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Full Length Research Paper

Callus formation and organogenesis of tomato (*Lycopersicon esculentum* Mill, C.V. Omdurman) induced by thidiazuron

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***In vitro* culture response was assessed in tomato (*Lycopersicon esculentum* Mill. c. v. Omdurman) for optimum callus induction and plantlet regeneration. Callus induction was achieved within seven to ten days directly on the cut surfaces of both hypocotyls and cotyledon explants cultured on Murashige and Skoog (MS) basal medium supplemented with α-naphthalene acetic acid (NAA), 2,4-dichloro-phenoxyacetic acid (2,4-D), Thidiazuron (TDZ) and benzyl adenine (BA) alone or in different combinations, but not in hormone free-medium. The highest callusing index (5.3) was obtained on hypocotyls explants cultured on MS medium supplemented with NAA at 0.5 mg/l followed by an index of 5.2 obtained from the same explant by using 0.1 mg/l NAA in combination with BAP at 0.5 mg/l. However, for the cotyledon explants, the highest callusing index (4.7) was obtained on MS medium supplemented with NAA at either 2.0 or 3.0 mg/l. After 8 weeks of culture, organogenesis was observed only on the explants cultured on medium containing different concentrations of TDZ alone or in combination with BAP. The best shoot formation (93%) was obtained for cotyledon explant callus induced on MS medium containing TDZ in combination with BAP both at 0.5 mg/l. The highest number (6) of shoot per explant was obtained when cotyledon explant callus was sub cultured on MS medium supplemented with 3.0 mg/l TDZ. Plain half strength of MS was found to be the best rooting medium, however, addition of IAA at 1.0 mg/l and IBA at 2.0 mg/l were found essential to induce highest number of roots (22.1 ± 0.9) and longer roots (11.0 ± 0.3 cm), respectively. This protocol would be useful to create somaclonal variation and utilize transgenic approaches for varietal improvement of tomato.**

Key words: *Lycopersicon esculentum*, callus induction, organogenesis, thidiazuron, acclimatization.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is a major vegetable crop that has achieved tremendous popularity over the last century and it is grown in almost every country of the world (Abu-El-Heba et al., 2008). In Sudan, tomato is important vegetable crop ranks second to onion among vegetable crops based on cultivated area (Ahmed et al., 2001). It is grown throughout the country where irrigation water and arable land are available and is mainly grown by small holders who employ relatively poor crop management practice (Abdelmageed et al., 2003). Tomato production in Sudan is adversely affected by wide ranges of biotic and abiotic stresses. The high temperatures during summer accompanied by low humidity limit the production of tomato to the cooler part of the year and leads to the seasonality of the crop production (Abdalla and Verkerk, 1968). Moreover, diseases infestations are notorious factors that reduce crop yields and inflate production costs. Tomato yellow leaf curl virus (TYLCV) disease and its vector, the whitefly *Bemisia tabaci* (Gennadius), are the major production constraints

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Abbreviations: NAA, α-Naphthalene acetic acid; MS, Murashige and Skoog basal medium; **2,4-D,** 2,4-dichloro-phenoxyacetic acid; **TDZ,** thidiazuron; **BA,** benzyladenine.

in the country (Yassin and Nour, 1965). TYLCV results in more than 70% yield reduction in Gezira, in central Sudan (Yassin, 1984; Yassin et al., 1982), and reportedly causes losses up to 100% in tomato in tropical and subtropical regions (Friedmann et al., 1998; Lapidot et al., 1997).

To attain sustainable tomato productions, such above mentioned constraints have been addressed by conventional breeding and enhanced management but it has resulted in limited commercial success. The integration of modern biotechnology like tissue culture into breeding programs may provide powerful tools to overcome these limitations.

Plant tissue culture techniques are recognized as useful instruments in crop improvement. *In vitro* culture is used in tomato in different biotechnological applications including production of virus free plants (Moghaieb et al., 1999) and genetic transformation (Park et al., 2003). The successful application of plant tissue culture presupposes the establishment of an efficient culture system, consisting of a competent genotype, explant source as well as optimal culture conditions (Plana et al., 2005). Tomato regeneration has been previously reported via organogenesis in several articles using different explants, such as leaf (McCormic et al., 1986; Gaffer et al., 1997; Öktem et al., 1999) and cotyledon (VanRoekel et al., 1993). In addition, Pozueta-Romero et al. (2001) regenerated shoots of three tomato cultivars from the hypocotyls after removing the primary and axillary meristems.

The experimental work was conducted to establish a reproducible protocol for callus induction and regeneration in Sudanese tomato cultivar Omdurman by using specific combinations of growth regulators including thidiazuron (TDZ). TDZ, a substituted phenylurea (N-phenyl-1,2,3-thidiazol-5-ylurea), is a potent bioregulant of *in vitro* morphogenesis (Murthy et al., 1998). The presence of TDZ, either alone or in combination with other growth regulators, is important for shoot organogenesis and somatic embryogenesis in a wide variety of plant species (Jiang et al., 2005). In this study, we report the importance of TDZ in inducing shoot regeneration from cotyledon and hypocotyls explants of Sudanese tomato cultivar Omdurman.

MATERIALS AND METHODS

Plant materials

Mature seeds of tomato (*L. esculentum* Mill., c.v. Omdurman) were obtained from the Agricultural research and Technology Corporation (ARTC) Wad Medani, Sudan and used as a source of explants throughout this experiment.

Surface sterilization and seed germination

Seeds were washed with continuously running tap water for 15 min. Under laminar flow cabinet seeds were disinfected with clorex® (Sodium hypochlorite 5.25%) with tween 20 for 15 min, then rinsed 4-5 times with sterile distilled water till the foam was completely removed. After surface sterilization, the seeds were inoculated on half-strength MS (Murashige and Skoog, 1962) medium and later transferred to condition with a 16 h photoperiod at 25°C ± 2. It was noticed that seeds started growing in dark and later they were transferred to light. It was observed that germination was possible after 10 - 12 days of culture.

Hypocotyls and cotyledon segments from 10 - 12 day-old *in vitro* raised seedlings were excised under aseptic conditions, the length of the hypocotyls was 1.0 cm. These explants were cultured on callus induction media and placed on pre-autoclaved MS basal medium supplemented with different growth regulators. Callus and regeneration from calluses were shown after 2 - 8 weeks of culture. Shoots from callus were separated; callus was removed and planted again on full and half MS medium containing different concentrations of auxins for root initiation. Rooted plants were transferred to green house and planted in soil under high moisture content.

Culture condition and data analysis

All the media used in this study were supplemented with 3% (w/v) sucrose, solidified with 0.8% (w/v) and the pH was adjusted to 5.8 before addition of the agar and autoclaving at 121°C and 15 lb psi for 15 min. Cultures were sub cultured at 4 weeks interval. Results were observed at regular intervals. To set numerical values for callusing index, scale rating from 0 to 9 was developed. The scale was defined as: 0- no tissue growth, 1- callus arising from one explant end, 2- callus arising from both explant ends, 3- callus arising from both explant ends and double the original explant size, 4- callus arising from both explant ends and triple the original explant size, 5- callus arising from both explant ends and four times the original explant size, 6- callus arising from both explant ends and five times the original explant size, 7- callus arising from both explant ends and six times the original explant size, 8- callus arising from both explant ends and seven times the original explant size, and 9- callus arising from both explants ends and eight times the original explant size.

All the experiments were repeated three times and standard errors of the means were calculated. Data were collected from three independent experiments and analyzed by using analysis of variance procedure (ANOVA) on excel computer program. Means were separated by Duncan's multiple range test (DMRT) (Duncan, 1955) and presented as average ± standard error (SE).

RESULTS AND DISCUSSION

The *in vitro* morphogenetic responses of cultured plants are affected by different components of the culture media and therefore, it is important to evaluate their effects on plant callus induction and regeneration (Gubis et al., 2004). Callus was initiated within 7 - 10 days directly on the cut surfaces of both hypoctyl and cotyledon explants cultured on MS basal medium supplemented with auxins (NAA and 2,4-D) and cytokinins (TDZ and BAP) alone or in different combinations, but not in hormone free-medium (Table 1). Callus response and callusing index were markedly affected by both the types of explant and growth regulators used. Different concentrations of auxins and cytokinins either singly or in combinations had a distinct effect on callus induction from both explants. Pal et al. (2007) reported that *in vitro* callus induction depends on the endogenous concentration of plant

Table 1. Effect of different concentrations of α-naphthalene acetic acid (NAA), 2, 4-dichlorophenoxy acetic acid (2, 4-D), benzyladenine (BA) and thidiazeron (TDZ) alone or in different combinations on the callus induction and regeneration of tomato (*Lycopersicon esculentum* Mill., c.v. Omdurman).

PGR	Concentration	Hypoctyl explant				Cotyledon explant			
		Callus response (%)	Callus index	Shoot formation (%)	Average no. of shoots	Callus formation (%)	Callus index	Shoots formation (%)	Average no. of shoots
2,4-D	0.0	0.0	0.0±0.0	0.0	0.0	0.0	0.0±0.0	0.0	0.0
	0.5	53.3	1.3±0.3	0.0	0.0	100	3.9±0.2	0.0	0.0
	1.0	60.0	1.1±0.3	0.0	0.0	80	1.4±0.3	0.0	0.0
	2.0	60.0	1.5±0.3	0.0	0.0	100	2.7±0.5	0.0	0.0
	3.0	80.0	1.9±0.4	0.0	0.0	100	2.3±0.5	0.0	0.0
NAA	0.5	86.6	5.3±0.4	0.0	0.0	100	2.7±0.1	0.0	0.0
	1.0	100	3.1±0.3	0.0	0.0	100	3.8±0.2	0.0	0.0
	2.0	93.3	2.3±0.2	0.0	0.0	75	4.7±0.1	0.0	0.0
	3.0	86.6	2.1±0.3	0.0	0.0	75	4.7±0.1	0.0	0.0
BAP	0.5	65.9	3.3±0.1	0.0	0.0	50.0	00±.00	0.0	0.0
	1.0	79.1	2.3±0.1	0.0	0.0	91.6	1.9±0.1	0.0	0.0
	2.0	100	1.7±0.1	0.0	0.0	66.6	2.4±0.1	0.0	0.0
	3.0	75.0	1.9±0.1	0.0	0.0	75.0	2.4±0.1	0.0	0.0
TDZ	0.5	50.0	2.7±0.3	0.0	0.0	58.3	1.8±0.1	25.0	3.0
	1.0	100	3.6±0.3	15.0	2.0	75.0	2.2±0.1	41.6	5.0
	2.0	100	3.8±0.09	12.5	2.0	86.6	2.2±0.1	50.0	5.0
	3.0	100	2.9±0.2	10.0	2.0	93.3	2.3±0.2	72.2	6.0
BAP/ NAA	0.1/0.1	100	2.4±0.2	0.0	0.0	100	1.4±0.1	0.0	0.0
	0.1/1.0	100	4.5±0.1	0.0	0.0	100	3.3±0.1	0.0	0.0
	0.5/0.1	100	5.2±0.1	0.0	0.0	100	4.6±0.1	0.0	0.0
	0.5/1.0	100	4.5±0.1	0.0	0.0	100	4±0.1	0.0	0.0
	1.0/0.1	100	4.3±0.1	0.0	0.0	100	4.5±0.1	0.0	0.0
	1.0/1.0	100	4.4±0.1	0.0	0.0	100	3.9±0.2	0.0	0.0
	2.0/0.1	100	4.3±0.1	0.0	0.0	100	4.2±0.2	0.0	0.0
	2.0/1.0	100	4.3±0.1	0.0	0.0	100	3.6±0.1	0.0	0.0

Table 1. Cont.

	0.1/0.1	100	4.0±0.3	0.0	0.0	100	2.5±0.04	0.0	0.0
	0.1/2.0	23.0	5.0±0.2	0.0	0.0	100	2.9±0.3	0.0	0.0
TDZ/	0.5/0.1	100	2.5±0.2	0.0	0.0	100	2.6±0.2	0.0	0.0
NAA	0.5/2.0	58.3	4.0±0.3	0.0	0.0	100	3.0±0.1	0.0	0.0
	1.0/0.1	100	3.3±0.2	0.0	0.0	100	3.2±0.5	0.0	0.0
	1.0/2.0	75.0	4.0 ±0.0	0.0	0.0	100	2.5±0.1	0.0	0.0
	2.0/0.5	100	4.1±0.2	0.0	0.0	100	2.9±0.4	0.0	0.0
	0.1/0.1	47.0	0.5±0.1	0.0	0.0	33.3	0.3±0.1	0.0	0.0
	0.1/2.0	100	2.7±0.2	0.0	0.0	100	2.9±0.2	0.0	0.0
	0.5/0.5	25.0	1.3±0.3	0.0	0.0	100	2.9±0.2	93.3	3.3
BAP/	0.5/2.0	100	4.5±0.3	0.0	0.0	100	4.2±0.2	33.3	3.4
TDZ	1.0/1.0	100	2.8±0.2	0.0	0.0	91.0	2.9±0.2	0.0	0.0
	1.0/2.0	100	2.3±0.2	0.0	0.0	100	1.9±0.09	0.0	0.0
	2.0/0.1	80.0	2.8±0.3	20.0	2.0	62.5	2.7±0.1	50.0	3.0
	2.0/2.0	31.2	1.3±0.1	5.0	1.0	75.0	2.8±0.2	33.3	3.2

Data represent the mean of three replicates with six explants for each treatment. Means followed by same letter do not differ statistically at p=0.05 according to the Duncan's multiple range test.

growth regulator as well as exogenously supplied growth regulator. Moreover, Nikam and Shitole (1998) reported that the growth regulator requirements for callus induction vary depending on the source of explant.

The highest callusing index (5.3) was obtained on hypoctyl explant cultured on MS medium supplemented with NAA at 0.5 mg/l (Figure 1a), followed by (5.2) obtained from the same explant by using 0.1 mg/l NAA in combination with BAP at 0.5 mg/l. However, for the cotyledon explant, the highest callusing index (4.7) was obtained on MS medium supplemented with NAA at either 2.0 (Figure 1b) or 3.0 mg/l (Table 1). This result is in agreement with Gulshan et al. (1981), who verified that, the medium containing 2.0 mg/l NAA was the most efficient for callus formation. 2,4-D is among the most widely used auxin used for *in vitro* callus induction in a wide range of plant species (Pal et al., 2007). However, in this study, 2,4-D was not found effective for callus induction in tomato.

Although sufficient callus was induced on both explants by all growth regulators used, but subsequent organogenesis was observed only on the callus induced on medium containing different concentrations of TDZ alone (Figures 1c and d) or in combination with BAP (Table 1). The best shoot formation (93%) was obtained for cotyledon explant callus induced and sub cultured on MS medium containing TDZ in combination with BAP both at 0.5 mg/l. The necessity of cytokinin for shoot initiation is well established (Beck and Coponetti, 1983; Evans et al., 1984). The highest number (6) of shoot per explant was obtained when cotyledon explant callus was sub cultured on MS medium supplemented with 3.0 mg/l TDZ.

In this study, TDZ showed the best result for shoot organogenesis than other growth regulators. TDZ, a synthetic phenylurea, is considered to be one of the most active cytokinins for shoot induction in plant tissue culture (Huetteman and Preece, 1993). Many reports declared that TDZ induces shoot regeneration better than other cytokinins (Thomas, 2003; Thomas and Puthur, 2004; Husain et al., 2007). In other study, Mok et al. (1982), declared that TDZ is involved in cytokinin metabolism. TDZ has been shown to induce accumulation of endogenous cytokinins (Murthy et al., 1995; Hutchinson et al., 1996). In addition to the cytokinin-like activity, Hutchinson et al., 1996 observed that, TDZ promoted auxin accumulation. Other studies found that TDZ affected auxin transport in hypocotyl tissues of *Pelargonium*

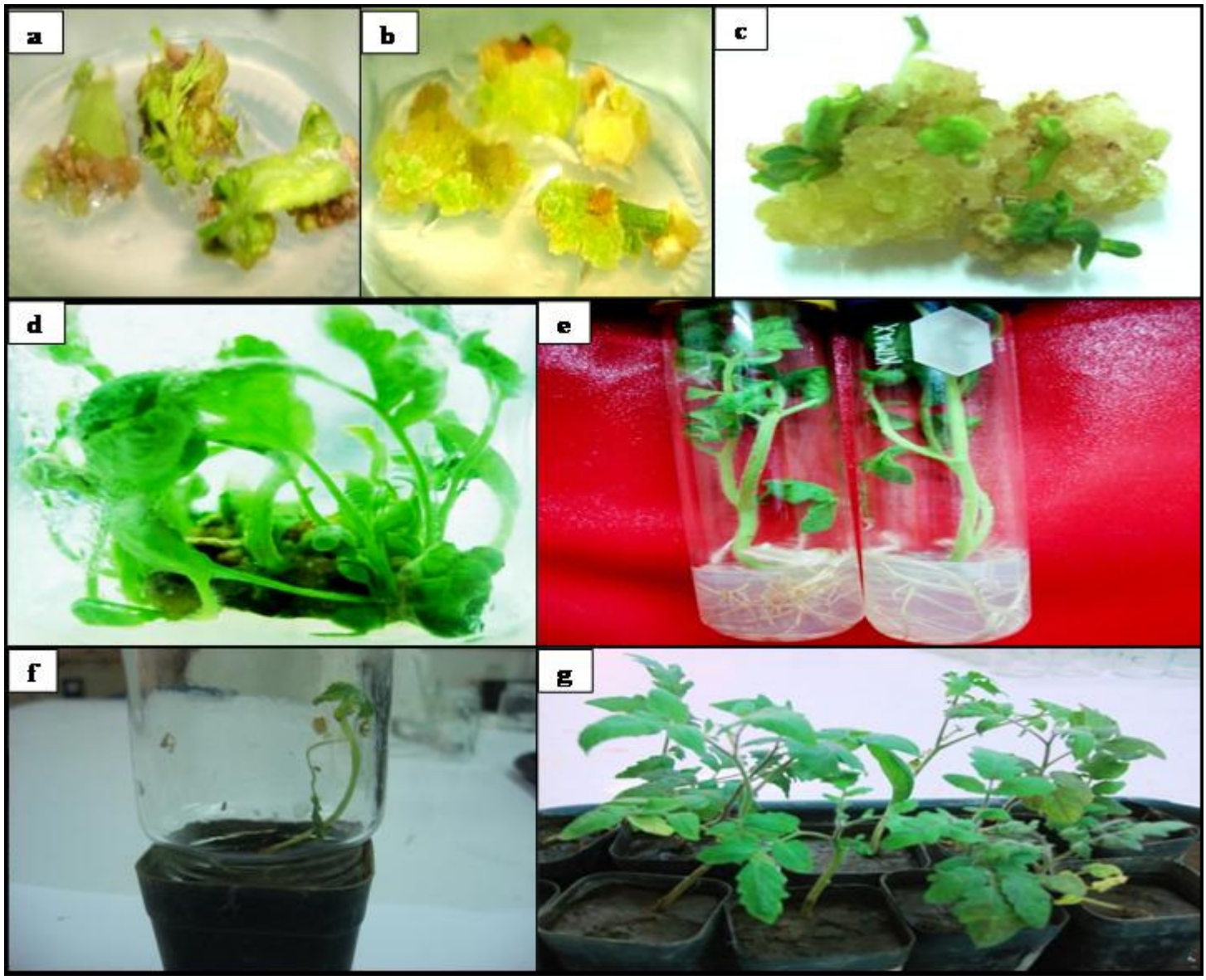

Figure 1. Plant regeneration from callus culture of tomato cv Omdrman. (a) Callus formation from cotyledon on MS medium containing 2.0mg/l NAA; (b) Callus formation from hypocotyls on MS medium containing 0.5mg/l NAA; (c) Hypoctyl and (d) Cotyledon explants after 8 weeks of incubation on a MS medium supplemented with 3.0 mg/l TDZ, showing the regeneration of shoots from the callus; and (e) regenerated plantlets with well developed roots induced on ½ MS+ 1.0 mg/l IAA. f - Acclimatization of plantlet under culture room conditions. g- Tomato plant established in soil under green house conditions.

hortorum (Murch and Saxena, 2001) and promoted the regeneration frequency by altering the levels of abscisic acid (Li and Yang, 1988), ethylene (Yip and Yang, 1986) and proline (Murch and Saxena, 1997). However, the mechanism of TDZ action in plant regeneration *in vitro* is not clear.

Here in this study, the best callus induction was obtained on hypocotyls. However, the cotyledon had better performance in regeneration through callus than hypoctyl explant indicating that, the cotyledonary tissue of tomato is an excellent explant for plant regeneration. Previous studies demonstrated that cotyledons of tomato were superior to other sources of explants, including hypocotyls, stems and leaves for promoting shoot organogenesis of tomato (Hamza and Chupeau, 1993; Van Roekel et al., 1993; Ling et al., 1998).

Induction of roots on calli-regenerated shoots is essential for successful establishment of the plantlet on the soil. 100% rooting was obtained in all hormones supplemented half strength MS medium. The best tomato rooting medium turned out to be half strength MS supplemented with any of the synthetic hormones (Table 2). In coherence with our result, Devi et al., 2008 reported that the best tomato rooting was obtained on half strength

Table 2. Effect of different concentrations of auxins and MS salt strength on rooting percentage, number of root per shoot and root length in tomato (*Lycopersicon esculentum* Mill., c.v. Omdurman)

PGR			Medium					
			Full MS			1/2MS		
IBA	IAA	NAA	Response (%)	Average no. of roots	Average length of roots	Response (%)	Average no. of roots	Average length of roots
0.0			44.4	6.7±0.6 hij	6.6± 0.4 fgh	90	10.0±1.0 fgh	7.4±0.3 ef
0.1			95	4.0±0.6 j	10.0 ±0.0 b	100	6.3±0.7 ij	9±0.3 c
0.5			96	7.9±0.9 ghi	6.4±0.5 ghi	100	7.8±0.6 ghi	10±0.0 b
1.0			100	17.6± 1.1 bc	6.5±0.1 fghi	100	7.3±0.5 hi	10±0.0 b
2.0			85	10.7±0.9 fg	5.8±0.2 hi	100	8.4±0.5 ghi	11±0.3 a
	0.1		96	11.8 ± 0.9 ef	5.6± 0.3 ij	100	7.5±0.4 hi	8.5±0.1 dc
	0.5		100	12.5±1.6 ef	4.7±0.2 jk	100	12.2± 1.7 ef	8±0.0 de
	1.0		100	14.1±1.1 de	3.8± 0.3 kl	100	22.1± 0.9 a	8±0.0 de
	2.0		100	14.1 ± 1.0 de	3.0±0 .0 lm	100	20.2±0.7 ab	8±0.0 de
		0.1	100	17± 0.8 cd	3.6±0.2 l	100	17.5±0.5 hi	6± 0.3 hi
		0.5	96	16.2± 0.8 cd	2.9±0.3 lm	100	21.2±0.8 a	3 ±0.0 lm
		1.0	83	8 ± 0.8 ghi	0.9 ±0.1 n	100	17.6±0.9 bc	2.5±0.1 m
		2.0	46	3.9± 0.6 j	0.5±0 .0 n	100	8.2± 0.9 ghi	1.2±0.06 n

Data represent the mean of three replicates with six explants for each treatment. Means followed by same letter do not differ statistically at p=0.05 according the Duncan's multiple range test.

MS basal medium. The highest mean number of roots/shoot (22.1 ± 0.9) was observed on half strength MS supplemented with IAA at 1.0 mg/l (Figure 1e), and longest root (11 ± 0.3 cm) observed in the same media containing IBA at 2.0 mg/l. Here in this study, 90% rooting was obtained on half strength MS basal medium without growth regulator indicating that this tomato genotype possessed sufficient level of endogenous auxin.

Since *in vitro* rooted plantlet are raised in the most congenial environmental conditions, hardening is imperative to ensure survival of the micro-propagated plants upon transfer to soil under natural conditions. Therefore, rooted plantlets were transferred to plastic pots containing autoclaved soil (soil: sand 2:1) and covered with glass bottle (Figure 1f) to maintain humidity, then kept under culture room conditions for one week. After three weeks, glass bottles were removed and transferred to green house and placed under shade until growth was observed. These plants exhibited 95% survival rate and all were morphologically normal (Figure 1g).

In conclusion, the results recorded during the present investigation clearly suggest that cotyledon explant obtained from 10 - 12 day-old seedlings of tomato are very important for efficient shoot regeneration. Furthermore, the present study underlines the importance of inclusion of TDZ in tomato regeneration media. Moreover, the present callus induction regeneration system would be important for genetic transformation and also has considerable potential to explore somaclonal variation as an alternative means of conventional hybridization.

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