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In vitro REGENERATION OF TOMATO (*Lycopersicon esculentum* Mill)

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ABSTRACT

A protocol was developed for shoot multiplication and regeneration in two tomato (*Lycopersicon esculentum*) cultivars. Shoots tips of about 2 cm length from *in vitro* establishment seedlings were used in the multiplication experiments. The explants were transferred into Murashige and Skoog media (MS medium) with (2.2, 4.4 µM) Benzyl adenine (BA), (9.2, 18.4 µM) Kinetin combined with 0.0, 2.7 µM Naphthalene acetic acid (NAA). Higher shoot number (8.4) was obtained with MS medium supplied with 18.6 µM Kinetin. For regeneration experiments, hypocotyl, cotyledon, stem, and leaf explants of both cultivars were used. Explants were cultured on MS media supplied with different levels of Naphthalene acetic acid (NAA) and Benzyl adenine (BA), Kinetin (Kin) and N-1,2,3-Thiadiazol-5-yl-N'-phenylurea(TDZ) Direct regeneration from the different expants was obtained on MS basal medium supplemented with TDZ at (0, 1, 2 and 4 µM) and NAA at (0, 2.7 and 5.4 µM), or BA at (0.0 or 2.2 µM) and Kinetin at (0.0, 2.3 µM). The highest shoot % (62.25) was obtained when Kinetin and BA were used at (2.3 and 2.2 µM) respectively. However, when NAA and TDZ were combined, 39.9 and 46.91% shoot regeneration was achieved with 2.7 and 4 µM, respectively for both Baladi and 593 cultivars. Very low shoot regeneration was observed with all NAA levels combined with 1 and 2 µM TDZ.

Keywords: Tomato; *Lycopersicon esculentum*; *in vitro*; regeneration; thidiazuron; growth regulators.

INTRODUCTION

Tomato (*Lycopersicon esculentum Mill*) is the most cultivated vegetable crop after potato (Nuez et al., 2013). Tomato is planted in almost 4 million hectares worldwide (Chaudhry et al., 2007). During the last two decades many biotechnological approaches have been focused on the improvement of tomato crop (Mandal et al., 2003). Different applications of *in vitro* culture is used in tomato production such as; virus free plant production (Moghaieb et al., 2004), genetic transformation (Ling et al., 1989) and in many fundamental research programmers (Arrillaga et al., 2001).

Plant tissue culture is an important tool in biotechnology and facilitator system for genetic transformation; However, *in vitro* regeneration ability of tomato remains the main limiting factor for efficient genetic transformation (Lima et al., 2004). Out of thousands of calli from two tomato varieties, five diploid plants were only regenerated after 15 months of subculturing (Zhao et al., 2014), in another study, only one hypocotyle differentiated a shoot under continuous red and far red light out of more than 500 explants (Lercari et al., 1999). On the other hand, multiple shoots were induced from leaf explants of Lycopersicon esculentum cultivar MicroTom, similar results were reported for several tomato cultivars with 3 mg L-1 BAP (Harish et al., 2101). A high shoot-regeneration capacity form different tomato lines was obtained when cotyledon explants were cultivated on medium containing 5.0 µM BAP (Arkita et al., 2013). TDZ was used in a liquid media in developing highly efficient system for regeneration of transformed tomato explants (Velcheva et al., 2005).

Regardless of the different results of regeneration, it is still essential to achieve a reliable *in vitro* system which can suit most tomato varieties.

Direct regeneration has been reported to vary with concentrations and combinations of hormones, culture media, temperature, light, genotype and explant used (Ichimura and Oda, 1995; Bahatia et al., 2004; Sheeja et al., 2004).

Therefore, the objective of this study is to establish an in vitro regeneration system of tomato of cultivated and local landrace cultivar using seedling explants.

MATERIALS AND METHODS

Plant Materials

Seeds of two tomato cultivars (Local landrace (Baladi) and 593) were used in this study, seeds of both cultivars were surface sterilized using 20% of Chlorox (sodium hypochlorite) for 15 minutes containing .1% (v/v) tween 20 as wetting agent, then were rinsed three time with sterile distilled water for five minutes every time.

Culture Media

Murashige and Skoog (1962) media (Duchefa Biochemical) was used as a basal media. The medium was supplemented with 30 mg/l of sucrose and 0.1 mg/l of myo-inistol, in addition of 8 mg/l of Agar. The PH was adjusted to (5.5), and then the medium was sterilized in the autoclave at 121C° for 21 minutes.

Tomato seeds were cultured in test tube containing 10 ml of MS basal media, two seed were planted in each test tube. Test tube were transferred to incubator and kept for (15-20) days at 22C° under 16 h day light of 40 µmol m-2s^{-1} illumination and 8 h dark period.

Multiplication Experiment

In this experiment, shoots tips of about 2 cm length were cut from the establishment seedlings after 15-20 days of germination, tips were transferred into MS basal media supplemented with NAA, combined with either Kinetin or BA (Tables 1 and 2), the cultures were incubated in a growth chamber at 22±1°C for two weeks with 16 h of photoperiod illumination of 40 µmol m-2s^{-1} supplied from cool white fluorescent. After four weeks of incubation, the numbers of shoots were recorded. Optimal hormonal combination was determined based on the result of these variables.

Regeneration Experiments

Effect of NAA and TDZ on shoot regeneration

In vitro Leaf explants of both cultivars were cultured onto MS media supplement with two different hormone levels (NAA at 0.0, 2.7, 5.4 µM and cytokinin (Thidiazuron) (TDZ) at 0.0, 1, 2, 4 µM) (Table 3).

Effect of BA and kinetin on shoot regeneration

Leal explants were culture onto medium supplemented with two cytokinin; BA at (0.0, 2.2 µM) and Kinetin at (0.0, 2.3 µM) as shown in (Table 4). Leaves were chosen as explants because of morphological uniformity. All cultured plates were incubated under complete dark conditions for 3 weeks, and then transferred to an incubator at 22C°±1 and 16 h daylight. After one month each plate was tested for the shoot regeneration.

Effect of explants on shoot and root regeneration

In this experiment, four different explants from the *in vitro* established plants of both cultivars were used (hypocotyl, cotyledon, stem, and leaf). Each explant was cut into two pieces and cultured into 9 cm diameter petridishes (4 segments for each plate) on MS basal media supplied with 2.7 µM NAA and 2 µM TDZ).

Rooting and Acclimatization

Regenerated shoots were excised and cultured on hormone free MS medium; the shoots were subcultured 2to 3 times on this medium. For rooting and acclimatization, elongated shoots of 3 to 4 cm length were excised and basally treated with T8 (commercial IBA rooting powder), the treated shoots were acclimatized as described by Abu-Qaoud, (2013).

Statistical Methods and Data Analysis

The combinations of the different growth regulator levels in multiplication experiments and type of explants and growth regulators levels in

regeneration experiments were considered as treatments. Treatments were arranged as factorial in a completely randomized design with 4 replicates in regeneration experiments and 10 replicates in multiplication experiments. Each experiment was repeated twice, the average of the two experiments was used for data analysis. Collected variable, were summarized and analyzed in one way analysis of variance, using SAS soft (SAS, 1990), comparative analysis were conducted for the significant results using LSD at .05 probability.

RESULTS

Seedling Growth

After 3 weeks, all seeds germinated successfully onto the basal medium (Fig. 1A), they continued to grow at this media for (3-4) weeks. All of seedlings were clean (no contamination was observed).

Multiplication Experiment

Effect of NAA and kinetin on shoot multiplication

The effect of different levels of NAA and Kinetin on the number of adventitious shoot produced of both tomato cultivars is shown in Table 1. Kinetin at 9.2 µM induced more shoot number per explant; the highest shoot number was achieved on medium containing 2.7 and 9.2 µM NAA and Kinetin for Balabi cultivar and 0.0 and 9.2 µM NAA and kinetin for 953 cultivar (Fig 1.B). More shoots were produced when higher levels of kinetin were used. However, when higher level of kinetin 18.4 µM was combined with 2.7 µM NAA, the shoot number was significantly decreased in both cultivars.

Effect of NAA and BA on shoot multiplication

The effect of different levels of NAA and BA on the number of adventitious shoot produced of both cultivars is shown in Table 2. Both BA and NAA exhibited a significant effect on the shoot number with significant interaction between hormonal levels. The highest shoot number (7.6 and 5.8) was obtained on medium containing 2.2 µM BA for both cultivars (Baladi and 593) respectively (Fig. 1C). However these numbers were not differ significantly from that produced on medium containing 4.4 µM BA for both cultivars. The lowest shoot number (3.7) was obtained with medium containing 4.4 and 2.7 µM of BA and NAA respectively in Baladi cultivar and 2.2 and 2.7 µM BA and NAA in 593 cultivar.

Table 1. Effect of different levels of NAA and Kinetin on shoot multiplication of two tomato cultivars

Growth regulator levels (µM)		Number of shoots per explants	
		Cultivar	
NAA	Kinetin	"Baladi"	593
0.0	9.2	6.0a	7.6a
	18.4	4.6b	5.7a
2.7	9.2	8.4a	6.5a
	18.4	3.6b	4.6b

Number followed by the same letter or letters are not significantly differ at 5% level according to (**LSD**) The least significant difference

Table 2. Effect of different levels of NAA and BA on shoot multiplication of two tomato cultivars

Growth regulator levels		Number of shoots per explants	
		Cultivar	
NAA	BA	"Baladi"	593
0.0	2.2	7.6a	5.8a
	4.4	5.0a	5.7a
2.7	2.2	3.9b	3.8b
	4.4	3.7b	4.2b

Number followed by the same letter or letters are not significantly differ at 5% level according to (**LSD**) The least significant difference

Regeneration Experiment

Effect of NAA and TDZ on Shoot Regeneration

Signification interaction between NAA and TDZ was observed on shoot percentage, the higher shoot percentage and average shoot number were obtained with the higher level of TDZ (4 µM) (Table 3). Maximum shoot percent (39.3 and 46.9) was obtained at 2.7 µM and4µM of NAA and TDZ respectively for both Baladi and 593 cultivars.(Fig 1.D). No regeneration was observed at these hormonal combinations (0.0, 0.0). (2.7, 0.0). (2.7, 1). (5.4, 0.0). (5.4, 1). (5.4, 2) µM of NAA and TDZ for "Baladi" and (0.0, 0.0). (5.4, 0.0). (5.4, 2) "593" respectively. For the number of produced shoots, similar trend to shoot percent in Baldi cultivar was observed. However the average shoot number was high (5) at level (0.0, 4 µM) of both NAA and TDZ respectively, for 593 cultivars.

Effect of BA and Kinetin on shoot and root regeneration

Maximum significant shoot percentage and average number of shoot (62.25, 3.66) were observed when BA and Kinetin were combined together for 593 cultivar and, The higher shoot regeneration percent was obtained when both BA and Kinetin were combined (33.4 and 1.5) for Baladi respectively. (Table 4, Fig. 1E),

Effect of explant on adventitious shoot and root formation

A significant interaction was observed between explant type and cultivars. In spite of the low regeneration percentage, stem explant of Baladi cultivar, exhibited significant higher shoot percentage (13.7), (Table 5), it resulted also in higher average number of shoot (1.5), however, hypocotyle explant possess a significant maximum root percent (65.8%), on the other hand, no significant difference was observed among the four explants on the average root number.

For 593 cultivar, Maximum shoot percentage was observed when stem was used as an explants (35.1%), followed by hypocotyle (19.8), but with significant difference between the two explants. In spite of the low regeneration percent obtained with leaf explants (3.4), significant maximum number of shoots was observed (6.39). Higher root percentage and average root number was observed when hypocotyle was used as an explant (56.5, 5), respectively (Fig. 1F).

Table 3. Effect of NAA and TDZ on shoot formation of tomato using leaf explants for both Baladi and 593 cultivars

Growth regulator		Cultivar			
		"Baladi"		593	
NAA	TDZ	Shoot %	Average number of shoot / reg. explant	Shoot %	Average number of shoot / reg. explant
0.0	0.0	0.0 b	0.0b	0.0c	0.0d
	1	3.8 b	1.5b	1.4c	1.0cd
	2	1.7 b	2.0b	21.7b	4.75a
	4	28.7a	4.43a	7.5 bc	5.0a
2.7	0.0	0.0b	0.0b	5.4 bc	1.0cd
	1	0.0b	0.0b	2.1bc	3.0abc
	2	3.3b	1.0b	2.8 bc	2.0bcd
	4	39.3a	4.38a	46.9a	3.59ab
5.4	0.0	0.0b	0.0b	0.0c	0.0d
	1	0.0b	0.0b	9.6 bc	3.5ab
	2	0.0b	0.0b	0.0c	0.0d
	4	16.9a	3.0 a	5.6bc	1.0cd

Number followed by the same letter or letters are not significantly differ at 5% level according to (**LSD**) The least significant difference

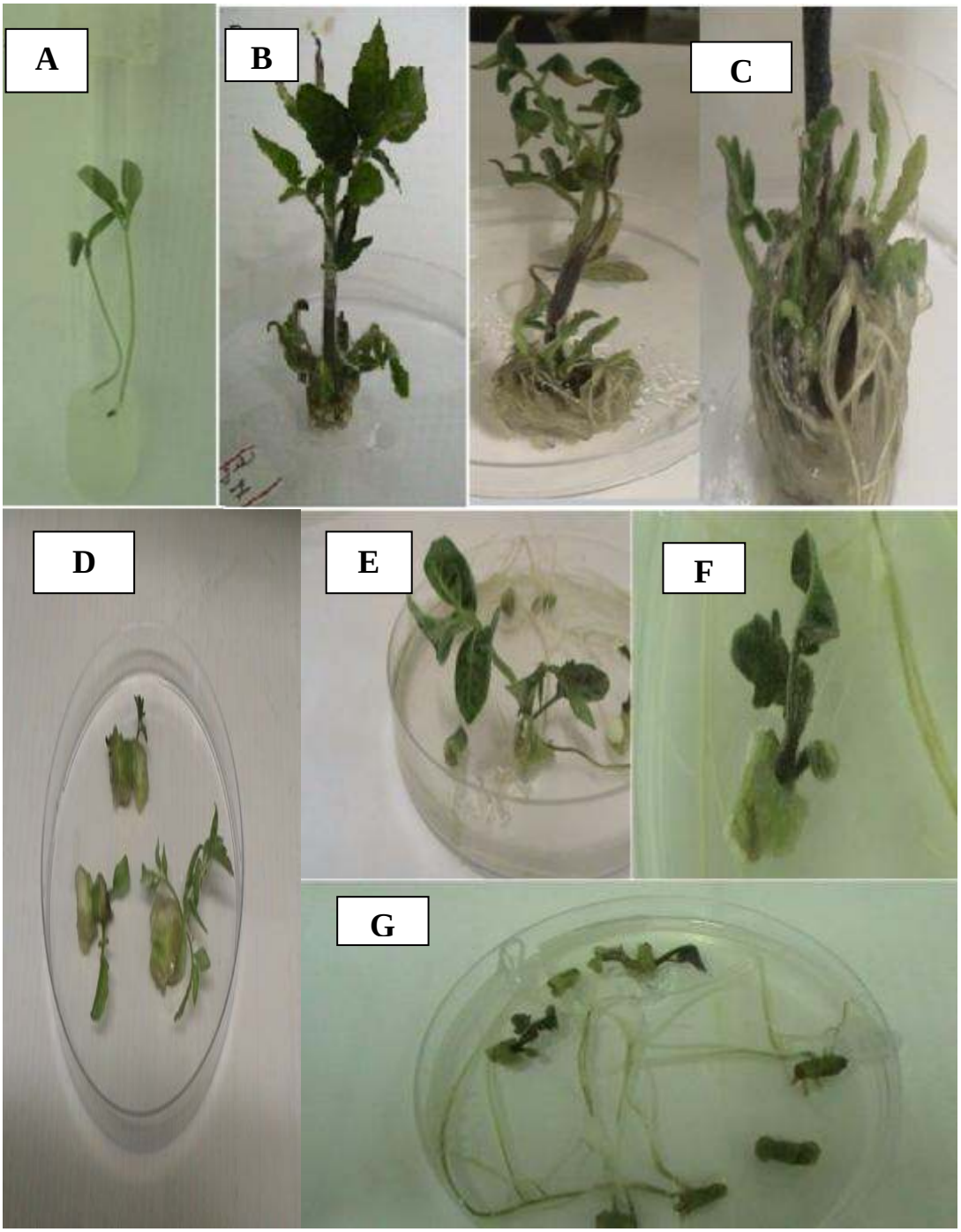

Fig. 1. A Growing tomato seeds on MS basal media. B+C: Multiplied shoot obtained on MS media containing 18.4 µM Kinetin and 0.0 µM. D-F: Shoot regeneration for leaf explant (cultivar 395). G: Regeneration from stem explants of Baladi culyivar

Table 4. Effect of BA and kinetin on shoot and root regeneration of tomato Baladi and 593 cultivars

Growth regulator	Cultivar			
	"Baladi"		593	
	Shoot %	Average number of shoot	Shoot %	Average number of shoot
2.3 µM Kinetin	2.4b	0.15b	6.65b	0.30b
2.2 µM BA	0.0b	0.0b	8.35b	0.25b
2.2 µM BA and 2.3 µM Kinetin	33.4a	1.5a	62.25a	3.66a

Number followed by the same letter or letters are not significantly differ at 5% level according to (**LSD**) The least significant difference

Table 5. The effect of different type of explants on shoot and root regeneration of tomato Baladh and 593 cultivar

Explant	Cultivar							
	"Baladi"				"593"			
	Shoot %	Average number of shoot	Root %	Average number of Root	Shoot %	Average number of shoot	Root %	Average number of root
Hypocotyl	3.6 b	0.21b	65.8a	6.36a	19.8b	1.19b	56.6a	5.0a
Cotyledon	2.56b	0.55b	6.7b	0.33b	2.92c	0.22b	25.61b	2.89b
Stem	13.7a	0.59b	4.40b	0.59b	35.1a	1.88b	4.70c	0.31c
Leaf	3.8b	1.5a	0.0b	0.0b	3.40c	6.39a	22.20b	2.42b

Number followed by the same letter or letters are not significantly differ at 5% level according to (**LSD**) The least significant difference

Rooting and Acclimatization

High percentage of the transferred shoots were rooted and successfully acclimatized (80 and 95%) for both Baladi and 593 cultivars respectively.

DISCUSSION

In vitro Seedling Growth

In our experiment, chlorox was used at 20% (v.v) to disinfest the seeds. This level was sufficient to prevent contamination without any effect on germination. Disinfestations of seeds by using Clorox have already been proved to be essential in tomato tissue culture and *in vitro* seed germination (Chaudhry et al., 2007).

Multiplication Experiments

Multiple shoots regeneration of both Baladi and 593 tomato cultivars were initiated from the shoot tip explants after (3-4) weeks of culture. The frequency of shoot number was influenced by both the type and concentration of hormones used. Micro-propagation has become a reliable and routine approach for large-scale rapid plant multiplication (Akbas et al., 2009). The dose of cytokinin is known to be critical in multiple shoots induction (Abdellatef and Khalafallah, 2207). kinetin was more effective than BA for multiple shoot production. Similar result was reported by Ishag et al. (2009) who proved that Kinetin at different concentrations can produce more multiple shoot when compared with BA and 2ip. Gubis et al. (2004) reported that the frequency of adventitious shoot regeneration depends on the type of explant and both the type and concentration of growth regulators.

In this study, when Kinetin was combined with NAA didn't show any positive response on shoot number and development; however, when it was used alone it was more effective. Results showed that the use of only BA on medium proved to be more beneficial than the combination of Auxin for shoot number.

Regeneration Experiments

In this study, we demonstrated the effect of cultivar, hormone and explant on adventitious shoot and root regeneration (Chaudhry et al., 2007) reported that the regeneration capacity is significantly influenced by cultivar and explant type.

In our study, we investigated the effect of NAA and TDZ on shoot formation, higher shoot percentage was obtained when TDZ was used at higher level (4 µM) combined with different concentration of NAA. This result agreed with that of other researchers (Velcheva, et al., (2005); Chaudhary et al., (2004) and Devi et al. (2008). Chaudhary et al., (2007), reported that auxins and cytokinins are involved in cell division and elongation, while cytokinin help in the process of differentiation, therefore, the appropriate concentration of these growth hormones is necessary for cell division and differentiation. They reported that MS medium containing 1.5 mg/L 2ip and 0.5 mg/l IAA exhibited the highest shoot regeneration because of the presence of Cytokinin.

These results are supported by Gubis et al., (2004) who reported shoot regeneration from hypocotyls on (MS) medium supplemented with 1 mg/l of zeatin and 0.1 mg/l of IAA. Similarly, Shivakumar et al., (2007) developed a regeneration protocol for tomato cultivars (ArkaSaurabh and ArkaVikas), using cotyledon and hypocotyls explants. Optimum regenerative response for all genotypes was obtained on MS medium supplemented with 2 mgl^{-1}BAP and 0.1mgl^{-1} IAA. Devi et al. (2008) reported, no shoot formation occurred on media containing 0.5 $mgl^{-1}$of IAA only.

The presence of high Cytokinin with low or equal amount of Auxin was confirmed by Gubis et al., (2003). Whereas, Raj et al., (2005) also used low levels of Auxin and Cytokinin for regeneration of leaf explants of the "Pusa Ruby tomato" (0.1 mg/l of IAA and 0.1mg/l of zeatin).

The low regeneration percentage obtained in this study compared to other researchers (Chaudhry, 2010), may be a cultivar effect, Chaudhry et al. (2007) recorded that the regeneration capacity is significantly influenced by cultivar and explant type. Differences in shoot regeneration percent in tomato among different tested cultivars were reported (Khan et al. 2006; Rashid and Bal, 2010; Arikita et al., (2013). Competence for *in vitro* regeneration was transferred from wild Lycopersicon species to the cultivated tomato [7]. In this study we demostrated the use of TDZ for shoot regeneration. TDZ was not widely used for tomato regeneration (Dhruva et al., 1978).

In this experiment, the effect of two levels and combinations of BA and Kinetin on shoot regeneration was investigated. The highest shoot percentage was obtained when both cytokinins were used at (2.2 and 2.3 µM), respectively. Our results supported the findings of other authors (Ichimura and Oda, 1995; Nogueria et al., 2001] who found that the most efficient medium for *in vitro* regeneration of tomato was MS medium supplemented with cytokinin only. A similar type of hormone was used by Rashid and Bal (2010) who observed that the optimal medium for plant regeneration was MS supplemented with 0.5 mg/l BAP and 0.5 mg/l Kinetin in two tomato genotypes. Brichkova et al., (2002). observed that the presence of two Cytokinin (BAP at 5.0 mg and Zeatin at 1.0 mg/l) contributed better to plant regeneration.

In our study, BA with cultivar 593 was better than Kinetin. The presence of BA in the culture medium has long been reported to promote shoot organogenesis in a large number of plant species (Mandal and Sheeja, 2003). Kartha et al., found that BA or zeatin alone induced shoot formation from leaf callus. Zeatin and BA were also found superior to kinetin for shoot formation from tomato leaf explants (Moghaieb et al., 2004).

In this study, we compared the shoot and root regeneration of different explants (hypocotyl, cotyledon, stem, and leaf). Stem induced more shoot regeneration followed by hypocotyl, leaf, and cotyledon. Most of the reports about adventitious regeneration in tomato deal with induction of regeneration in hypocotyls or cotyledon (Locey, 1981; Motte et al., 2013). Similar result was also reported by Jabeen et al. (2005), who stated that the higher shoot

regeneration were obtained from shoot tip followed by hypocotyl and leaf disc. In contrary, Ghada et al., (2008). reported rapid regeneration of tomato plants using hypocotyle with part of cotyledon. These results are supported by the results of Faria and Illg (1996). in which they reported, a maximum shoot formation obtained from the tomato hypocotyls. Similar results was reported by other researches (Lima et al., 2004; Khan et al., 2006).

In our study, among the various explants used, hypocotyl was best in terms of average number of shoot, which could probably be attributed to the age compared to other explant leaf and stem. Younger explants showed better callus induction and organogenetic response. The result is consistent with the earlier findings (Locey, 1981).

Our results were inconsistent to other finding in which cotyledons were superior for shoot regeneration to other source of explants, including hypocotyls, stems and leaves (Hamza and Chupeau, 1993; VanRoekel et al., 1993; Ling et al., 1998). Similar results was reported by Nogueira et al., (2001), who observed high regeneration frequency with cotyledon explants of two tomato genotypes (Santa Clara or its natural mutant Firme), respectively.

In this study, Rooting and acclimatization of regenerated shoots, was simply achieved using a simple protocol. Ouyang et al., (2003) observed that the high rooting % was obtained onto media supplied with (0.5 and 1 mg/l) level of auxin; he suggested that tomato possesses high levels of endogenous auxins.

It is known that establishing an efficient regeneration protocol is a difficult process and requires optimization of multiple factors that influence the regeneration capacity. Here, we studies the effect of cultivar, growth regulators and explants type on regeneration of two tomato cultivars. The molecular process of shoot induction in still unclear and under investigation. Through a detailed analysis of the different aspects of shoot regeneration, Motte et al., (2013) tried to reveal hinge points and novel candidate genes that may be targeted to increase shoot regeneration capacity. Investigations have recently been empowered by the identification of key genes that function in regeneration, involved in hormonal biosynthesis, transport, signaling, and hormone interactions (Su and Zhang, 2014).

The result of this study is considered as initial findings on the multiplication and regeneration of local cultivar and land race of tomato in Palestine, more investigations are needed to improve the multiplication and regeneration tomato cultivar. In addition ex-vitro studies are also needed to evaluate the growth and performance of the regeneration plants.

CONCLUSION

Both BA and Kinetin were used to induce shoot multiplication in two tomato cultivars, however, kinetin was more effective than BA. On the other hand, higher shoot regeneration was obtained when TDZ was used at 4 µM combined with different concentrations of NAA**.** The highest shoot percentage was obtained when both (BA and Kinetin) were used at (2.2 and 2.3 µM), respectively. Different explants exhibited different regeneration capacity, stem induced more shoot regeneration followed by hypocotyl, leaf, and cotyledon.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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