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ASSESSMENT OF CARBON SOURCES ON *IN VITRO* SHOOT REGENERATION IN TOMATO

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An innovative approach for *in vitro* shoot regeneration by both direct and indirect means was developed in three tomato genotypes culturing hypocotyls and leaf discs explants on MS and N6 basal media fortified with various concentrations of carbon sources (sucrose and sorbitol) individually, accumulatively and also in amalgamation with various plant growth regulators. No response of *in vitro* shoot regeneration was recorded in all the genotypes by the individual application of carbon sources in both MS and N6 basal media. On the other hand, their accumulative effect rapidly enhanced the *in vitro* shoot regeneration frequency in all the genotypes. The highest shoot organogenesis frequency (100, 99.00 and 97.69%) was recorded in Rio Grande, Roma and Moneymaker, respectively on MS medium fortified with carbon sources (30: 30 g/l) culturing hypocotyls. Supplementation of sucrose: sorbitol (30: 30 g/l) in N6 medium along with different PGRs (0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP) produced the highest shoot regeneration frequency (96.33, 92.69 and 88.74%) in Roma, Rio Grande and Moneymaker culturing leaf discs. Our findings suggest an alternative approach as hormone-free protocol for *in vitro* shoot regeneration in tomato that would save the resources with regard to hormonal costs and time.

Keywords: Hypocotyls, *in vitro* regeneration, explant, sucrose, sorbitol, *Solanum lycopersicum*

***Abbreviations*:** BAP: 6-benzylaminopurine; CIM: Callus induction medium; CS: Carbon sources; IAA: Indole-3-acetic acid; Kin: Kinetin; MS: Murashige and Skoog medium (1962); NAA: Naphthalene acetic acid; PGRs: Plant growth regulators; RM: Regeneration medium; ZEA: Zeatin

INTRODUCTION

Tomato is considered as an important vegetable throughout the world and is a multipurpose food. It is more essential for human due to its lycopene that prevents the prostate, lung, breast and skin cancer. Lycopene is also a potent antioxidant that saves our body cells from damage (Romer *et al.*, 2000). Tomato also contains vitamin B6, folate, niacin, potassium and fiber in large quantities that protect from heart diseases (Muir *et al.*, 2001).

During the previous three decades, this crop has been improved by the application of biotechnological approaches to ensure the large production in order to meet the ever-increasing requirements of commercial market and to get benefits of its nutritional values (Pandey *et al.*, 2011). These approaches have produced transgenic plants through tissue culture procedures applying plant growth regulators (Cortina and Culianez-Macia, 2004). The one problem that occurs in externally applied hormones is too much loss of resources and time. An alternative method for *in vitro* shoot regeneration in tomato was used to regenerate the tomato plants. As the indirect method of plantlet regeneration requires a long period of time for maintaining the callus cultures. Therefore, the organogesis potential of embryogenic calli is considerably limited which might be due to somaclonal variation (Bouman and De Klerk, 2001).

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).

Hence, very first time, we report an efficient, rapid, economical and hormone free procedure for tomato. The aim of our study was to investigate the effect of sucrose and

sorbitol individually, accumulatively and also in combination with various plant growth regulators in MS (Murashige and Skoog, 1962) and N6 (Chu, 1978) basal media. The *in vitro* shoot regeneration was compared on the basis of shoot organogenesis frequency, *in vitro* shoot length and the number of shoots primordial when carbon sources were fortified with only basal media and amalgamated with various plant growth regulators in order to standardize a method for *in vitro* shoot regeneration of tomato plants by the destitute of plant growth regulators.

MATERIALS AND METHODS

***Plant material and culture conditions*:** Seeds of tomato (*Solanum lycopersicum*) cultivars; Rio Grande, Moneymaker and Roma were provided by Horticultural Research Institute (HRI), NARC, Islamabad. The mature seeds of Rio Grande, Moneymaker and Roma were drenched in sterilized water for twenty-four hours at 4°C for breaking seeds dormancy. The seeds were disinfected with 70% (v/v) ethyl alcohol for one minute and then in 5.25% sodium hypochlorite at 10, 20, 30, 40 and 50% (v/v) with (2 drops/100 ml) of Tween-20 for twenty minutes. Subsequently the seeds were washed five times with sterilized water to remove the traces of clorox from the seeds. The seeds were dried on autoclaved filter paper for fifteen minutes and cultured on MS (1962) medium supplemented with 30 g/l sucrose and 7 g/l agar. The pH of the medium was maintained at 5.7 before autoclaving. The cultures were kept in the dark conditions for about five days (until germination) and then put under sixteen hours' photoperiod, 25±2ºC temperature, 50 $\mu molm^{-2}s^{-1}$ fluorescence light and 65-70% relative humidity. Two to three weeks old *in vitro* seedlings were used for *in vitro* shoot regeneration.

***Evaluation of individual effects of sucrose and sorbitol in MS and N6 basal media on organogenesis*:** In this experiment, 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 Murashige and Skoog (1962) basal medium amalgamated with various concentrations of carbon sources (sucrose and sorbitol) individually (Table 1). Similarly, the explants were cultured on N6 basal medium with diverse concentrations of sucrose and sorbitol in separate combinations (10-40 mg/l) (Table 2).

***Culture of explants on MS and N6 basal media with sucrose and sorbitol accumulatively*:** In this experiment, the hypocotyls and leaf discs were cultured on a regeneration media (MS basal medium supplemented with various combinations of carbon sources; sucrose: sorbitol (Table 1). Similarly the explants were cultured on another regeneration media (N6 basal medium fortified with various combinations of sucrose and sorbitol (Table 2). The pH of all media was set to 5.7 with HCl (1N) or NaOH (1N) and autoclaving was done at 121ºC for fifteen minutes. The culture was shifted to growth room at 25±2ºC and 50 $\mu molm^{-2}s^{-1}$ fluorescent light with sixteen hours light and eight hours dark photoperiod and 65-70% relative humidity. After twenty days of culture, the *in vitro* shoot regeneration was compared on the basis of

Table 1. Type I shoot induction media used for *in vitro* shoot regeneration by assessing various concentrations of sucrose and sorbitol individually and accumulatively in MS basal medium

Regeneration media	Composition
RMS1	4.3 g/l MS salts and vitamins, 15 g/l sucrose ± 15 g/l sorbitol
RMS2	4.3 g/l MS salts and vitamins, 20 g/l sucrose ± 20 g/l sorbitol
RMS3	4.3 g/l MS salts and vitamins, 25 g/l sucrose ± 25 g/l sorbitol
RMS4	4.3 g/l MS salts and vitamins, 30 g/l sucrose ± 30 g/l sorbitol
RMS5	4.3 g/l MS salts and vitamins, 35 g/l sucrose ± 35 g/l sorbitol
RMS6	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 2. Type II shoot induction media used for *in vitro* shoot regeneration by assessing various concentrations of sucrose and sorbitol individually and accumulatively in N6 basal medium

Regeneration media	Composition
RMN1	4.0 g/l N6 salts and vitamins, 15 g/l sucrose ± 15 g/l sorbitol
RMN2	4.0 g/l N6 salts and vitamins, 20 g/l sucrose ± 20 g/l sorbitol
RMN3	4.0 g/l N6 salts and vitamins, 25 g/l sucrose ± 25 g/l sorbitol
RMN4	4.0 g/l N6 salts and vitamins, 30 g/l sucrose ± 30 g/l sorbitol
RMN5	4.0 g/l N6 salts and vitamins, 35 g/l sucrose ± 35 g/l sorbitol
RMN6	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

Table 3. Type III 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, 15: 15 (g/l) sucrose: sorbitol
RM_{2A}	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 20: 20 (g/l) sucrose: sorbitol
RM_{3A}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP, 25: 25 (g/l) sucrose: sorbitol
RM_{4A}	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP, 30: 30 (g/l) sucrose: sorbitol
RM_{5A}	MS, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA3, 35: 35 (g/l) sucrose: sorbitol
RM_{6A}	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 4. Type IV 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, 15: 15 (g/l) sucrose: sorbitol
RM_{2B}	N6, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP, 20: 20 (g/l) sucrose: sorbitol
RM_{3B}	N6, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP, 25: 25 (g/l) sucrose: sorbitol
RM_{4B}	N6, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP, 30: 30 (g/l) sucrose: sorbitol
RM_{5B}	N6, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA3, 35: 35 (g/l) sucrose: sorbitol
RM_{6B}	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

organogenesis frequency (%) and the *in vitro* shoot length (cm) for each treatment combination using both types of explants in three tomato genotypes.

***Organogenesis on MS and N6 media having sucrose and sorbitol along with various PGRs*:** The embryogenic calli of three tomato genotypes were obtained by culturing the hypocotyls and leaf discs on CIM (MS salts, sucrose 3% and supplemented with IAA 2.0 mg/l and BAP 2.5 mg/l) and plant agar 0.7%. 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 cytokinins; 6-benzyl amino purine (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, indole-3-acetic acid (IAA, 0.1 – 1.0 mg/l) and gibberellins, (GA_3, 0.5 mg/l) were added in MS and N6 media with sucrose and sorbitol and put in jars (height; 12 cm & diameter; 8 cm). The hypocotyls and leaf discs-derived calli were cultured on these regeneration media. For all the genotypes, four explants per jar and 3-4 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 at 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.

***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; the 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.

***Root formation*:** The regenerated shoots of tomato about 3-5 cm in length; obtained by both direct and indirect means were excised and washed with sterilized water to remove the agar. Subsequently, they were shifted to root induction medium (MS salts 4.3 g/l, sucrose 30 g/l, Nitsch vitamins, IBA (0.2–0.4 mg/l), pH 5.7 and solidified with agar 0.7% in sterilized jars (12 × 8 cm).

***Acclimatization of plantlets in greenhouse*:** 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 photoperiod and 65-70% relative humidity). The plantlets were irrigated at 2-3 days interval until for three weeks. The plantlets were then transferred to larger pots and maintained in a greenhouse under normal conditions until they reached to maturity stage and bore fruits. After maturity, the plants were harvested and survival rate was recorded.

***Statistical analysis*:** All the experiments were performed in completely randomized design (CRD). The values are the mean ± standard deviation for all the observations. Six treatments for *in vitro* shoot regeneration frequency, *in vitro* shoot length and multiple shoot primordial were employed

and repeated three times. For all the experimental treatments, data were 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) by using Statistical Software; The Statistix v. 8.1 (Analytical Software, 2005). The mean values by the different letters within a column are statistically different at 5% level of significance (LSD Test).

RESULTS

***Bulking of explants after decontamination*:** 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 a few ml of 70% ethanol for one minute. After removal of ethanol, these seeds were treated with five different concentrations of clorox (Table 5) for twenty minutes and then cultured on MS plain medium. Results revealed that the lowest contamination frequency (1.04, 2.08 and 0%) was recorded in Rio Grande, Moneymaker and Roma, respectively applying 50% Clorox. Similarly at 40% clorox, lower contamination percentage (2.08, 4.85 and 2.97%) was found in Rio Grande, Moneymaker and Roma (Table 5). Forty and fifty percent clorox was then appraised on the *in vitro* seed germination percentage in all the cultivars. The higher germination percentage (88.09, 79.75 and 76.19%) was secured in Rio Grande, Moneymaker and Roma after sterilizing the seeds with forty percent clorox as compared to fifty percent Clorox (Table 6).

***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 in tomato. The hypocotyls and leaf discs were excised from fifteen days old *in vitro* seedlings and used as explants sources. The explants were cultured on MS medium supplemented with various levels of sorbitol and sucrose in separate experiments (Table 1). Similarly, the explants were cultured on N6 medium having the same concentrations of sucrose and sorbitol individually (Table 2). No regeneration response was noticed in all the genotypes from both types of explants even after thirty days of culturing.

***Organogenesis was rapidly enhanced by the application of carbon sources in MS basal media in absence of PGRs*:** The synergistic effect of sucrose and sorbitol was investigated in three cultivars of tomato culturing 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 plant growth regulators (Table 1). The synergism of carbon sources showed significant effects on *in vitro* shoot regeneration frequency and shoot length. Both the organogenesis frequency and 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, 99 and 97.69%) was recorded in Rio Grande, Roma and

Table 5. Assessment of different concentrations of clorox (v/v) on contamination frequency (%) in tomato

Percentage of clorox (v/v) used	No. of contaminated seeds			No. of non-contaminated seeds			Contamination frequency (%)		
	Rio Grande	Money-maker	Roma	Rio Grande	Money-maker	Roma	Rio Grande	Money-maker	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. The total number of seeds (n) 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. Coefficient of variation was 17.26 (ANOVA).

Table 6. Assessment of clorox (40 and 50%) on germination frequency (%) in tomato

Clorox % (v/v)	No. of germinated seeds			No. of contaminated seeds			Germination percentage (%)		
	Rio Grande	Money-maker	Roma	Rio Grande	Money-maker	Roma	Rio Grande	Money-maker	Roma
40	148.00	134.00	128.00	5.00	4.00	6.00	$88.09^{a} \pm 4.79$	$79.75^{b} \pm 4.51$	$76.19^{c} \pm 3.19$
50	121.00	103.00	91.00	3.00	2.00	4.00	$72.02^{d} \pm 3.01$	$61.31^{e} \pm 2.20$	$54.16^{f} \pm 2.02$

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. The total number of seeds (n) inoculated for all the cultivars was one hundred and sixty-eight. Coefficient of variation was 2.15 (ANOVA)

Moneymaker on RMS4 culturing hypocotyls. Similarly, the leaf discs-derived highest organogenesis frequency (100, 95.86 and 93.48%) was recorded in Rio Grande, Roma and Moneymaker on RMS4 (Table 7; Fig. 1 A, B and C). The highest *in vitro* shoot length (9.66 and 8.63 cm) was secured in Rio Grande using hypocotyls and leaf discs, respectively. It was followed by Roma where the best shoot length (8.79 and 8.17 cm) was recorded from hypocotyls and leaf discs. Similarly, the maximum shoot length (7.48 and 7.25 cm) was achieved in Moneymaker from hypocotyls and leaf discs (Figs. 3 & 4).

***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 shoot regeneration percentage 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 varieties and carbon sources. The explants showed swelling within three to four days and initiation of shoots occurred after seven days of culturing using optimum level of carbon sources in regeneration medium. The highest regeneration percentage (96.33 and 93.69%) was recorded in Rio Grande from hypocotyls and leaf discs on RMN4 (N6 basal medium supplemented with sucrose: sorbitol; 30: 30 g/l). It was followed by Roma (94.45 and 88.66%) and Moneymaker (92.75 and 86.56%) on RMN4 (Table 8; Fig. 1 D, E and F). Similarly, the highest *in vitro* shoot length (11.12 and 10.89 cm) was obtained in Rio Grande followed by Roma (10.49 and 9.76 cm) and Moneymaker (9.93 and 9.19 cm) from hypocotyls and leaf discs (Figs. 5 & 6). The concentration of carbohydrate sources higher than 30 g/l had inhibitory role to both shoot regeneration frequency and shoot length (Table 8; Figs. 5 & 6).

***Influence of carbon sources in MS medium along with various PGRs on organogenesis and multiple shooting*:** During this experiment, different ratio of sucrose and sorbitol were amalgamated with various plant growth regulators (Table 3) to compare the efficiencies of carbon sources supplemented with basal media only as well as with plant growth regulators. The different treatments having sucrose and sorbitol along with plant growth regulators produced multiple shoots in all the cultivars. Due to mass 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

Table 7. Assessment of synergistic effect of sucrose and sorbitol in MS basal media without PGRs on *in vitro* shoot regeneration in tomato

MS basal media fortified with (sucrose: sorbitol) (g/l)	*In vitro* shoot regeneration frequency culturing hypocotyls (%)			*In vitro* shoot regeneration frequency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
15 : 15	42.65± 4.41r	49.54±3.04q	51.91±2.99pq	32.89±3.23tu	40.66±2.9rs	56.26±3.76p
20 : 20	73.59±5.00lmn	62.92±5.17o	78.35±4.49j-l	68.00±5.33no	54.83±2.91pq	70.79±4.23mn
25 : 25	86.25±6.2fg-i	77.25±3.5kl	83.44±4.2g-j	79.63±5.43jk	70.18±2.5n	86.44±4.08f-h
30 : 30	100.00 ± 0.00a	97.69±3.98a-c	99.00±1.72ab	100.00±0.00a	93.48±4.54b-e	95.86±3.95a-d
35 : 35	95.72±4.01a-d	90.15±2.17d-f	92.56±4.46c-e	88.49±5.31e-g	81.25±3.77h-k	89.00±4.19e-g
40 : 40	89.32±5.71e-g	84.00±4.01g-j	80.35±3.32i-k	83.73±5.1g-j	76.39±2.7k-m	73.42±3.71l-n

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences ($P \leq 0.05$). The values after ± sign demonstrate standard deviation. Coefficient of variation was 5.77 (ANOVA).

Table 8. Assessment of synergistic effect of sucrose and sorbitol in N6 basal media without PGRs on *in vitro* shoot regeneration in tomato

N6 basal media fortified with (sucrose: sorbitol) (g/l)	*In vitro* shoot regeneration frequency culturing hypocotyls (%)			*In vitro* shoot regeneration frequency culturing leaf discs (%)		
	Rio Grande	Moneymaker	Roma	Rio Grande	Moneymaker	Roma
15 : 15	48.66±4.16q-s	32.92±3.68v-y	38.42±3.82t-w	57.49±3.93n-q	41.75±4.17s-v	45.58±3.65r-t
20 : 20	69.45±4.87j-m	54.69±3.93o-r	60.34±3.87m-o	73.82±5.94h-k	50.00±4.00p-s	65.42±3.85k-n
25 : 25	91.00±4.33a-d	77.62±4.49f-j	84.00±4.00c-g	89.75±4.17a-e	73.00±5.00i-l	78.25±4.05f-j
30 : 30	96.33±3.68a	92.75±4.27a-c	94.45±5.76ab	93.69±4.85ab	86.42±4.54b-f	88.66±5.61a-e
35 : 35	88.76±5.25a-e	82.54±5.79d-h	90.12±5.49a-e	78.33±4.80f-j	75.85±4.34g-j	81.00±5.65e-i
40 : 40	66.51±3.43k-n	70.63±3.49j-l	80.85±4.67e-i	58.00±4.00n-p	63.93±3.77l-o	73.85±3.29h-k

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences ($P \leq 0.05$). The values after ± sign demonstrate standard deviation. Coefficient of variation was 9.56 (ANOVA).

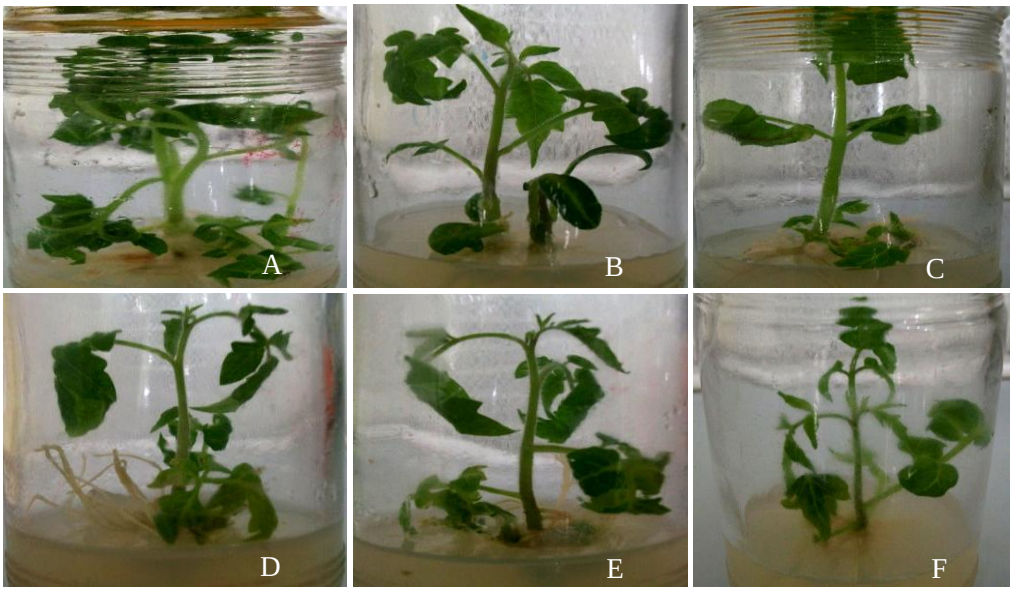

Figure 1. Direct shoot organogenesis in tomato on MS and N6 basal media fortified with sucrose and sorbitol: A, B and C, *In vitro* shoot regeneration in Rio Grande, Moneymaker and Roma, respectively, on MS basal media supplemented with sucrose: sorbitol (30: 30 g/l); D, E and F, Shoot organogenesis in Rio Grande, Moneymaker and Roma on N6 basal media supplemented with sucrose: sorbitol (30: 30 g/l)

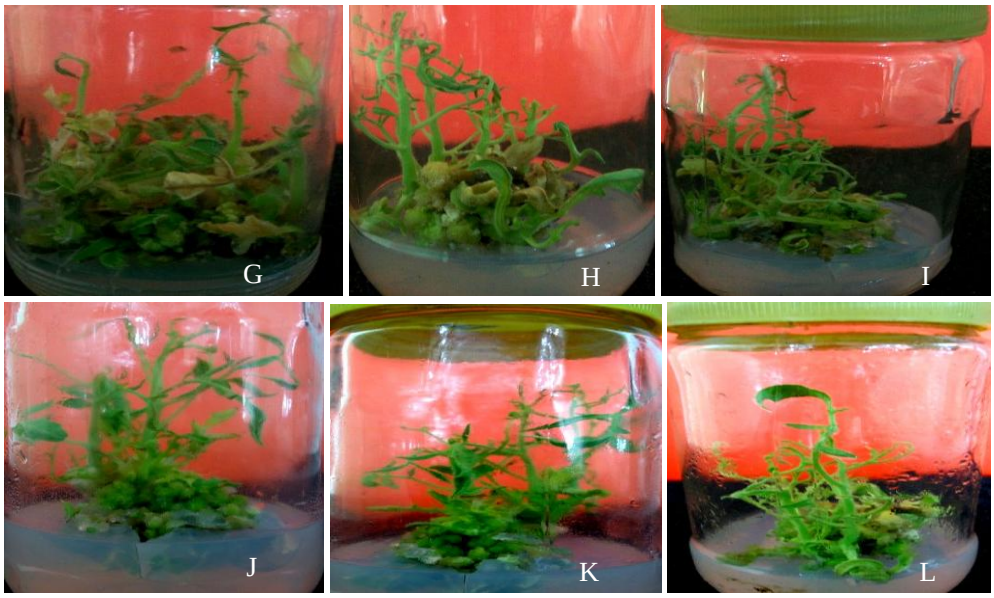

Figure 2. Indirect shoot regeneration in tomato on MS and N6 basal media having sucrose and sorbitol along with various PGRs: G, H and I, Shoot organogenesis in Rio Grande, Moneymaker and Roma, respectively on MS basal media + sucrose: sorbitol (30: 30 g/l) + 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP; J, K and L, *In vitro* shoot regeneration in Rio Grande, Moneymaker and Roma on N6 basal media fortified with sucrose: sorbitol (30: 30 g/l) and IAA (0.1 mg/l) + ZEA (1.0 mg/l) + BAP (2.0 mg/l)

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. The highest organogenesis frequency and more number of primordial shoots were recorded on RM_{4A} in all genotypes from both types of

Table 9. Assessment of synergistic effect of sucrose and sorbitol in MS basal media fortified with various PGRs on *in vitro* shoot regeneration in tomato

Regeneration 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}	50.69±2.31s-u	47.82±2.84t-v	53.69±3.73q-s	58.44±3.90n-q	52.34± 3.17r-t	62.82±3.83l-n
RM_{2A}	58.85±2.37n-q	55.62±4.14p-s	57.00±4.12o-r	66.00±4.23j-m	59.49±2.95n-p	61.63±5.00lm-o
RM_{3A}	76.00±3.74d-h	76.42±6.36d-g	81.00±5.23cd	79.52±5.11c-e	73.23±3.73f-i	84.00±5.97bc
RM_{4A}	80.35±4.34cd	79.00±5.82c-f	88.00±5.6ab	86.85±3.83ab	82.55±5.94bc	90.72±5.15a
RM_{5A}	63.92±3.32k-n	71.65±5.27g-j	66.33±3.24j-l	72.68±3.46g-i	68.78±7.79i-k	75.45±5.11d-h
RM_{6A}	60.00±3.58n-p	60.33±4.58m-p	74.00±4.14e-i	68.79±4.00i-k	64.00±4.55k-n	70.41±4.83h-j

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences (P≤0.05). The values after ± sign indicate standard deviation. Coefficient of variation was 4.24 (ANOVA).

explants (Table 9). In this type of indirect shoot regeneration, leaf discs were found more responsive giving better results than that of hypocotyls. The highest regeneration frequency (90.72, 86.85 and 82.55%) was recorded in Roma, Rio Grande and Moneymaker from leaf disc-derived explants (Table 9; Fig. 2 G, H and I). The highest number of shoot primordial (14, 12) was recorded in Rio Grande followed by Roma (13, 11) and Moneymaker (12, 10) culturing leaf discs and hypocotyls on RM4A (Figs. 7 & 8).

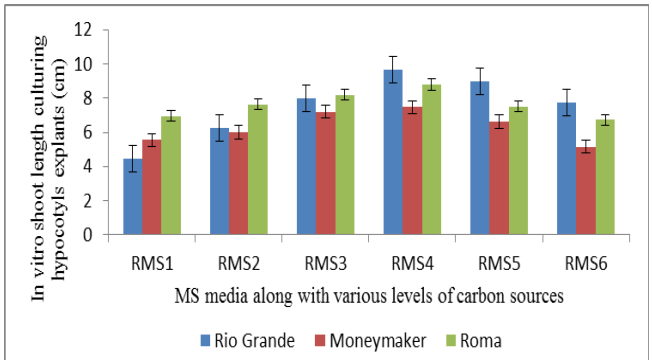

Figure 3. Effect of various levels of sucrose and sorbitol in MS basal media on *in vitro* shoot length culturing hypocotyls explants

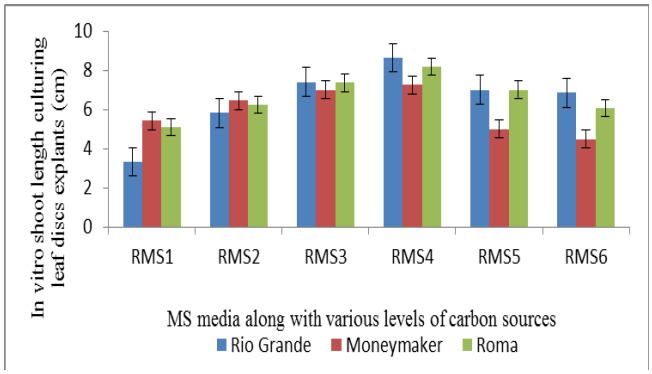

Figure 4. Effect of various levels of sucrose and sorbitol in MS basal media on *in vitro* shoot length culturing leaf discs explants

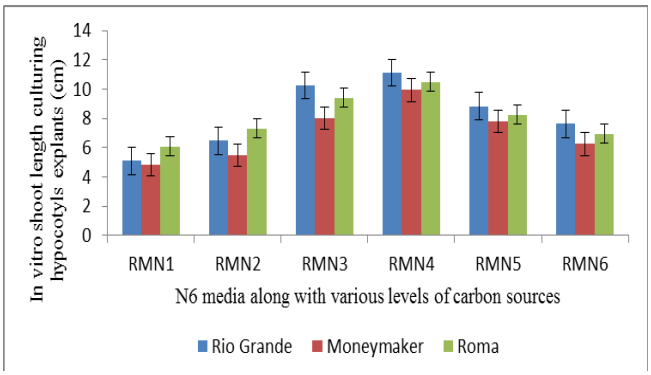

Figure 5. Effect of various levels of sucrose and sorbitol in N6 basal media on *in vitro* shoot length culturing hypocotyls explants

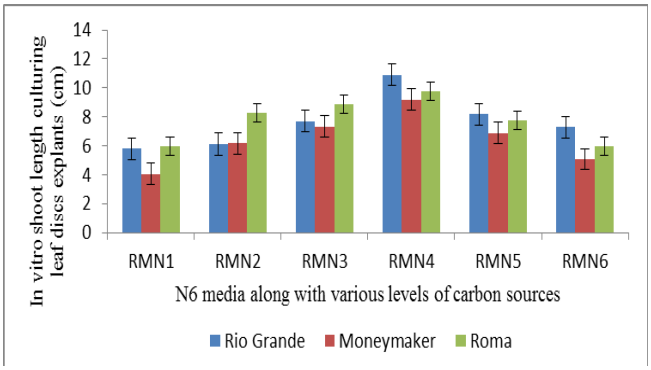

Figure 6. Effect of various levels of sucrose and sorbitol in N6 basal media on *in vitro* shoot length culturing leaf discs explants

The *in vitro* shoot regeneration frequency and primordial shoot number was decreased applying sucrose: sorbitol more or less than (30: 30 g/l) (Table 9; Figs. 7 & 8).

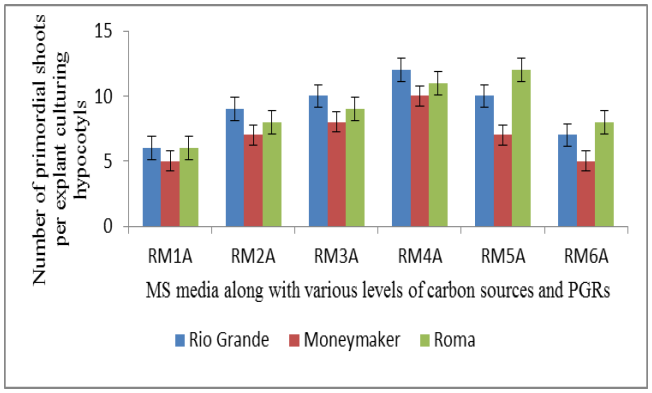

Figure 7. Effect of various levels of sucrose and sorbitol in MS basal media along with various PGRs on *in vitro* shoot number culturing hypocotyls

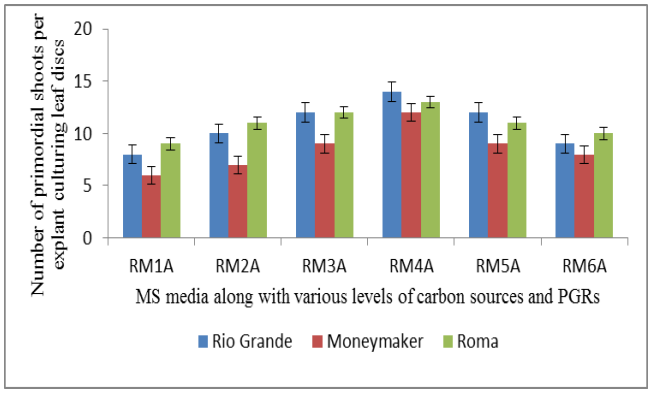

Figure 8. Effect of various levels of sucrose and sorbitol in MS basal media along with various PGRs on *in vitro* shoot number culturing leaf discs

***Mutual effect of sucrose and sorbitol in N6 medium along with various PGRs on in vitro shoot regeneration and multiple shooting*:** To investigate the mutual effect of carbohydrate sources on *in vitro* shoot regeneration frequency and multiple shooting, N6 basal medium was fortified with different concentrations of sucrose and sorbitol along with various levels of PGRs (Table 4). In this experiment calli formation was occurred from the cut ends of explants and then these calli were maintained after every twelve to fifteen days on the same culture media for successful shoot regeneration. The highest organogenesis frequency was recorded in Roma (96.33 and 92.25%) followed by Rio Grande (92.69 and 87.79%) and Moneymaker (88.74 and 83%) from leaf discs and hypocotyls on RM_{4B} (Table 10; Fig. 2 J, K and L). Similarly, the highest number of primordial shoots (16, 15 and 14) was obtained in Roma, Rio Grande and Moneymaker culturing leaf discs (Fig. 9). 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.

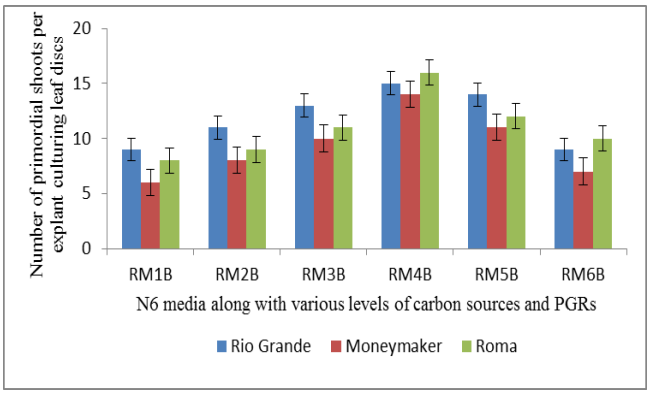

Figure 9. Effect of various levels of sucrose and sorbitol in N6 basal media along with various PGRs on *in vitro* shoot number culturing leaf discs

DISCUSSION

Surface sterilization is an important step that inhibits the

Table 10. Assessment of synergistic effect of sucrose and sorbitol in N6 basal media fortified with various PGRs on *in vitro* shoot regeneration in tomato

Regeneration 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}	52.72±6.66v	40.00±4.70wx	43.74±4.14w	57.43±5.80u	48.69±4.84v	52.75±3.51v
RM_{2B}	63.92±5.99r-t	65.43±5.20qr	76.49±4.36i-l	70.25±4.79m-p	73.58±3.66l-n	78.25±4.17i-k
RM_{3B}	77.64±5.14i-l	70.66±5.19m-o	81.00 ±3.77g-i	83.56±3.74e-h	79.45±4.80h-j	86.41±5.61d-f
RM_{4B}	87.79±4.85c-e	83.00±6.07f-h	92.25±5.40a-c	92.69±5.34ab	88.74±4.21b-d	96.33±3.85a
RM_{5B}	74.45±3.82k-m	69.00±3.81n-q	78.36±4.57i-k	80.37±5.77g-j	75.86±4.04j-l	84.66±3.11d-g
RM_{6B}	66.00±5.74p-r	63.59±4.53r-t	59.33± 3.73tu	64.82±4.83q-s	60.66±4.19s-u	67.84±4.64o-r

Each data is the average of three replicates. Mean values superscripted with different letters show significant differences (P≤0.05). The values after ± sign represent standard deviation. Coefficient of variation was 4.71 (ANOVA).

growth of microorganisms. The most widely used chemical for seed surface sterilization of tomato is sodium hypochlorite (NaOCl) (Steinitz and Bilavendran, 2011). Superior germination percentage is prerequisite for plant tissue culture (Sakhanokho *et al.,* 2001). Mature seeds of tomato genotypes namely Rio Grande, Moneymaker and Roma were surface sterilized with 40% clorox (v/v). The germination frequency ranging from 76.19–88.09% was recorded in these genotypes. Our findings demonstrated clearly that appropriate concentration of clorox with Tween-20 increased the seeds germination percentage by removing contamination. Chetty *et al.* (2012) reported 25% sodium hypochlorite with 0.1 % Tween 20 for sterilization of seeds of tomato cv. Micro-Tom for twenty minutes.

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 are 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 (Petersen *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.

In direct regeneration, the hypocotyls yielded more shoot regeneration frequency and also more shoot length 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 standard medium (MS or N6 basal media fortified with only carbon sources devoid of exogenously 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 was found better than MS in producing maximum shoot regeneration frequency, shoot length and number of primordial shoots. Our findings are in accordance with Sheeja *et al.* (2004) who reported that the highest regeneration frequency, shoot length and number of shoots were recorded on N6 than that on 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 are 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 proclaimed that the regeneration frequency was 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 was 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 effect of sucrose, sorbitol and glucose individually and in combination 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. The 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).

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 induction.

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 coincides 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 was 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 was reported that multiple shoot proliferation was 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 are 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.

***Conclusion*:** We have developed a novel procedure that will limit the hurdles of earlier reported *in vitro* shoot regeneration protocols in tomato. The significance of this procedure 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 by 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 by using *Agrobacterium*-mediated genetic transformation.

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