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In vitro selection for tomato plants for drought tolerance via callus culture under polyethylene glycol (PEG) and mannitol treatments

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Abstract: Significant differences were found between tomato genotypes for their ability for callus formation on the two types of explants on the different types of media. The highest genotype for callus formation was Super Marmand (77.10%) and the lowest genotype for callus formation was 12 M (56.3 %). M_2 medium (83%) could be recommended as appropriate medium for callus induction for all genotypes. Concerning the type of explants the results showed that cotyledon leaf explants (70.39%) were better than hypocotyls explants (66.67%) for callus formation. In the present experiment the mean numbers of regenerated shoots showed highly significant differences between genotypes. Amcostar exhibited large number of shoots under all factors studied as compared to other genotypes. The effect of genotypes and media interaction on plant regeneration from callus was highly significant. The best combination for indirect regeneration was Amcostar genotype on M_4 medium (93.8% with a mean 9.7). The growth dynamic of callus tissue was evaluated during the growing period of 30 days on the basic medium MS supplemented with different concentration of PEG, The concentrations of PEG in the medium showed a great effect on the growth value at the end of 30 days growing period. The mass of callus and shoots regenerated directly from explants were evaluated after 60 days of growing on regeneration MS medium supplemented with different concentration of PEG. The highest dry weight was achieved when the Peto-86 explants were cultivated on MS supplemented with 75.00 gram PEG. Shoots regeneration frequency of cotyledon segment of tomato genotype ranged from 12.00 to 82.40 %. The highest shoot regeneration was achieved on the explants of Super Strain B it was 2.14 shoot per explants. The mean number of shoots per explants was decreased by increasing the concentration of mannitol in the medium the mean numbers of shoots per explants were 1.811, 1.59, 1.50, 1.34 and 1.43 for the Mannitol concentration 0.0, 25.0, 50.0, 75.0 and 100.0 mM/L, respectively. The regeneration capability it's self-affected strongly by the increasing of mannitol in the medium.

Introduction

Plant biotechnology could help plant breeders by creating and manipulating genetic variability. The contribution of plant biotechnology in plant breeding includes improving both crop quantity and quality. This improvement can be achieved by preventing infection with pathogens and parasites, increasing the plant tolerance against biotic and a biotic stress. Genetic variation in tomato is required to breed cultivars that are highly resistant to several biotic and abiotic stress conditions ((Kuckuck *et al.* 1991, Larkin, 1996 and Bhatia *et al*., 2004).

Abiotic stress limits crop productivity, and plays a major role in determining the distribution of plant species across different types of environments. Abiotic stress and its effects on plants in both natural and agricultural settings is a topic that is receiving increasing attention because of the potential impacts of climate change on rainfall patterns and temperature extremes, salinization of agricultural lands by irrigation, and the overall need to maintain or increase agricultural productivity on marginal lands (Araus *et al.* 2002; Boyer 1982).

Water deficit is one of the most important environmental disturbances, which influence the distribution of many species from year to year. The tissue culture method is a novel approach to this problem; the main idea is that cultivated cells are used as the selection units rather than whole plants (Moffat, 1996, Butenko and Kuchku 1979). The method is based on spontaneous or induced mutants induced *in vitro* after which resistant cells influenced by a selective agent are selected and plants are subsequently regenerated from the surviving resistant cells.

Polyethylene glycol, a non penetrable and nontoxic osmotic, lowers the water potential of the medium and has been used to simulate drought stress in plants. Cells adapted to PEG caused deficit of water have been isolated in *Lycopersicon esculentum*. (Bressan, *et al.*1981; Handa, *et al.* 1982, 1983).

PEG –adapted cell lines of *Lycopersicon esculentum* have shown a high level of tolerance to salt stress as compared to non-adapted cells. PEG in water may be controlled primarily by the metric forces of ethylene oxide sub units of the PEG polymer. The term atrium is proposed for PEG in soil – plant – water relation studies. A series of experiments with mannitol, a sugar alcohol often by for simulation of osmotic stress, were performed with excised wheat embryos, rape seedlings and potato stem segments grown *in vitro* by Lipavska and Dick (1996). The aim of this study is producing tomato plants from tissue cultures highly tolerate to drought using PEG (poly ethylene glycol) and manitol as osomo regulators.

Materials and Methods

***In vitro* selection procedure:**

In this experiment different concentrations of polyethylene glycol 6000 (PEG) or mannitol were added to the culture medium. A primary experiments was carried using seeds of Castle rock, peto-86, super strain B, Amcostar and super Marmand genotypes which germinated on MS Murashige and Skoog (1962) medium free hormone medium supplemented with different

concentrations of PEG (0, 25, 50, 75 and 100g/L). After 30 days from culturing explants on the selective medium, the fresh weight and, dry weight in mg were measured. Moreover, the growth rate relative to fresh and dry weight also measured. In the case of explants developed directly to form callus and shoots,the fresh and dry weights were measured 60days after explants culturing.

Indirect regeneration under abiotic stress (PEG, mannitol)

Two weeks old growing seedlings carrying the two cotyledon leaves were used as source of explants. The M2 medium (Table1) was used for callus induction, after 8 weeks growth period, calli were transferred into the regeneration medium (M_4) supplemented with different concentrations of PEG (0.0, 25.0, 50.0, 75.0 and 100.0 g/L) or mannitol (0.0, 25.0, 50.0, 75.0 and 100.0 mM /L), respectively. After four weeks, the mean numbers of regenerated shoots / replicate were recorded.

Direct regeneration under abiotic stress (PEG, mannitol)

In this experiment the cotyledon leaf (explants) were excised from two weeks old tomato seedlings and cultured directly on the regeneration medium (R1) (Table2) supplemented with different concentrations of polyethylene glycol 6000 PEG (0, 25, 50, 75 and 100 gram/l or mannitol (0, 25, 50, 75 and 100 mM), respectively (Table 2). After four weeks, the mean numbers of regenerated shoots / replicate were recorded.

Table 1: The composition of different media used in the present study for callus induction and regeneration (indirect regeneration) of tomato plants.

Medium code	Medium composition
M_1	MS + 1 mg/L 2.4D + 1 mg/L Kin
M_2	MS + 2 mg/L BAP + 0.2 mg/L NAA
M_3	MS + 5 mg/L BAP + 0.25 mg/L IAA
M_4	MS + 2 mg/L BAP + 0.6 mg/L IAA

Where: BAP = 6-Benzyladenine IAA = Indole-3-acetic acid
NAA = Naphthalene acetic acid 2, 4- D = 2, 4-dichlorophenoxyacetic acid
Kin = Kinetin MS = Modified Murashinge and Skoog medium

Table 2: The composition of different media used in the present investigation for regeneration from tomato explants (direct regeneration).

Medium code	Medium composition
R_1	MS + 0.2mg/L NAA + 2mg/L Kinetin
R_2	MS + 0.2mg/L NAA + 2mg/L BAP
R_3	MS + 0.25mg/L IAA + 5mg/L BAP

Results and Discussion

Callus growth dynamic:

The growth dynamic of callus tissue was evaluated during the growing period of 30 days on MS medium supplemented with different concentration of PEG. The fresh weights of callus at the end of 30 days growth period for five genotypes Peto-86, Castle Rock, Super Strain-B, Amcostar and Super Marmand were 2.44, 2.63, 1.64, 1.75 and 1.48 g / culture, respectively (Table 3) (Fig. 1) .

The concentration of PEG in the medium have a great effect on callus fresh weight and growth value, the fresh weights of callus after 30 day of growth of the five genotypes on the PEG concentration (0.0, 25.0, 50.0, 75.0, and 100.0 g / Liter were 2.65, 2.43, 1.99, 1.63, and 1.20 gram / culture respectively. The concentrations of PEG in the medium showed a great effect on the growth value at the end of 30 days growing period. The growth values for callus growing on MS medium relative to the control were 52.26, 32.20, 53.04, 37.72 and 63.84 % for the Peto-86, Castle Rock, Super Strain-B, Amcostar and Super Marmand, respectively (Table4)and Fig(1).

Table 3: Callus fresh weight in gram of five tomato genotypes after 30 days of growing on MS medium supplemented with different PEG concentrations

Genotypes	Callus fresh weight in gram on different; PEG concentrations in medium.					Means*
	00	25	50	75	100	
Peto-86	3.12	2.75	2.52	2.17	1.63	2.44 A
Castle-Rock	3.86	3.23	2.50	2.33	1.24	2.63 A
Super-Strain-B	2.07	2.12	1.80	1.12	1.07	1.64 B
Amcostar	2.43	2.34	1.86	1.23	0.92	1.75 B
Super- Marmand	1.77	1.68	1.54	1.28	1.13	1.48 B
Means	2.65A	2.43A	1.99B	1.63B	1.20C	1.98

$LSD_{0.05}$ G. 0.344 C. 0.344
*Means followed with the same letters are not significant

Table 4: Growth values of 30 days old calli of five tomato genotypes grown on MS medium supplemented with 4 different PEG concentrations relative to growth value of control.

Genotypes	Callus growth value relative to control on different PEG concentrations in medium.				
	00	25	50	75	100
Peto-86	100	88.14	80.79	69.64	52.26
Castle-Rock	100	83.70	64.89	60.40	32.20
Super-Strain-B	100	102.61	87.12	65.33	53.04
Amcostar	100	96.33	76.67	50.87	37.72
Super- Marmand	100	94.92	81.92	72.32	63.84
Mean	100	93.14	78.28	63.71	47.81

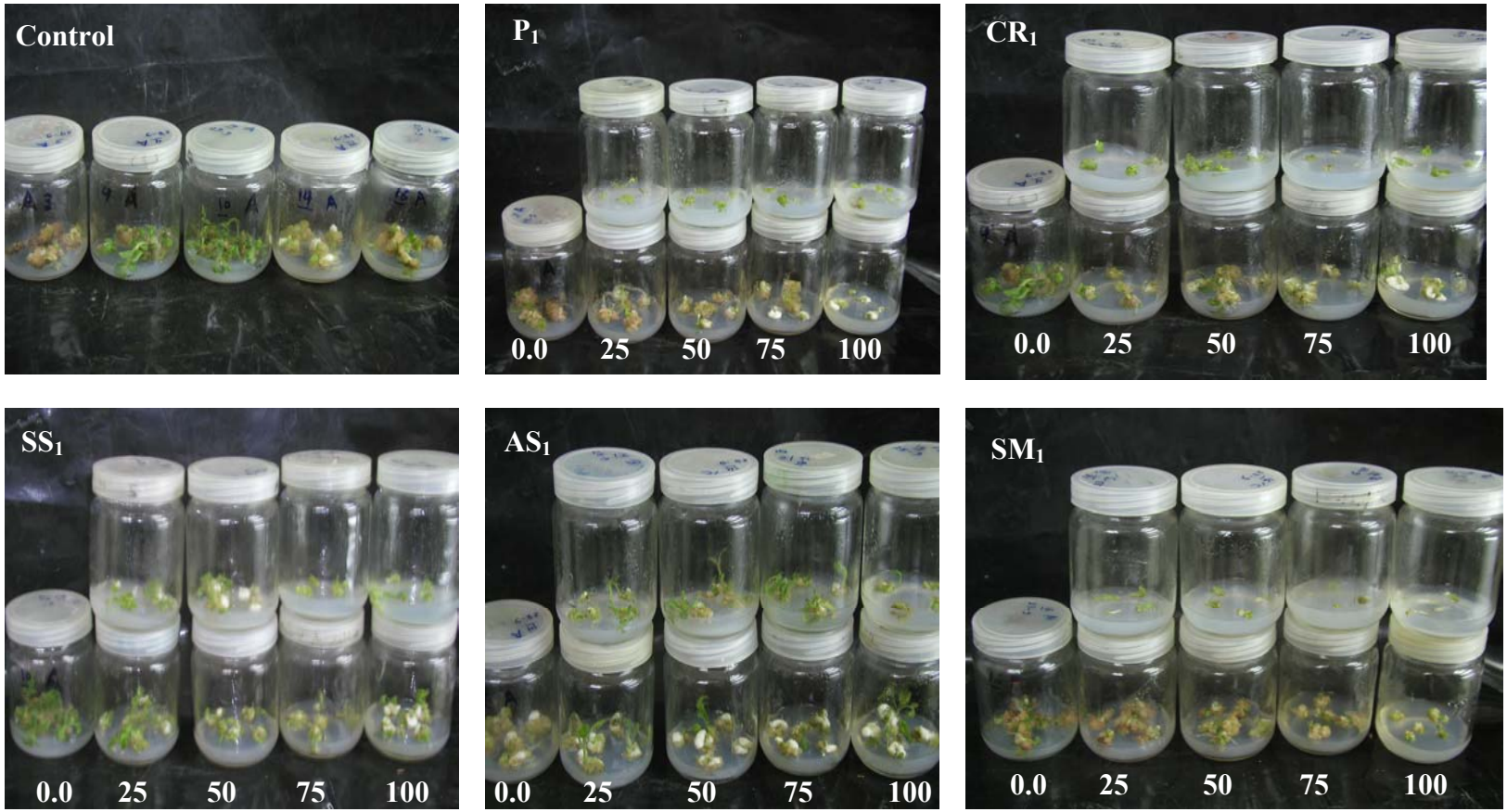

Fig. (5): *In vitro* selection for PEG (in g/l, up row) and mannitol (in mM, down row) -tolerant and direct regeneration of tomato plants on MR_1 medium with different concentrations (0, 25, 50, 75, 100). From up left to down right: Five tomato genotypes on MR_1 (PEG & Mannitol)-free medium, Peto 86 (P_1), Castle Rock (CR_1), Super Strain B (SS_1), Amcostar (AS_1) and Super Marmand (SM_1).

Callus growth and mass of shoots from direct regeneration

Callus growth and mass of shoots:

The mass of callus and shoots regenerated directly from explants were evaluated after 60 days of growing on regeneration MS medium supplemented with different concentration of PEG. The fresh weight of the explants and its callus and shoot formed after 60 days was measured in gram (Table 5). The means of callus and shoots of different genotypes of tomato after 60 day growing period were 3.48, 5.14, 5.83, 3.81, and 4.51 gram / explants for the 0.00, 25.00, 50.00, 75.00, and 100.00 gram / Liter.

The highest dry weight was achieved when the Peto-86 explants were cultivated on MS supplemented with 75.00 gram PEG. The means of dry weight of callus for the different genotypes ranged between 34.1 to 44.35 mg / culture Table 6. These means were 42.8, 36.7, 44.35, 39.3, and 34.1 mg / culture for Peto-86, Castle Rock, Super Strain-B, Amcostar, and Super Marmand, respectively, while the means of dry weight for different concentration 00.0, 25.00, 50.00, 75.00, and 100.00 gram/liter PEG were 1, 38.65, 45.05, 38.05, and 46.4 mg / culture.

Table 5: Fresh weight in gram of explants and their produced calli and shoots of five tomato genotypes after 60 days of growing on MS medium supplemented with 5 different concentrations of PEG.

Genotypes	Callus fresh weight in g. on MS medium supplemented with different concentrations of PEG in g. / Liter					Means*
	00	25	50	75	100	
Peto-86	5.52 BCD	4.19 BCDEFG	5.16 BCDEF	4.77 BCDEF	3.90 BCDEFG	4.71 AB
Castle-Rock	3.26 DEFG	5.28 BCDE	4.84 BCDEF	3.48 CDEFG	3.68 CDEFG	4.11 BC
Super-Strain-B	1.51 G	5.14 BCDEF	8.499 A	4.56 BCDEF	8.29 A	5.60 A
Amcostar	3.53 CDEF	6.22 ABC	6.560 AB	3.56 CDEFG	4.32 BCDEF	4.84 AB
Super-Marmand	3.59 CDEFG	4.86 BCDEF	4.12 BCDEFG	2.66 EFG	2.36 FG	3.52 C
Means	3.48 C	5.14 AB	5.83 A	3.81 C	4.51 BC	

*Means followed with the same letters are not significant.

Table 6: The dry weight in mg/culture of explants and their produced calli and shoots of five tomato genotypes after 60 days of growing on MS medium supplemented with 5 different concentrations of PEG.

Genotypes	Explants and their produced calli and shoots dry weight in mg. on MS medium supplemented with different concentrations of PEG in g. / L.					Means**
	00.0	25.00	50.00	75.00	100.00	
Peto-86	39.80 CDEF	36.80 CDEF	45.4 BCD	47.65 BC	44.75 BCD	42.8 A
Castle-Rock	31.95 DEFG	38.3 CDEF	39.05 CDEF	35.4 CDEF	38.85 CDEF	36.7 B
Super-Strain-B	20.15 G	37.4 CDEF	54.85 B	40.35 CDE	69.0 A	44.35 A
Amcostar	25.2 FG	40.95 CDE	47.15 BC	35.5 CDEF	47.8 BC	39.3 AB
Super- Marmand	28.95 EFG	39.9CDE	38.70 CDEF	31.35 DEFG	31.6 DEFG	34.1 B
Means	29.10 C	38.65 B	45.05 A	38.05 B	46.4 A	

*Means followed with the same letters are not significant.

Plant regeneration under PEG treatments:-

The averages of shoots regenerated from cotyledon segment explants of different genotypes (Direct regeneration were (8.00, 25.00, 43.00, 51.00 and 11.00 %) for Peto 86 , Castle Rock , Super Strain-B , Amcostar and Super Marmand , respectively .In the same time this averages for shoots regenerated from callus culture (indirect regeneration) were (64.0, 82.0, 91.0, 95.0 and 64.0 %) for Peto- 86 , Castle Rock , Super Strain , Amcostar and Super Marmand genotypes , respectively On the other hand the influence of PEG on the shoots regeneration frequencies was very high. For shoot regeneration from cotyledon segments (direct regeneration) the average percentages of regenerated shoots over all genotypes were 65.00, 32.00, 20.00, 21.00 and 10.00 % on the PEG concentrations 0.0 , 25 , 50 , 75 and 100 gm/L, respectively (Table 7) .

The average percentages of calli producing shoots over all genotypes (indirect regeneration) were 91.00, 79.00, 76.00, 81.00 and 69.00 % on PEG concentration 0, 25, 50, 75 and 100 gm/L in the medium (Table 8). The analysis of variance indicates that there were significant differences between different concentrations of PEG on shoot formation. The obtained results (Table 7, 8) revealed that, the frequency of either direct or indirect regeneration was reduced for all tested tomato genotypes by increasing the concentration of PEG in the medium.

The frequency of direct shoot regeneration was reduced from 65 % to 10 % when the PEG concentration was increased from 0.0 mg/l to 100 mg/l, respectively (Table 7). Also, the frequency of indirect shoot regeneration was reduced from 91 % at 0.0 mg/l PEG to 69.0 % at 100 mg/l PEG (Table 8).

Generally, the frequency of shoot regeneration from calli (indirect regeneration) was higher than that from cotyledon segments (direct regeneration) for all tested tomato genotypes (Tables 7, 8). Moreover, analysis of variance showed that, the interaction between genotypes and PEG concentrations on either direct or indirect regeneration was significant. The highest frequencies of indirect regeneration were 95% for Amcostar and 91% for Super Strain B. This finding is in a good agreement with the result of Ray *et al.* (1982) who worked on tomato cells culture for water stress, Brown and Hellebust (1978); Handa *et al.* (1983); Le Rudulier *et al* .(1984) and Adams *et al.* (1992) whom reported that the polyethylene Glycol is often used as on osmoticum to induce water stress in plant tissues. The additional PEG released by freezing and elevated temperatures accounted

for 2 to 11 % of the total callus PEG contained, this was assumed to have been released form the simplest of the plasmalema. The other 89 to 98 % of the total callus PEG was considered apoplastic.

Table7: Effect of tomato genotypes and PEG concentration on the mean of direct shoot regeneration frequencies.

Genotypes		PEG concentration g/l					Mean*	
		0.0	25	50	75	100	No	%
Peto-86c		1.088 FGHI	0.812 HI	0.812 HI	0.914 GHI	0.71 I	0.867	8.0
Castle Rock		1.782 BC	1.088 FGHI	1.262 DEFG	0.71 I	0.812 HI	1.161	25.0
Super Strain B		2.070 AB	1.552 CDEF	1.088 FGHI	1.218 EFGH	1.088 FGHI	1.403	43.0
Amcostar		2.038 A	1.364 CDEFG	1.494 CDEF	1.724 BCD	1.146 FGHI	1.607	51.0
Super Marmand		1.640 BCDE	0.71 I	0.71 I	0.71 I	0.71 I	0.896	11.0
Mean	No	1.778	1.105	1.073	1.55	0.893		
	%	65 %	32.0 %	20.0	21.0	10.0		

LSD 0.05 = 0.4124 *Means followed with the same letters are not significant.

Table 8: Effect of tomato genotypes and PEG concentration on the mean of indirect shoot regeneration frequencies.

Genotypes	PEG concentration g/l					Mean*	
	Control	25.0	50.0	75.0	100.0	No	%
Peto-86c	2.60 CDEF	2.80 BCDE	2.80 BCDE	2.60 CDEF	2.00 GE	2.56 C	64.0
Castle Rock	4.00 A	3.80 AB	3.40 ABC	3.00 ABCDE	2.20 DEF	3.28 B	82.0
Super Strain B	4.00 A	3.20 ABCD	3.40 ABC	3.80 AB	3.40 ABC	3.64 AB	91.0
Amcostar	4.00 A	3.80 AB	3.80 AB	4.00 A	3.40 ABC	3.80 A	95.0
Super Marmand	3.60 ABC	2.20 CEF	1.60 F	2.80 BCDE	2.60 CDEF	2.56 C	64.0
Mean	3.64 A	3.16 BC	3.04 BC	3.24 AB	2.76 C		
%	91.00	79.00	76.00	81.00	69.00		

$LSD_{0.05}$ = 0.9289 *Means followed with the same letter is not significant.

Plant regeneration under mannitol treatments

The mean of number of regenerated shoots and its frequencies are given in Table (9,10) and Fig (1). Results in Table (9) showed that shoots were regenerated from explants cotyledon segment (direct regeneration) of all tomato tested genotypes on MR_1 medium supplemented with various concentrations of mannitol, the frequency of shoot regeneration was varied between genotypes to the same concentration of mannitol in the MS selective medium. Shoots regeneration frequency of cotyledon segment of tomato genotypes were ranged from 12.00 to 82.40 %. The analysis of variance showed that there is a significant difference between genotypes in their capability to regenerate shoots from cotyledon segments.The mean numbers of regenerated shoot per explants were 0.99, 1.53, 2.14, 2.06 and 0.952 for Peto 86, Castle Rock, Super Strain-B, Amcostar and Super Marmand genotype, respectively (Table 9). The highest shoot regeneration (2.14 shoot per explants) was achieved on the explants of Super Strain-B.Concerning the effect of mannitol concentration on regeneration,, data in Table (9) showed that mean number of shoots per explants was decreased by increasing the concentration of mannitol in the medium. The mean numbers of shoots/explant were 1.81, 1.59, 1.50, 1.34 and 1.43 for the mannitol concentration 00, 25, 50, 75 and 100 mM/L, respectively (Table 9).

Table 9: Effect of tomato genotypes and mannitol concentration on the direct shoot regeneration frequencies.

Genotypes	Number of direct shoot regenerated /culture on MS mediu supplemented with different mannitol concentrations mM/liter					Mean*	
	00	25	50	75	100	No	%
Peto-86	1.160	0.986	1.088	0.812	0.914	0.992 C	12.00
Castle Rock	1.890	1.530	1.674	1.160	1.406	1.532 B	40.80
Super Strain B	2.350	2.258	1.962	1.962	2.166	2.140 A	82.40
Amcostar	2.112	2.196	2.066	1.962	1.970	2.061 A	74.40
Super Marmand	1.544	0.986	0.710	0.812	0.710	0.952 C	9.60
Mean	1.811 A	1.591 B	1.500 BC	1.342 C	1.433 BC		
%	59.2	47.2	41.6	19.20	38.4		

$LSD_{0.05}$ = 0.1665 *Means followed with the same letters not significant

The regeneration frequencies were 59.2, 47.2, 41.6, 19.2 and 38.4 % respectively for 00, 25, 50, 75 and 100 mM/l. Moreover a significant difference was observed between the mannitol concentrations in their effect on regeneration ability.

Regarding the indirect shoot regeneration from calli culture, results in Table (10) showed that highly significant differences were found between genotypes on forming shoots. Moreover all concentration of mannitol affected the regeneration frequencies and reduced it (Table10). High concentration of mannitol in the regeneration medium, reduced the number of regenerated shoot/calli was decreased for different tomato genotypes. The mean number of shoots/calli was ranged between 2.64 to 6.20 (Table 10). The shoot regeneration frequencies were 39.0 %, 62.5 %, 77.50 %, 77.00 % and

33.00 % for Peto- 86, Castle Rock, Super Strain B, Amcostar and Super Marmand genotypes, respectively.

Concerning the effect of mannitol concentration in the regeneration medium, data in (Table 10) showed that, by adding 100 mM mannitol / Liter of MS medium the regeneration frequencies was reduced from 81.50 (control) to 57.00. The means number of shoot/calli were 6.52, 3.76, 4.00, 4.28 and 4.48 for 00, 25, 50, 75 and 100 mM/L mannitol, respectively. Statistical analysis showed that, significant differences were found between genotypes under investigation for their regeneration ability. Moreover clear differences were found between the applied concentrations of mannitol and its relation with the regeneration capability.

Table 10: Effect of tomato genotypes and mannitol concentrations on indirect shoot regeneration frequencies.

Genotypes	Number of indirect shoot regenerated /culture on MS medium supplemented with different mannitol concentrations mM/l.					Mean*	
	00	25	50	75	100	No	%
Peto-86c	4.000 DEF	2.800 FGHI	3.000 FGH	2.800 FGHI	2.600 FGHI	3.04	39.0
Castle Rock	7.400 AB	3.400 EFG	4.000 DEF	4.800 CDE	5.400CD	5.00	62.5
Super Strain B	7.600 AB	4.800 CDE	5.200 CD	6.200 BC	7.200 AB	6.20	77.5
Amcostar	7.200 A	8.000 C	5.600 C	5.600 C	5.800 C	6.16	77.00
Super Marmand	5.600 C	2.200 GHI	2.200 GHI	1.800 GHI	1.400 I	2.64	33.00
Mean	6.52	3.76	4.00	4.28	4.48		
%	81.50	48.50	50.50	53.0	57.00		

LSD .0.05 = 1.289 *The means followed with the same letter are not significant.

References

Adams, P., Thomas, J.C., Vernon, D.M., Bohnert, H.J. & Jensen, R.G. 1992. Distinct cellular and organismic responses to salt stress. Plant Cell Physiol. **33**, 1215-1223.

Araus, J.L., Slafer, G.A., Reynolds, M.P. & Royo, C. 2002. Plant breeding and drought in C-3 cereals: what should we breed for ?. Ann. Bot. **89**, 925-940.

Bhatia, P., Ashwath, N., Senaratna T. & Midmore, D. 2004. Tissue culture studies of tomato (*lycopersicon esculentum*) plant cell , Tissue and organ culture **78**, 1-21.

Boyer, J.S. 1982. Plant productivity and enviroment. Science, **218**, 443-448.

Bressan, R.A., Hasegawa, P.M. & Handa, A.K. 1981. Resistance of cultured higher plant cells to polyethylene glycol induced water stress. Plant Sci. Lett. **21**, 23-30.

Brown, L.M. & Hellebust, J.A. 197). Sorbitol and proline as intracellular osmotic solutes in the green alga *Sticnococcus bicillaris*. Can. J. Bot. **56**, 676-679.

Butenko, R.G., Kuchko, A.A. 1979) Physiological Aspects of Procurement, Cultivation, and Hybridization of Isolated Potato Protoplasts. Soviet Plant Physiology. **26**, 901-909.

Gulati, A. & Jaiwal, K.P. 1994. *In vitro* selection and characterization of Vigna radiate cell-line resistant to PEG-induced drought stress. Acta Physiologia Plantarum **16**, 53-60.

Handa, A.K., Bressan, R.A., Handa, S., Hasegawa, P.M. 1982. Characteristics of cultured tomato cells after prolonged exposure to medium containing polyethylene glycol. Plant Physiol. **69**, 514-521.

Handa, S., Bressan, R.A., Handa, A.K., Carpita, N.C. & Hasegawa, P.M. 1983. Solutes contributing to osmotic adjustment in cultured plant cells adapted to water stress. Plant Physiol. **73**, 834-843.

Kuckuck, H., Kobabe, G. & Wenzel, G. 1991. Fundamentals of plant Breeding. Springer-Verlag, Berlin.

Larkin, P.J. 1996. Plant breeding – assessing past and expected contributions from classical and somaclonal mutagenesis, somatic hybridization and genetic engineering of 2nd Asia-Pacific Conference on plant Cell and Tissue Culture, July 28- August 1, 1996, Beijing, China, 17-24.

Larkin, P.J. & Seoweroft, W.R. 1981. Somaclonal variation a novel source of variability from cell cultures for plant improvement. Theoretical and applied Genetics. **60**, 197-212.

Le Rudulier, D., Strom, A.R., Dandekar, A.M., Smith, L.T. & Valentine, R.C. 1984. Molecular biology of osmoregulation. Science. **224**, 1064-1088.

Lipavska, H. & Dick, V. 1996. Uptake of mannitol from the media by *in vitro* grown plants. Plant cell, Tissue and Organ Culture **45**, 103-107.

Moffat, A.S. 1996. Ecology: Biodiversity is a boon to ecosystems, not species. Science. **271**, 1497.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol.Plant. **15**, 473-497.

Ray, Bressan, A., Handa, Avtar K., Handa, Sangtta. & Hasegawa, Paul M. 1982. Growth and water relations of cultured tomato cells after adjustment to low external water potentials. Plant Physiol. **70**, 1303-1309.