contents (µg/g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Proline contents in non-transgenic plants			Proline contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$87.80^{kl} \pm 28.90$	$68.69^{lmn} \pm 24.58$	$105.20^{k} \pm 28.88$	$328.10^{f} \pm 53.24$	$246.41^{h} \pm 52.92$	$381.90^{e} \pm 42.25$
6 ºC	$256.78^{gh} \pm 72.46$	$166.02^{j} \pm 47.19$	$204.81^{i} \pm 36.40$	$432.20^{d} \pm 58.95$	$375.69^{e} \pm 67.71$	$483.40^{c} \pm 48.15$
8 ºC	$367.20^{e} \pm 63.11$	$282.49^{g} \pm 53.37$	$353.46^{ef} \pm 40.84$	$645.50^{a} \pm 54.88$	$586.52^{b} \pm 75.13$	$674.94^{a} \pm 52.10$
25 ºC	$60.73^{lmn} \pm 19.49$	$45.33^{mn} \pm 17.13$	$73.68^{k-n} \pm 15.78$	$62.80^{lmn} \pm 18.59$	$42.78^{n} \pm 13.57$	$76.93^{klm} \pm 18.08$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 31.72 at $p \leq 0.05$.

Table 4.46 Comparison of total soluble sugar contents (mg/g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Total soluble sugar contents in non-transgenic plants			Total soluble sugar contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$15.49^{no} \pm 1.33$	$12.64^{op} \pm 0.31$	$18.26^{n} \pm 0.94$	$52.81^{h} \pm 2.52$	$40.72^{i} \pm 2.2$	$57.54^{g} \pm 2.72$
6 ºC	$29.92^{l} \pm 1.8$	$26.09^{m} \pm 1.37$	$34.77^{jk} \pm 2.7$	$69.32^{e} \pm 1.9$	$63.57^{f} \pm 1.86$	$79.81^{c} \pm 4.65$
8 ºC	$37.24^{j} \pm 1.98$	$32.07^{kl} \pm 1.71$	$43.56^{i} \pm 2.55$	$85.63^{b} \pm 3.35$	$75.03^{d} \pm 2.33$	$90.75^{a} \pm 5.61$
25 ºC	$7.25^{r} \pm 0.31$	$3.96^{s} \pm 0.34$	$9.87^{pqr} \pm 0.49$	$7.8^{qr} \pm 0.3$	$3.62^{s} \pm 0.51$	$10.7^{pq} \pm 0.71$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 3.25 at $p \leq 0.05$.

4.19.4 Chlorophyll contents (mg/g FW)

Data concerning chlorophyll "a" presented in Table 4.47 demonstrated that there were significant differences in chlorophyll "a" contents after various chilling treatments (4, 6 and 8 ºC). The chlorophyll "a" contents relatively decreased by lowering the cold treatments for seven days. There were no obvious differences among transgenic and NT plants of three tomato genotypes at 25 ºC. After chilling treatment (8 ºC), the highest level of chlorophyll "a" (10.15, 8.16 and 7.64 mg/g FW) was recorded in transgenic plants of Roma, Rio Grande and Moneymaker as compared to their NT plants that exhibited 3.42, 3.96 and 2.19 mg/g, respectively. Whereas, the minimum chlorophyll "a" levels (3.39, 2.94 and 1.89 mg/g) were recorded in transgenic plants of Roma, Rio Grande and Moneymaker that were higher than that of their NT counterparts at 4 ºC.

Data about chlorophyll "b" contents presented in Table 4.48 showed that chilling stress significantly affected chlorophyll "b" contents in both types of plants. The chlorophyll "b" contents of Roma transgenic line (8.11, 9.12 and 16.97 mg/g) were statistically higher than that of its NT plants (3.61, 6.56 and 10.89 mg/g) at 4, 6 and 8 ºC. It was followed by transgenic line of Rio Grande where chlorophyll "b" contents (3.97, 9.38 and 14.61 mg/g) were recorded at 4, 6 and 8 ºC that were comparable to chlorophyll "b" contents (1.12, 5.96 and 6.83 mg/g) of its counterparts. Similarly, the lowest contents of chlorophyll "b" (1.92, 7.85 and 11.96 mg/g) were obtained in transgenic line of Moneymaker at 4, 6 and 8 ºC that were higher to its NT counterparts that exhibited 0.87, 3.88 and 4.98 mg/g chlorophyll "b" contents.

Total chlorophyll contents of transgenic and NT plants of three tomato genotypes were extracted after exposing to three chilling stresses to distinguish among transgenic and NT plants (Figs. 4.29, 4.30 & 4.31). At 25 ºC, there were no obvious differences in total chlorophyll contents among transgenic and NT plants of three tomato lines. After exposing to chilling stress, the total chlorophyll contents rapidly enhanced in transgenic lines. The highest contents of total chlorophyll (23.38 mg/g) were secured in transgenic Roma plants comparable to its counterparts that exhibited 12.11 mg/g total chlorophyll contents after exposing the plants at 8 ºC (Table 4.49). It was followed by Rio Grande transgenic line which exhibited 19 mg/g total chlorophyll contents, while its NT plants

gave 10.69 mg/g at 8 ºC. The lowest total chlorophyll contents (3.64 mg/g) were recorded in Moneymaker transgenic line that was higher than that of its counterparts that gave 1.73 mg/g total chlorophyll contents (Table 4.49). The greater contents of chlorophyll in transgenic tomato lines than that of their NT plants indicated the greater stability of chlorophyll that correlated with their tolerance to cold stress with reduced damage.

4.19.5 Carotenoid contents (mg/g FW)

The transgenic plants of three tomato genotypes with *CBF3/DREB1A* gene and their counterparts were compared on the basis of carotenoid contents under various chilling treatments (4, 6 and 8 ºC). Without any stress, the carotenoid contents were found almost similar in *CBF3* and NT plants of all the lines investigated. Upon cold treatment, the transgenic plants had significantly ($p \leq 0.05$) higher carotenoid contents than that of NT plants (Table 4.50). In some transgenic tomato lines, the carotenoid contents increased up to three times than that of their NT plants. The highest carotenoid contents (2.41, 5.16 and 7.98 mg/g FW) were recorded in transgenic plants of Roma at 4, 6 and 8 ºC, respectively that were comparable to its NT plants that gave 1.41, 3.05 and 5.94 mg/g carotenoid contents. It was followed by transgenic Rio Grande where maximum carotenoid contents (1.93, 3.91 and 6.85 mg/g) were obtained at 4, 6 and 8 ºC that were higher than that of their counterparts exhibiting 0.84, 2.61 and 4.74 mg/g carotenoid contents (Table 4.50). The lowest carotenoid contents (0.98, 4.18 and 6.09 mg/g) were secured in Moneymaker transgenic plants that were higher than that of their NT plants at 4, 6 and 8 ºC.

4.19.6 Ascorbic acid contents (mg/100 g FW)

The selected T_2 transgenic and NT tomato lines were examined to check their chilling tolerance on the basis of ascorbic acid (AsA) contents. Under normal temperature (25 ºC), *DREB1A* plants and NT plants did not exhibit any significant differences in AsA contents. However, after chilling stress, clear differences were noticed in susceptibility and cellular injury of NT plants in terms of necrosis and shoots collapsing at all low

Table 4.47 Comparison of chlorophyll "a" contents (mg/g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Chlorophyll "a" contents in non-transgenic plants			Chlorophyll "a" contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$1.000^{mn} \pm 0.32$	$0.98^{mn} \pm 0.21$	$0.78^{n} \pm 0.32$	$2.94^{hij} \pm 0.48$	$1.89^{kl} \pm 0.35$	$3.39^{ghi} \pm 0.64$
6 ºC	$2.62^{ijk} \pm 0.53$	$1.97^{kl} \pm 0.38$	$1.38^{lmn} \pm 0.38$	$6.34^{c} \pm 0.74$	$4.49^{de} \pm 0.62$	$7.72^{b} \pm 1.25$
8 ºC	$3.96^{d-g} \pm 0.67$	$2.19^{jkl} \pm 0.51$	$3.42^{f-i} \pm 0.54$	$8.16^{b} \pm 1.33$	$7.64^{b} \pm 1.21$	$10.15^{a} \pm 1.47$
25 ºC	$3.72^{e-h} \pm 0.53$	$1.72^{lm} \pm 0.42$	$4.23^{def} \pm 0.63$	$3.84^{efg} \pm 0.39$	$1.85^{kl} \pm 0.32$	$4.69^{d} \pm 0.61$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.83 at $p \leq 0.05$.

Table 4.48 Comparison of chlorophyll "b" contents (mg/g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Chlorophyll "b" contents in non-transgenic plants			Chlorophyll "b" contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$1.12^{no} \pm 0.42$	$0.87^{o} \pm 0.11$	$3.61^{kl} \pm 0.32$	$3.97^{jk} \pm 0.48$	$1.92^{mn} \pm 0.18$	$8.11^{f} \pm 0.5$
6 ºC	$5.96^{gh} \pm 0.68$	$3.88^{jk} \pm 0.29$	$6.56^{g} \pm 0.65$	$9.38^{e} \pm 1.06$	$7.85^{f} \pm 0.35$	$9.12^{e} \pm 0.46$
8 ºC	$6.83^{g} \pm 0.8$	$4.98^{hi} \pm 0.44$	$10.89^{d} \pm 0.74$	$14.61^{b} \pm 0.88$	$11.96^{c} \pm 0.28$	$16.97^{a} \pm 1.52$
25 ºC	$1.51^{no} \pm 0.41$	$2.85^{lm} \pm 0.35$	$4.85^{ij} \pm 0.42$	$1.85^{mno} \pm 0.06$	$3.33^{kl} \pm 0.52$	$5.05^{hi} \pm 0.34$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.007 at $p \leq 0.05$.

Table 4.49 Comparison of total chlorophyll contents (mg/g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Total chlorophyll contents in non-transgenic plants			Total chlorophyll contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$3.73^{lm} \pm 1.25$	$1.73^{n} \pm 0.73$	$3.29^{m} \pm 0.41$	$7.29^{hi} \pm 2.05$	$3.64^{lm} \pm 1.41$	$8.84^{g} \pm 1.14$
6 ºC	$7.12^{hi} \pm 1.76$	$4.28^{lm} \pm 1.37$	$8.08^{gh} \pm 1.78$	$14.38^{d} \pm 3.16$	$11.58^{ef} \pm 2.28$	$19.68^{b} \pm 2.41$
8 ºC	$10.69^{f} \pm 2.11$	$7.07^{h} \pm 1.71$	$12.11^{e} \pm 1.69$	$19.00^{b} \pm 3.23$	$16.26^{c} \pm 3.04$	$23.38^{a} \pm 2.05$
25 ºC	$6.18^{ij} \pm 1.29$	$4.64^{kl} \pm 1.21$	$10.49^{f} \pm 1.38$	$6.51^{ij} \pm 1.4$	$5.68^{jk} \pm 1.41$	$11.38^{ef} \pm 1.41$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.33 at $p \leq 0.05$.

temperatures (4, 6 and 8 ºC) leading to plant death within few days. In case of transgenic plants, no severe symptoms of wilting after chilling treatments were observed and plants continued their normal growth once maintained to optimum culture conditions (Figs. 4.29, 4.30 & 4.31). The highest level of AsA (43.72, 67.24 and 89.79 mg/100 g) was recorded in Roma transgenic line which exhibited 48, 38.04 and 49.65% increase in AsA contents over NT plants followed by Rio Grande transgenic line (37.64, 62.75 and 85.74 mg/100 g) which showed 78.55, 93.01 and 64.31% increase in AsA levels over NT plants at 4, 6 and 8 ºC (Table 4.51). Similarly, the lowest level of AsA (40.53, 57.84 and 79.18 mg/100 g) was obtained in Moneymaker transgenic line which indicated 68.52, 59.2 and 65.23% increase in AsA contents over NT plants at 4, 6 and 8 ºC. The normal growth of all these transgenic lines under cold stress showed that high AsA contents could counteract the lethal effects of reactive oxygen species (ROS) by overexpressing *DREB1A*.

4.19.7 Malondialdehyde contents (nmol/g FW)

Three weeks old T_2 transgenic tomato lines along with NT plants were incubated at 4, 6 and 8 ºC for seven days to check malondialdehyde (MDA) contents. The NT plants exhibited severe wilting five days post-treatment, while transgenic lines were not severely affected and showed normal growth at all chilling stresses (Figs. 4.29, 4.30 & 4.31). At normal temperature, there were no marked differences among the values of MDA contents of all the transgenic lines in both transgenic and NT plants that ranged from 7.29 – 9.19 nmol/g FW. After chilling treatments, MDA contents in leaves of all the lines increased significantly, but the rate of increment being too lower in transgenic plants compared to NT ones (Table 4.52). The values of MDA in transgenic Roma were 41.02, 28.69 and 16.33% lower than that of NT plants followed by Rio Grande whose values of MDA in transgenic plants were 40.45, 29.97 and 19.68% lower than that of its counterparts at 4, 6 and 8 ºC. Similarly, the value of MDA contents (38.64, 32.45 and 22.88%) were recorded in transgenic Moneymaker that were lower than that of its NT plants at 4, 6 and 8 ºC, respectively. These results suggest that transgenic tomato lines reduced membrane damage by accumulating lower MDA contents, thus maintaining membrane integrity at low temperatures.

Fig. 4.29 Evaluation of cold tolerance in transgenic and NT plants of tomato genotype i.e. Rio Grande under various low temperature regimes. Three week old seedlings of transgenic and NT plants were exposed to cold stresses for seven days and maintained at 25 ºC for 1 day. Photographs were taken 9 days after the exposure of various cold stresses (A) First three NT plants from left showing necrosis, while transgenic plants are lush green at 4 ºC (B) Phenotypically, first three NT plants from left are showing severe wilting, whereas transgenic plants demonstrate normal growth at 6 ºC (C) Morphologically, first three NT plants from left show more wilting as compared to their counterparts which are showing normal growth at 8 ºC

Fig. 4.30 Evaluation of cold tolerance in transgenic and NT plants of tomato genotype i.e. Moneymaker under various low temperature regimes. To produce transgenic tomato (*Solanum lycopersicum*), we used lip9 inducible promoter to overexpress *Arabidopsis CBF3* gene in tomato (*Solanum lycopersicum* cv. Moneymaker) (A) Phenotypes of first three lip9:*CBF3* tomato plants from right with respect to NT plants after exposure to cold stress (4 ºC) (B) Cold tolerance of first five lip9:*CBF3* tomato plants from right after exposure to a temperature of 6 ºC for 7-d and returned to normal temperature for one day as compared to NT plants (C) Phenotypes of first three tomato transgenic plants from right overexpressing *CBF3* gene whose leaves did not exhibit significantly curling and wilting as compared to NT plants after exposure to a temperature of 8 ºC. Photographs are indicatives of 3 independent experiments (n = 35 for each experiment)

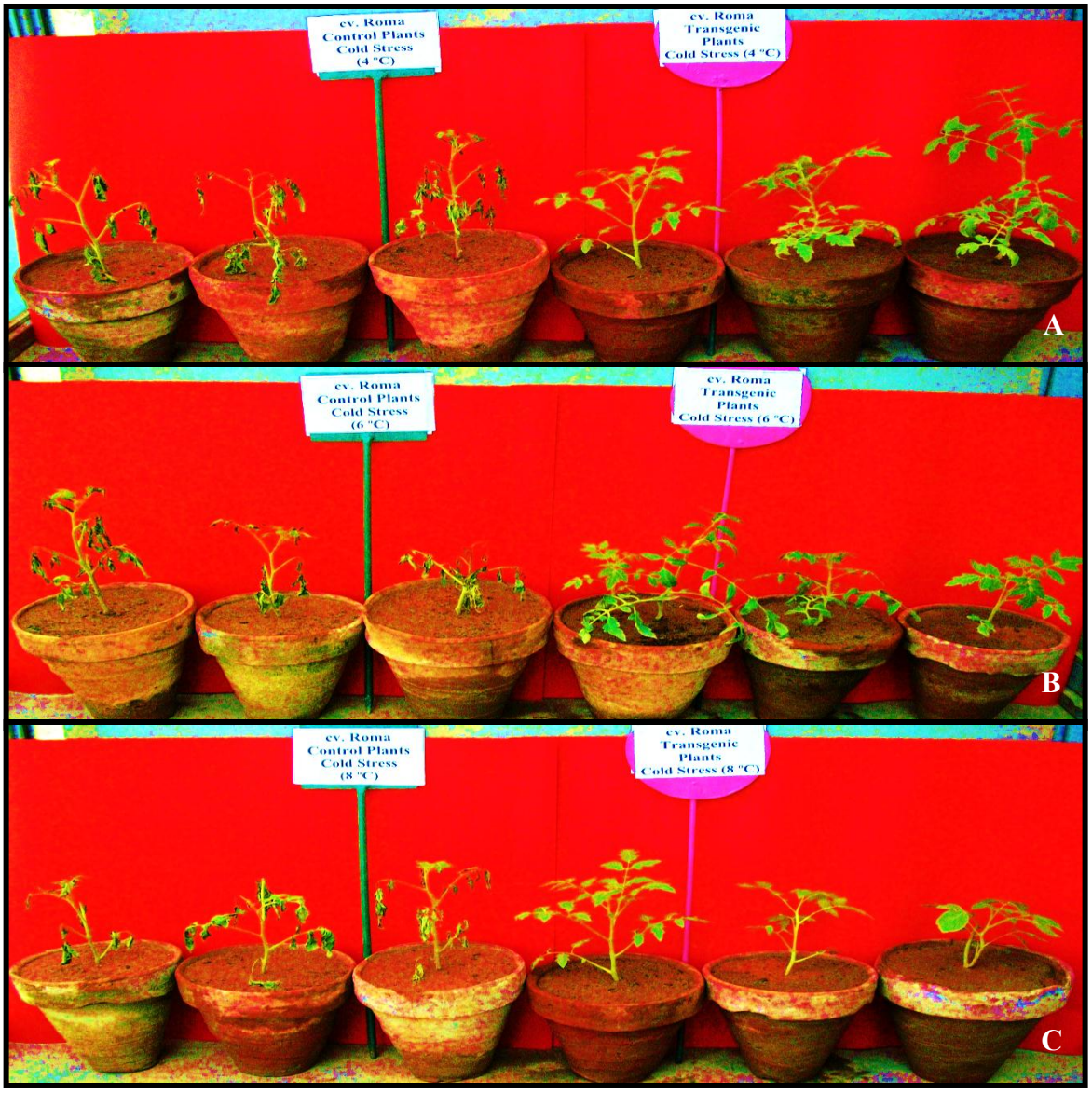

Fig. 4.31 Evaluation of cold tolerance in transgenic and NT plants of tomato genotype i.e. Roma under various low temperature regimes (A) Phenotypic responses of first three transgenic plants from right and NT plants, where NT plants showed severe wilting symptoms as compared to transgenic plants after 4 °C cold stress (B) Phenotypic changes of first three NT plants from left and transgenic plants by exposing to low temperature (6 °C) for seven days followed by maintenance at 25 °C for one day (C) Phenotypes of first three NT plants from left showing clearly the wilting and dried leaves symptoms compared to their counterparts showing almost normal growth at 8 °C temperature stress

Table 4.50 Comparison of carotenoid contents (mg/g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Carotenoid contents in non-transgenic plants			Carotenoid contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$0.84^{kl} \pm 0.12$	$0.23^{l} \pm 0.1$	$1.41^{ijk} \pm 0.41$	$1.93^{hij} \pm 0.24$	$0.98^{k} \pm 0.23$	$2.41^{gh} \pm 0.31$
6 ºC	$2.61^{gh} \pm 0.35$	$1.28^{jk} \pm 0.44$	$3.05^{g} \pm 0.34$	$3.91^{f} \pm 0.55$	$4.18^{ef} \pm 0.46$	$5.16^{d} \pm 0.15$
8 ºC	$4.74^{de} \pm 0.45$	$2.39^{gh} \pm 0.53$	$5.94^{c} \pm 0.6$	$6.85^{b} \pm 1.05$	$6.09^{c} \pm 0.18$	$7.98^{a} \pm 0.59$
25 ºC	$1.11^{k} \pm 0.4$	$2.05^{hi} \pm 0.09$	$2.91^{g} \pm 0.42$	$1.15^{k} \pm 0.1$	$2.15^{h} \pm 0.33$	$3.00^{g} \pm 0.51$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 0.68 at $p \leq 0.05$.

Table 4.51 Comparison of ascorbic acid (AsA) contents (mg/100 g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Ascorbic acid contents in non-transgenic plants			Ascorbic acid contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$21.08^{mn} \pm 1.68$	$24.05^{m} \pm 0.7$	$29.54^{l} \pm 0.51$	$37.64^{jk} \pm 1.06$	$40.53^{ij} \pm 2.15$	$43.72^{i} \pm 3.04$
6 ºC	$32.51^{l} \pm 0.95$	$36.33^{k} \pm 1.26$	$48.71^{gh} \pm 1.34$	$62.75^{e} \pm 2.63$	$57.84^{f} \pm 1.6$	$67.24^{d} \pm 4.46$
8 ºC	$52.18^{g} \pm 1.3$	$47.92^{h} \pm 1.47$	$60.00^{ef} \pm 1.62$	$85.74^{b} \pm 4.67$	$79.18^{c} \pm 4.7$	$89.79^{a} \pm 5.54$
25 ºC	$17.83^{n} \pm 0.34$	$11.36^{o} \pm 0.42$	$23.35^{m} \pm 1.38$	$17.56^{n} \pm 0.4$	$11.79^{o} \pm 0.83$	$23.95^{m} \pm 1.92$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 3.76 at $p \leq 0.05$.

Table 4.52 Comparison of malondialdehyde (MDA) contents (nmol/g FW) of T_2 transgenic and NT plants under cold stresses in tomato

Chilling stress	Malondialdehyde contents in non-transgenic plants			Malondialdehyde contents in transgenic plants		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
4 ºC	$18.17^{b} \pm 1.18$	$15.63^{c} \pm 0.79$	$20.22^{a} \pm 1.14$	$10.81^{e} \pm 0.73$	$9.59^{fg} \pm 0.66$	$11.92^{d} \pm 0.59$
6 ºC	$6.93^{j} \pm 0.27$	$11.02^{de} \pm 1.00$	$14.94^{c} \pm 1.09$	$4.86^{kl} \pm 0.19$	$7.45^{ij} \pm 0.54$	$10.66^{ef} \pm 0.52$
8 ºC	$5.78^{k} \pm 0.19$	$2.83^{no} \pm 0.34$	$3.98^{lm} \pm 0.34$	$4.64^{l} \pm 0.12$	$2.19^{o} \pm 0.19$	$3.33^{mn} \pm 0.19$
25 ºC	$7.54^{ij} \pm 0.38$	$8.86^{gh} \pm 0.74$	$8.92^{gh} \pm 0.96$	$7.29^{ij} \pm 0.52$	$8.27^{hi} \pm 0.57$	$9.18^{gh} \pm 0.67$

The values are means of three plants. Mean values following by the different letters show significant differences ($p \leq 0.05$). The values after ± sign demonstrate standard deviation (n = 3). LSD value was 1.09 at $p \leq 0.05$.

V. DISCUSSION

5.1 Seed sterilization and germination

The most widely used chemical for seed surface sterilization of tomato is sodium hypochlorite (NaOCl) (Mercado *et al.,* 2000; Steinitz and Bilavendran, 2011). Superior germination frequency is prerequite for plant tissue culture (Sakhanokho *et al.,* 2001). During this study, 40% clorox (v/v) was found to be optimal for seed sterilization with the highest germination frequency ranging from 76.19 – 88.09% in three tomato genotypes. Our findings suggested clorox (40%) along with Tween 20 that enhanced seed germination frequency removing contamination. Chetty *et al.* (2012) reported 25% NaOCl with 0.1% Tween 20 for sterilization of seeds of cv. Micro-Tom for twenty minutes. Two to three weeks old *in vitro* seedlings of three tomato genotypes were used as explant sources. Eighteen days old *in vitro* plants were reported for callus induction and *in vitro* shoot regeneration by Hu & Philips (2001). Our findings were in contrast with that of Reda *et al.* (2004) who reported six days old *in vitro* seedling for callus induction and *in vitro* shoot regeneration. It might be due to difference in genotypes. The younger explants exhibit better response than that of older ones because culturing of older tissues causes browning in culture medium. The excision of older explants initiates the tissues to remove phenolic compounds that are easily oxidized. These oxidative products are toxic and cause necrosis leading to explants death (Zenkteler and Kwasna, 2007).

5.2 Hypocotyls and leaf discs as the best explants sources

The explants of tomato present a model system that can provide the fundamental knowledge about the expression of competence for *in vitro* shoot regeneration during tissue culture studies (Lercari *et al.,* 1999; Bertram and Lercari, 2000). It has been reported that explants have a direct effect on callus induction and *in vitro* shoot regeneration (Schween and Schwenke, 2003). In tomato tissue culturing, hypocotyls have been reported as one of the best explants source for callus induction and shoot regeneration (De Faria *et al.,* 2002; Lercari and Bertram, 2004). Similarly, leaf discs have also been reported for callus formation and *in vitro* shoot regeneration in previous reports

(Sigareva *et al.*, 2004; Mamidala and Nanna, 2009; Roy *et al.*, 2011). In our studies, hypocotyls and leaf discs were found the most responsive explants producing the highest shoot regeneration frequency and more number of shoot primordial per explants. The similar type of study was carried out by Gubis *et al.* (2003) who investigated the effect of six explants on shoot organogenesis in 13 cultivars of tomato (*Solanum lycopersicum* Mill.). According to this report, the *in vitro* shoot regeneration capacity significantly affected by the explant types and they inferred that hypocotyls and epicotyls produced the highest regeneration frequency (100%) yielding 6.3 and 6.5 number of primordial shoot per explant. Lalage *et al.* (2007) scrutinized three types of explants namely cotyledon, young leaf and hypocotyls and proposed that young leaf was the most suitable source of explants for callus induction in tomato. Gubis *et al.* (2004) tested the effect of hypocotyls and cotyledons explants on *in vitro* shoot regeneration in tomato and recommended hypocotyls for efficient *in vitro* shoot regeneration and more number of shoots per explant than that of cotyledons. Our findings were in parallelism with the report of Moghaleb *et al.* (1999) who outlined that hypocotyls produced 70.2% *in vitro* shoot regeneration frequency compared to cotyledons explants that yielded only 35.3% shoot regeneration in tomato. Our findings were not in agreement with El-Bakry *et al.* (2002) who suggested cotyledons for *in vitro* morphogenesis in tomato. This contrast might be due to variation in genotypes examined.

5.3 Effect of auxin and cytokinin combination on callus induction

The callus induction is greatly influenced by various genotypes, explants, common media and hormonal concentrations (Applewhite *et al.*, 1994; Nikam and Shitole, 1998). Our findings were consistent with the results by Baste *et al.* (2007) who reported that there were differential responses of callus induction and regeneration in diverse genotypes of tomato. During the present study, MS medium fortified with 2.0 mg/l IAA and 2.5 mg/l BAP gave the highest callus induction frequency. The low frequencies of callus induction were recorded on MS medium supplemented with IAA (1.0 mg/l), NAA (1.0 mg/l), Kin (2.0 mg/l) and also with IAA - 2,4-D (0.5 – 2.0 mg/l) from both types of explants. The hormonal combinations of NAA and BAP were also used by Oh *et al.* (2006) and Soria-Guerra *et al.* (2007) that correlated with our findings.

In our studies**,** it has been proved that BAP played a significant role for callus induction in combination with auxins in various tomato cultivars. On the contrary, the media having only auxin in the form of 2, 4-D at 3.0 mg/l and 4.0 mg/l did not show any callus induction response in all the genotypes. Plevnes *et al.* (2006) reported maximum callus induction on MS medium supplemented with 2.0 mg/l IAA and 1.0 mg/l BAP which was converse to our findings. Our findings clearly demonstrate that the best calli induction frequency can be achieved with higher concentration of cytokinins than that of auxins. Our findings were consistent with the earlier report by Raj *et al.* (2005) who concluded that callus was usually formed on medium fortified with higher cytokinins to moderate level of auxin. Lambe *et al.* (1997) ascribed that callus could be produced at the cut surfaces of dicotyledoneous explants by the propitious use of auxin and cytokinin and *in vitro* shoot regeneration was enhanced by altering the hormonal ratio. According to their findings, the long term plant tissue culture results in the huge loss of totipotency of organogensis to initiate cell differentiation.

Our findings were inconsistent with the report by Leljak-Levanic *et al.* (2004) who described that maximum calli induction was recorded on MS medium supplemented with 2,4-D which has been widely used auxin for *in vitro* callus induction in many crop species. In our study, varying levels of auxin (IAA, NAA) rather than 2,4-D proved to be critical in yielding morphogenic calli in all the genotypes examined. These variations in callus induction might be due to difference in genetic composition of genotypes. Brasileiro *et al.* (1999) working on callus induction and organogenesis in tomato through various hormonal regimes were able to produce anther callus on media supplemented with BAP (1.0 mg/l) and NAA (1.0 mg/l). Lalage *et al.* (2007) scrutinized the combinational influence of auxin and cytokinin on callus induction frequency in five tomato genotypes culturing cotyledon, hypocotyls and leaf explants. According to this report, cent percent callus formation was obtained on MS medium containing NAA (2.0 mg/l) and BAP (0.5 mg/l) from all the explants in five genotypes. These results are dissonant with our findings because in our study, more levels of cytokinin than that of auxin were found suitable for efficient callus induction in tomato. It might be due to variation in genotypes.

5.4 Effect of silver nitrate on callus induction

During this study, different concentrations of silver nitrate ($AgNO_3$) along with various hormonal regimes were investigated on callus induction frequency in three tomato genotypes. All the genotypes responded differently on various levels of $AgNO_3$. In case of cv. Moneymaker, the highest callogenesis was recorded on 10 mg/l $AgNO_3$. While in Roma and Rio Grande, the best callus induction frequency was obtained on 12.5 mg/l and 15 mg/l $AgNO_3$, respectively. The current study has clearly illustrated the effect of $AgNO_3$ on callus induction in tomato culturing hypocotyls and leaf discs explants. The hypocotyls performed better in producing percent green calli than that of leaf discs. Our findings were in line with Rakshit *et al.* (2010) who carried out research in five inbred lines of maize for callus induction and *in vitro* shoot regeneration and reported $AgNO_3$ (15 mg/l) supplemented medium for efficient organogenic calli induction culturing fourteen days old embryos. The effect of $AgNO_3$ was tested on callus proliferation in *Hevea brasiliensis* and it was found that 20 mg/l $AgNO_3$ improved the callus texture which in turn enhanced the embryogenic callus induction frequency (Kala *et al.*, 2013). It has been reported that collar region of hypocotyls explants produced yellow friable embryogenic callus in *Coffea dewevrei* on half strength MS medium complemented with 6.8 mg/l $AgNO_3$ in combination with IAA and BA (Sridevi and Giridhar, 2014). Fei *et al.* (2000) suggested 10 mg/l $AgNO_3$ for embryogenic callus induction yielding the highest callus induction frequency (79.9%) in buffalograss. Purnhauser and Gyulai (1993) conducted a tissue culture study on wheat, triticale, rape and tobacco and reported that $AgNO_3$ (17 mg/l) strongly enhanced the number of morphogenic calli upto 68% and more than 90% calli of *Brassica napus* were green on $AgNO_3$ supplemented medium. Lua Figueiredo *et al.* (2000) investigated the effect of $AgNO_3$ on callus induction and reported that the callus cultures devoid of $AgNO_3$ reduced the callus fresh weight and compact texture, while supplementation of $AgNO_3$ (17 mg/l) to media having 2, 4-D or NAA enhanced callus growth within three to four weeks reducing the tissue browning. Kothari-Chajer *et al.* (2008) reported that inorganic nutrients such as $CoCl_2$ and $AgNO_3$ had a stimulatory influence on callus induction in *Paspalum scrobiculatum* and *Eleusine coracana*. According to their report, MS medium augmented with $AgNO_3$ produced

highly embryogenic and friable callus in both plants. $AgNO_3$ was crucial for the maintenance of callus culture. $AgNO_3$ (2.0 mg/l) yielded the highest callus induction in cabbage (*Brassica oleracea* var. capitata) and stimulated the shoot organogenesis from cotyledons explants in Chinese cabbage (Achar, 2002). Williams *et al.* (1990) investigated the effect of $AgNO_3$ on organogenic calli and long-term culture in *Brassica oleracea* and reported that $AgNO_3$ (3.0 mg/l) significantly enhanced the callus growth and permitted the callus culture for long time duration. They also reported that calli became dead culturing the hypocotyls explants on media devoid of $AgNO_3$, while $AgNO_3$ supplemented medium grew green calli vigorously.

5.5 Effect of cobalt chloride on callus induction

The effect of cobalt chloride ($CoCl_2$) along with various combinations of plant growth regulators was evaluated on callus induction frequency in three tomato genotypes. During this study, it was found that callus induction frequency significantly improved at all the concentrations of $CoCl_2$. We obtained the highest CIF in cv. Rio Grande and Roma with the application of 5.0 mg/l $CoCl_2$, while in cv. Moneymaker, the best callogenesis was recorded on 4.25 mg/l cobalt chloride in combination with different hormonal regimes. In the previous study, the effect of $CoCl_2$ was explored on *in vitro* cultures of *Coffea canephora* and found that MS medium enriched with 3.4 – 6.8 mg/l $CoCl_2$ along with BA (1.1 µM) and IAA (2.85 µM) yielded yellow and friable embryogenic calli from cut edges of cotyledonary leaves and hypocotyls (Kumar *et al.,* 2007). The efficiency of callus induction in recalcitrant barley was also improved by modified nutrient levels in MS basal medium inclusive of picloram (20.70 µM), NH_4NO_3 (10.30 mM), KH_2PO_4 (6.25 mM), Na_2MoO_4 (2.06 µM), glycine (26.64 µM) and $CoCl_2$ (0.55 µM) giving morphogenic calli within six weeks (Chauhan and Kothari, 2004). Amarasinghe (2009) carried out research on *in vitro* performance of nine indica rice varieties evaluating the effect of $CoCl_2$ and concluded that the rate of calli production was significantly higher in all the tested varieties on MS basal medium augmented with 5-10 mg/l $CoCl_2$. Kothari-Chajer *et al.* (2008) examined the role of $CoCl_2$ on callus induction in *Paspalum scrobiculatum* and *Eleusine coracana*. According to them, $CoCl_2$

had a stimulatory effect on callus formation in both plants and yielded friable and embryogenic calli.

5.6 Effect of auxin and cytokinin combination on shoot organogenesis

The *in vitro* shoot regeneration in tomato is directly affected by common media, media composition, time of maintaining the explants on multiplication media and genotypes (Mohamed *et al.,* 2006; Vinoth *et al.,* 2012). Therefore, it was necessary to understand the factors having a key role in establishing an efficient tissue culture protocol in tomato. During our study, all the genotypes exhibited the diverse responses in their regeneration ability, which has been confirmed in the earlier report by Tomsone *et al.,* 2004). Fresh weight and days to callus induction are directly affected by the concentrations of plant growth regulators (Lalage *et al.,* 2007). In tomato the *in vitro* shoot regeneration occurs mainly from calli obtained from de-differentiation of hypocotyls, cotyledons and leaf discs explants (Compton and Veilleux, 1991). For *in vitro* shoot regeneration, the most widely used media supplementing with higher concentration of cytokinin than that of auxin have been confirmed by many researchers (Faisal *et al.,* 2012; Hamama *et al.,* 2012). Auxin-cytokinin interactions have differential responses on *in vitro* shoot regeneration but always in a synergistic manner (Amoo and Staden, 2013). During our experiment, the regeneration frequencies increased with the increasing concentration of BAP from 1-3 mg/l. Coherent with our findings, Sheeja and Mandal (2003) reported that the highest shoot induction frequency was secured on the MS basal medium supplemented with IAA (0.5 mg/l) and BAP (2.0 mg/l). Moghaleb *et al.* (1999) investigated the effects of genotypes and explants directly or indirectly on *in vitro* plant regeneration in tomato and reported that different genotypes showed different percent regeneration responses due to their diverse genetic background. According to their findings, hypocotyls produced higher *in vitro* shoot regeneration frequency (70.2%) as compared to cotyledonary leaves (35.3%). Similarly, Pozueta-Romero *et al.* (2001) developed an efficient and rapid *in vitro* shoot regeneration protocol for three tomato genotypes culturing radicle, cotyledon and hypocotyls as explants. This article reported that proximal part of hypocotyls had an efficient regenerative capability producing more number of shoots per explants as compared to other types of explants used. As

summarized by El-Bakry (2002), the hormonal regime of IAA (0.2 mg/l) and BAP (2.0 mg/l) produced the highest shoot regeneration frequency (94.1%) when ten genotypes of tomato were scrutinized for their capability of producing *in vitro* primordial shoots. Our findings were contrary to the results of Gubis *et al.* (2003) who reported 100% regeneration from hypocotyls but in our studies, the highest regeneration was 69%. It might be due to difference in media, genotypes and explants used. From our experiments, we found that BAP was more efficient in producing *in vitro* shoot regeneration and multiplication, but long exposure to BAP arrested the capability of shoot multiplication. In many studies, it has been reported that zeatin alone or in combination with auxin is the best plant hormone for regeneration (Tripathi *et al.,* 2013). We suggest BAP for the best regeneration response in tomato and it is also more economical than that of zeatin.

5.7 Effect of silver nitrate on *in vitro* shoot regeneration

The differentiation capability of tissues is blocked if ethylene accumulates in the intercellular spaces forming aerenchyma (Gaspar, 1986). The ethylene action is inhibited by Ag+ ions and this inhibitory influence of silver ion is due to intrusion with ethylene binding (Beyer, 1979; Burgos and Alburquerque, 2003). Due to its inhibitory action on ethylene, silver nitrate improves *in vitro* shoot regeneration from calli clumps reducing tissue browning (Ptak *et al.,* 2010; Slater *et al.,* 2011; Nookaraju and Agrawal, 2012). During this study, the highest *in vitro* shoot regeneration frequency was recorded on media fortified with 8.5 mg/l $AgNO_3$ in cv. Moneymaker. While in cv. Rio Grande and Roma, the best shoot regeneration was secured on media having 10.0 mg/l $AgNO_3$. Our findings indicate that effect of $AgNO_3$ is genotype-dependent which is harmonious with the earlier report by Anantasaran and Kanchanapoom (2008). The promotive effect of $AgNO_3$ in callus induction and *in vitro* shoot regeneration has been narrated in some previous studies in various crops such as cauliflower (*Brassica oleracea*), cassava (*Manihot esculenta*), sunflower (*Helianthus annuus*), cabbage (*Brassica oleracea*) and Azuki bean (*Phaseolus angularis*) (Zobayed *et al.,* 1999; Zhang *et al.,* 2001; Yordanov *et al.,* 2002; Achar, 2002; Mohamed *et al.,* 2006). The $AgNO_3$ (10 mg/l) was utilized in *Coffea canephora* and significant increase (60%) in the number of embryos per explants was noticed. Conversely, the inhibitory effect of $AgNO_3$ on *in vitro* shoot regeneration

was also observed due to its higher levels (Fuentes *et al.*, 2000). Rashid (2002) conducted a study on minor millet (*Paspalum scrobiculatum*) for somatic embryogenesis and concluded that $AgNO_3$ at 10 mg/l increased the embryogenesis frequency (76%) as compared to control (53%). Kanwar *et al.* (2010) examined the effect of $AgNO_3$ on the number of shoots per calli clumps in *Punica granatum* and reported the highest number of *in vitro* shoots per cotyledon-derived calli (11.26) on MS medium augmented with NAA (6 µM), BAP (8 µM), GA_3 (6 µM) and $AgNO_3$ (24 µM). They concluded that high concentration of $AgNO_3$ (40 and 48 µM) inhibited the number of shoots per calli clumps. In agreement with our findings, 8.5 mg/l $AgNO_3$ was reported by Brar *et al.* (1999) who investigated the effect of $AgNO_3$ on shoot organogenesis frequency of cowpea culturing cotyledonary explants and found twofold regeneration frequency from calli clumps in comparison to the control. Our results clearly indicated that BAP had harmonious effect with $AgNO_3$ in the enhancement of *in vitro* shoot regeneration. Our approach was consistent with Cruz de Carvalho *et al.* (2000) who reported 63.8% shoot development in *Phaseolus vulgaris* on MS media along with BAP, but it increased up to 100% applying BAP in supplementation with $AgNO_3$. In another report, the necrosis was noticed in callus cultures of rice and maize due to ethylene biosynthesis and this necrosis was diminished by the supplementation of $AgNO_3$ and thereby improved the callus proliferation (Ptak *et al.*, 2010). The exact mechanism of reducing necrosis in callus cultures is not yet clear but it may be due to decline in ethylene production. Chugh and Khurana (2003) reported that the explants of wheat were more embryogenic on regeneration medium supplemented with $AgNO_3$ (10 mg/l) along with TIBA (1 mg/l) and kinetin (0.4 mg/l). By the addition of $AgNO_3$, a significant improvement of somatic embryogenesis and subsequent regeneration were recorded in wheat. In Peanut (*Arachis hypogaea* L.), the multiple shoot formation improved by the addition of $AgNO_3$ along with hormonal regimes culturing shoot tip explants (Ozudogru *et al.*, 2013).

5.8 Effect of cobalt chloride on *in vitro* shoot regeneration

Many physiological processes in plants are influenced by a gaseous hormone; ethylene. The callus growth, embryogenesis and *in vitro* shoot regeneration are suppressed by ethylene produced by cultured explants (Seong *et al.*, 2005). The

application of ethylene inhibitors have been found essential in suppression of ethylene biosynthesis. It has been reported that $CoCl_2$ is a powerful suppressor of ethylene production by blocking the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene and hence improves the regenerative potential of many crops (Chraibi *et al.*, 1992; Fuentes *et al.,* 2000). *In vitro* culturing of Habanero pepper was improved by the inhibition of ethylene by $CoCl_2$, but its high concentration was toxic for the plantlets (Santana-Buzzy *et al.,* 2006). During this study, the highest *in vitro* shoot regeneration frequency was recorded in cvs. Rio Grande and Roma on SIM containing $CoCl_2$ (5.0 mg/l). The leaf discs-derived calli were more organogenic on this regeneration medium and several small plantlets were produced from the calli clumps within 2-3 weeks. While in case of cv. Moneymaker, the best organogenesis was obtained on 4.25 mg/l $CoCl_2$ along with hormonal combinations. Our findings were parallel to the previous studies in petunia by Dimasi-Theriou *et al.* (1993) who investigated the effect of $CoCl_2$ on the number of shoots per leaf explants and reported that the number of shoots decreased with the application of high levels of $CoCl_2$. Plus *et al.* (1993) examined the effect of $CoCl_2$ on *in vitro* plant regeneration in pearl millet (*Pennisetum americanum*) and suggested 8.5 mg/l $CoCl_2$ into SIM for efficient development of plants from embryogenic tissues. The previous report by Meskaoui and Tremblay (1999) has illustrated that the use of $CoCl_2$ at all concentrations decreased the ethylene level as well as the number of somatic embryos in Black spruce (*Picea mariana*). Similarly, the effect of ethylene was tested on rose shoot cultures and found that the multiplication rate was high in the containers where ethylene accumulation was low due to inhibitory action of $CoCl_2$, due to which the multiple shoot regeneration enhanced in rose (Kevers *et al.,* 1992). In another study, the impact of $CoCl_2$ was investigated on shoot multiplication in *Capsicum frutescens* and found that exogenous application of 5.0 mg/l $CoCl_2$ significantly enhanced the number of shoots and shoot length after forty-five days of culturing (Sharma *et al.,* 2008). The use of $CoCl_2$ (8.5 mg/l) increased the somatic embryogeneis and number of embryos in *Daucus carota* because the number of embryos was directly related to the ethylene inhibition level, while the high concentration of $CoCl_2$ (17 mg/l) had a toxic effect inhibiting cell survival and browning of cotyledon also occurred (Roustan *et al.,* 1989).

Chae *et al.* (2012) established an efficient and reproducible *in vitro* shoot regeneration protocol in Gloxinia (*Sinningia speciosa*) optimizing $CoCl_2$ concentrations and suggested SIM supplementing BAP (2 mg/l), NAA (0.1 mg/l) and $CoCl_2$ (1.0 mg/l) improved significantly the shoot regeneration frequency and number of shoots by 12% as compared to control. Similarly, Brar *et al.* (1999) reported that $CoCl_2$ had a promotive effect on *in vitro* shoot regeneration and enhanced the number of shoots produced per cotyledon explants at 4.24 mg/l in cowpea.

5.9 Individual effects of sucrose and sorbitol on shoot organogenesis

The individual effect of sucrose and sorbitol was investigated on shoot organogenesis in Rio Grande, Moneymaker and Roma. Various concentrations of these carbon sources were augmented with MS and N6 basal media in different experiments. It was noticed that no regeneration was recorded in all the genotypes. Our findings were consistent with the earlier report, where effect of sucrose and sorbitol was scrutinized on induction of plant regeneration in Miscanthus x ogiformis Honda'Giganteus' and it was reported that medium containing only sucrose or sorbitol could not considerably enhance the *in vitro* shoot regeneration (Peterson *et al.,* 1999). This research group inferred that poor stimulation of shoot organogenesis by sucrose or sorbitol was due to weak hydrolysis of these carbon sources during autoclaving and culturing, but the exact mechanism of stimulatory effect was not known. Similarly, Nowak *et al.* (2004) ascertained that osmotic strength of medium was modified by the concentration of carbohydrates. Therefore, the media could be recognized as a dynamic system in which the accessibility of sugars and osmotic adjustment changed regularly and differently for sucrose and sorbitol, depending upon the concentration supplemented at the start of culture. They could alter the action of cytokinins and auxins or may inhibit the osmotic nature.

5.10 Sucrose and sorbitol in mutual combination were efficient for direct shoot organogenesis

During this experiment, combinational effect of sucrose and sorbitol was found to be efficient for yielding the highest *in vitro* shoot regeneration culturing hypocotyls and leaf discs in tomato. It was noticed that *in vitro* shoot regeneration frequency and shoot length were higher using hypocotyls than that of leaf discs on both MS and N6 basal media. These diverse responses of explants to sucrose and sorbitol might be due to the capability of different developmental stages to metabolize various sugars. These explants were differentiated and elongated into shoots within fifteen days. Hence, the shoot organogenesis on basal media fortified with only carbon sources limited the time frame from forty to fifteen days as compared to carbon sources-supplemented regeneration media along with PGRs. Likewise, higher regeneration frequency was recorded in our standardized medium (MS or N6 basal media fortified with only carbon sources devoid of exogenous applied PGRs) compared to regeneration medium having carbon sources along with various combinations of PGRs. In addition, sucrose and sobitol combinational effect was compared when these carbon sources were augmented with N6 or MS basal inorganic salts. N6 medium was found better in producing maximum shoot regeneration frequency, shoot length and number of primordial shoots than that of MS medium. Our findings were in accordance with Sheeja *et al.* (2004) who reported that the highest regeneration frequency, shoot length and number of shoots were recorded on N6 medium as compared to MS medium in three cultivars of tomato. The sugar absorption rate depends not only on types of species, explants, carbon source and its concentration, but also on the basal medium itself. For example, solid and liquid media have different osmotic conditions and this property of medium determines the accessibility of sugars for cultured tissues (Nowak *et al.*, 2004). Our findings were supported by the earlier research report by Cho *et al.* (2004) who investigated the effect of sucrose and sorbitol individually and accumulatively in rice culturing scutellum-derived calli and inferred that the regeneration frequency improved by the synergistic effect of sucrose and sorbitol in MS and N6 basal media as compared to individual effect of carbon sources and it was also inferred that MS basal media was superior to that of N6 basal media for *in vitro*

shoot regeneration in rice. Similarly, the individual effect of glucose and sorbitol was assessed on regeneration potential in *Pharbitis nil* culturing hypocotyls explants on MS basal media supplemented with 11.0 – 22.0 μM/l BA and 0.55 μM/l NAA and it was claimed that shoot and root organogenesis strongly inhibited by the individual influence of glucose or sorbitol from hypocotyls explants (Alina *et al.*, 2006). Our findings were also in parallel with the previous research studies by Hossain *et al.* (2013) who scrutinized the individual and accumulative effect of sucrose, sorbitol and glucose on *in vitro* shoot formation in banana cultivar Sabri and argued that no promotive response was given by glucose and sorbitol on shoot regeneration when applied alone, but their combination with sucrose in a ratio of 1: 1 yielded precocious shoot formation. Our proposed procedure mainly focuses on direct shoot regeneration by avoiding callus phase because long term plant tissue culture loses the potential of organogenic totipotency. The rationale behind this study is that epigenetic alteration occurs if the culture is maintained for a prolonged period of time that induces irretrievable genetic changes that eventually harm the totipotency in plants (Plana *et al.*, 2006).

5.11 Mutual effect of sucrose and sorbitol along with various PGRs enhances indirect shoot regeneration in tomato

The presence of carbon sources is inevitable for tissue culture media because they have determining influence on the induction of floral stimulus (Jana and Shekhawat, 2011). Generally sucrose is being used as an effective carbon source for *in vitro* morphogenesis in various plants. But in the present study, the mutual effect of sucrose and sorbitol along with various plant growth regulators was scrutinized on *in vitro* shoot regeneration and multiple shoot formation. The sucrose and sorbitol in augmented concentration (30: 30 g/l) were found to be the best carbon sources that critically enhanced the growth rate of tomato calli for maximum indirect shoot regeneration in all the genotypes investigated. This finding coincided with the earlier report in turfgrass by Cao *et al.* (2006) who reported that sucrose (30 g/l) and sorbitol (10 - 15 g/l) in regeneration media (modified from MS basal medium) improved the plantlet regeneration and sorbitol served as an osmoregulator to stimulate an appropriate cell status for efficient shoot regeneration. The enhancement of shoot organogenesis by applying

sorbitol might also be due to triglycerides accumulation (Sairam *et al.,* 2003). But in our study, the callus formation was done within eight days on medium containing appropriate concentration of sucrose and sorbitol. Shahsavari (2011) investigated the effect of sorbitol on *in vitro* shoot regeneration in rice and reported that regeneration frequency increased up to 40 - 45% in low regeneration capacity of rice cultivars by applying appropriate level of sobitol (20 g/l) along with hormonal regimes; NAA (0.5 mg/l), Kin (2.0 mg/l) and BAP (2.0 mg/l) but the regeneration frequency was detrimental on higher concentration of sorbitol. An *in vitro* culture study was conducted on pear and it was reported that multiple shoot proliferation rapidly enhanced by the application of sorbitol (20 - 30 g/l) (Kadota *et al.,* 2001). Kumar *et al.* (2010) examined the effect of sorbitol on *in vitro* shoot regeneration in rice through calli phases and reported that shoot induction medium i.e. MS medium having sorbitol (20 g/l), NAA (1.0 mg/l) and kinetin (4.0 mg/l) exhibited the best regeneration response and concluded that sorbitol was found to be critical for organogenesis. During our experiment, the calli tissues became dead by continuous incubation on the same regeneration media (with sucrose and sorbitol) that resulted in complete loss of morphogenesis. Our observations were in parallel to earlier report by Walker and Parrot (2001) who conducted a tissue culture study in soybean and concluded that long exposure of culture on same medium could result in complete failure of *in vitro* morphogenesis.

5.12 Factors affecting tissue culture based transformation

5.12.1 Effect of seedling age

Age of seedlings is one of the crucial aspects in transformation experiments affecting the efficacy of *Agrobacterium* infection (Solis *et al.,* 2003). To evaluate the susceptibility of *Solanum lycopersicum* seedlings to gene transfer via *Agrobacterium tumefaciens*, various ages of *in vitro* seedling (5, 10, 15, 20 and 25 days) were investigated. Among these ages, fifteen days old seedlings were found to be the most sensitive to *Agrobacterium* infection that showed the maximum transformation efficiency (TE) from both hypocotyls and leaf discs explants in all the genotypes. The comprehensive investigation revealed that gene transfer efficiency decreased by increasing more than fifteen days old seedlings. TE was approximately two times higher

in fifteen-days-old seedlings than that of twenty-five days old seedlings (Table 4.14). Our results demonstrated that younger seedlings (15-days old) were more effective in gene transfer than that of older ones. It might be due to the reason that younger seedlings were more susceptible to *Agrobacteium* and might also be due to increase in active surface area i.e. more number of dividing plant cells facilitating efficient T-DNA transfer in younger seedlings than that of older ones, consistent with the other recent reports (Chakrabarty *et al.,* 2002; Ribas *et al.,* 2011; Duan *et al.,* 2012). Sharma *et al.* (2009) optimized an efficient *Agrobacterium*-mediated transformation protocol culturing cotyledons from ten days old seedlings and reported the highest TE (41.4%) in tomato cv. Pusa Ruby. Li *et al.* (2009) used Fast *Agrobacterium*-mediated Seedling Transformation (FAST) technique for transient gene expression analysis in seedlings of *Arabidopsis*. The cotyledons from 3 - 5 days old seedlings were employed in transformation experiments and more than 50% TE was obtained. They also found that older seedlings (more than seven days old) had a sharp decline in TE. Duan *et al.* (2010) reported that twenty days old seedling proved to be the best age of explants for TE that ranged between 1.00 to 4.08%. Our findings were not consistent with the earlier report by Arcos-Ortega *et al.* (2010) who evaluated the influence of seedling age over TE in *Capsicum chinense* Jacq. The maximum TE (55%) secured culturing four weeks old leaf explants, while it decreased and serious tissue necrosis occurred when 8-week old explants were cultured. Hence, it is clear from this study that age of *in vitro* seedlings has a significant effect on TE in tomato.

5.12.2 Effect of pre-culture period

The pre-culture period is an important factor that significantly affects the TE in tomato (Singh *et al.,* 2011; Rai *et al.*, 2012). TE is closely linked with cell division or dedifferentiation (Arias *et al.,* 2006). The major limiting factor in regeneration and TE is the phase of plant cell cycle. It has been reported that TE of cells with nuclei at the S phase of the cycle was higher (Lai and Chen, 2002; Pena *et al.,* 2004). The delivery of foreign DNA through *Agrobacterium tumefaciens* occurs through S phase of cell cycle, therefore the actively growing cells particularly in S phase are the most responsive for plant genetic transformation via *Agrobacterium.* As the plant genetic transformation is greatly influenced by the cell cycle dynamics, but no practical means have been devised

to positively affect the TE by directly motivating the cell cycle (Gordon-Kamm *et al.*, 2002). Several studies have revealed the animation of "window of competence"; a time during which the plant cells are more vulnerable to *Agrobacterium tumefaciens* transformation. It has been suggested that during the pre-culturing stage, the plant cells become more competent for transformation by physiological adaptation (Costa *et al.*, 2002). The pre-culture period increases the regeneration percentage by eliminating the hypersensitive response of explants induced by *Agrobacterium* in co-cultivation stage (Arcos-Ortega *et al.,* 2010). Therefore, in order to control the hypersensitive response of hypocotyls and leaf discs by co-cultivation and to improve the TE, various pre-culture periods (0, 24, 48, 72 and 96 hours) were investigated in three tomato genotypes. The pre-culture period of 48 hours yielded the highest TE from both hypocotyls and leaf discs explants. The minimum TE was recorded by direct culturing of explants on co-cultivation media. However, the pre-culture period extending from forty-eight hours did not yield a significant change in regeneration and TE in all the genotypes. Our findings were supported by the earlier reports in which the highest TE recorded at forty-eight hours pre-culture period (Ellul *et al.,* 2003; Cortina and Culianez-Macia, 2004; Sharma *et al.,* 2009; Gao *et al.,* 2009; Cruz-Mendivil *et al.,* 2011). In contrast, Park *et al.* (2003) established an efficient *Agrobacterium*-mediated transformation system in tomato culturing cotyledons and hypocotyls explants from 10-day-old seedlings and ascertained that pre-culturing of these explants for twenty-four hours gave the highest TE (20%). These differences might be due to difference in *Agrobacterium* strain, type and age of explants and genotypes. Rai *et al.* (2012) optimized an efficient *Agrobacterium*-mediated transformation protocol for tomato cultivars using cotyledons explants and reported that six days pre-culturing period significantly improved the TE upto 44.3% in tomato. Similarly, Dan *et al.* (2006) conducted a study on tomato cv. MicroTom and reported that pre-culturing of cotyledon on pre-cultured medium for 24-h decreased the TE and promoted browning of some tissues. While freshly isolated cotyledon explants improved the TE. These diverse observations might be due to disparity of tomato genotypes and explants used.

5.12.3 Effect of bacterial density

Bacterial density plays very important role in transformation, and its optimization is considered to be a critical factor for securing maximum TE in tomato (Gao *et al.*, 2009). Therefore, various bacterial densities (0.1, 0.2, 0.3, 0.4 and 0.5) at 600 $_{nm}$ were investigated to acquire maximum TE in three tomato genotypes. Among various bacterial densities examined, $OD_{600\ nm}$ = 0.2 was found to be optimal level for obtaining maximum efficiency of gene transfer in all the genotypes. The further increase of OD beyond 0.2 significantly decreased the TE and promoted uncontrolled bacterial growth along with explants necrosis. Our findings coincided with the earlier report by Qiu *et al.* (2007) who engineered tomato cv. Micro-Tom with *CsZCD* gene utilizing an improved transformation protocol via *Agrobacterium* and reported that among various *Agrobacterium* concentrations, $OD_{600\ nm}$ = 0.2 was found to be the best level for securing the highest TE (20.87%) in tomato. Dan *et al.* (2006) generated tomato (cv. Micro-Tom) transgenic plants through *Agrobacterium*-mediated method, based on bacterial titers and argued that at OD reading of 1.0, the explants death occurred due to bacterial overgrowth, while the intermediate *Agrobacterium* titer ($OD_{600\ nm}$ = 0.1 – 0.5) was found to be optimal for efficient genetic transformation in tomato and no significant differences were noticed between TE at $OD_{600\ nm}$ = 0.1 and at $OD_{600\ nm}$ = 0.5. They also claimed that $OD_{600\ nm}$ = 0.1 was used in further transformation experiments. Contrary to our findings, high concentration of OD increased the TE in some species as supported by Guo *et al.* (2012) who optimized various factors for efficient gene transfer in tomato cv. Micro-Tom and concluded that *Agrobacterium* suspension ($OD_{600\ nm}$ = 0.5) along with other optimized parameters yielded the maximum TE (3.9%) culturing cotyledonary explants. Similarly, Cruz-Mendivil *et al.* (2011) conducted transformation study and inferred that leaf explants co-infected with bacterial density ($OD_{600\ nm}$ = 0.5) produced the maximum TE (19.1%) in tomato cv. Micro-Tom. Wu *et al.* (2006) assessed various experimental factors on transformation efficiency in tomato and reported that $OD_{600\ nm}$ = 0.15 was found to be efficient bacterial concentration in obtaining the highest efficiency of transient expression (90.5%) in tomato cv. Lichun. These differences might be due to diverse explants and genotypes.

5.12.4 Effect of acetosyringone

The acetosyringone (AS) plays a key role in natural infection of plant species by *Agrobacterium tumefaciens* activating Vir genes of Ti plasmid which can easily transfer T-DNA region into plant DNA (Lipp Joao and Brown, 1993; Cortina and Culianez-Macia, 2004; Wu *et al.,* 2006). Pitzschke and Hirt (2010) identified several steps involved for studying the interaction between *Agrobacterium* and plant cells such as recognition and expression of Vir genes, targeting of Vir factors, transfer and integration of T-DNA into host cells. The Vir region (30 - 40 kb) of Ti plasmid is responsible for T-DNA transfer into host plant cells. This region is made up of six necessary operons (Vir A, Vir B, Vir C, Vir D, Vir E and Vir G) and two unnecessary operons (Vir F and Vir H). The only two operons (Vir A and Vir G) constitute two component systems (Vir A/Vir G) that activates the transcription of other Vir genes (Riva *et al.,* 1998). This Vir A/Vir G system recognizes a diversity of phenolic compounds such as AS that behaves as the inducers of Vir gene expression for stable genetic transformation. *Agrobacterium* strains showed different sensitivity responses to various concentrations of AS (Subramanyam *et al.,* 2011). Similarly, TE varies with different concentration of AS and their interaction with various genotypes. Although, AS like compounds are secreted by dicotyledonous plants themselves but the addition of AS in inoculation and co-cultivation media improves tomato TE (Mahmoudian *et al.*, 2002). Therefore, for the improvement of TE, different concentrations of AS (0 - 100 µM) were added in inoculation and co-cultivation media and were scrutinized in three tomato genotypes. In our study, 60 µM AS concentration enhanced TE in all the genotypes, while further increase of AS gradually decreased the transformation efficiencies. Our findings assisted the findings of Wu *et al.*, 2006 who recorded the highest TE (73.2%) with the application of 50 µM AS using *Agrobacterium tumefaciens* strain LBA4404 culturing hypocotyls and cotyledons of ten days old seedlings. Guo *et al.* (2012) used 100 µM acetosyringone in culture medium for *Agrobacterium* strain, EHA105 inoculating cotyledonary explants of tomato cv. Micro-Tom and obtained 5.1% TE. AS (100 µM) has also been reported in other previous studies (Gao *et al.,* 2009). Our results clearly demonstrated the effect of low levels of AS on regeneration and TE along with high levels which adversely affected the regeneration

and subsequent TE in tomato due to overgrowth of *Agrobacterium*. The high levels of AS in tomato for improved transformation have also been reported in previous reports by Cortina and Culianez-Macia, 2004; Fuentes *et al.*, 2008 who used 200 µM AS concentration and obtained transformation efficiencies ranging from12.5% to 30%. This variation might be due to differences in explants type, genotypes, *Agrobacterium* strains and co-cultivation conditions.

5.12.5 Effect of infection period

A peculiar infection period plays an important role for T-DNA transfer into plant genome as low infection duration ensures very less TE due to improper association of *Agrobacterium* with host tissues. While high infection time generates hypertonic conditions due to excessive growth of bacteria that ultimately burst the plant cells (Dan *et al.*, 2006; Thiruvengadam *et al.*, 2013). During this study, various infection durations (2, 3, 5, 8 and 10 min) were tested for obtaining maximum TE culturing hypocotyls and leaf discs from fifteen days old *in vitro* seedlings. Among these infection periods, 3 min at $OD_{600\ nm}$ = 0.2 was proved to be the best for explants survival and maximum TE. Above 3 min infection duration, excessive bacterial growth occurred. No transformant was achieved when explants were infected with *Agrobacterium* culture only for one min, indicating that there was no attachment of bacteria with the explants. Our findings indicated a specific range of *Agrobacterium* density and infection period for obtaining maximum TE. Guo *et al.* (2012) optimized various transformation factors in tomato cv. Micro-Tom culturing cotyledon explants and concluded that bacterial concentration and infection time had significant effect on contamination, necrosis and ultimately TE. They recommended 5 min infection period for maximum TE (5.1%) in Micro-Tom. Similarly, Rai *et al.* (2012) developed an efficient procedure for genetic transformation of tomato culturing cotyledonary explants from six days old *in vitro* seedlings and suggested 5 min infection duration for maximum TE (44.3%). This is contradictory to our findings, as in our experiment, necrosis started in explants at five min infection duration. Wu *et al.* (2006) optimized various transformation factors in tomato (cv. Lichun) and pointed out that inoculation period of ten minutes was optimum for obtaining maximum TE (27%) in tomato. Higher infection period of 30 min has been reported in the previous report by

Sharma *et al.* (2009) who developed *Agrobacterium*-mediated system for tomato transformation culturing cotyledonary explants. This inconsistency of infection duration might be due to different genotypes and bacterial strain used.

5.12.6 Effect of co-cultivation media pH

The proper pH of co-cultivation media is crucial for efficient genetic transformation through *Agrobacterium tumefaciens* in tomato. The gene transfer ability of *Agrobacterium* is mainly dependent upon the Vir genes activity in Ti plasmid (Wu *et al.*, 2006; Gao *et al.*, 2009). It has been reported that the Vir genes can be highly expressed at low pH (Ogaki *et al.*, 2008). Therefore, the co-cultivation medium with low pH and with phenolic compounds such as acetosyringone were helpful for efficient gene transfer in tomato (Dang and Wei, 2007; Gao *et al.*, 2009). It has been reported that optimal pH of co-cultivation medium is also dependent on the type of explants used in the transformation experiments (San *et al.*, 2011). During this study, among varying levels of pH of co-cultivation media tested, pH of 5.6 and 5.7 were found optimum for the highest TE culturing hypocotyls and leaf discs, respectively. Our results were supported by the findings of Mondal *et al.* (2001) who reported that pH (5.6) of the co-cultivation medium had encouraging effects on TE. Rai *et al.* (2012) optimized an *Agrobacterium*-mediated genetic transformation system in three tomato cultivars culturing cotyledon explants and recommended that co-cultivation medium with pH 5.0 enhanced TE in all the genotypes. In ramie (*Boehmeria nivea* L.), the effects of pH and acetosyringone on stable genetic transformation were evaluated and found that co-cultivation medium with 50 mg/l acetosyringone and 5.9 pH enhanced the gene transfer efficiency from 10.5 to 24.7% in five varieties of ramie (Wang *et al.*, 2012). The co-cultivation medium with varying pH levels and efficiency of three different *Agrobacterium tumefaciens* strains were investigated in elite inbred lines of maize via culturing immature embryos. It was found that EHA105 proved to be better than those of LBA4404 and GV3101 and the highest TE (2.35 to 5.26%) was recorded on co-cultivation medium with an acidic pH (5.4) in dark conditions at 22 ºC (Huang and Wei, 2005). An efficient *Agrobacterium*-mediated transformation system was developed by optimizing co-cultivation medium's pH in soybean (*Glycine max* L.) by Dang and Wei

(2007). It was found that pH of co-cultivation medium and *Agrobacterium* strain had a greater influence on TE. The acidic medium (pH 5.4) of co-cultivation was found to be optimal for the highest transformation efficiency (4.29 to 18%) culturing embryonic tip in seven cultivars of soybean.

5.12.7 Effect of cefotaxime sodium

The growth of *Agrobacterium* in the medium after co-cultivation adversely affects TE. The bacterial elimination and regeneration of transformed tissues on medium with appropriate PGRs and selective agents are the most critical steps for efficient transformation system (Klee, 2000). Due to minimal toxicity on plant tissues, claforan® has been widely used to avoid the overgrowth of bacteria in *Agrobacterium*-mediated transformation experiments (Fuentes *et al.*, 2008). In this study, six different levels of cefotaxime sodium were assessed to control the excessive growth of *Agrobacterium* and found that 500 mg/l cefotaxime sodium was optimum for efficient decontamination of bacteria without affecting plant growth. Below this level, uncontrolled bacterial growth along with retarded plant growth noticed in all the genotypes. Above this level, no bacterial growth was found but the regeneration efficiencies of both hypocotyls and leaf discs explants became very low due to high pressure of antibiotic on tissues. Our results were in line with the findings of Roy *et al.* (2006); Ahsan *et al.* (2007) who used 500 mg/l claforan® for the elimination of bacterial growth in tomato cvs. Pusa Ruby and Punjab Upma. Our results were contradictory with Bihao *et al.* (2012) who used 400 mg/l claforan® in the medium for the elimination of *Agrobacterium* on infected explants. Other groups of scientists reported that bacterial growth might be efficiently controlled by using 300 mg/l cefotaxime sodium (Pozueta-Romero *et al.*, 2001; Kisaka and Kida, 2003; Cortina and Culianez-Macia, 2004). These differences might be due to disparity in *Agrobacterium* strain, genotypes and explants used. El-Siddig *et al.* (2009) evaluated the effect of claforan® on regeneration and TE of tomato cv. CastleRock by using bacterial strain LBA4404. According to this article, 250 mg/l cefotaxime sodium played an inhibitory role on growth of bacteria and no harmful effects on regeneration of tomato cv. CastleRock were noticed. Vidya *et al.* (2000) optimized transformation protocol for tomato cv. Pusa Ruby using *Agrobacterium* strain LBA4404 and cotyledons as explants

and pointed out that claforan® at 400 mg/l in the medium was the most effective dose in eliminating the adhering bacteria.

5.12.8 Effect of co-cultivation duration

Optimum co-cultivation duration plays an important role in efficient T-DNA transfer in host genome. During the process of *Agrobacterium*-mediated T-DNA transfer, high necrosis and poor survival rate of target plant tissues occur that are linked to hypersensitive defense reactions in plants due to bacterial infection (Mercuri *et al.,* 2000; Chakrabarty *et al.,* 2002). The plant defense mechanisms against *Agrobacterium* yielded a quick and huge generation of reactive oxygen species at the infection site in target plant species that ultimately result in plant cell death (Das *et al.,* 2002). Therefore, it was necessary to optimize the co-cultivation period; a possible way for improving the efficiency of *Agrobacterium*-mediated genetic transformation of tomato. The optimization of co-cultivation period is one of the most important steps for T-DNA transfer, bacterial attachment and stable integration of the foreign gene in infected explants (Li *et al.,* 2011; Ren *et al.,* 2012). The co-cultivation duration has been reported to influence the regeneration capacity and subsequent TE in various plant species (Shilpa *et al.,* 2010; Seo *et al.,* 2011). During this study, various co-cultivation periods (24, 48, 72 and 96 hours) along with constant OD and infection time were investigated for obtaining maximum transformants and found that 48 hours co-cultivation duration was optimum for the highest TE in three tomato cultivars. Below this period, TE decreased dramatically due to poor bacterial growth and above this period, TE along with regeneration lowered due to bacterial overgrowth. Our results were in agreement with Dan *et al.* (2006); Singh *et al.* (2011); Cruz-Mendivil *et al.* (2011) who reported that forty-eight hours of co-cultivation was optimum for tomato transformation, while contradictory to other reports by Qiu *et al.,* 2007; Fuentes *et al.,* 2008; Gao *et al.,* 2009, where maximum TE obtained at seventy-two hours of co-cultivation. This difference might be due to difference in tomato cultivars, *Agrobacterium* strain used, infection time and explants used. Guo *et al.* (2012) recommended twenty-four hours co-cultivation period for maximum TE culturing cotyledonary-derived explants of tomato cv. Micro-

Tom keeping 5 min incubation time and 0.5×10^8 cells/ml ($OD_{600\ nm}$ = 0.5) *Agrobacterium* suspension.

5.12.9 Effect of pre-selection period

The pre-selection period is the most critical step for transformation experiments as it increases the percent recovery of transformants between the co-cultivation and selection phases (Husaini, 2010). The regeneration of transformants is hampered by the selective agents. In order to avoid the direct shock of infected cells to such selective agents, a delay period (pre-selection) is required for the recovery of transformed cells from infection process that gives more time for stable integration of desired gene and also for the expression of selection marker gene (Alsheikh *et al.*, 2002; Zhao *et al.*, 2004). During the pre-selection period, the explants produced the fragile calli that enhanced the transformation efficiency (Khan *et al.,* 2003). Therefore, different pre-selection periods were tested in three tomato genotypes for optimization. During the present study, it was found that hypocotyls and leaf discs incubated for six days on pre-selection medium were appropriate for explants rehabilitation from transformation treatment. The survival frequencies of hygromycin-selected explants forming shoots were higher at 6 days of pre-selection period. This survival rate of selected explants decreased with an increase of pre-selection period. On the contrary, the explants that were directly transferred on hygromycin grew slightly and their growth arrested with tissues decay, ultimately no transgenic plant obtained. Our findings were supported by the previous reports in which pre-selection period of seven days produced the highest efficiency of gene transfer in tomato genotypes (Jabeen *et al.,* 2009; Afroz *et al.,* 2011). Sun *et al.* (2011) studied the genetic transformation and regeneration of pear using leaf segments and reported that the explants shifted to a pre-selection medium containing 100 mg/l cefotaxime and 100 mg/l carbenicillin for three days in the dark at 25 ºC yielded the highest TE of 53.5%. Another research investigation was carried out on efficient leaf disc transformation and regeneration in transgenic strawberry plants applying pre- and post- agro-infection strategies. During this study, importance of different parameters affecting TE was analyzed. It was found that among various factors studied, pre-selection phase was the most critical factor influencing TE. The maximum TE (31.25%) was recorded on five

days pre-selection period (Husaini, 2010). Khan *et al.* (2003) conducted a study on shoot regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation in five varieties of *Brassica napus* and found that a pre-selection period of seven days after co-cultivation was necessary for efficient regeneration and transformation. The maximum regeneration (92%) and transformation (24%) were recorded in five varieties of *Brassica napus*. Contrary to our findings, Thiruvengadam *et al.* (2012) conducted a study on early flowering in transgenic orchid by overexpressing oncidium MADS box (*OMADS1*) gene via *Agrobacterium*-mediated transformation and achieved maximum TE (9%) on three weeks pre-selection period. Similarly, Shivakumar *et al.* (2007) ascertained that pre-selection period of five days following selection pressure from cotyledons and hypocotyls-derived transformation in tomato was optimum for efficient TE. It was also reported by Rai *et al.* (2012) that during the selection process, a very low selection pressure might allow the highest regeneration capacity to transformed cells with the support of cross-feeding from the neighboring non-transformed tissues.

5.12.10 Effect of hygromycin

Hygromycin phosphotransferase (*Hpt*) has been widely used as a plant selectable marker gene for genetic transformation of tomato (Afroz *et al.*, 2011; Li *et al.*, 2013; Alvarez Viveros *et al.*, 2013). Hygromycin plays a critical factor for selecting transformed tissues as it inhibits transcription and translation, ultimately leading to the death of non-transformed tissues. It acts as an amino glycoside antibiotic that blocks plant regenerating potential (Jadhav Pravin *et al.*, 2011). The concentration of hygromycin was found to be tissue specific and it was mandatory to optimize the lethal dose of hygromycin for tissue culture dependent method of transformation (Datta *et al.*, 2004). During present study, we found that 35 mg/l hygromycin caused the death of all hypocotyls and leaf discs in all the genotypes tested and also the regeneration potential of the genotypes was inversely related to hygromycin levels. Roy *et al.* (2006) transformed *bspA* gene in tomato cv. Pusa Ruby and reported the browning of explants at 40 mg/l hygromycin within one week which ultimately stopped the regeneration of explants. Shah *et al.* (2010) engineered tomato plants overexpressing *ech42* gene against fungal pathogens. They revealed that hygromycin (25 mg/l) distinguished the transformed plants

because the non-transformed plants were pale with poor growth of roots, while transformed plants remained green with efficient root growth. Li *et al.* (2013) reported *MsrB* genes as plant selection markers in tomato transformation and compared the selection efficiency of methyl viologen and hygromycin system in tomato and ascertained that hygromycin (20 mg/l) showed 3.1% selection efficiency as compared to methyl viologen which gave 2.1% selection efficiency in tomato transformants. Guo *et al.* (2012) optimized various factors influencing tomato transformation using *Agrobacterium*-mediated method and reported 24 mg/l hygromycin as lethal dose for transformants in Micro-Tom tomato.

5.13 Factors affecting *in planta* transformation

5.13.1 Investigation of hygromycin sensitivity level for *in planta* transformation

Suitable selection marker is an important tool for transgenic plant production by selecting only transformed plants from a huge mass of non-transformed cells (Yau and Stewart, 2013). Therefore, it was crucial to optimize the concentration of an appropriate plant selection agent that limited the growth of non-transgenic plants and improved the selection of transgenic plants. In earlier studies, hygromycin has been extensively used as a selection marker in tomato transgenic plant production (Li *et al.,* 2013; Xin *et al.,* 2014; Viveros *et al.,* 2013). Hygromycin is a powerful antibiotic that limits polypeptide elongation during protein synthesis in non-transformed plants, while transformed plants with metabolites can turn over the effect of selection agent (Olhoft *et al.,* 2003). During this study, various concentrations of hygromycin were tested and 35 mg/l was found as a lethal dose of hygromycin at which all the leaves of plantlets showed necrosis and ultimately plant death occurred. Our findings were contradictory with those of Hiei and Komari (2006) who developed an efficient protocol for indica rice and reported that 75 mg/l hygromycin was appropriate for primary selection of transgenic plants. Girhepuje and Shinde (2011) used hygromycin sensitivity test to confirm the inheritance of transgene in tomato T_1 and T_2 generations using 50 mg/l hygromycin and reported that transgenic lines overexpressing *chi194* gene were highly resistant to fungal pathogen; *lycopersici*. Ghedira *et al.* (2013) used floral dip method of *in planta* transformation to achieve *Arabidopsis* transgenic plants using hygromycin (20 mg/l) as a plant selective

marker and disclosed that all wild type plants became dead and only transformed plants were survived under the use of selection marker.

5.13.2 Effect of OD on *in planta* transformation

The optical density indicating *Agrobacterium* cell density is related to cell number in the inoculum and it has been found to be a significant factor which directly affects TE (Dutt and Grosser, 2009). The excessive mass of bacterial cell can generate stress and affect the regeneration potential of plant cells, while lower mass can decrease the tendency of T-DNA transfer into host genome (Mondal *et al.,* 2001; Wroblewski *et al.,* 2005). Therefore, it was a dire need to optimize the bacterial cell density for obtaining the maximum *in planta* TE in tomato. During present study, the highest *in planta* TE was recorded at $OD_{600\ nm}$ = 1.0 with significant variations among all the genotypes. An efficient and rapid agroinjection method of *in planta* transformation was developed to ensure quick expression of transgene in tomato fruits and was reported that optical density ($OD_{600\ nm}$ = 0.1) improved the transient gene expression in tomato (Orzaez *et al.,* 2006). Another study has been conducted on the transformation of *Maesa lanceolata* through *Agrobacterium*-infection *in planta* transformation for both transient and stable gene expression. It was concluded that optimum bacterial density ($OD_{600\ nm}$ = 1.0) significantly increased TE (Faizal and Geelen, 2012). Trieu *et al.* (2000) modelled a novel system of *in planta* transformation via seedlings infiltration in *Medicago truncatula* and argued that $OD_{600\ nm}$ of 1.6 was optimum for securing efficient TE, ranged from 2.9 – 27.6%. Moreover, the analysis of T_2 generation indicated that the transformants were stable and followed by Mendelian fashion.

5.13.3 Effect of acetosyringone on *in planta* transformation

Acetosyringone is exogenously applied phenolic compound for the activation of *vir* genes in *Agrobacterium* that are crucial for the delivery of T-DNA into plant genome (Mayavan *et al.,* 2013; Kumar *et al.,* 2014). Another philosophy of improved TE by applying an optimum concentration of acetosyringone lies behind the reprogramming of incompetent cells of *Agrobacterium* to become competent one by perturbing the defense signal transduction measures induced by *Agrobacterium* in plant cells (Kumria *et al.,*

2001). It has been reported that monocotyledonous plants can't produce these phenolic compounds and even though they execute, the levels are too low to act as signals (Subramanyam *et al.,* 2011). On the contrary, dicotyledonous plants can produce phenolic compounds but the addition of acetosyringone improves TE (Stachel *et al.,* 1985; Subramanyam *et al.,* 2013). The proposed study was envisaged to improve *in planta* TE in tomato by optimizing various concentrations of acetosyringone in infection medium and after investigation, 60 µM aceotosyringe was found to be paramount level for the highest TE. It is obvious from our results that *in planta* TE is indirectly related with the optimum concentration of aceotosyringone in the infection medium. Incongruous with our findings, Orzaez *et al.* (2006) developed agroinjection method of *in planta* in tomato via fruit stylar apex. This technology could revitalize the crop improvement of tomato to meet its ever increasing demand due to shorten the time by direct transfer of transgenes in fruit. They concluded that acetosyringone (200 µM) in infiltration medium improved the transient gene expression which has become a valuable tool for crop improvement. Similarly, acetosyringone (200 µM) have been reported for *in planta* transformation in lentil (Mahmoudian *et al.,* 2002), alfalfa (Weeks *et al.,* 2008) and wheat (Zale *et al.,* 2009). This discrepancy of acetosyringone might be due to different genotypes, bacterial strain and explants used. In previous research study, various parameters affecting microinjection *in planta* transformation of tomato have been optimized and concluded that infection medium fortified with acetosyringone (100 mM) exhibited 28.68% TE as compared to control plants that gave only 2.6% TE by microinjecting one-day-old germinated seeds (Vinoth *et al.,* 2013). Similarly, a recent report has modelled the *in planta* seed transformation via *Agrobacterium* in brinjal (*Solanum melongena* L.) and revealed that infiltration and co-cultivation media fortified with acetosyringone (100 µM) remarkably increased TE (16.38%) in brinjal (Subramanyam *et al.,* 2013).

5.13.4 Effect of incubation period on *in planta* transformation

Incubation period is a critical factor that incredibly influences explants survival and TE because it is directly related with the sensitivity of explants and virulence of bacterial strain (Carvalho *et al.,* 2004; Orzaez *et al.,* 2006). Short time period is not

favorable for attachment and T-DNA transfer by *Agrobacterium* into plant genome, while long period of infection might injure the explants resulting in necrosis, due to which competence of T-DNA transfer would be limited (Duan *et al.*, 2013). In order to scrutinize the precise time period for *Agrobacterium* incubation, various infections regimes were applied to improve *in planta* TE in tomato and it was noticed that twenty minutes incubation period produced the highest TE in all the genotypes tested. Our results clearly demonstrated that regeneration of explants showed a parallelism with the incubation period and thereby its optimization was crucial for securing efficient *in planta* transformation system in tomato. A high-throughput *in planta* transformation was developed in switchgrass (*Panicum virgatum*) for transient expression by incubating seedlings with bacterial strain AGL1 for thirty minutes. It was concluded that this novel system promoted the gene expression in switchgrass by significantly enhancing the GUS expression percentage from 6 – 54% with the application of 100 μM acetosyringone (Chen *et al.,* 2010). Keshamma *et al.* (2012) conducted a study on *in planta* transformation in field bean by inoculating embryo exes from 2-day-old seedlings as explants and ascertained that incubation period of one hour proved to be effective for efficient transformation in field bean with stability of foreign genes through three successive generations. Another successful tissue culture-independent *in planta* approach was developed for revitalizing the genetic improvement in *Capsicum annuum* using 4-day-old seedlings and found that forty minute incubation period for intercotyledonary region injured with a needle yielded the highest TE in bell pepper (Kumar *et al.,* 2009). Similarly, Rao *et al.* (2008) developed a simple *in planta* method of transformation via *Agrobacterium*-mediated in pigeon pea to overcome the problem of recalcitrance of regeneration culturing apical meristem from 2-day-old seedlings as explants and concluded that infection time of one hour was optimum for rapid and quick *in planta* transformation in pigeon pea and they also reported that more than one hour incubation period resulted in browning and necrosis in the seedlings.

5.13.5 Effect of co-cultivation period on *in planta* transformation

Co-cultivation period is an important factor that significantly affects TE because T-DNA transfer into plant genome via *Agrobacterium* is a lengthy phenomenon that takes

a few hours to some days depending on plant species, type of explants and culturing environment (Mayavan *et al.,* 2013). The long period of co-cultivation although increases the transformation efficiency but may result in tissue necrosis due to which the explants death can occur, while short period of co-cultivation reduces the potential of T-DNA transfer due to less number of bacteria (Montoro and Rattana, 2003; Folta and Dhingra, 2006). During current study, the 48-h co-cultivation period with 60 µM acetosyringone yielded the highest *in planta* TE ranging from 5.49 – 8.28% in three tomato transgenic lines. A comprehensive study was conducted on transformation of soybean via *Agrobacterium*-mediated culturing embryonic tips emerging from one-day-old germinating seedlings and noticed that co-cultivation duration of 48-h improved the transient *GUS* gene expression, while long period of co-cultivation limited the regenerating capability of embryonic tips because these were more sensitive explants to *Agrobacterium tumefaciens* (Liu *et al.,* 2004). In another study, a tissue-culture independent transformation protocol in brinjal has been elaborated using seeds as explants and unveiled that 72-h co-cultivation period and 100 µM acetosyringone were paramount levels for securing the highest TE (43.88%) in brinjal. It was also narrated that this method didn't involve time consuming steps and circumvented the problems arising during plant regeneration *in vitro* culture (Subramanyam *et al.,* 2013). Mahmoudian *et al.* (2002) developed a convenient vacuum infiltration transformation protocol in lentil based on 72-h co-cultivation of cotyledonary nodes with *Agrobacterium tumefaciens* and inferred that this method gave the highest transient GUS expression in lentil. This discrepancy of co-cultivation period between their findings and ours might be due to different genotypes, bacterial strain and explants used. Ogawa and Mii (2005) reported 72-h co-cultivation for the evaluation of 12 β-lactam antibiotics in tobacco. The method established in this manuscript used leaf tissues as explants for *in planta* transformation in order to scrutinize the phytotoxic effects of LBA4404 and EHA101 in tobacco.

5.13.6 Effect of different growth media on seedling establishment

Vermiculite improves the quality of soil and thereby, it is known as soil conditioner and it also surrounds the soil particles and in this sense it acts as a matrix in soil mixes. Moreover, it is an inexpensive material and possesses excellent water holding

capacity (76%) (Chaudhary *et al.,* 2012). The vermiculite growing medium is better because it provides greater oxygenation due to its lower compaction compared to other plant growing media (Padilla *et al.,* 2007). However, the equal proportion of soil and vermiculite has been reported to work efficiently in plant initial establishment and growth (Mondal *et al.,* 2004). Earlier reports have revealed the significance of soil: vermiculite growth medium for successful seedling establishment in tomato (Khare *et al.,* 2010; Vinoth *et al.,* 2012; Sinha and Rajam, 2013). Vinoth *et al.* (2013) developed an efficient tissue culture independent *Agrobacterium*-mediated transformation system in tomato and argued that sterilized sand: soil: vermiculite (1: 2: 1) proved to be the best growth medium for successful plantlet formation. Our findings were in parallelism with those of Hazarika and Rajam (2011) who raised T_1 transgenic tomato seedlings on soil: vermiculite (1: 1) growth medium and reported that soil and vermiculite in equal ratio found efficient in yielding the highest germination frequency ranging from 35 – 88% in transgenic lines. The small plantlets with well-developed roots were also acclimatized on soil: vermiculite (1: 1) medium for one week in tissue culture room and then transferred to glasshouse under controlled conditions. On the contrary, Mahesh *et al.* (2013) germinated T_2 transgenic and control lines on MS modified medium and then transferred these seedlings into little earthenware pots having soil and vermiculite mixture in equal proportion to ensure proper plant growth and development.

In this study, among various protocols optimized for *in vitro* shoot regeneration in tomato, MS media supplemented with sucrose: sorbitol (30: 30 g/l) produced the highest *in vitro* shoot regeneration frequency (100, 97.69 and 99%) in cv. Rio Grande, Moneymaker and Roma, respectively as compared to other protocols optimized for *in vitro* shoot regeneration. The significance of this approach is that it avoids callus development phase, and ultimately reduces the abnormal plant development due to somaclonal variations. Likewise, the jeopardy of contamination was also reduced following one simple explants culturing step, avoiding the maintenance of callus cultures again and again. By using this procedure, we can appreciably save potential resources in terms of hormonal costs and time. Based on our findings, we propose this hormone-free

approach for *in vitro* shoot regeneration purposes for the development of stress tolerant cultivars of tomato using *Agrobacterium*-mediated genetic transformation.

We have optimized two transformation methods; one is tissue culture based and the other is a novel tissue culture independent method via *Agrobacterium*-mediated system. By employing these optimized methods, we have successfully transformed three tomato genotypes transferring *AtDREB1A* gene under the control of an inducible promoter lip9. Tissue culture based optimized transformation method gave 6.66 – 18.32% TE using hypocotyls and 4.44 – 15% TE culturing leaf discs in all the tested genotypes. The tissue-culture independent transformation method (*in planta*) yielded 3.82 – 8.28% TE. Based on our findings, we recommend *in planta* method for tomato transformation. Because it took less time (32 days) in transforming tomato cultivars as compared to tissue culture based transformation method (126 days). Moreover, *in planta* method signifies less cost and requires no sterilized working environment as compared to tissue culture based method that needs high cost as well as highly sterilized working environment.

5.14 Comparison of transgenic and NT plants on morphological basis

5.14.1 Plant height

During this study, *AtCBF3* transgenic plants showed no distinct phenotypic variations of plant height with respect to their isogenic NT plants at normal growth conditions. Our findings were in contrast with those of Jin *et al.* (2012) who engineered tomato with *PL1* gene and claimed that transgenic tomato plants had reduced plant height than that of control plants. Bo *et al.* (2006) ascertained the conflicting results with our findings and argued that chrysanthemum plants transformed with *rd29A:AtDREB1A* showed more plant height than that of NT plants. Consistent with our findings, Singh *et al.* (2011) engineered tomato cv. Shalimar with *At-CBF1* gene driven by *rd29A* inducible promoter against chilling stress and reported that transgenic tomato plants had no significant differences for plant height as compared to NT plants, without any chilling stress.

5.14.2 Number of leaves per plant

In this study, tomato transgenic plants carrying *AtCBF3* gene produced non-significantly more number of leaves per plant as compared to NT plants. Our findings were in line with the previous report on tomato plants by Garcia-Hurtado *et al.* (2012) who produced transgenic tomato overexpressing *CcGA20ox1* and reported that number of leaves in transgenic lines was higher than that of WT plants. Our findings were not supported by Safdar *et al.* (2013) who generated transgenic tomato cv. Rio Grande overexpressing yeast halotolerance genes (*HALI* and *HALII*) and concluded that six transgenic lines had lower number of leaves compared to control line. This might be due to difference in genotypes.

5.14.3 Fresh weight per plant

The present findings didn't support the role of *AtDREB1A* gene in the enhancement of fresh weight in three tomato transgenic lines under normal growth conditions. It might be due to the reason that the promoter lip9 didn't induce the expression of *AtDREB1A* because of its cold inducible nature. Our findings were consistent with the earlier preposition by Garcia-Hurtado *et al.* (2012) who reported that transgenic tomato plants overexpressing *CcGA20ox1* gene produced the same fresh weight with WT plants under normal growth environment. Similarly, Bo *et al.* (2006) narrated that tomato transgenic plants overexpressing *AtDREB1A* under the influence of 35S promoter exhibited unwanted growth phenotypes like reduced fresh weight as compared to control plants. This undesirable phenotype could be prohibited by the replacement of 35S CaMV with inducible promoter (rd29A) in transgenic *Arabidopsis* plants overexpressing *DREB1A* (Kasuga *et al.,* 1999). On the contrary, another research group investigated the effect of *HALI* and *HALII* on fresh weight in transgenic tomato and proposed that transgenic lines improved fresh weight and water contents compared to NT plants (Safdar *et al.,* 2013).

5.14.4 Dry weight per plant

During current study, T_2 transgenic plants carrying *AtCBF3* and NT plants were compared on the basis of dry weight (g/plant) in three tomato genotypes at 25 °C and it was confirmed that transgenic plants didn't exhibit any marked differences in dry weight with respect to NT plants. Our findings were confirmatory with the earlier studies by Zhao *et al.* (2007) who attributed that the phenotype of transgenic *Festuca arundinacea* plants overexressing *rd29A:AtCBF3* neither exhibit growth retardation nor growth improvement with respect to control plants i.e. showed no significant differences with control plants for dry weight per plant. Similarly, Gupta and Rajam (2013) transformed tomato with *mtlD* gene and reported 40 - 71% increase in DW as compared to control plants. In contrary, Dai *et al.* (1999) engineered tomato plants with *AtHXK1* gene and concluded that growth and dry weight of transgenic plants were inversely related with the overexpression of hexokinase activity in photosynthetic tissues, which might be due to inhibition of *CAB1* (sugar-responsive photosynthetic gene) (Jang *et al.,* 1997). These differences between our findings and previous reports might be due to difference in transgene, promoter and genotypes tested.

5.14.5 Root length

The root growth is a crucial parameter in plant stressful environment, as roots act as an engine for meeting the requirements of transpiration and making water available to plants (Liu and Huang, 2000). The outcomes of this study revealed that under normal growth conditions transgenic lines produced almost the same root length with that of their isogenic counterparts due to lack of expression of *AtCBF3* gene at normal temperature, which were converse with the achievements of Saad *et al.* (2010) who conducted greenhouse study of transgenic tobacco carrying *AlSAP* gene and reported a small increase in root length as compared to control plants when studied under normal growth conditions. Another greenhouse investigation of transgenic tomato was carried out by Garcia-Hurtado *et al.* (2012) who assessed the greenhouse behavior of transgenic tomato plants carrying gibberellin 20-oxidase (*CcGA20ox1*) isolated from citrus. This research team reported significant increase in transgenic plant characteristic such as root length under non-stressed environment compared with WT plants. Similarly, Eriksson *et al.*

(2000); Vidal *et al.* (2001); Phillips (2004) documented the longer roots compared with their isogenic counterparts overexpressing *GA20ox* in various plant species. These differences between our findings and previous research reports might be due to disparity in transgene, promoter, genotypes and growth conditions.

5.14.6 Days to flowering

To assess the overexpression effects of *CBF3* gene on vegetative as well as on reproductive growth in three tomato genotypes, we analyzed the transgenic plants possessing a single copy of gene and found that they showed non-significantly a longer period of time to flower initiation when compared with NT plants. In this study, although delay in flowering was recorded in transgenic plants, but the overall growth rate was similar to that of isogenic counterparts. Contrary to our findings, earlier research by Ellul *et al.* (2004) demonstrated that overexpression of *AtAPETALA1* in tomato reduced its flowering time without affecting the average plant production and proposed that flowering started after the production of six leaves in transgenic plants compared with their isogenic counterparts that flowered after the production of eleven leaves. Similarly, Gilmour *et al.* (2000) analyzed the growth parameters of transgenic *Arabidopsis* harboring *CBF3* gene under controlled environment and pointed out the significant differences among transgenic and NT plants with respect to flowering time and also transgenic plants took a longer period of time to flowering than that of control plants.

5.14.7 Number of flowers per plant

Our study clearly demonstrated that transgenic lines produced almost same number of flowers per plant compared with NT plants without any chilling stress. Our findings were converse with the previous proposition by Hammond and Zhao (2009) who reported the overexpression of *pkv* (protein kinase-viroid induced) gene in tobacco cv. Xanthi with more number of flowers per plant and more height in transgenic plants than that of NT plants. Similarly, tobacco transgenic plants were developed by transforming *rolD* gene; that enhanced the flowering process and higher number of flowers produced in transgenic lines as compared to their isogenic counterparts. These variations might be due to transgene, promoter, genotypes and growth conditions.

5.14.8 First fruit set

During this study, we compared the three tomato transgenic lines and NT plants on the basis of first fruit set (days) at normal growth conditions and found that transgenic lines produced fruits within the same period of time compared with NT plants. Similar to our findings, outcomes of the previous report by Singh *et al.* (2011) indicated that transgenic tomato var. Shalimar overexpressing *AtCBF1* gene did not exhibit any marked differences in morpho-agronomical characteristics such as growth habit, fruit shape and days to fruiting compared with NT plants. Our findings were not at par with those of Komakhin *et al.* (2010) who engineered tomato cv. Marglob with *NLS-recA-licBM3* gene and performed comparative analysis of transgenic and control plants. According to this report, all the transformants yielded fruit 10-15 days earlier than that of control plants and the transgene did not exhibit any adverse effect on pollen fertility. Our morphological analysis was inconsistent with earlier research report, where the morphological analysis illustrated the early reproductive phase in transgenic plants of tobacco than that of NT plants (Mauro *et al.,* 1996). This might be due to higher accumulation of proline or depletion of ornithine in transgenic tobacco (Bettini *et al.,* 2003). These differences might be due to diverse transgenes, genotypes tested and variation in culture conditions.

5.14.9 Number of fruit per plant

During this study, we noticed no significant role of *AtCBF3* gene on the number of fruit per plant without any chilling stress, thereby no significant variations were found in number of fruit per plant of three tomato transgenic lines as compared to their isogenic NT plants. Inconsistent with the previous findings by Jin *et al.* (2012) who documented that overexpression of *PL1* fusion gene in tomato producing more number of fruit per plant compared with their WT plants. According to this research group, no negative effects were observed on the growth of sixteen tomato transgenic lines due to overexpression of *PL1* gene. A similar greenhouse study was conducted for the aim of analyzing tomato transgenic plants harboring *rolD* gene on the basis of morpho-biochemical parameters and concluded that the most-striking morphological effect of transgenics production was a significant increase in fruit number per plant as compared to NT plants, which might be due to higher number of flowers per transgenic plants and also

due to shortening of plant life cycle that shifted the plants from flowers to fruits (Bettini *et al.,* 2003).

5.14.10 Fruit diameter

In this study, we noticed that transgenic lines produced non-significantly smaller fruit size in term of fruit diameter as compared to their isogenic counterparts. Kevany *et al.* (2008) found confirmatory results with our findings and ascertained that overexpression of *LeETR4* gene in transgenic tomato didn't exhibit any adverse effects on fruit size and yield. This research group reported a smaller fruit size of transgenic lines compared with fruits of control plants, but there was no statistical differences among transgenic and control plants with respect to fruit size. Similarly, Singh *et al.* (2011) conducted a comparative analysis of morpho-agronomical characteristics of tomato transgenic plants carrying *AtCBF1* gene and reported a non-significant variations in fruit diameter among transgenics and NT plants. Another research group (Me *et al.,* 2007) obtained contradictory results with our findings, who reported significant effect of *LeETR2* expression on the enhancement of fruit size in transgenic lines as compared to WT plants. These contradictions might be due to diverse transgenes, genotypes tested and variation in culture conditions.

5.14.11 Fruit weight

This study demonstrated that the fruit weight of 4-week old transgenic lines was not associated with *lip9:CBF3* activity at normal temperature and almost same fruit weight of transgenic lines was recorded with respect to their NT plants. Our findings were at par with those of Bettini *et al.* (2003) who revealed that tomato transgenic cv. Tondino overexpressing *rolD* gene produced no significant differences for mean fruit weight (g) as compared to isogenic control plants. Our findings were not confirmatory with that of Ellul *et al.* (2004) who carried out comparative analysis of transgenic tomato plants harboring *AP1* gene and reported that transgenic line produced fertile fruit that had relatively lower mean weight (g) than that of control plants. Similarly, Singh *et al.* (2011) generated marker-free transgenic tomatoes with *AtCBF1* gene and proposed that the average fruit weight of transgenic plants was comparatively lower than that of NT plants.

Me *et al.* (2007) obtained contradictory results with our findings who reported significantly heavier fruit of transgenic lines than that of WT plants.

5.14.12 Number of seeds per fruit

In order to determine the role of *AtCBF3* gene on the ultimate yield, a comparative analysis was conducted on three tomato transgenic lines and respective NT plants and found that the number of seeds per fruit in transgenic lines was non-significantly lower, resulting in fewer yield of transgenic lines compared with NT ones. Contrary to our findings, a controlled-environment study of *Arabidopsis* transgenic plants was carried out by Gilmour *et al.* (2000) who investigated the morpho-physiological performance of transgenic plants overexpressing *CBF3* gene. According to their findings, number of seeds per fruit in transgenics was significantly lower than that of control plants, which might be due to overexpressing *CBF3* gene in least parts of transgenics that produced less axillary shoots.

The outcomes of this detailed morphological study of three tomato transgenic lines clearly demonstrated that the expression of *CBF3* gene didn't seem to be linked with agronomic and yield parameters in tomato. It might be due to the reason that lip9; a cold inducible promoter could not induce the expression of *CBF3* gene under normal growth conditions and hence no significant differences were recorded among transgenic and NT plants.

5.15 Evaluation of cold tolerance on physiological basis

5.15.1 Stomatal Conductance

Solanum lycopersicum is a cold sensitive crop that shows various injuries at temperature below 10 ºC (Park *et al.,* 2004). The degree of cold tolerance in tomato is correlated with its tendency to stomatal closure during chilling stress (Aroca *et al.,* 2001, 2003; Bloom *et al.,* 2004). The stomatal behavior during chilling is related to water status within the leaf and integrity of membrane system. The flow of water declines during cold stress due to which stomata become closed (Hubbard *et al.,* 2001; Matzner and Comstock, 2001). Photosynthesis is greatly influenced by stomatal closure under cold stress due to which CO_2 supply to the leaf becomes limited and ultimately electron

transport chain of photosynthesis is reduced, resulting in the production of reactive oxygen species (ROS) (Gill *et al.,* 2013). It has been reported that the first symptom of low temperature stress occurs as a stomatal closure that inhibits photosynthesis (Zhang *et al.,* 2012). During this study, the overexpression of *CBF3/DREB1A* gene in tomato resulted in the enhancement of stomatal conductance that contributed towards improvement of cold tolerance. This is an agreement with the earlier reports which indicated the role of *CBF3* gene in tomato under cold stress (Maruyama *et al.,* 2004 & 2009; Lissarre *et al.,* 2010). In present scenario, Zhang *et al.* (2012) examined the effect of *betA* gene in cotton and found that overexpression of this gene enhanced the synthesis of glycinebetaine (GB) that exhibited the improved tolerance against chilling stress. Under chilling stress, the transgenic plants had higher stomatal conductance and ultimately more photosynthesis than that of control plants by the production of higher levels of GB. Our findings were in confirmatory with the previous studies by Dahal *et al.* (2012) who investigated the effect of CBFs transcription factors (*BnCBF17*) in *Brassica napus* at low temperature stress (5 ºC) and concluded that overexpression of *BnCBF17* increased photosynthetic rate in transgenic plants by increasing CO_2 assimilation and stomatal conductance as compared to non-acclimated wild type plants. Gilmour *et al.* (2000, 2004) ascribed the function of *Arabidopsis* CBF3 transcriptional activators under cold stress that mimicked the phenotypic, physiological and biochemical variations in transgenic plants that were evident in their non-transgenic counterparts.

5.15.2 Transpiration rate

The transpiration rate is an essential phenomenon that affects the water relation in plants. Water acts as a solvent and medium for many physiological and biochemical activities such as photosynthesis, uptake of nutrients, transportation of materials and cooling effects that have direct influence on plant growth and development (Farooq *et al.,* 2009; Guana *et al.,* 2011). The high transpiration rate increases water uptake and turgor pressure that allow more supply of CO_2 to the leaf due to greater stomatal aperture as well as rapid cell division and enlargement (Brini *et al.,* 2007). During this study, the transpiration rate of transgenic lines was significantly higher than that of NT plants due to over-expression of *DREB1A* gene that regulated the other genes involved in cold

tolerance. Due to lower transpiration rate in NT plants, their nutrients absorption efficiency was reduced, ultimately they suffered from wilting. However, the process of transpiration regulation by *DREB1A* in transgenic plants is unclear. Due to decreased transpiration rate, many metabolic processes like photosynthesis, respiration, uptake and transport of ions are changed. These changes result in the production of ROS (H_2O_2, O_2^-, OH and OH_2) that cause damage to DNA, RNA, and protein and cause injuries to cell membranes (Lupinkova and Komenda, 2004; Nishiyama *et al.*, 2006). The overexpression of *DREB1A* produces antioxidants [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO) and ascorbate peroxidase (APX)] that detoxify these ROS and save the plants from oxidative damage (Suzuki and Mittler, 2006; Li *et al.*, 2011; Gupta *et al.*, 2012). An α-expansin gene (*VfEXPA1*) has been found to regulate the stomatal conductance, cloned from epidermal strips of *Vicia faba* L. and was transformed in tobacco. Due to overexpression of *VfEXPA1* gene, the transgenic tobacco plants indicated the enhanced levels of transpiration and photosynthetic rates than that of WT plants (Cheng *et al.*, 2011). Zhang *et al.* (2012) scrutinized the chilling tolerance in cotton transferring *betA* gene from *Escherichia coli* and narrated that there were no significant differences in transpiration rate of transgenic and wild type plants under normal temperature but after exposure to 5 °C for twenty-four hours, the transgenic lines exhibited higher transpiration rate than that of respective WT lines due to overexpression of the *betA* producing more glycinebetain contents in transgenic lines.

5.15.3 CO_2 concentration rate

Under chilling stress, the CO_2 concentration rate in intercellular spaces is reduced due to stomatal closure leading to the impairment of the functions of chloroplast (Sonoike, 1998). Due to improper functioning of chloroplast, PSI photoinhibition occurs and NADP production in Calvin cycle is limited, resulting in the breakdown of electron transport chain between PSI and PSII and destruction of Rubisco activity (Ishibashi *et al.*, 1997; Bechtold *et al.*, 2005; Huang *et al.*, 2012). Consequently, the leaf photosynthetic efficiency is depressed leading to the synthesis of ROS (Shao *et al.*, 2007). ROS are more reactive and causes injuries to chloroplast, nucleic acids and proteins due to oxidative

stress. Plants hold antioxidant enzymes to protect their photosynthetic machinery by scavenging these toxic free radicals and also acting as signaling molecules to regulate the expression of cold defense genes (Hasanuzzaman *et al.,* 2012; Gill *et al.,* 2013). It has been reported that stomata become closed under chilling stress, limiting the CO_2 concentration rates; consequently decline of photosynthesis occurs in chilling sensitive plants (DeRidder and Crafts-Brandner, 2008). During this study, the transgenic tomato plants had elevated leaf CO_2 concentration rate than that of NT plants. The overexpression of *DREB1A* produced various antioxidant enzymes such as SOD, POD, CAT, PPO and APX (Suzuki and Mittler, 2006; Li *et al.,* 2011; Gupta *et al.,* 2012). Bruggemann *et al.* (1992) outlined the influence of long term chilling stress (6 - 10 ºC) on photosynthesis functions in young tomato plants and reported that at 6 ºC, impairment of starch formation was common in chilled plants, while in unchilled plants it increased up to eight-fold of the contents recorded in chilled plants. They also suggested that the activity of ribulose-1, 5-biphosphate carboxylase enzyme was irreversibly decreased resulting in the strong decrease of photosynthesis in chilled leaves. Zhang *et al.* (2012) assumed that the overexpression of *betA* gene enhanced glycinebetaine in cotton under chilling stress that protected the cell membrane integrity against chilling stress. They claimed that the transgenic plants had higher CO_2 concentration rate, stomatal conductance and electron transport chain of PSII resulting in efficient photosynthetic rate.

5.15.4 Photosynthetic rate

Stressful environments cause alterations in many physicochemical and molecular processes in plants. Photosynthesis; a physiological process is also severely altered by stressed environment (Ashraf and Harris, 2013). Photosynthesis is inhibited by photoinhibition due to stomatal closure under cold stress (Aro *et al.,* 1993; DeRidder and CraftsBrandner, 2008). Coherent with our findings, Pino *et al.* (2008) suggested that cold tolerance regulated by endogenous CBF pathway which altered the various processes related to plants structure, biochemistry and physiology. The previous studies on photosynthetic rate have shown that low temperature can change many photosynthetic products and ectopic *CBF3/DREB1A* expression can modify the regulation of several genes involved in the process of photosynthesis (Savitch *et al.,* 2005; Goulas *et al.,*

2006). An enhanced overexpression of *DREB1A* resulting in increased photosynthetic rate, have been reported in tobacco and *Brasica napus* (Kasuga *et al.,* 2004; Savitch *et al.,* 2005). In present study, transgenic tomato lines exhibited improved photosynthetic rates under cold condition compared to NT plants. Relative to NT, transgenic tomato plants showed significant variation in the photosynthetic rate under chilling stress and Roma transgenic line exhibited the highest photosynthetic rate at various chilling stresses. Congruous with our findings, Sui *et al.* (2007) reported that overexpression of *LeGPAT* transgene significantly improved net photosynthetic rate in tomato as compared to NT plants. Moreover, maximal photochemical efficiency of transgenic plants decreased more slowly under cold stress (4 °C) but recovered more rapidly under normal conditions than that of NT plants. Another research group investigated the effect of *AtCBF* gene on physiological changes in transgenic and WT plants of potato and concluded that transgenic plants improved chilling tolerance due to enhanced photosynthetic potential compared to WT plants and they also pointed out that potato transgenic plants were dark green due to higher chlorophyll contents and more number of stomata than that of WT plants (Pino *et al.,* 2008). Our findings coincided with those of Sun *et al.* (2010) who engineered tobacco plants overexpressing tomato *tAPX* gene. The transformants had higher photosynthetic rate and more protection of photosynthesis apparatus under cold stress than that of WT plants. They also ascertained that transgenic lines had elevated level of photochemical efficiency than that of WT plants under low temperature stress.

5.15.5 Relative water contents

Relative water contents (RWC) is an essential parameter for screening cold tolerance in plants because more RWC indicates that plants have more water holding capacity that makes them more adaptive in cold environment (Gupta *et al.,* 2012). Our results demonstrated that Roma transgenic line was more tolerant to cold due to higher RWC than that of other two transgenic lines. The NT plants exhibited wilting after cold stress due to low RWC, while transgenic plants showed normal growth due to more RWC and turgor recovery. The high RWC in tomato transgenic lines was persistent with the likelihood that the overexpression of *DREB1A* increased the capacity for cold tolerance in tomato. In previous study, chilling stress tolerance was improved in tomato by

transforming *At-DREB1A* gene under the control of cold inducible promoter RD29A. Upon exposure to low temperature (4 ºC) for three days, T_1 transgenic line improved chilling tolerance by increasing RWC compared to NT plants (Singh *et al.,* 2011). Our findings coincided with the earlier proposition by Khare *et al.* (2010) who examined the influence of *mtlD* gene in transgenic tomato line after exposure to chilling stress (4 ºC). They reported that non-transformed plants died after cold stress, while transformed plants showed tolerance due to overexpression of *mtlD* gene accumulating higher RWC than that of NT plants. A late embryogenesis abundant (*LEA*) gene; *CbLEA* was isolated from *Chorispora bungeana* and introduced into tobacco whose overexpression exhibited cold tolerance by significantly higher RWC compared with their counterparts. The degree of chilling tolerance in tobacco was associated with high accumulation of *CbLEA* protein that protected the transgenic plants from cold injury (Zhang *et al.,* 2009). Yanez *et al.* (2009) reported that bZIP transcription factor regulated cold inducible gene *SlAREB1* and overexpression of this gene played an essential role in cold tolerance accumulating more RWC in *Solanum* genus.

5.15.6 Leaf osmotic potential

Chilling stress reduces the membrane viscosity and decelerates metabolism leading to the production of free radicals and oxidative stress that change the normal activities of macromolecules and decrease the osmotic potential (Xiong *et al.,* 2002). Chilling stress also affects the water relations in a cell as well as in the whole plant that creates ice crystals, due to which dehydration stress is also produced (Beck *et al.,* 2007). In order to maintain water potential, the plant cell must regulate its internal osmotic potential to enhance the mechanism of stress tolerance (Khare *et al.,* 2010). During this study, *DREB1A* transgenic tomato lines showed significantly lower leaf osmotic potential (Ψs) than that of their counterparts, and therefore maintained higher RWC. Under chilling stress, low Ψs of transgenic plants involved in more water assimilation maintaining cell turgor, which increased higher RWC than that of NT plants (Zhang *et al.,* 2012). The cold responsive transcription factor/gene transferred by genetic engineering encodes an enzyme that is involved in pathways leading to production of osmolytes and proteins in plant that cope with environmental stress (Zhu, 2001;

Prabhavathi *et al.*, 2002). These osmoprotectants (proline, glycinebetaine and polyamines etc) accumulate in sufficient quantities and help in adjusting osmotic potential, scavenging toxic free radicals, stabilizing the structure of macromolecules, enhancing the activities of protein chaperones and maintaining ionic balance and membrane integrity (Rajam *et al.*, 1998; Grover *et al.*, 1999; Diamant *et al.*, 2001). Khare *et al.* (2010) engineered tomato plants overexpressing the bacterial *mtlD* gene against chilling stress. They pointed out that osmoprotectants were produced due to *mtlD* over-expression that maintained the osmotic potential and prevented the tomato transgenic plants from chilling injury. Zhang *et al.* (2012) developed chilling tolerant cotton plants transferring *betA* gene and argued that overexpression of *betA* produced glycinebetaine which demonstrated the positive correlation with chilling stress. They also reported that transgenic plants had lower leaf osmotic potential than that of WT plants after exposure to chilling stress, and maintained more RWC and thereby indicated the chilling tolerance.

5.16 Evaluation of cold tolerance on biochemical basis

5.16.1 Membrane leakage

Cell membrane is considered as a primary target of cold stress and its stability and integrity is the main concern in plants under chilling stress environment (Wang *et al.*, 2010). During metabolism of chloroplast and mitochondria, ROS are generated that rupture the cell membrane (Apse and Blumwald, 2002). The integrity of plasma membrane is adversely affected by ions leakage which is an inherent trait of plants and necessary for appropriate functioning of cells under chilling stress (Feng *et al.*, 2003; Zhang *et al.* 2012). Usually, chilling stress damages cell membrane with alteration in composition of fatty acid phospholipids, destruction of cell structures, impairment of photosynthesis and disruption of protein assembly (Mirdehghan *et al.*, 2007; Singh *et al.*, 2011). The cold responsive transcription factor (CBF3) encodes an enzyme that is involved in pathways leading to production of osmolytes (Zhu, 2001; Prabhavathi *et al.*, 2002). These osmoprotectants (proline, glycinebetaine and polyamines etc) accumulate in sufficient quantities and help in membrane integrity (Rajam *et al.*, 1998; Grover *et al.*, 1999; Diamant *et al.*, 2001). Moreover, overexpression of *DREB1A* also produces antioxidants [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT),

polyphenol oxidase (PPO) and ascorbate peroxidase (APX)] that detoxify ROS, save the plants from oxidative damage and ultimately improve membrane integrity (Suzuki and Mittler, 2006; Li et al., 2011; Gupta *et al.*, 2012). During this study, the electrolyte leakage (an essential parameter for determining membrane damage) was statistically higher in NT plants than that of transgenic plants. During this study, we noticed that overexpression of *CBF3* gene could efficiently limit tissue injury determined by chlorosis that delayed in transgenic lines, while it was evident in NT plants. Our results demonstrate the retention of membrane integrity by reducing ion leakage in transgenic lines under low temperature. Our findings were confirmatory with Singh *et al.* (2011) who developed cold tolerance in tomato transforming *At-CBF1* gene and reported considerable decrease in membrane injury in transgenic plants as compared to their counterparts under cold stress (4 ºC) for three days. Similarly, Khare *et al.* (2010) suggested the mannitol-1-phosphate dehydrogenase gene involvement in cold tolerance due to significant decrease in electrolyte leakage in transgenic tomato plants. In another study, C-repeat binding factor (*CBF3*) gene was identified from *Lolium perenne* (L.), designed as *LpDREB1A/LpCBF3* and transformed in *Arabidopsis*. Overexpression of *LpDREB1A* induced *AtDREB1A/CBF3* expression of target COR genes in *Arabidopsis* and facilitated freezing tolerance by reducing the ion leakage in transgenic plants (Xiong and Fei, 2006).

5.16.2 Proline contents

The chilling injury in plants can be coped with the production of osmolytes such as proline (Claussen, 2005; Foyer and Noctor, 2009; Li *et al.*, 2013). The earlier reports have revealed positive correlation between cold tolerance and proline accumulation in various crops (Szabados and Savoure, 2010; Akram and Ashraf, 2013; Patade *et al.*, 2013). Proline performs multiple functions in generating chilling tolerance in plants; it mediates osmotic adjustment under water-deficit situation (Verbruggen and Hermans, 2008; Lee *et al.*, 2014), stabilizes proteins and membranes ensuring membrane integrity (Szabados and Savoure, 2010), scavenges ROS by acting as a partial antioxidant (Theocharis *et al.*, 2011; Bhandari and Nayyar, 2014) enhances the expression of genes related to osmotic-stress (Iyer and Caplan, 1998; Szabados and Savoure, 2010), maintains

NAD^+/NADH ratio, increases photochemical activity of photosystem II (Theocharis *et al.,* 2012) and decreases MDA contents (Kishor *et al.,* 2005). The urge of our research work was to measure proline contents under various low temperature regimes in transgenic and NT tomato plants. It was found that low temperature (4 ºC) induced severe wilting in NT plants due to osmotic stress, while transgenic plants didn't show any wilting and retained normal growth after 24-h recovery period due to overexpression of *CBF3* gene resulting in the higher accumulation of proline in transgenic plants. Our findings were in confirmatory with the earlier reports by Gilmour *et al.* (2000); Gong *et al.* (2002) who ascertained the overexpression of *CBF3* gene in *Arabidopsis thaliana* which promoted proline accumulation and ultimately cold acclimation. In another study, cold tolerance improved in maize by the accumulation of proline and glycinebetaine, and reduced lipid peroxidation (Chen *et al.,* 2000). Similarly, Cook *et al.* (2004) suggested the involvement of *Arabidopsis CBF3* gene in improving chilling tolerance in transgenic *Arabidopsis* due to enhanced expression of targeted *P5CS2* gene and reported higher proline contents compared to non-acclimated plants. Fan *et al.* (2012) engineered sweet potato with *BADH* gene (isolated from *Spinacia oleracea*) and claimed that overexpression of this gene enhanced the production of proline and betaine which lowered down the production of ROS and enhanced antioxidant enzymes activities due to which transgenic sweet potato plants became more adaptive to chilling stress. Similarly, overexpression of rice *Osmyb4* gene was achieved in spring rape (*Brassica napus*) at cold stress (4 °C) and transgenic plants showed the improved cold tolerance, which was obvious from the accumulation of more biomass, proline contents and antioxidants compared to WT plants (Gomaa *et al.,* 2012).

5.16.3 Total soluble sugar contents

Plants can protect themselves from chilling injury by the accumulation of compatible solutes like total soluble sugars (TSS). TSS save plant cells from damage through many ways; acting as osmoprotectants, a source of nutrients, controlling plant metabolism and protecting cellular membranes by interrelating with lipid bilayer (Ristic and Ashworth 1993; Wanner and Junttila, 1999; Shao *et al.,* 2006). Moreover, TSS play an essential role in signal transduction as primary messengers (Wang *et al.,* 2008; Xiao *et*

al., 2009). During present study, three tomato transgenic lines were developed by transforming cold inducible gene *DREB1A/CBF3* under the control of inducible promoter; lip9. We were interested to confirm the response of transgenic tomato plants by over-expressing *CBF3* gene under various low temperature stresses. Transgenic and NT plants of these genotypes were incubated at 0 °C for 2-3 days followed by recovery at 25 °C. It was noticed that neither control nor transgenic plants exhibited tolerance against freezing temperature and died. Subsequently, TSS contents were extracted from transgenic and NT plants of all the genotypes under normal temperature. There were no significant differences between transgenic and NT plants of all the genotypes. However, after different chilling stresses, TSS (sucrose, glucose, fructose and galactose) increased in all the transgenic lines due to cold inducible expression of *DREB1A*. Our findings were in parallelism with the earlier report of Hsieh *et al.* (2002) who conducted study on *AtDREB1A* heterology expression for enhanced chilling tolerance in transgenic tomato. The transgenic tomatoes developed in our research study were also exposed to chilling stresses for seven days. It was found that transformed tomato plants were unable to survive at freezing temperature. It might be due to lack of expression of cold responsive homologs in transformed tomatoes, confirming that *AtCBF3* gene enhanced chilling tolerance, but not freezing tolerance in transgenic tomato lines. In another study, cotton *GhDREB* gene was isolated and transferred into wheat under the control of an inducible promoter; *rd29A*. The functional analysis demonstrated that transformed plants had elevated tolerance to chilling stresses accumulating more TSS contents and chlorophyll contents in leaves (Gao *et al.,* 2009). The deficiency of soluble sugar contents resulted in the freezing sensitivity of *Arabidopsis gi-3* mutant. Wild type plants accumulated higher levels of soluble sugars than that of *gi-3* mutants and produced tolerance under chilling stress (Cao *et al.,* 2007).

5.16.4 Chlorophyll contents

In plants molecular machinery, chlorophyll acts as a major biochemical factor that is critical for photosynthesis; a process in which water molecule is converted to oxygen and chlorophyll performs its function in this redox chemistry to generate an electronic state by absorbing light and transferring excitation energy to photosystems reaction

centre for generating energy to plants (Heathcote *et al.,* 2002; Ferreira *et al.*, 2004; Yano *et al.,* 2006). During this study, there were no obvious differences in chlorophyll contents (Chl a, b and total chlorophyll) among transgenic and NT plants at normal temperature. Under low temperature, the chlorophyll contents increased in both transgenic and NT plants, but the rate of increase was higher in transgenics than those of NT plants. The elevated degree of cold tolerance in tomato transgenic lines might be due to maintaining more chlorophyll contents and normal photosynthetic apparatus under chilling stresses (Zhang *et al.,* 2011). This result denotes that the incorporated trait is quite functional in T_2 transgenic tomato plants. Visual symptoms of chilling induced injury like leaf wilting and necrosis were noticed in NT plants due to less chlorophyll synthesis capacity, compared to transgenic plants. The evidence behind plant necrosis under chilling stress is the inhibition of Rubisco enzyme activity, involved in C_3 cycle. Moreover, the activity of fructose 1, 6-bisphosphatase (FBPase) for regulating glycolysis is also limited (Kingston-Smith *et al.,* 1997). The inactivation of these enzymes demonstrates that chilling induces changes in proteins such as binding of inhibitors in Rubisco resulting in loss of photosynthesis (Edmondson *et al.,* 1990; Portis, 1992). In plants, photosynthetic potential directly depends upon the amount of chlorophyll contents present in leaves (Schlemmer *et al.,* 2005). The overexpression of *DREB1A* improved survival rates of transgenic plants under cold conditions enhancing chlorophyll contents. Zhang *et al.* (2011) investigated the role of *SlGMEs* under chilling stress in tomato and reported that *SlGMEs* transgenic tomato plants improved cold tolerance based on minimum loss of chlorophyll contents. Gupta *et al.* (2012) reported that *cbf1* gene driven by inducible promoter RD29A improved chilling stress tolerance in transgenic cucumber under cold stress (4 °C), while the NT plants didn't survive and ultimately died. A similar type of study was conducted on the improvement of wheat against cold stress transferring *GhDREB* gene. The overexpression of *GhDREB* improved tolerance against cold stress by producing elevated chlorophyll contents in leaves (Gao *et al.,* 2009). Our findings also suggested that *DREB1A* could be a model candidate for developing cold tolerance in various crops.

5.16.5 Carotenoid contents

The carotenoids play an essential role in harvesting light energy for photosynthesis and protect chlorophyll from photo-oxidation due to excess light energy by limiting ROS produced during photosynthesis (Aluru *et al.*, 2006; Heath *et al.*, 2013). Carotenoids are manufactured within the plastids from phytoene and catalyzed by indispensable phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) enzymes (Carol and Kuntz, 2001). Besides their self-protective functions as antioxidants, they also act as precursors to phytohormone like abscisic acid (ABA) which plays key role in seed development, regulation of stomata and plant responses to cold stress (Anderson *et al.*, 2004; Yamaguchi-Shinozaki and Shinozaki, 2006; Zeng *et al.*, 2013). *CBF3*/*DREB1A* is induced by chilling stress at transcriptional level in an ABA-independent manner encoding proteins that save plants against chilling injury. Hitherto, it is unclear why cold responsive *CBF3* in an ABA-independent manner is related to ABA accumulation (Kim, 2007). During this study, there were no marked differences in carotenoid contents among transgenic and NT tomato lines under normal temperature. Upon exposure to chilling stress, there was almost three-fold increase in carotenoid contents in two transformed lines, compared with their NT counterparts due to overexpression of *DREB1A* gene. Gerjets and Sandmann (2006) cloned two genes; *crtR* and *crtO* ketolase from cyanobacterium *Synechocystis* and transferred into potatoes. The transgenic potatoes accumulated more carotenoids than that of wild type plants, indicating the improvement of crop quality with normal photosynthesis and primary metabolism. A gene; lutein epsilon-ring hydroxylase (*LeLUT1*) was isolated from tomato and transformed into tobacco for cold tolerance. The transgenic tobacco exhibited elevated level of carotenoid contents under cold conditions. Due to higher levels of carotenoid, the transgenic plants exhibited lower contents of H_2O_2, O_2^-, MDA, less ion leakage and and higher levels of chlorophyll fluorescence, oxidizable P_{700} (PSI) and net photosynthetic rate than that of WT plants. Due to overexpression of *LeLUT1,* the photoinhibition, photooxidation and photosynthetic sensitivity decreased in transgenic tobacco plants (Zhou *et al.*, 2013).

5.16.6 Ascorbic acid contents

The chilling stress increases the production of toxic free radicals that cause oxidative damage and change the response of antioxidant enzyme system in plant cells. The degree of these biochemical changes may differ depending on the plants tolerance or sensitivity against chilling stress (Iseri *et al.,* 2013). Ascorbic acid (AsA) is an essential antioxidant that detoxifies ROS generated during cold stress, and it also acts as a co-factor for many enzymes (Eltayeb *et al.,* 2007; Kim *et al.,* 2013). AsA has been reported to regulate cell division and growth by acting as a signal transduction molecule (Green and Fry, 2005). AsA also performs its functions in various physiological processes such as photosynthesis, photo-protection, hormones production and cell wall biosynthesis (Conklin and Barth, 2004). In order to investigate the chilling tolerance in tomato by over-expressing *DREB1A*, we measured AsA contents in leaves of plants exposed to various chilling stresses. The measurement of this antioxidant exhibited obvious discrepancies among transgenic and NT tomato plants at all chilling stresses. Present study reports an enhanced accumulation of AsA in transgenic tomato lines than that of their NT counterparts under various cold stresses. Our findings were consistent with the results of Sun *et al.* (2010) who narrated that transgenic tobacco over-expressing *StAPX* resulted in the enhanced levels of AsA that played a significant role in scavenging ROS under various environmental stresses and guarded the plant cells against their toxic effects. It has been revealed that overexpression of *SlGME1* and *SlGME2* resulted in the greater accumulation of total AsA that enhanced chilling tolerance and higher survival rate in transgenic tomato plants (Zhang *et al.,* 2011). In another study by Maruthasalam *et al.* (2010) constitutive over-expression of glucose oxidase (*GO*) conferred cold tolerance potential in transgenic tobacco plants by the activation of defense system. This defense system was associated with higher activities of antioxidants especially AsA, CAT and APX. On the other hand, control plants showed chilling injury symptoms including necrosis of various plant parts that led to plant death. Similarly, Miura *et al.* (2012) confirmed the overexpression of a transcription factor *SlICE1* in *Solanum lycopersicum* which enhanced the accumulation of antioxidants including β-carotene, lycopene and AsA conferring protection against free radicals generated during cold stress.

5.16.7 Malondialdehyde (MDA) contents

Malondialdehyde (MDA) is an important parameter for measuring lipid peroxidation and efficiency of antioxidant enzyme system in plant tissues and acts as an index for membrane injury in tomato (Li *et al.,* 2013). MDA is an end product of unsaturated fatty acids decomposition in phospholipids and its degree of production indicates the extent of cell injury by toxic free radicals (Song *et al.,* 2011). When the level of unsaturated FAs decrease in membranes, the membranes viscosity is elevated resulting in the oxidation of membrane proteins that lead to the decrease of electrical field strength in cell membranes. Due to these changes, the membrane-bound enzymes become inactive, which disturb the cell metabolism and ultimately plant death may occur (Popov *et al.,* 2006). In order to unravel the mystery of enhanced cold tolerance in transgenic tomato lines, we compared MDA contents of transgenic and NT tomato plants and found that the overexpression of *DREB1A* in transgenic tomato conferred tolerance against cold stress by lowering down the MDA production. Consistent with the findings of Zhang *et al*. (2011) who expressed *SlGMEs* in tomato and argued that after cold treatment (4 ºC) for seven days, MDA contents in transformed plants became lower and likewise, more plant survival rate recorded in transformed plants as compared to their NT counterparts. Similarly, Liu *et al.* (2013) examined the influence of cold stress on the transgenic tomato plants that inducibly overexpressed *Lefad7* gene and inferred that wild type plants exhibited more MDA contents than that of transgenic plants. They also reported lower ROS contents along with higher antioxidant enzyme activities which enhanced the chilling tolerance by altering the membrane lipids composition in transgenic plants as compared to WT plants. An earlier report by Zhang *et al.* (2009) implied that overexpression of *AtIpk2b* enhanced cold tolerance in tomato producing much lower level of MDA contents in transgenic plants. In another study, Wang *et al.* (2008) engineered tobacco plants overexpressing *MpAFP149* gene and reported that T_1 transformants accumulated lower MDA contents and membrane leakage compared with WT plants and exhibited more protection and tolerance to cold stress. Similarly, Zhu *et al.* (2010) confirmed that transgenic *Arabidopsis* overexpressing *ThpI* gene showed enhanced cold tolerance by producing lower MDA contents than that of WT plants.

Zhang *et al.* (2004) conducted a similar type of study and reported that like *Arabidopsis CBF1-3* genes, tomato possesses 3 CBF homologs namely *Lycopersicum esculentum CBF1-3* (*LeCBF1-3*). Among them, only *LeCBF1* was functional in cold stress. They transformed *LeCBF1* in *Arabidopsis* under the control of 35S constitutive promoter and found aberrant growth and phenotypes of *Arabidopsis* transgenic plants. But the overexpression of *LeCBF1* induced the expression of cold regulated (COR) genes and hence increased freezing tolerance in *Arabidopsis* without any chilling stimulus. From this experiment, they inferred that *LeCBF1* was an active homolog of *AtCBF* genes. To prove these logic consequences, they performed another experiment in which they transformed *LeCBF1* or *AtCBF3* in tomato under constitutive 35S promoter and reported inhibitory effects of *LeCBF1* or *AtCBF3* on growth as well as yield characteristics including stunted growth, delay in flowering and fewer numbers of fruit per plants in transgenic plants as compared to their isogenic control plants. From this experiment, they argumented that tomato transgenic plants didn't exhibit any freezing tolerance. But our research study is contrasting from that of Zhang *et al.* (2004) and signifies the importance of lip9 inducible promoter which triggers *AtDREB1A* gene to regulate the expression of multiple cold stress responsive genes to create chilling tolerance in tomato with no abnormal phenotypic changes. From present study, we also concluded that under normal growth conditions, no distinguishable differences were noticed in transgenic and NT tomato plants. But under various levels of chilling stress, improved chilling tolerance was noticed in all the transgenic lines with no inhibitory effects of *AtDREB1A* gene on growth as well as yield characteristics of all the transgenic lines.

VI. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Tomato (*Solanum lycopersicum* Mill.) has relatively short life span and it is considered as an important vegetable crop in Pakistan due to its high economic value. Due to this reason, its area under cultivation has been increasing day by day. During the year 2009-10, area under tomato cultivation was 50,000 hectares with an annual production was estimated at about 476800 tons. Its area under cultivation was increased to 52,300 hectares with an annual production of 530,000 tons during the year 2011-12. However, its supplies are extensively reduced during winter season because it is the most sensitive to cold and its germination, growth, respiration, photosynthesis and flowering are severely affected below 12 °C.

This study was carried out to develop cold tolerance through the incorporation of *CBF3* gene in three tomato genotypes namely Rio Grande, Moneymaker and Roma. Various tissue culture protocols were optimized for callus induction, direct and indirect shoot regeneration in order to get successful genetic transformation. The seeds were surface-sterilized with an optimized level of clorox (40%). Five different types of explants (cotyledons, epicotyls, leaf discs, internodes and hypocotyls) from 15-d old *in vitro* seedlings were evaluated for their regeneration potential. Among these explants, hypocotyls and leaf discs were found to be the best ones producing the highest regeneration frequency and mean number of primordial shoots per explants.

Different types and concentrations of PGRs, $AgNO_3$ and $CoCl_2$ were investigated for callus induction, *in vitro* shoot regeneration and the mean number of primordial shoots per explants. The maximum callus induction frequency was recorded using hypocotyls, while *in vitro* shoot regeneration frequency and the mean number of primordial shoots per explants were significantly higher when leaf discs were used as explants in all the genotypes. The maximum callus induction frequency (67.48%) was recorded in cv. Rio Grande on CIM_6 (MS medium supplemented with 2.0 mg/l IAA and 2.5 mg/l BAP). The callus induction frequency was increased up to 75.65 and 91.83% in cv. Rio Grande when CIM_6 medium was fortified with 5.5 mg/l $CoCl_2$ and 15.0 mg/l

$AgNO_3$, respectively. It was followed by cv. Moneymaker whose maximum callus induction (58.23%) was produced on CIM_4 (MS medium enriched with 2.0 mg/l NAA and 2.0 mg/l BAP) which was enhanced up to 68 and 82.66% when CIM_4 was fortified with 3.5 mg/l $CoCl_2$ and 10.0 mg/l $AgNO_3$. In case of cv. Roma, the maximum callus induction (62%) was obtained on CIM_5 (MS medium augmented with 1.0 mg/l NAA and 2.5 mg/l BAP) which was increased up to 73.66 and 88.33% when CIM_5 was supplemented with 4.5 mg/l $CoCl_2$ and 12.5 mg/l $AgNO_3$. Similarly, *in vitro* shoot regeneration frequency on MS media supplemented with 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP significantly enhanced with the addition of 8.0 – 10.0 mg/l AgNO3 in all the cultivars i.e. in cv. Rio Grande (96.65%) followed by Roma (92.66%) and Moneymaker (90%) using leaf discs-derived calli. $AgNO_3$ also had promotive effect on induction of multiple shoots per explants. The maximum mean number of multiple shoots per explants (12.33) were recorded on MS media supplemented with IAA – ZEA – BAP (0.1 - 1.0 - 2.0) (mg/l) along with $AgNO_3$ (10.0 mg/l) in Roma followed by Moneymaker (11.00) and Rio Grande (10.66) using leaf discs as explant sources. Likewise, shoot induction media (SIM) supplemented with $CoCl_2$ (4.0 – 5.0 mg/l) significantly enhanced *in vitro* shoot regeneration frequency and the mean number of primordial shoots per explants compared to SIM devoid of $CoCl_2$.

Direct and indirect *in vitro* shoot regeneration was also assessed on MS and N6 basal media supplemented with various concentrations of sucrose and sorbitol along with or without various PGRs. The hypocotyls performed better in direct shoot regeneration, while leaf discs were found to be efficient in calli-derived shoot regeneration in all the genotypes. MS medium performed better than that of N6 medium in producing direct shoot regeneration, while N6 medium gave better performance in indirect shoot regeneration. The highest *in vitro* shoot regeneration frequency (either direct or indirect means) was recorded when sucrose: sorbitol was used in 30: 30 (g/l) accumulatively. The highest direct shoot regeneration frequency (100, 97.69 and 99%) was recorded in Rio Grande, Moneymaker and Roma, respectively on MS medium fortified with only carbon sources. Similarly, the highest calli derived shoot regeneration frequency (96.33, 92.69 and 88.74%) was obtained in Roma, Rio Grande and Moneymaker on N6 basal media

along with sucrose and sorbitol (30: 30 g/l) fortified with IAA (0.1 mg/l), ZEA (1.0 mg/l) and BAP (2.0 mg/l). Similarly, more shoot length and number of shoots primordial were recorded on N6 basal medium as compared to MS basal medium in both direct and indirect shoot regeneration from both types of explants in all the genotypes. These optimized tissue culture protocols were employed in transformation experiments incorporating the cold tolerant gene in three tomato cultivars via *Agrobacterium*-mediated transformation system.

Various transformation factors were also optimized for obtaining maximum gene transfer efficiency. Fifteen days old *in vitro* seedlings, 48-h pre-culture period, $OD_{600\ nm}$ = 0.2 of bacterial culture, 3-min infection time, 60 µM acetosyringone, pH 5.6 of co-cultivation medium and 48-h co-cultivation period were found to be optimum for efficient transformation in all the genotypes examined. After co-cultivation, the explants were incubated for 6-d on a pre-selection medium having 500 mg/l cefotaxime and then they were transferred on a selection medium supplemented with 35 mg/l hygromycin. These optimized conditions gave 6.66 – 18.32% TE using hypocotyls and 4.44 – 15% TE culturing leaf discs in all the tested genotypes. The tissue-culture independent transformation method (*in planta*) was also performed optimizing various important parameters. The soil: vermiculite (1: 1) growth medium, 35 mg/l hygromycin level, $OD_{600\ nm}$ = 1.0, 60 µM acetosyringone, 20 min incubation period and 48-h co-cultivation period were found to be the best for *in planta* transformation system yielding 3.82 – 8.28% TE.

The T_2 transgenic lines were compared with their isogenic NT plants on the basis of morphological characteristics. The outcomes of this detailed morphological study of three tomato transgenic lines clearly demonstrated that the expression of *CBF3* gene didn't seem to be linked with agronomic as well as yield parameters in tomato. It might be due to the reason that lip9; a cold inducible promoter could not induce the expression of *CBF3* gene under normal growth conditions and hence no significant differences were recorded among transgenic and NT plants.

The transgenic plants and their isogenic counterparts of all the genotypes were exposed to different low temperature stresses (4, 6 and 8 °C) for seven days and then

returned to 25 °C for recovery under controlled conditions in glasshouse and then transgenic lines were screened for cold tolerance on the basis of physiological parameters. At normal temperature (25 ºC), no marked differences were found among transgenic and NT plants for all the physiological characteristics. But after cold stresses, stomatal conductance (gs), transpiration rate (E), CO_2 concentration rate (Ci), photosynthetic rate (A) and relative water contents (%) enhanced significantly in all the transgenic lines due to overexpression of *DREB1A* compared to NT counterparts. While the leaf osmotic potential (Ψs) in both types of plants decreased significantly with the increased chilling stress. The rate of decrease of Ψs was higher in transgenic lines than that of NT ones upon exposure to various low temperature stresses.

The tomato transgenic lines were also screened for their chilling tolerance on the basis of biochemical characteristics. Under non-stressed conditions, no significant differences were recorded among 3 weeks old transgenic and NT lines with respect to all biochemical parameters. Increase in chilling stress was accompanied by the increase in ion leakage (%) and malondialdehyde contents (nmol/g FW) in the leaves of all transgenic lines but the rate of increase was too lower than that of NT plants. The proline contents (µg/g FW), total soluble sugar contents (mg/g FW), chlorophyll contents (mg/g FW), carotenoid contents (mg/g FW) and ascorbic acid contents (mg/100 g FW) improved under cold stress compared to NT plants, but the rate of contents of all these parameters gradually decreased by increasing the low temperature from 4 – 8 ºC.

6.2 Conclusions

In vitro morphogenesis is greatly influenced by a gaseous plant growth regulator (ethylene). The findings of our research work indicate that ethylene is linked with the suppression of *in vitro* morphogenesis in tomato. $AgNO_3$ and $CoCl_2$ interact with ethylene and enhance the callus induction, *in vitro* shoot regeneration frequency and the mean number of primordial shoot in tomato. Among various explants tested, hypocotyls and leaf discs were found to be more responsive for callogenesis and shoot organogenesis. We have developed an innovative approach for *in vitro* shoot regeneration using 30: 30 (g/l) sucrose and sorbitol accumulatively in MS and N6 basal media. The significance of this approach is that it avoids callus development phase, and ultimately

reduces the abnormal plant development due to somaclonal variations. Likewise, the jeopardy of contamination was also reduced following one simple explants culturing step, avoiding the maintenance of callus cultures again and again. By using this procedure, we can appreciably save potential resources in terms of hormonal costs and time. Based on our findings, we propose this hormone-free approach for *in vitro* shoot regeneration purposes for the development of stress tolerant cultivars of tomato using *Agrobacterium*-mediated genetic transformation. We have optimized two transformation methods; one is tissue culture based and the other one is a novel tissue culture independent method using seeds as explants via *Agrobacterium*-mediated system. By employing these optimized methods, we have successfully transformed three tomato genotypes transferring *CBF3* gene under the control of an inducible promoter lip9. The detailed morphological study of three tomato transgenic lines clearly demonstrated that the expression of *CBF3* gene didn't seem to be linked with agronomic as well as yield parameters at normal temperature. No marked differences were recorded among transgenic and NT plants under normal growth conditions. But under cold stress, transgenic lines exhibited chilling tolerance in term of improved membrane stability, stomatal conductance, transpiration rate, CO_2 concentration rate, photosynthetic rate, relative water contents, proline contents, total soluble sugar contents, chlorophyll contents, carotenoid contents and ascorbic contents due to inducible expression of *CBF3* gene. The Roma transgenic line showed better chilling tolerance as compared to Rio Grande and Moneymaker at all low temperature stresses.

6.3 Recommendations

Further studies regarding reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), singlet oxygen ($^1O_2$) and hydroxyl radical (OH) that rupture cell membranes and alter the gene expression during cold stress, can be extended in tomato transgenic lines. From present studies, it is also recommended that antioxidants [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO) and ascorbate peroxidase (APX)] that detoxify these ROS and save the plants from oxidative damage needs to be conducted.

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APPENDICES

Appendix 1. ANOVA for the effect of clorox on contamination frequency

S.O.V	DF	SS	MS	F	P
Treatment	4	3105.63	776.406	228.50	0.0000
Variety	2	191.25	95.623	28.14	0.0000
Treatment*Variety	8	61.02	7.628	2.24	0.0543
Error	28	95.14	3.398		
Total	44	3471.19			
Grand Mean 10.682	CV 17.26				

Appendix 2. ANOVA for the effect of clorox on germination frequency

S.O.V	DF	SS	MS	F	P
Treatment	1	529.53	529.534	53.16	0.0000
Variety	2	543.22	271.608	27.26	0.0001
Treatment*Variety	2	80.33	40.163	4.03	0.0520
Error	10	99.62	9.962		
Total	17	1324.70			
Grand Mean 78.434	CV 4.02				

Appendix 3. ANOVA for regeneration frequency of different explants

S.O.F	DF	SS	MS	F	P
Explant	4	13073.9	3268.48	3888.84	0.0000
Variety	2	1068.1	534.07	635.43	0.0000
Explant*Variety	8	16.1	2.01	2.39	0.0418
Error	28	23.5	0.84		
Total	44	14183.8			
Grand Mean 47.400	CV 1.93				

Appendix 4. ANOVA for the number of shoots by different explants

S.O.V	DF	SS	MS	F	P
Explant	4	196.163	49.0408	152.47	0.0000
Variety	2	4.884	2.4420	7.59	0.0023
Explant*Variety	8	9.230	1.1537	3.59	0.0055
Error	28	9.006	0.3216		
Total	44	225.243			
Grand Mean 5.6218	CV 10.09				

Appendix 5. ANOVA for the effect of various PGRs on callus induction

S.O.V	DF	SS	MS	F	P
Explant	1	345.1	345.09	252.81	0.0000
Treatment	7	64010.6	9144.38	6699.16	0.0000
Variety	2	1040.8	520.38	381.23	0.0000
Explant*Treatment	7	131.8	18.83	13.80	0.0000
Explant*Variety	2	19.9	9.93	7.27	0.0012
Treatment*Variety	14	2357.7	168.41	123.38	0.0000
Explant*Treatment*Variety	14	49.6	3.54	2.59	0.0033
Error	94	128.3	1.37		
Total	143	68084.7			
Grand Mean 31.837	CV 3.67				

Appendix 6. ANOVA for the effect of AgNO3 along with PGRs on callus induction

S.O.V	DF	SS	MS	F	P
Explant	1	151.2	151.17	25.31	0.0000
Treatment	7	53404.9	7629.28	1277.14	0.0000
Variety	2	309.7	154.83	25.92	0.0000
Explant*Treatment	7	126.2	18.03	3.02	0.0066
Explant*Variety	2	38.4	19.22	3.22	0.0445
Treatment*Variety	14	4957.9	354.14	59.28	0.0000
Explant*Treatment*Variety	14	204.8	14.63	2.45	0.0055
Error	94	561.5	5.97		
Total	143	60728.1			

Grand Mean 55.410 CV 4.41

Appendix 7. ANOVA for the effect of $CoCl_2$ along with PGRs on callus induction

S.O.V	DF	SS	MS	F	P
Explant	1	432.6	432.57	252.44	0.0000
Treatment	7	29403.2	4200.45	2451.30	0.0000
Variety	2	966.3	483.17	281.97	0.0000
Explant*Treatment	7	18.7	2.67	1.56	0.1573
Explant*Variety	2	7.1	3.53	2.06	0.1331
Treatment*Variety	14	7810.2	557.87	325.56	0.0000
Explant*Treatment*Variety	14	60.4	4.32	2.52	0.0043
Error	94	161.1	1.71		
Total	143	38975.3			

Grand Mean 44.986 CV 2.91

Appendix 8. ANOVA for the effect of various PGRs on *in vitro* shoot regeneration

S.O.V	DF	SS	MS	F	P
Explant	1	372.2	372.20	214.92	0.0000
Treatment	10	94811.3	9481.13	5474.59	0.0000
Variety	2	462.1	231.07	133.42	0.0000
Explant*Treatment	10	62.0	6.20	3.58	0.0003
Explant*Variety	2	2.1	1.07	0.62	0.5415
Treatment*Variety	20	2736.4	136.82	79.00	0.0000
Explant*Treatment*Variety	20	80.0	4.00	2.31	0.0026
Error	130	225.1	1.73		
Total	197	98815.2			

Grand Mean 33.373 CV 3.94

Appendix 9. ANOVA for the effect of various PGRs on the number of primordial shoots per explants

S.O.V	DF	SS	MS	F	P
Explant	1	8.490	8.4899	15.18	0.0002
Treatment	10	884.818	88.4818	158.18	0.0000
Variety	2	10.343	5.1717	9.25	0.0002
Explant*Treatment	10	1.566	0.1566	0.28	0.9847
Explant*Variety	2	0.040	0.0202	0.04	0.9645
Treatment*Variety	20	11.879	0.5939	1.06	0.3973
Explant*Treatment*Variety	20	0.404	0.0202	0.04	1.0000
Error	130	72.717	0.5594		
Total	197	997.540			

Grand Mean 2.6414 CV 28.31

Appendix 10. ANOVA for the effect of AgNO3 along with PGRs on *in vitro* shoot regeneration

Source	DF	SS	MS	F	P
Explant	1	466	465.58	212.09	0.0000
Treatment	10	97856	9785.59	4457.78	0.0000
Variety	2	480	240.13	109.39	0.0000
Explant*Treatment	10	42	4.20	1.91	0.0487
Explant*Variety	2	11	5.43	2.47	0.0883
Treatment*Variety	20	3993	199.63	90.94	0.0000
Explant*Treatment*Variety	20	94	4.72	2.15	0.0056
Error	130	285	2.20		
Total	197	103537			

Grand Mean 57.846 CV 2.56

Appendix 11. ANOVA for the effect of $AgNO_3$ along with PGRs on the number of primordial shoots per explants

S.O.V	DF	SS	MS	F	P
Explant	1	19.41	19.414	19.34	0.0000
Treatment	10	1695.12	169.512	168.84	0.0000
Variety	2	35.85	17.924	17.85	0.0000
Explant*Treatment	10	1.36	0.136	0.14	0.9992
Explant*Variety	2	0.07	0.035	0.04	0.9654
Treatment*Variety	20	63.82	3.191	3.18	0.0000
Explant*Treatment*Variety	20	1.82	0.091	0.09	1.0000
Error	130	130.52	1.004		
Total	197	1949.45			

Grand Mean 6.1515 CV 16.29

Appendix 12. ANOVA for the effect of $CoCl_2$ along with PGRs on *in vitro* shoot regeneration

S.O.V	DF	SS	MS	F	P
Explant	1	229.6	229.61	75.25	0.0000
Treatment	10	84330.4	8433.04	2763.65	0.0000
Variety	2	236.5	118.26	38.76	0.0000
Explant*Treatment	10	62.5	6.25	2.05	0.0332
Explant*Variety	2	9.5	4.77	1.56	0.2132
Treatment*Variety	20	1047.6	52.38	17.17	0.0000
Explant*Treatment*Variety	20	102.9	5.15	1.69	0.0433
Error	130	396.7	3.05		
Total	197	86823.5			

Grand Mean 46.528 CV 3.75

Appendix 13. ANOVA for the effect of $CoCl_2$ along with PGRs on the number of primordial shoots per explants

S.O.V	DF	SS	MS	F	P
Explant	1	8.081	8.0808	12.56	0.0005
Treatment	10	599.535	59.9535	93.20	0.0000
Variety	2	17.162	8.5808	13.34	0.0000
Explant*Treatment	10	1.919	0.1919	0.30	0.9805
Explant*Variety	2	0.010	0.0051	0.01	0.9922
Treatment*Variety	20	15.283	0.7641	1.19	0.2748
Explant*Treatment*Variety	20	1.990	0.0995	0.15	1.0000
Error	130	83.626	0.6433		
Total	197	729.313			

Grand Mean 2.3232 CV 34.52

Appendix 14. ANOVA for synergistic effect of sucrose and sorbitol in MS media without PGRs on *in vitro* shoot regeneration

S.O.V	DF	SS	MS	F	P
Explant	1	548	547.8	40.34	0.0000
Treatment	7	108008	15429.7	1136.37	0.0000
Variety	2	1232	616.2	45.38	0.0000
Explant*Treatment	7	306	43.8	3.22	0.0042
Explant*Variety	2	156	78.0	5.75	0.0044
Treatment*Variety	14	2877	205.5	15.13	0.0000
Explant*Treatment*Variety	14	476	34.0	2.51	0.0045
Error	94	1276	13.6		
Total	143	114938			

Grand Mean 63.870 CV 5.77

Appendix 15. ANOVA for synergistic effect of sucrose and sorbitol in MS media without PGRs on *in vitro* shoot length

S.O.V	DF	SS	MS	F	P
Explant	1	17.479	17.4794	26.34	0.0000
Treatment	7	441.647	63.0924	95.09	0.0000
Variety	2	9.437	4.7186	7.11	0.0013
Explant*Treatment	7	4.762	0.6803	1.03	0.4188
Explant*Variety	2	3.756	1.8780	2.83	0.0640
Treatment*Variety	14	61.283	4.3773	6.60	0.0000
Explant*Treatment*Variety	14	6.974	0.4982	0.75	0.7182
Error	94	62.370	0.6635		
Total	143	610.420			

Grand Mean 5.9067 CV 13.79

Appendix 16. ANOVA for synergistic effect of sucrose and sorbitol in N6 media without PGRs on *in vitro* shoot regeneration

S.O.V	DF	SS	MS	F	P
Explant	1	149	148.6	4.51	0.0362
Treatment	7	91592	13084.6	397.58	0.0000
Variety	2	1923	961.6	29.22	0.0000
Explant*Treatment	7	941	134.5	4.09	0.0006
Explant*Variety	2	46	22.8	0.69	0.5021
Treatment*Variety	14	2381	170.1	5.17	0.0000
Explant*Treatment*Variety	14	843	60.2	1.83	0.0452
Error	94	3094	32.9		
Total	143	101642			

Grand Mean 59.993 CV 9.56

Appendix 17. ANOVA for synergistic effect of sucrose and sorbitol in N6 media without PGRs on *in vitro* shoot length

S.O.V	DF	SS	MS	F	P
Explant	1	7.558	7.558	13.20	0.0005
Treatment	7	724.528	103.504	180.82	0.0000
Variety	2	36.711	18.356	32.07	0.0000
Explant*Treatment	7	8.488	1.213	2.12	0.0489
Explant*Variety	2	0.442	0.221	0.39	0.6806
Treatment*Variety	14	16.133	1.152	2.01	0.0246
Explant*Treatment*Variety	14	13.373	0.955	1.67	0.0755
Error	94	53.806	0.572		
Total	143	861.477			

Grand Mean 6.5351 CV 11.58

Appendix 18. ANOVA for synergistic effect of sucrose and sorbitol in MS media along with PGRs on *in vitro* shoot regeneration

S.O.V	DF	SS	MS	F	P
Explant	1	514.2	514.16	76.63	0.0000
Treatment	7	37765.7	5395.10	804.08	0.0000
Variety	2	1013.0	506.48	75.49	0.0000
Explant*Treatment	7	174.8	24.98	3.72	0.0013
Explant*Variety	2	150.8	75.41	11.24	0.0000
Treatment*Variety	14	793.2	56.66	8.44	0.0000
Explant*Treatment*Variety	14	246.2	17.58	2.62	0.0030
Error	94	630.7	6.71		
Total	143	42308.4			

Grand Mean 61.146 CV 4.24

Appendix 19. ANOVA for synergistic effect of sucrose and sorbitol in MS media along with PGRs on the number of primordial shoots per explant

S.O.V	DF	SS	MS	F	P
Explant	1	95.06	95.063	59.33	0.0000
Treatment	7	1275.94	182.277	113.75	0.0000
Variety	2	94.62	47.312	29.53	0.0000
Explant*Treatment	7	10.44	1.491	0.93	0.4868
Explant*Variety	2	0.38	0.188	0.12	0.8897
Treatment*Variety	14	42.37	3.027	1.89	0.0372
Explant*Treatment*Variety	14	30.63	2.188	1.37	0.1859
Error	94	150.63	1.602		
Total	143	1717.44			

Grand Mean 7.7708 CV 16.29

Appendix 20. ANOVA for synergistic effect of sucrose and sorbitol in N6 media along with PGRs on *in vitro* shoot regeneration

S.O.V	DF	SS	MS	F	P
Explant	1	931.7	931.7	113.74	0.0000
Treatment	7	72146.7	10306.7	1258.23	0.0000
Variety	2	991.0	495.5	60.49	0.0000
Explant*Treatment	7	124.2	17.7	2.17	0.0442
Explant*Variety	2	22.6	11.3	1.38	0.2573
Treatment*Variety	14	681.5	48.7	5.94	0.0000
Explant*Treatment*Variety	14	290.5	20.7	2.53	0.0041
Error	94	770.0	8.2		
Total	143	77223.0			

Grand Mean 60.824 CV 4.71

Appendix 21. ANOVA for synergistic effect of sucrose and sorbitol in N6 media along with PGRs on the number of primordial shoots per explant

S.O.V	DF	SS	MS	F	P
Explant	1	42.25	42.250	13.85	0.0003
Treatment	7	1972.00	281.714	92.32	0.0000
Variety	2	73.63	36.813	12.06	0.0000
Explant*Treatment	7	35.75	5.107	1.67	0.1249
Explant*Variety	2	9.13	4.563	1.50	0.2295
Treatment*Variety	14	26.38	1.884	0.62	0.8445
Explant*Treatment*Variety	14	50.87	3.634	1.19	0.2953
Error	94	286.83	3.051		
Total	143	2502.00			

Grand Mean 8.3333 CV 20.96

Appendix 22. ANOVA for the effect of seedling age on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	228.26	228.261	1061.05	0.0000
Treatment	4	1227.73	306.931	1426.74	0.0000
Variety	2	5.02	2.512	11.68	0.0001
Explant*Treatment	4	19.10	4.774	22.19	0.0000
Explant*Variety	2	0.87	0.436	2.03	0.1407
Treatment*Variety	8	25.50	3.187	14.81	0.0000
Explant*Treatment*Variety	8	4.68	0.585	2.72	0.0129
Error	58	12.48	0.215		
Total	89	1523.68			

Grand Mean 6.9392 CV 6.68

Appendix 23. ANOVA for the effect of pre-culture period on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	28.437	28.437	119.79	0.0000
Treatment	4	761.333	190.333	801.77	0.0000
Variety	2	7.033	3.517	14.81	0.0000
Explant*Treatment	4	1.281	0.320	1.35	0.2628
Explant*Variety	2	1.151	0.576	2.42	0.0974
Treatment*Variety	8	24.980	3.123	13.15	0.0000
Explant*Treatment*Variety	8	4.867	0.608	2.56	0.0182
Error	58	13.769	0.237		
Total	89	843.294			

Grand Mean 4.4288 CV 11.00

Appendix 24. ANOVA for the effect of OD on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	48.210	48.210	204.45	0.0000
Treatment	4	417.415	104.354	442.54	0.0000
Variety	2	14.406	7.203	30.55	0.0000
Explant*Treatment	4	16.710	4.177	17.72	0.0000
Explant*Variety	2	4.227	2.113	8.96	0.0004
Treatment*Variety	8	9.048	1.131	4.80	0.0001
Explant*Treatment*Variety	8	4.338	0.542	2.30	0.0326
Error	58	13.677	0.236		
Total	89	528.242			

Grand Mean 3.2659 CV 14.87

Appendix 25. ANOVA for the effect of acetosyringone on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	123.44	123.435	311.03	0.0000
Treatment	5	2056.08	411.217	1036.18	0.0000
Variety	2	21.86	10.928	27.54	0.0000
Explant*Treatment	5	13.58	2.716	6.84	0.0000
Explant*Variety	2	0.06	0.030	0.08	0.9276
Treatment*Variety	10	24.46	2.446	6.16	0.0000
Explant*Treatment*Variety	10	12.50	1.250	3.15	0.0022
Error	70	27.78	0.397		
Total	107	2280.13			

Grand Mean 7.8796 CV 7.99

Appendix 26. ANOVA for the effect of infection duration on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	37.610	37.610	217.14	0.0000
Treatment	4	700.824	175.206	1011.52	0.0000
Variety	2	3.255	1.627	9.39	0.0003
Explant*Treatment	4	11.252	2.813	16.24	0.0000
Explant*Variety	2	1.252	0.626	3.62	0.0331
Treatment*Variety	8	17.001	2.125	12.27	0.0000
Explant*Treatment*Variety	8	3.600	0.450	2.60	0.0169
Error	58	10.046	0.173		
Total	89	784.845			

Grand Mean 3.8669 CV 10.76

Appendix 27. ANOVA for the effect of co-cultivation media pH on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	402.91	402.907	559.37	0.0000
Treatment	5	1068.78	213.757	296.76	0.0000
Variety	2	7.78	3.889	5.40	0.0066
Explant*Treatment	5	94.43	18.887	26.22	0.0000
Explant*Variety	2	22.25	11.126	15.45	0.0000
Treatment*Variety	10	36.59	3.659	5.08	0.0000
Explant*Treatment*Variety	10	20.42	2.042	2.83	0.0052
Error	70	50.42	0.720		
Total	107	1705.93			

Grand Mean 7.8406 CV 10.82

Appendix 28. ANOVA for the effect of cefotaxime sodium on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	124.87	124.872	151.25	0.0000
Treatment	5	1153.18	230.637	279.36	0.0000
Variety	2	25.87	12.934	15.67	0.0000
Explant*Treatment	5	1.79	0.359	0.43	0.8228
Explant*Variety	2	24.88	12.441	15.07	0.0000
Treatment*Variety	10	15.70	1.570	1.90	0.0594
Explant*Treatment*Variety	10	20.00	2.000	2.42	0.0154
Error	70	57.79	0.826		
Total	107	1424.44			

Grand Mean 9.4664 CV 9.60

Appendix 29. ANOVA for the effect of co-cultivation period on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	90.41	90.406	666.93	0.0000
Treatment	3	796.56	265.521	1958.76	0.0000
Variety	2	74.62	37.310	275.24	0.0000
Explant*Treatment	3	7.00	2.334	17.22	0.0000
Explant*Variety	2	0.53	0.266	1.96	0.1518
Treatment*Variety	6	27.24	4.540	33.49	0.0000
Explant*Treatment*Variety	6	1.93	0.321	2.37	0.0444
Error	46	6.24	0.136		
Total	71	1004.64			

Grand Mean 6.4989 CV 5.67

Appendix 30. ANOVA for the effect of pre-selection period on transformation efficiency

S.O.V	DF	SS	MS	F	P
Explant	1	79.45	79.449	217.41	0.0000
Treatment	4	1090.52	272.629	746.06	0.0000
Variety	2	43.63	21.813	59.69	0.0000
Explant*Treatment	4	44.30	11.074	30.30	0.0000
Explant*Variety	2	8.96	4.480	12.26	0.0000
Treatment*Variety	8	23.98	2.997	8.20	0.0000
Explant*Treatment*Variety	8	6.45	0.806	2.21	0.0399
Error	58	21.19	0.365		
Total	89	1318.63			
Grand Mean 4.0922	CV 14.77				

Appendix 31. ANOVA for the effect of hygromycin on *in vitro* shoot regeneration

S.O.V	DF	SS	MS	F	P
Explant	1	39.6	39.6	33.20	0.0000
Treatment	4	72467.9	18117.0	15178.1	0.0000
Variety	2	1193.6	596.8	499.98	0.0000
Explant*Treatment	4	232.7	58.2	48.74	0.0000
Explant*Variety	2	10.8	5.4	4.52	0.0149
Treatment*Variety	8	956.9	119.6	100.20	0.0000
Explant*Treatment*Variety	8	24.0	3.0	2.51	0.0203
Error	58	69.2	1.2		
Total	89	74997.7			
Grand Mean 26.578	CV 4.11				

Appendix 32. ANOVA for the effect of growth media on germination frequency

S.O.V	DF	SS	MS	F	P
Treatment	2	1319.00	659.500	260.86	0.0000
Variety	2	399.88	199.939	79.08	0.0000
Treatment*Variety	4	44.63	11.156	4.41	0.0136
Error	16	40.45	2.528		
Total	26	2052.39			
Grand Mean 75.152	CV 2.12				

Appendix 33. ANOVA for the effect of growth media on seedling establishment

S.O.V	DF	SS	MS	F	P
Treatment	2	1676.60	838.299	504.60	0.0000
Variety	2	184.76	92.378	55.61	0.0000
Treatment*Variety	4	22.38	5.594	3.37	0.0352
Error	16	26.58	1.661		
Total	26	2179.18			
Grand Mean 72.946	CV 1.77				

Appendix 34. ANOVA for the effect of OD on *in planta* transformation efficiency

S.O.V	DF	SS	MS	F	P
Treatment	3	77.934	25.9781	38.25	0.0000
Variety	2	14.786	7.3929	10.88	0.0005
Treatment*Variety	6	2.992	0.4987	0.73	0.6274
Error	22	14.942	0.6792		
Total	35	114.291			
Grand Mean 3.1675	CV 26.02				

Appendix 35. ANOVA for the effect of incubation period on *in planta* transformation efficiency

S.O.V	DF	SS	MS	F	P
Treatment	3	97.942	32.6473	34.32	0.0000
Variety	2	34.612	17.3058	18.19	0.0000
Treatment*Variety	6	1.849	0.3081	0.32	0.9173
Error	22	20.927	0.9512		
Total	35	156.240			
Grand Mean 3.7167		CV 26.24			

Appendix 36. ANOVA for comparison of transgenic and NT plants on the basis of plant height

S.O.V	DF	SS	MS	F	P
Plants	1	3.754	3.754	0.55	0.4759
Variety	2	566.306	283.153	41.38	0.0000
Plants*Variety	2	11.331	5.665	0.83	0.4648
Error	10	68.424	6.842		
Total	17	700.150			
Grand Mean 65.623		CV 3.99			

Appendix 37. ANOVA for comparison of transgenic and NT plants on the basis number of leaves per plant

S.O.V	DF	SS	MS	F	P
Plants	1	17.39	17.385	2.72	0.1299
Variety	2	1506.79	753.393	118.01	0.0000
Plants*Variety	2	1.09	0.546	0.09	0.9187
Error	10	63.84	6.384		
Total	17	1669.05			
Grand Mean 34.021		CV 7.43			

Appendix 38. ANOVA for comparison of transgenic and NT plants on the basis of fresh weight

S.O.V	DF	SS	MS	F	P
Plants	1	19.99	19.992	2.54	0.1421
Variety	2	1111.73	555.863	70.63	0.0000
Plants*Variety	2	1.60	0.800	0.10	0.9043
Error	10	78.70	7.870		
Total	17	1490.84			
Grand Mean 54.519		CV 5.15			

Appendix 39. ANOVA for comparison of transgenic and NT plants on the basis of dry weight

S.O.V	DF	SS	MS	F	P
Plants	1	2.1701	2.17014	3.52	0.0902
Variety	2	8.5797	4.28984	6.95	0.0128
Plants*Variety	2	0.0187	0.00934	0.02	0.9850
Error	10	6.1696	0.61696		
Total	17	30.6702			
Grand Mean 5.7528		CV 13.65			

Appendix 40. ANOVA for comparison of transgenic and NT plants on the basis of root length

S.O.V	DF	SS	MS	F	P
Plants	1	16.283	16.2830	1.33	0.2750
Variety	2	181.245	90.6225	7.42	0.0106
Plants*Variety	2	0.331	0.1654	0.01	0.9866
Error	10	122.090	12.2090		
Total	17	344.196			

Grand Mean 21.726 CV 16.08

Appendix 41. ANOVA for comparison of transgenic and NT plants on the basis of days to flowering

S.O.V	DF	SS	MS	F	P
Plants	1	18.000	18.000	0.59	0.4592
Variety	2	481.000	240.500	7.92	0.0087
Plants*Variety	2	3.000	1.500	0.05	0.9520
Error	10	303.667	30.367		
Total	17	886.000			

Grand Mean 60.333 CV 9.13

Appendix 42. ANOVA for comparison of transgenic and NT plants on the basis of number of flowers per plant

S.O.V	DF	SS	MS	F	P
Plants	1	22.178	22.178	0.93	0.3572
Variety	2	522.801	261.401	10.98	0.0030
Plants*Variety	2	1.338	0.669	0.03	0.9724
Error	10	238.030	23.803		
Total	17	793.744			

Grand Mean 30.600 CV 15.94

Appendix 43. ANOVA for comparison of transgenic and NT plants on the basis of first fruit set

S.O.V	DF	SS	MS	F	P
Plants	1	18.000	18.000	0.55	0.4769
Variety	2	397.000	198.500	6.02	0.0192
Plants*Variety	2	3.000	1.500	0.05	0.9557
Error	10	329.667	32.967		
Total	17	768.000			

Grand Mean 69.333 CV 8.28

Appendix 44. ANOVA for comparison of transgenic and NT plants on the basis of number of fruit per plant

S.O.V	DF	SS	MS	F	P
Plants	1	39.783	39.783	2.89	0.1198
Variety	2	356.201	178.101	12.95	0.0017
Plants*Variety	2	0.140	0.070	0.01	0.9949
Error	10	137.541	13.754		
Total	17	576.750			

Grand Mean 17.500 CV 21.19

Appendix 45. ANOVA for comparison of transgenic and NT plants on the basis of fruit diameter

S.O.V	DF	SS	MS	F	P
Plants	1	3.1000	3.10005	3.65	0.0851
Variety	2	11.3293	5.66465	6.67	0.0144
Plants*Variety	2	0.1893	0.09465	0.11	0.8956
Error	10	8.4918	0.84918		
Total	17	24.5111			

Grand Mean 4.7417 CV 19.43

Appendix 46. ANOVA for comparison of transgenic and NT plants on the basis of fruit mean weight

S.O.V	DF	SS	MS	F	P
Plants	1	27.380	27.380	1.35	0.2723
Variety	2	272.666	136.333	6.72	0.0141
Plants*Variety	2	1.817	0.908	0.04	0.9564
Error	10	202.895	20.290		
Total	17	567.747			

Grand Mean 36.593 CV 12.31

Appendix 47. ANOVA for comparison of transgenic and NT plants on the basis of number of seeds per fruit

S.O.V	DF	SS	MS	F	P
Plants	1	82.776	82.776	1.67	0.2247
Variety	2	281.836	140.918	2.85	0.1048
Plants*Variety	2	3.255	1.628	0.03	0.9677
Error	10	494.282	49.428		
Total	17	938.799			

Grand Mean 118.09 CV 5.95

Appendix 48. ANOVA for the effect of various chilling stresses on stomatal conductance of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	3.31961	3.31961	968.37	0.0000
Treatment	3	0.33374	0.11125	32.45	0.0000
Variety	2	0.11777	0.05888	17.18	0.0000
Plants*Treatment	3	1.21247	0.40416	117.90	0.0000
Plants*Variety	2	0.04972	0.02486	7.25	0.0018
Treatment*Variety	6	0.02355	0.00393	1.15	0.3520
Plants*Treatment*Variety	6	0.04547	0.00758	2.21	0.0588
Error	46	0.15769	0.00343		
Total	71	5.26699			

Grand Mean 0.3303 CV 17.73

Appendix 49. ANOVA for the effect of various chilling stresses on transpiration rate of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	3.034	3.0340	316.76	0.0000
Treatment	3	103.234	34.4114	3592.68	0.0000
Variety	2	6.368	3.1838	332.40	0.0000
Plants*Treatment	3	1.277	0.4258	44.46	0.0000
Plants*Variety	2	0.040	0.0199	2.08	0.1367
Treatment*Variety	6	2.580	0.4300	44.89	0.0000
Plants*Treatment*Variety	6	0.162	0.0271	2.83	0.0199
Error	46	0.441	0.0096		
Total	71	117.466			

Grand Mean 1.8131 CV 5.40

Appendix 50. ANOVA for the effect of various chilling stresses on CO_2 concentration rate of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	33616	33616.1	481.24	0.0000
Treatment	3	188748	62915.9	900.69	0.0000
Variety	2	20064	10032.2	143.62	0.0000
Plants*Treatment	3	15397	5132.5	73.48	0.0000
Plants*Variety	2	500	250.2	3.58	0.0358
Treatment*Variety	6	2097	349.5	5.00	0.0005
Plants*Treatment*Variety	6	2427	404.5	5.79	0.0001
Error	46	3213	69.9		
Total	71	278929			

Grand Mean 181.91 CV 4.59

Appendix 51. ANOVA for the effect of various chilling stresses on photosynthetic rate of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	113.60	113.60	319.64	0.0000
Treatment	3	6036.49	2012.16	5661.62	0.0000
Variety	2	226.77	113.39	319.04	0.0000
Plants*Treatment	3	77.82	25.94	72.99	0.0000
Plants*Variety	2	1.99	0.99	2.80	0.0713
Treatment*Variety	6	333.89	55.65	156.58	0.0000
Plants*Treatment*Variety	6	8.50	1.42	3.99	0.0027
Error	46	16.35	0.36		
Total	71	6816.27			

Grand Mean 10.762 CV 5.54

Appendix 52. ANOVA for the effect of various chilling stresses on relative water contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	1809.3	1809.3	194.37	0.0000
Treatment	3	36339.0	12113.0	1301.30	0.0000
Variety	2	927.3	463.6	49.81	0.0000
Plants*Treatment	3	629.9	210.0	22.56	0.0000
Plants*Variety	2	65.9	32.9	3.54	0.0372
Treatment*Variety	6	334.8	55.8	6.00	0.0001
Plants*Treatment*Variety	6	167.9	28.0	3.01	0.0146
Error	46	428.2	9.3		
Total	71	42390.5			

Grand Mean 55.468 CV 5.50

Appendix 53. ANOVA for the effect of various chilling stresses on leaf osmotic potential

S.O.V	DF	SS	MS	F	P
Plants	1	0.59042	0.59042	122.27	0.0000
Treatment	3	3.02848	1.00949	209.05	0.0000
Variety	2	0.05984	0.02992	6.20	0.0041
Plants*Treatment	3	0.22990	0.07663	15.87	0.0000
Plants*Variety	2	0.04389	0.02194	4.54	0.0158
Treatment*Variety	6	0.23186	0.03864	8.00	0.0000
Plants*Treatment*Variety	6	0.06639	0.01107	2.29	0.0511
Error	46	0.22213	0.00483		
Total	71	4.53058			

Grand Mean 0.6406 CV 10.85

Appendix 54. ANOVA for the effect of various chilling stresses on membrane leakage of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	13037.4	13037.4	2966.28	0.0000
Treatment	3	8848.8	2949.6	671.09	0.0000
Variety	2	1607.4	803.7	182.85	0.0000
Plants*Treatment	3	5048.4	1682.8	382.87	0.0000
Plants*Variety	2	58.8	29.4	6.69	0.0028
Treatment*Variety	6	371.2	61.9	14.07	0.0000
Plants*Treatment*Variety	6	81.6	13.6	3.09	0.0125
Error	46	202.2	4.4		
Total	71	29408.8			

Grand Mean 32.269 CV 6.50

Appendix 55. ANOVA for the effect of various chilling stresses on proline contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	641263	641263	1720.68	0.0000
Treatment	3	1747962	582654	1563.42	0.0000
Variety	2	60917	30459	81.73	0.0000
Plants*Treatment	3	228723	76241	204.58	0.0000
Plants*Variety	2	8805	4403	11.81	0.0001
Treatment*Variety	6	9164	1527	4.10	0.0022
Plants*Treatment*Variety	6	8444	1407	3.78	0.0039
Error	46	17143	373		
Total	71	2806337			

Grand Mean 267.06 CV 7.23

Appendix 56. ANOVA for the effect of various chilling stresses on total soluble sugar contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	16762.5	16762.5	4265.38	0.0000
Treatment	3	29676.2	9892.1	2517.13	0.0000
Variety	2	1441.4	720.7	183.39	0.0000
Plants*Treatment	3	5747.6	1915.9	487.51	0.0000
Plants*Variety	2	116.7	58.3	14.84	0.0000
Treatment*Variety	6	111.5	18.6	4.73	0.0008
Plants*Treatment*Variety	6	62.4	10.4	2.65	0.0273
Error	46	180.8	3.9		
Total	71	54170.0			

Grand Mean 37.852 CV 5.24

Appendix 57. ANOVA for the effect of various chilling stresses on chlorophyll "a" contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	154.294	154.294	592.62	0.0000
Treatment	3	155.974	51.991	199.69	0.0000
Variety	2	34.595	17.298	66.44	0.0000
Plants*Treatment	3	74.163	24.721	94.95	0.0000
Plants*Variety	2	11.209	5.604	21.53	0.0000
Treatment*Variety	6	7.323	1.221	4.69	0.0008
Plants*Treatment*Variety	6	7.344	1.224	4.70	0.0008
Error	46	11.976	0.260		
Total	71	469.005			

Grand Mean 3.7947 CV 13.45

Appendix 58. ANOVA for the effect of various chilling stresses on chlorophyll "b" contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	202.07	202.072	537.49	0.0000
Treatment	3	749.52	249.839	664.55	0.0000
Variety	2	151.51	75.755	201.50	0.0000
Plants*Treatment	3	100.38	33.460	89.00	0.0000
Plants*Variety	2	0.68	0.342	0.91	0.4099
Treatment*Variety	6	9.97	8.328	22.15	0.0000
Plants*Treatment*Variety	6	11.99	1.999	5.32	0.0003
Error	46	17.29	0.376		
Total	71	1283.60			

Grand Mean 6.1681 CV 9.94

Appendix 59. ANOVA for the effect of various chilling stresses on total chlorophyll contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	581.63	581.632	887.41	0.0000
Treatment	3	1008.40	336.133	512.84	0.0000
Variety	2	336.94	168.470	257.04	0.0000
Plants*Treatment	3	237.77	79.256	120.92	0.0000
Plants*Variety	2	24.30	12.148	18.54	0.0000
Treatment*Variety	6	31.41	5.236	7.99	0.0000
Plants*Treatment*Variety	6	11.67	1.945	2.97	0.0156
Error	46	30.15	0.655		
Total	71	2400.40			

Grand Mean 9.4597 CV 8.56

Appendix 60. ANOVA for the effect of various chilling stresses on carotenoid contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	37.109	37.1091	214.41	0.0000
Treatment	3	197.416	65.8054	380.21	0.0000
Variety	2	30.863	15.4314	89.16	0.0000
Plants*Treatment	3	17.669	5.8898	34.03	0.0000
Plants*Variety	2	1.730	0.8651	5.00	0.0109
Treatment*Variety	6	12.435	2.0725	11.97	0.0000
Plants*Treatment*Variety	6	2.930	0.4884	2.82	0.0201
Error	46	7.961	0.1731		
Total	71	309.160			

Grand Mean 3.0979 CV 13.43

Appendix 61. ANOVA for the effect of various chilling stresses on ascorbic acid contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	5666.3	5666.33	1081.27	0.0000
Treatment	3	26870.1	8956.71	1709.16	0.0000
Variety	2	1224.1	612.05	116.79	0.0000
Plants*Treatment	3	2396.3	798.75	152.42	0.0000
Plants*Variety	2	55.1	27.55	5.26	0.0088
Treatment*Variety	6	212.3	35.38	6.75	0.0000
Plants*Treatment*Variety	6	73.1	12.18	2.32	0.0483
Error	46	241.1	5.24		
Total	71	36778.2			

Grand Mean 42.610 CV 5.37

Appendix 62. ANOVA for the effect of various chilling stresses on malondialdehyde contents of transgenic and NT plants

S.O.V	DF	SS	MS	F	P
Plants	1	150.02	150.020	336.81	0.0000
Treatment	3	1020.59	340.196	763.76	0.0000
Variety	2	74.24	37.120	83.34	0.0000
Plants*Treatment	3	137.75	45.918	103.09	0.0000
Plants*Variety	2	1.14	0.572	1.28	0.2868
Treatment*Variety	6	135.54	22.591	50.72	0.0000
Plants*Treatment*Variety	6	7.35	1.224	2.75	0.0229
Error	46	20.49	0.445		
Total	71	1547.63			

Grand Mean 8.9632 CV 7.45

Appendix 63. Probe purification for Southern blot hybridization

1. Fifty microliter of amplified PCR product (649 bp) was run on 1.5% agarose gel (50 watts power, 50 volt and 100 mA current) for 40 min and visualized using a UV transilluminator.

2. The gel with amplified product band was cut with surgical blade and put in an autoclaved eppendorf tube and purification was done using QIAquick PCR Purification Kit (QIAGEN, catalog # 28104) and protocol.

3. The buffer PBI (600 µl) was added and the tube was incubated in an incubator at 56 ºC, 500 rpm for 10 min.

4. The mixture was transferred into the column and centrifuged at 14000 rpm for 1 min and flowthrough was discarded.

5. The washing was done with buffer PE (750 µl) containing ethanol (750 µl) and the mixture was spun at 14000 rpm for 1 min and the filtrate was discarded.

6. Subsequently, the column was placed into a clean RNase-free centrifuge tube and 50 µl buffer EB (10 mM Tris.Cl, pH 8.5) was added to the centre of the column to elute.

7. The mixture was incubated into the centrifuge machine for 5 min and centrifugation was done at 14000 rpm for 1 min. Now it was purified probe for using in southern blot hybridization.

PUBLICATIONS FROM THIS STUDY

- **Sabir Hussain Shah**, Shaukat Ali and Ghulam Muhammad Ali. 2013. A novel approach for rapid *in vitro* morphogenesis in tomato (*Solanum lycopersicum* Mill.) with the application of cobalt chloride. European Academic Research. 1 (9): 2702-2721. **(IF: 0.485)**
- **Sabir Hussain Shah**, Shaukat Ali, Sohail Ahmad Jan and Ghulam Muhammad Ali. 2014. Assessment of carbon sources on *in vitro* shoot regeneration in tomato. Pakistan Journal of Agricultural Sciences. 51(1): 197-207. **(IF: 1.054)**
- **Sabir Hussain Shah**, Shaukat Ali, Sohail Ahmad Jan, Jalal-ud-Din and Ghulam Muhammad Ali. 2014. Assessment of silver nitrate on callus induction and *in vitro* shoots regeneration in tomato (*Solanum lycopersicum* Mill.). Pakistan Journal of Botany. 46(6): 2163-2172 **(IF: 1.207)**
- **Sabir Hussain Shah**, Shaukat Ali, Sohail Ahmad Jan, Jalal-ud-Din and Ghulam Muhammad Ali. 2015. Piercing and incubation method of *in planta* transformation producing stable transgenic plants by overexpressing *DREB1A* gene in tomato (*Solanum lycopersicum* Mill.). Plant Cell, Tissue and Organ Culture. 120: 1139-1157 **(IF: 2.612)**
- **Sabir Hussain Shah**, Shaukat Ali, Sohail Ahmad Jan, Jalal-ud-Din and Ghulam Muhammad Ali. 2015. Callus induction, *in vitro* shoot regeneration and hairy root formation by the assessment of various plant growth regulators in tomato (*Solanum lycopersicum* Mill.). The Journal of Animal & Plant Sciences. 25(2): 528-538 **(IF: 0.549)**