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In Vitro Growth Media Effect for Regeneration of Tomato (*Lycopersicon esculentum*) and Evaluation of the Salt Tolerance Activity of Callus

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Abstract. The identification of the best culture medium and the most suitable tomato variety was performed by *In vitro* regeneration. In the present study, attempts have been made to develop an efficient protocol for regeneration of V_1 (ROMA VF), V_2 (Baromasi Hybrid Tomato: JHOLOK) and V_3 (Tomato Hybrid F_1 JAGUR) varieties with their tolerance against abiotic stress (salt) to obtain stress tolerant tomato. The cotyledon induced in MS medium which was supplemented with T_1 (MS+1mg/l BA+0.5mg/l NAA), T_2 (MS + 1.5mg/l BA + 1mg/l NAA) and T_3 (2mg/l BA + 1.5mg/l NAA) from seeds of those varieties. Maximum percentage (75%) of cotyledon induction was observed in T1 for V1, V2 and T3 for V3 respectively. Cotyledon derived from different concentrations of BA and NAA were cultured on MS medium supplemented with T_3 (1.5 mg/l NAA, 2 mg/l BA) and TL (2.0 mg/l Kinetin) for plantlet regeneration. It was observed that MS media supplemented with T_3 + TL produced lowest percentage of shoot (20%), callus (10%) and root (0%) in case of V_2 and highest percentage of shoot (80%), callus(60%) and root (20%) in case of V_3. Callus were transferred to the regeneration medium supplemented with NaCl (0 to 75mM) in order to examine their responses to salinity, the above three varieties, showed a significant decline in the callus growth. Present studies have shown that Hybrid F_1 JAGUR variety is more responsive in regeneration and salt tolerant than others.

Key words: Tomato, Cotyledons, *In vitro* regeneration, MS media, Plant growth regulators.

INTRODUCTION

Tomato (*Lycopersicon esculentum*. Mill) is regarded as the 2nd most important vegetable crop in the world after potato (Bhatia *et al.,* 2004a; Foolad, 2004). It is one of the most important protective foods as it possesses appreciable quantities of vitamins and minerals and sometime rightly referred to as poor man's orange (Devi *et al*. 2008). Botanical name of tomato is *Lypecorsicon esculemtun* and belongs to family Lycopersicae. Tomato belongs to the family Solanaceae by its nature of a perennial plant but is commercially cultivated as an annual crop. It is considered as important dietary vegetable crop. As it is short duration crop and gives high yield, it is important from economic point of view and hence area under its cultivation is increasing day by day. Tomatoes require a minimum temperature of 10^0C and high light intensity to grow. Tomato is used in preserved products like ketch-up, sauce, chutney, soup, paste etc. Tomato is a rich source of minerals, vitamins and organic acid, essential amino acids and dietary fibers. Tomato is known as productive as well as protective food. It is a rich source of vitamin A and C; it also contains minerals like iron, phosphorus.Tomato contains Lycopene and Beta-carotene pigments and is also cholesterol free (Block *et al*., 1992; Gerster, 1997; Rao and Agarwal, 2000).

Tissue culture is an important tool of biotechnology, which can be used to subject of research. Several researchers have reported about adventitious regeneration in tomato deal with induction of shoots on hypocotyls, apical meristem, cotyledons, stems petioles, leaves, anthers and inflorescences explants (Moghaleb *et al*. 1999, Raziuddin *et al*. 2004, Brichkova *et al*. 2002; Compton and Veilleux 1991). However, the improvement of the adventitious shoot regeneration system using tissue culture methods of tomato plants is still important due to the diverse morphogenic potential of different genotypes (Tomsone *et al*. 2004). The main objective of this research work is to comparative investigation of the effect of different growth media composition of three tomato varieties protocol for regeneration and study on their tolerance against different salt concentration.

MATERIALS AND METHODS

This research work was conducted at the Department of Genetic Engineering and Biotechnology, Shahjalal University of Science & Technology (SUST), Sylhet- 3114, Bangladesh

Explants collection

The explants material ROMA VF, Baromasi Hybrid Tomato (JHOLOK) and Tomato Hybrid F_1 (JAGUR) varieties seeds for the experiment was collected from local market in Sylhet. Mature seeds of three tomato (*Lycopersicon esculemtun)* varieties were used as explants for cotyledon induction, shoot regeneration and callus production experiments.

Explant selection

In explants selection diseases and unhealthy seeds are avoided.

Explant sterilization

Mature seeds were surface sterilized with 70% ethyl alcohol for 30 sec, and then for 12-15 min with 5% sodium hypochlorite (commercial bleach is mainly hypochlorite). Surface sterilized seeds were rinsed several times with sterile distilled water before inoculation on cotyledons induction medium.

Sterilization of equipments

Glasswares were scrubbed with brush in a hot detergent bath. They were washed thoroughly with tap water and then rinsed two to three times with distilled water and if necessary autoclaved shortly in order to liquefy the agar and to kill any contaminates that may be present. All the glasswares that needed

cleaning were soaked in detergent water for 2 hours followed by repeated washing in tap water to remove components of detergent. Petri dishes, beaker etc. were wrapped with aluminum foil and left in the autoclaved at 121°C, 15 1b, for 20 minutes for sterilization.

Media formulation for tomato (Lycopersicon esculentum)

Cotyledons induction medium is consisting of MS basal organic and inorganic components supplemented with 3.0% sucrose and BA at (1.0, 1.5, 2.0) mg/l with NAA at (0.5, 1.0, 1.5) mg/l media were prepared and autoclaved at 15 pound square inch (psi) for twenty minutes at 121°c. The pH of the media was adjusted at 5.8-6.0 by adding 1M NaOH. The cotyledon part was transfer into the fresh media T_3 supplemented with 2mg/l kinetin for the highest shoot, callus and root formation with different combination of hormones media and then callus of three varieties were transferred into the salt media for tolerance test.

Culture conditions

Cultures were incubated at 27 ± 2° C under light provided by white fluorescent tubes giving the intensity of about 2000 lux for 16 hours/day. The explants were subcultured with the same media every three weeks interval.

RESULTS

This experiment involves induction of shoot from three varieties V_1-(ROMA VF Tomato), V_2-(Baromasi Hybrid Tomato: JHOLOK) and V_3 - (Tomato Hybrid F_1 JAGUR) of tomato seeds. For this purpose seeds were inoculated in regenerated media. Cotyledons regeneration started within one week of transfer of seeds to three regeneration media T_1 (MS + 1mg/l BA + 0.5mg/l NAA), T_2 (MS + 1.5mg/l BA + 1.0mg/l NAA) and T_3 (MS + 2mg/l BA + 1.5mg/l NAA). Visible cotyledons formation was noted within 2 weeks on the cultivars. (Wang *et al*., 1987) who reported that high concentration of cytokine and low concentration of auxin

promoted plantlet regeneration. Ten days after initial culturing of explants, numbers of shoot/explants data we recollected from the experiment. The data collected on cotyledons initiation response of explants are percentages. (Table 1) shown that T_1 media is very effective in V_1 and V_2, and T_3 media is very effective in V_3 for cotyledons induction (Figure: 1a, 1b, 1c).

After 10 days, cotyledons were transferred into the fresh media T3 supplemented with 2mg/L kinetin .Within 3 weeks; shoots were covered with green buds and also leaf. Some plantlet also shows the callus in the media. Most efficient media for shoot regeneration are T_3 media supplemented with MS + 2.0mg/l BA + 1.5mg/l NAA and TL media supplemented with MS + 2.0 mg/l kinetin (Figure: 1d, 1e and 1f). In the table (Table 2), V_3 is most efficient variety and shows that the highest shoot, callus and root formation.

Effects of salinity on callus growth:

The embryogenic callus of three verities was cultured onto the best callus induction medium. Three-week-old callus was divided into pieces of 1.5 gm. These pieces were transferred onto the same medium supplemented with different NaCl concentrations, that is, 0, 25, 50 and 75 mM, for salt stress responses. At the end of the two-week period, the callus was taken for growth analysis. For the callus growth analysis, the fresh weights of callus were recorded at the beginning and the end of the culture period. The relative growth was calculated on the basis of the initial and final growths. Then the most effective plantlets were transferred into the T_3 + TL media for further growth and callus of three varieties were transferred into the salt media for tolerance test. As the NaCl concentration in the medium increased, there was a decrease in callus fresh weight (Table 3).

The fresh weights of callus of the three genotypes on the NaCl concentration (0.0, 25.0, 50.0, 75.0 mM) were in the medium showed a great effect on callus fresh

weight and the growth value at the end of two weeks growing period. The growth values for callus growing on MS medium were best in V_3.

DISCUSSION

The morphogenesis response seems to be highly dependent plant growth regulators used in the media, which is again cultivar and genotypic specific. To overcome these problems certain modern approaches of gene manipulation might be required, in which *in vitro* regeneration of the transformed cells is an important prerequisite. A good *in vitro* plant regeneration system may also assist in further improvement of the commercially important varieties for salt tolerance via genetic engineering. The development of a cost effective and efficient protocol for mass propagation of high quality seedlings via tomato tissue culture could help lower the price per seedling.

The success in tomato regeneration response has been found to depend largely on genotype, explants, and plant growth regulator used in culture medium (Bhatia *et al.*, 2004a). Plant growth regulators affect morphogenic tomato cultures (Branca *et al.,* 1994). For tomato regeneration, a wide variety of plant growth regulators have been used with varying concentrations. Many cytokinine and auxin combinations could induce shoot proliferation in tomato from different source of explants. Benzyl adenine (BA) + Naphthalene acetic acid (NAA) are the best for callus induction from shoot tips and also found that kinetin (Kin) are the best for regeneration shoots.

Studied the effect of different growth regulators and plant regeneration of tomato explants, the best regeneration medium was the Murashige and Skoog (MS) medium supplemented with 2 mg/l of BA and 1.5 mg/l NAA and (75-80)% frequency of regeneration was observed when cotyledon explants were used. Callus formation from cotyledon is best when gotten in T_3 + TL and the maximum percentage of shoot formation. Reported that cotyledon is the best explants source for callus formation and regeneration. Although we identified the best varieties and best medium for tomato shoot production among the

varieties and media tested in this investigation, the significant variety x medium interaction indicates that best variety. V_1 and V_2 do not perform best against all media. Similarly, the best medium T3 does not perform best for all varieties. This significant interaction will allow us to identify the best specific variety V_3 and medium combination for clonal propagation or transformation study.

Salinity affects yield quality and quantity, so that yield characters must be taken into account when breeding for salinity tolerance. But not only yield-related characters are important. As salinity affects almost every aspect of the physiology and biochemistry of the plant, the enhancement of crop salt tolerance will require the combination of several to many physiological traits (Cuartero and Fernandez-Munoz, 1999), not simply those directly influencing yield. Na+ in salt-stressed plants is taken up by the roots and is accumulated in the whole plant depending on: whether or not the cultivars are salt-tolerant or salt-sensitive (Alpaslan *et al.*1999; Dionisio- Sese and Tobita(1998); Golldack *et al.*(2003); Lefevre *et al.*(2001), salt concentrations in the growing media Lefevre *et al.(*2001); Ndayiragije and Lutts(2006), plant organelles Shah *et al.*(2002); Ueda *et al.*(2006), exposure times Lefevre *et al*. (2001); Mitsuya *et al.*(2003a); Parker *et al.*(2006); Ueda *et al.*(2006), growing season Asch *et al*.(1999), and if they are grown in conditions of light or darkness Mitsuya *et al.*(2003b), while K+ usually decreases. There are some reports which indicate the low Na+ accumulation via limited Na+ absorption by H+- ATPase Roy *et al.*(2005) and H+-PPase activities Liu *et al.*(2006), Na+/H+ antiporter in both plasma membranes and vacuolar membranes Fukuda *et al.*(1999); Fukuda *et al.*(2004); Martinez-Atienza *et al.*(2007); Ohta *et al.*(2002); Zhao *et al.*(2006), leading to lower salt concentration in salt tolerant varieties than those in salt sensitive varieties. In addition, the Na+ accumulation in tomato crops has been effectively utilized to identify salt-tolerant or salt-sensitive varieties Golldack *et al.*(2003); Zeng (2005); Zeng *et al.*(2004).

Tam and Lang(2003) reported that Induced mutation in vitro was applied to increase levels of genetic variability in cell culture. It was found that the

optimum medium was well response to callus production. Parts of the calli were transferred to NaCl supplemented medium in an attempt to produce physiological variants from somaclones and evaluated some physiological aspects of cellular adaptation in response to salinity among genotypes. High Na/Ca ratio in the saline environment may impair the selectivity of the root membrane and results in positive accumulation of sodium in the, root and shoot Muhammad *et al.(*1987) However, limited information is available on the response of tomato to Ca to Mg ratios in the growth medium. The aim of the present study was to measure the effect of saline medium on the growth of tomato varieties varying in salt tolerance. The increase in shoot sodium might be due to the increasing concentration of sodium in the rooting medium which ultimately resulted its excessive uptake by plant Muhammed (1986) or decrease in efficiency of Na-exclusion mechanism. There was normal plant regeneration in the no-stress medium, but increased NaCl concentration in medium decreased percent plant regeneration in tomato varities. (Tab.C, Fig.j,k).

From this study it is revealed that the V_3 is more salt tolerant then another two and the three tomato varieties have good regeneration and callus induction ability on T_3 + TL containing media. In conclusion, based on the proposed methods and the experiments conducted so far, it was found that media containing 2.0 mg/l BA + 1.5 NAA mg/l would be the best media for tomato shoots regeneration, the result being consistent with prior scientific observations. For future transformation experiments only one medium will be used out of the three already tested in the tissue culture experiment. Similarly, since the V_3 performed the best, the future experiments will be focused on this variety. V_3 is the most suitable genotype for genetic transformation studies. Its calli will be used as recipients of salt tolerance activity. V_3 (Tomato Hybrid F_1 JAGUR) tomato is more responsive on T_3 (2.0 mg/l BA + 1.5 mg/l NAA) medium with 2.0 mg/l Kinetin to tissue culture. Using mature seeds explants, cotyledon formation, regeneration of plantlets were attained. Thus it will be suitable for use in the future as source of target material for genetic transformation of tomato.

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Table 1: Percentage of cotyledons induction from seeds of V_1, V_2, and V_3 by using MS media after 10 days.

Media name	No. of seeds	Cotyledons induction from V_1 seeds	% of Cotyledons induction of V_1	Cotyledons induction from V_1 seeds	% of Cotyledons induction of V_2	Cotyledons induction from V_3 seeds	% of Cotyledons induction of V_3
T_1	8	6	75	6	75	0	0
T_2	8	4	50	0	0	4	50
T_3	8	2	25	4	50	6	75

Table 2: Formation of shoot, callus and root in different varieties on T_3 + TL media.

Variety	**Number of cotyledons**	**Shoot**	**% of shoot formation**	**Callus**	**% of callus formation**	**Root**	**% of root formation**
V_1	12	4	33.3	2	16.6	1	8.33
V_2	10	2	20	1	10	0	0
V_3	10	8	80	6	60	2	20

Table 3: Salt tolerance activity of different varieties on different NaCl concentrations.

Media	Dry weight of callus before inoculation(gm)	Dry weight of callus after 2 weeks(gm)		
		V_1	V_2	V_3
TR_1	1.5	1.31	1.45	2.23
TR_2	1.5	0.92	1.31	1.42
TR_3	1.5	0.58	1.25	1.25
TR_4	1.5	0.47	0.90	0.85

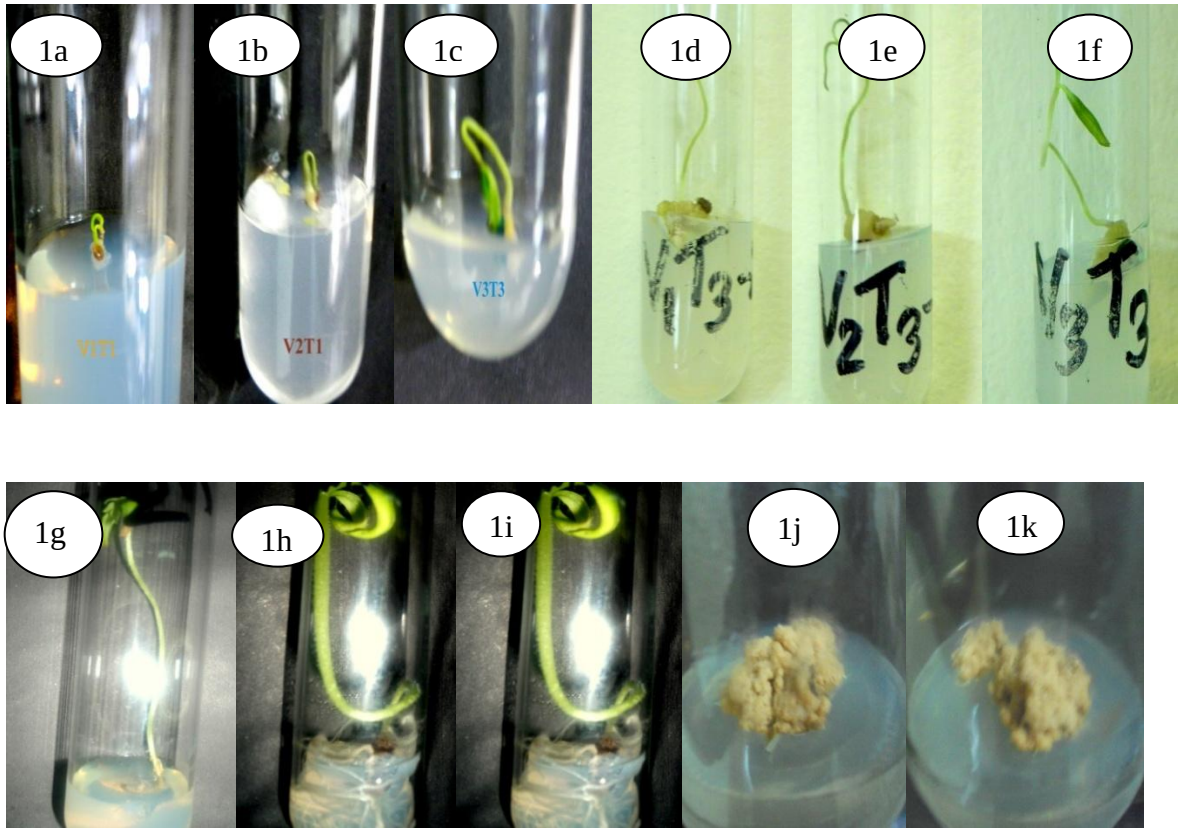

Figure 1: 1a, 1b and 1c Regeneration of V_1, V_2 and V_3 cotyledon in MS Media after 10 days; 1d, 1e, and 1f formation of shoot, callus and root in different varieties on T_3 + TL media; 1g, 1h, and 1i Shoot and root formation after 10 days of V_1, V_2, and V_3 in (T_3 + TL) media; 1j Callus (1.5 mg weight) on salt media; and 1k Callus (in decreasing weight) on salt media after 2 weeks.