استحداث الكالس وتجديد النبات في أربعة أصناف محلية من البندورة (*Solanum Lycopersicum* L.)

Callus Induction and Regeneration Responses of Four Local Tomato Varieties (*Solanum Lycopersicum* L.)

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الملخص

تم تنفيذ البحث في مخبر زراعة الأنسجة النباتية في السويداء التابع للهيئة العامة للبحوث العلمية الزراعية (GCSAR) خلال الأعوام 2018 و2019 بهدف وضع بروتوكول فعال لاستحداث الكالس وتجديد النبات من أربعة أصناف محلية من البندورة. تم دراسة تأثير كل من الخزعات النباتية وهي الوريقات الفلقية والسويقات الجنينية والعنق الجذري (والمأخوذة من البادرات بعمر 15 يوماً) وتركيز منظمات النمو في الوسط في استحداث الكالس وتجديد النبات. لوحظت أفضل استجابة لاستحداث الكالس من خزعات العنق الجذري المزروعة على وسط MS مضافاً إليه 2 ملغ/ل BAP+ 0.2 ملغ/لNAA. كما تفوقت الخزعات النباتية للعنق الجذري على الوريقات الفلقية والسويقات الجنينية في كافة الأصناف المدروسة حيث بلغ متوسط نسبة استحداث الكالس 88.47، 64.47، 45.73% بالترتيب، وتميز الكالس المتشكل بقوام هش وحبيبي وبلون أخضر داكن وبمتوسط وزن 1.866غ وحجم 355.85 ملم^3. لم تسجل فروقاً معنوية بين أصناف البندورة المدروسة فيما يتعلق بنسبة استحداث الكالس على الرغم من أن الصنف بسكنتا قد سجل أعلى نسبة مقارنة بباقي الأصناف. أما فيما يتعلق بنسبة تجديد النبات فقد سجلت أعلى نسبة للتجديد (78.71%) وأكثر عدد للنموات (4.45 نمو/الكالس) على الوسط MS المضاف إليه 2 ملغ/ل BPA+0.5 ملغ/ل IAA. وقد أعطى الصنف ضهر الجبل أفضل قدرة على التجديد من الكالس (50.38%) وأكثر عدد للنموات (3.39 نمو/كالس). بينما أعطى الصنف بريح نموات أطول (0.373 سم) بدون فروق معنوية بين الأصناف.

الكلمات المفتاحية: البندورة، التجديد، الخزعات النباتية، زراعة الكالس، العنق الجذري، منظمات النمو.

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Abstract

The study was carried out at Sweida research center/ the General Commission for Scientific Agriculture Research (GCSAR)/ Syria during 2018 and 2019 in order to develop an efficient protocol for callus induction and plant regeneration of four local tomatoes varieties. Cotyledons, hypocotyls and root crown explants were extracted from well developed seedlings and used as explants to evaluate their capacity for callus induction. Different concentrations and combinations of plant growth regulators (PGRs) were added to MS medium to evaluate their efficacy on callus induction and shoot initiation. The best response for callus induction was observed from root crown cultured on MS medium supplemented with 2 mg/L BAP+0.2 mg/L NAA. Root crown explants gave the highest callogenesis compared to cotyledons and hypocotyls in all studied varieties where the mean callus induction frequency was 88.47, 64.47 and % 45.73 respectively. Hard friable, nodular and dark green callus was obtained from root crown explants with a mean callus fresh weight (1.866 g) and size (355.85 mm^3). No significant differences were recorded among tomato varieties in respect to callogenesis, even though Baskanta recorded a higher callus induction frequency compared to other varieties. Furthermore, the highest percentage of shoot regeneration (78.71%) and the highest number of shoots (4.45 shoots) were recorded on (2 mg/L BAP+0.5 mg/L IAA). Daher-Aljabal gave the best response for regeneration in terms of percentage (50.38%) and shoot number per callus (3.39). While the tallest shoot was 0.373 cm in Brieh with no significant differences among varieties.

Key Words: Callus culture, Explants, Plant Growth Regulators, Regeneration, Root crown, Tomato.

Introduction

Tomato *(Solanum Lycopersicum* L.) is the second most consumed vegetable in the world (Iqbal et al., 2019) after potato (Rashid et al., 2012). It is grown in every country in the world, in outdoor fields, greenhouses and net houses (Bredy *et al.*, 2015). Recently, this crop has gained huge popularity due to its anti-cancer and antioxidant characteristics (Khuong *et al.*, 2013). All tomato species are diploid (2N = 2X = 24) and have the same chromosome number and structure and it is one of the most genetically characterized higher plant species (Foolad, 2007) with the smallest genome size (953 Mb) among *Solanaceae* (Arumuganathan and Earle, 1991). So that, tomato crop is ideal for genomic studies and plant breeding (Lammerts *et al.*, 2011) because it's easily grown, has a short life cycle and easy to manipulate (Costa and Heuvelink, 2005). However, traditional methods for tomato breeding can be expensive and fastidious due to the time and facilities required by each breeding generation and to the problems with the selection of suitable standards for cultivating (Khuong et al., 2013). Therefore, 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 (Abdel-Raheem et al., 2007). *In vitro* culture is used in tomato in different biotechnological applications (Magdoleen et al., 2010) Establishment of an efficient tissue culture protocol is an advantage of cell and tissue culture for genetic improvement (Sheeja et al., 2004). In tissue culture technique, callus has great potential due to produced genetic variability which is very important in breeding program (AL-Hussaini et al.,2015). Obtaining plantlets using callus induction from plant tissue culture techniques has motivated many researchers to select tomato as model species for the advancement of several genetic characters (Hanur and Krishnareddy, 2016). *In vitro* regeneration of cultivated tomato has been a subject

of research because of the commercial value of the crop and its amenability for further improvement via genetic manipulation (Rao and Kalyani, 2014) However, the success of *in vitro* plant regeneration depends on many factors (Manawadu et al., 2014) In tomato, callus induction and plant regeneration in tomato is genotype, explant, growth regulator and medium dependent (Papry et al., 2016) The most influential factors of *in vitro* plant growth are the interaction and balance between the plant growth regulators endogenously and exogenously by cultured cells (Nasution and Nasution, 2019). However, the regeneration response of tomato to plant growth regulators (PGRs) has been observed to be highly genotype-specific, and as such, the type and concentration suitable for one genotype may not be optimal for others (Bhatia et al., 2005).

Many tissue culture studies have been conducted on tomato and different explants sources have been used for callogenesis and regeneration. Various hormonal combinations are used to induce callus and regeneration (Ishag et al., 2009). The degree of response of explants has been reported by Durzan (1984) in order of leaves, cotyledon, hypocotyls, whereas Plastira and Perdikaris (1997) had reported in the order of hypocotyls, cotyledon and leaves. (Harish et al., 2010). Somaclonal variation through callus culture has been possible to generate useful genetic variation for desired traits. However, few studies have been carried out on local Syrian tomato varieties to enhance callus induction and no one of these studies tested the regeneration of plantlets from callus and the factors that affecting callus induction and plant regeneration. So, the present study was undertaken to investigate the effect of explants type and media composition on *in vitro* callus induction and regeneration for four local tomato varieties. Root crown explants were tested for their efficiency for callus induction and plant regeneration.

Materials & Methods

Research site and plant material:

The experiments were carried out at Sweida Agricultural Research Center in General Commission for Scientific Agricultural Research (GCSAR)/Syria, during 2018-2019. Four local tomato varieties were used from the gene bank of GCSAR (Daher-Aljabal, Brieh, Baskanta and Daraa). Seeds were surface-sterilized by washing under running tap water, then immersed in 0.6g/l topsin M (fungicide) for 15 min. and rinsed three times with distilled water. Seeds then were sterilized with 1% (v/v) sodium hypochlorite (NaOCl) for 7 min., rinsed three times with autoclaved distilled water under aseptic conditions. The seeds were dried on autoclaved filter papers for 15min. then cultured in sterilized Petri dishes (10 seeds/dish, Figure1a) containing MS basal medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 7g/l agar. Cultures were kept in a growth room for germination (Figure1b)

Callus induction and growth:

Cotyledons, hypocotyls and root crowns explants of 15-days-old seedlings (Figure1c) were used in order to select the best explants for callus induction. Explants were cut into small pieces of about 3-5 mm segments (Figure 2a,b,c,d) and cultured in aseptic conditions on callus formation medium which are MS basal medium supplemented with 30 g/l sucrose and 7g/l agar and different concentration of 2,4-D (2,4 dichlorophenoxy acetic acid), NAA (1-naphthalene acetic acid), IAA (Indol-3-acetic acid) and BAP (6-Benzylaminopurine). Ten different media were tested for callus induction named MS1 to MS10 (Table 1). The pH of the medium was adjusted to 5.8 then autoclaved at 120 °C and 1.04 kg/cm^2 pressure for 20 min. Three replications per treatment were used for each tomato variety, 15 explants in

each replication. Callus formation was monitored weekly. 8 weeks after incubation, the following parameters were recorded:

$$\text{Callus formation frequency} = \frac{\text{Number of callus-producing explants}}{\text{Total number of explants cultured}} \times 100$$

(Durrani et al., 2017)

-Callus fresh weight (g) by recording the difference between flasks before and after 40 days of incubation (Rakshit et al., 2007).

-Callus size (length*width*height mm^3) by measuring the length, width and height of callus before sub-culture (Rakshit et al., 2007).

-Callus morphology: texture (compact or friable) and color (creamy, light green, green, pale yellow, light brown). (Almaarri, 2018)

Regeneration stage:

Best explants were selected for further callus proliferation and callus were subcultured three times every 3-4 weeks intervals on the best callus induction medium to obtain enough callus for regeneration stage. Healthy callus were sub cultured on eleven regeneration media (MS basal medium supplemented with different concentrations of PGRs (plant growth regulators) named R1 to R11 in addition to control R0 (hormone-free medium) in order to assess the regeneration capacity of different tomato varieties (Table 1). Regeneration percentage, number of days for shoot formation, number of regenerated shoots from callus and shoot length (cm) were recorded. Regenerated shoots were multiplied on MS medium supplemented with 1mg/L BAP to obtain enough number of shoots then regenerated shoots of 1.5 cm were transferred to a rooting medium (1/2MS basal medium supplemented with l mg/L IBA, indole-3-butyric acid) for 60 days. Rooted plantlets were cultured in pots filled with a mixture of perlite: peat moss (1:1) for acclimatization. Cultures were maintained at 24±2 ºC under 16:8 h photoperiod provided by fluorescent tubes with light intensity of 40 µmol m^{-2} s^{-1}.

Table 1. Composition of culture media used for callus induction and plant regeneration of studied tomato varieties

Medium code for callus induction	Composition	Medium code for regeneration stage	Composition
MS1	1.75mg/L 2,4-D	**R0**	**Without hormones**
MS2	1.75 mg/L 2,4-D+0.2 mg/L IAA	**R1**	**1 mg/L BAP+0.2 mg/L NAA**
MS3	1 mg/L NAA+0.1 mg/L BAP	**R2**	**2 mg/L BAP+0.5 mg/L NAA**
MS4	1.75 mg/L NAA+0.2 mg/L BAP	**R3**	**2 mg/L Kin+0.2 mg/L IAA**
MS5	2 mg/L BAP+0.2 mg/L NAA	**R4**	**1 mg/L BAP+0.2 mg/L IAA**
MS6	0.5 mg/L 2,4-D	**R5**	**2 mg/L BAP+0.5 mg/L IAA**
MS7	1.5 mg/L 2,4-D	**R6**	**2 mg/L Kin+0.2 mg/L NAA**
MS8	2.5 mg/L 2,4-D	**R7**	**2 mg/L Kin+0.5 mg/L NAA**
MS9	1.75 mg/L IAA	**R8**	**1 mg/L Zeatin**
MS10	1.75 mg/L NAA	**R9**	**1 mg/L 2ip**
		R10	**2 mg/L BAP+0.2 mg/L IAA**
		R11	**2 mg/L BAP**

Each treatment was replicated three times with 15 explants in each replication

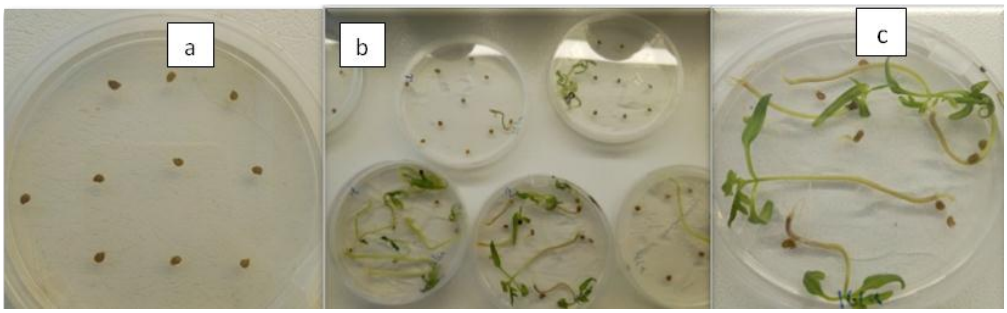

Figure1: a: seed cultured on MS medium. b: geminated seeds. c: seedlings used as explants source

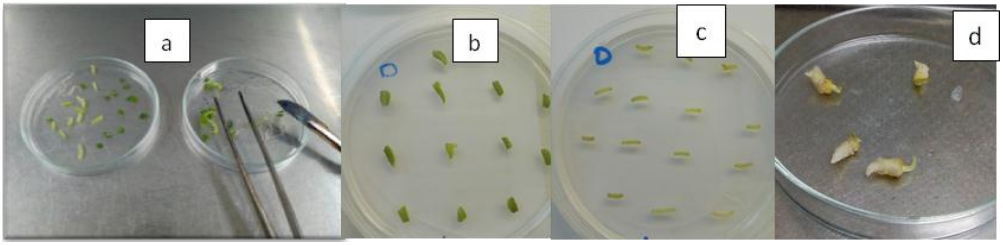

Figure 2: a: seedlings were cut into explants aseptically, b: cotyledon. c: hypocotyls and d: root crown explants

Statistical analysis:

The experiments were performed as completely randomized design (CRD) in a factorial system. Data were subjected to ANOVA analysis, mean values were compared according to at least significant difference test (LSD) with p value ≤0.01. The obtained results were statistically analyzed using GenStat12 program.

Results

Callus induction frequency

Seeds of studied tomato varieties were germinated on MS medium after 15 days of culture with a germination percentage of 100%, 80%, 80%, 80% for Baskanta, Daraa, Brieh, Daher-Aljabal, respectively. Seedlings of 5-8 cm were cut aseptically into three parts: cotyledons, hypocotyls and root crowns (which were tested for the first time in this research). Explants were cultured on induction media. Data were analyzed after 8weeks of culture and the results showed that among all 2,4-D concentrations, 1.75 mg/L (MS1) found to be the most effective concentration of 2,4-D for callus formation from cotyledons and hypocotyls which exceeded significantly the other tested concentrations (0.5, 1.5, 2.5 mg/L) as can be seen in Figure 3 . On the other hand, using IAA (1.75mg/L) or NAA(1.75mg/L) alone in the medium led to rhizogenesis after 7days of culture and callus was pale yellow to brown in color and compact in texture (Figure 6 a,b.). According to these findings, media MS6, MS7, MS8, MS9 and MS10 which contain 2.4-D, IAA, NAA alone were excluded from further work and MS1 (1.75mg/L 2,4-D) was selected over other media supplemented with 2,4-D to be compared with the other tested media. As shown in Figure 4, root crown explants gave the highest callogenesis compared to cotyledons and hypocotyls in all studied varieties

Based on the overall responses of explants on all treatment, a statistical difference was found among MS formulations. When cotyledons and hypocotyls used as explants MS1(1.75mg/L 2,4-D) was found to be the best medium for callus induction (64.48%, 45.73%) followed by MS3 (45.16%, 40.3%), MS4 (23.23%, 20.06%) and MS5 (6.84%, 5.28%) respectively, with significant differences among tested media. The same media didn't response to callus induction in the same way when root crowns explants used regardless of the tomato variety. Results presented in Table 1 showed the responses of tomato varieties for callus formation when root crowns cultured on MS5 medium supplemented with 2mg/L BAP+0.2 mg/L NAA, where the mean callus induction frequency was (88.48%) followed by cotyledons (64.75%) than hypocotyls (45.73%) as it can be seen in Figure 5a. On the other hand, the highest callogenesis (96.67%) was recorded in the variety Brieh when culturing root crowns explants on MS5 followed by Daraa (88.38%), Baskanta (80.84&) and Daher-Aljabal (80%). Regarding tomato variety responses, Baskanta showed to be the best response variety for callogenesis as a mean of callus induction frequency for all explants used followed by Daraa(70.14%), Daher-Aljabal (60.09%) and Brieh (59.39%) as presented in Figure 5b.

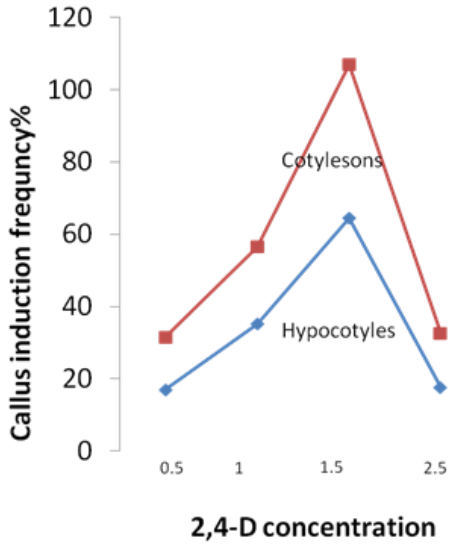

Figure3. Effect of different 2,4-D concentration on callus induction frequency

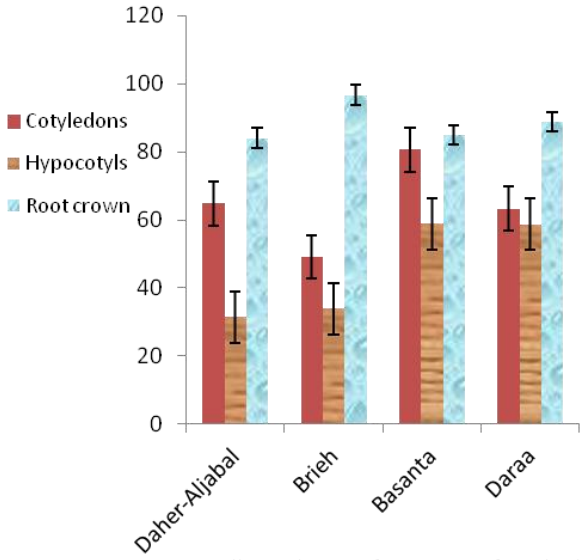

Figure 4. Callus induction frequency of studied tomato lines according to explant type

Callus color varied from creamy, pale yellow (Figure 6c), brown (Figure 6d), light green to green, whereas the texture was friable or compact depending on the media, explants and tomato variety. Hard friable, nodular and dark green callus was observed in all tomato varieties on MS5 medium when root crowns used as explants (Figure 6g,h), while hard friable callus varied in color from creamy to light green were observed when cotyledons (Figure 6f) and hypocotyls (Figure 6ke) cultured on MS10.

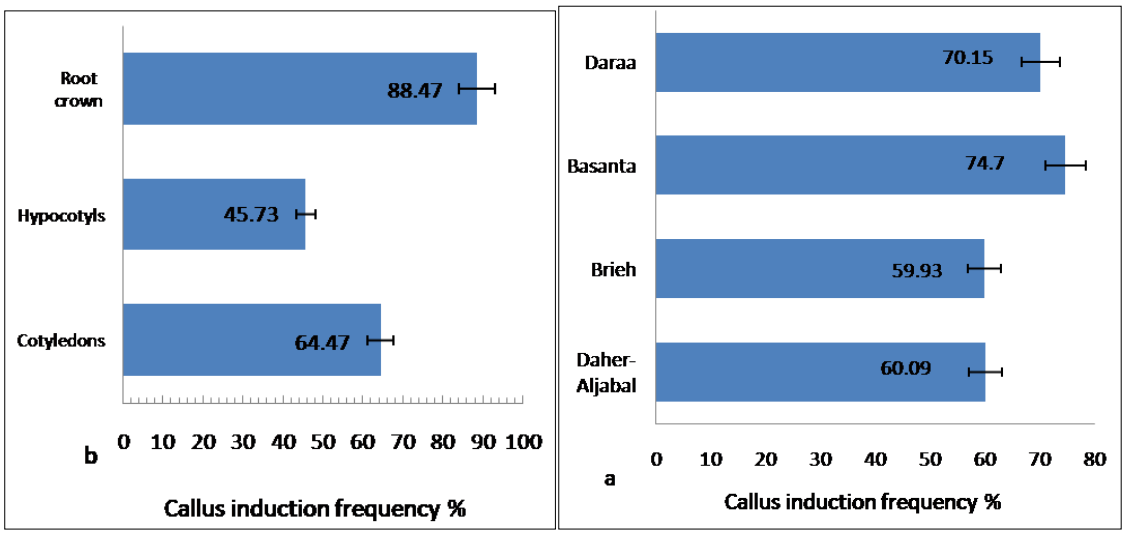

Figure 5 a, b Callus induction frequency (%) in tomato varieties

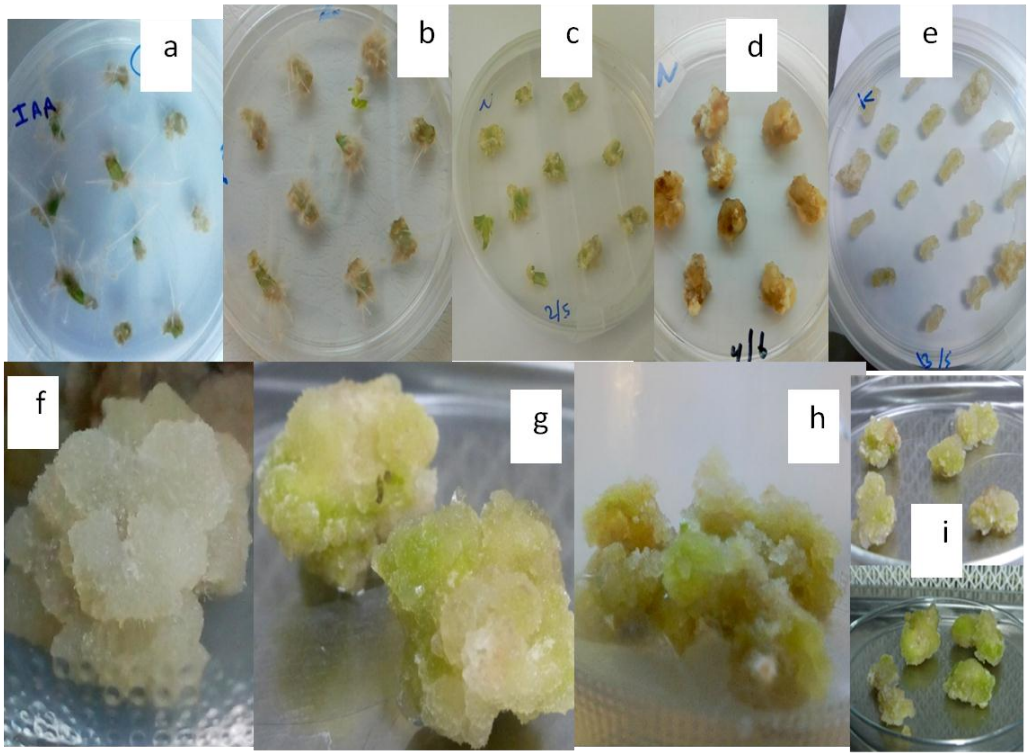

Figure 6: Rhizogenesis formation on a. 1.75mg/L IAA, b. 1.75mg/L NAA.

c. Pale yellow callus. d. Brown callus. e. Callus initiated from hypocotyls.

f. Callus initiated from cotyledons. g+h. Callus initiated from the root crown.

i. Callus subculture.

Table 2. The effect of different explants type and different media composition on callus induction and morphology in four tomato varieties:

Explant type	Media composition	Tomato variety								
		Daher-Aljabal		Brieh		Baskanta		Daraa		
		Callus induction frequency	Callus color& Texture	Callus induction frequency	Callus color& Texture	Callus induction frequency	Callus color& Texture	Callus induction frequency	Callus color& texture	**Mean**
Cotyledones	MS1	64.83 a	LCrF	49.18a	LCrF	80.56a	LCrF	63.33a	LGF	**64.47a**
	MS2	30.07 b	CrC	34.82ab	CrC	47.58ab	PYC	53.14ab	Cr.C	**41.39b**
	MS3	42.5 ab	LGF	22.20b	CrF	66.13ab	CrF	49.82ab	LGF	**45.16b**
	MS4	21.67 b	CrC	14.14b	CrC	30.5bc	CrC	26.62b	CrC	**23.23c**
	MS5	0 c	——	2.22bc	CrC	16.25c	LCrC	8.89bc	CrC	**6.84c**
Mean		31.81A		24.51A		48.20A		40.36A		
$LSD_{0.01}$ (line)		29.22		23.99		43.13		44		
$LSD_{0.01}$ (Explant)										**15.41**
$LSD_{0.01}$(Medium)										**30.82**
$LSD_{0.01}$ (explant*medium)										**13.78**
Hypocotyls	MS1	31.44a	CrF	33.91a	CrF	58.80a	LGF	58.75a	LGF	**45.7a**
	MS2	29.62a	LBF	30.08a	LBF	45.95ab	CrC	55.56a	CrC	**40.3a**
	MS3	11.67ab	CrF	13.67b	CrF	30.68ab	LGF	24.22ab	LGF	**20.06b**
	MS4	5.55b	BC	4.06b	BF	21.11ab	CrC	15.61b	CrC	**9.92b**
	MS5	0.00b	——	0.00b	CrC	14.48b	CrC	0.00b	——	**5.26b**
Mean		15.65A		16.35A		34.21A		30.82A		
$LSD_{0.01}$		28.43		36.12		56.30		52.55		
$LSD_{0.01}$(explant)										**17.11**
$LSD_{0.01}$(medium)										**19.13**
$LSD_{0.01}$ (explant*medium)										**38.27**
Root crowns	MS1	6b	LCrF	8.89b	CrF	6b	CrF	11.49b	Cr.F	**8.09b**
	MS3	0b	——	0b	——	17.67b	PYC	0b	——	**4.42b**
	MS4	0b	——	2.22b	BC	6b	LBC	0b	——	**2.05b**
	MS5	0b	——	2.2b	BC	18.33b	LBC	0b	——	**5.14b**
		84a	GNF	96.67a	GNF	84.85a	GNF	88.38a	GNF	**88.48a**
Mean		18A		22A		26.57A		19.97A		
$LSD_{0.01}$ (line)		16.08		9.18		20.07		15.01		
$LSD_{0.01}$(explant)										**5.946**
$LSD_{0.01}$(medium)										**6.646**
$LSD_{0.01}$ (explant*medium)										**13.293**

The different letters in the same column or row refer to significant difference among treatment ($p \leq 0.01$)
Cr= creamy, PY= pal yellow, LB= light brown, B=brown,LG=light green, G=green. N=nodular, C=compact, F=friable

Callus weight and size

The present study investigated the importance of explants type on callus quality in terms of weight and size as illustrated in Table 3. callus were proliferated and sub cultured for another 8 weeks with intervals 15-21 days by using MS1(1.75mg/L 2,4-D) for callogenesis from cotyledons and hypocotyls and MS5(2 mg/L BAP+0.2 mg/L NAA) for callogenesis from root crowns explants. The maximum growth of callus in terms of fresh weight (1.866g) and size (355.85 mm^3) was obtained when root crowns used as explants at 2 mg/L BAP+0.2 mg/L NAA(MS5) for all varieties which exceeded significantly ($p \leq 0.01$) the other explants as represented in Table 3. Additionally, mean callus weight and size were affected significantly with tomato variety where Brieh formed a callus of 216.52 mm^3 size and weighed 0.97g in average.

Table 3. Effect of explants type and media composition on callus fresh weight and size of four local tomato varieties:

Tomato variety	Callus fresh weight (g)				Callus size (mm^3)			
	Cotyledons	Hypocotyls	Root crown	Mean	Cotyledons	hypocotyls	root crown	Mean
Daher-Aljabal	0.342a	0.311a	1.38b	0.95a	16.15b	10.11a	228.19bc	133.29b
Brieh	**0.534**a	0.482 a	**2.12**a	0.97a	69.21a	49.31a	555.94 a	216.52a
Baskanta	0.405a	0.351a	1.89ab	0.71a	49.14ab	39.99a	265.62 c	105.78b
Daraa	0.419a	0.338a	**2.08** a	0.92a	44.29 ab	34.18a	372.62ab	123.01b
Mean	0.427 B	0.370B	1.866A		44.71B	33.39B	355.85A	
$LSD_{0.01}$ (explant)				0.262	$LSD_{0.01}$ (explant)			62.04
$LSD_{0.01}$ (line)				0.302	$LSD_{0.01}$ (line)			71.63
$LSD_{0.01}$ (explant*line)				0.526	$LSD_{0.01}$ (explant*line)			124.07

Values in the table are the mean of three replications. Means were separated with Duncan's. The different letters in column or row indicate a significant difference among treatment ($p \leq 0.01$)

Plant regeneration stage

- Regeneration percentage:

Healthy callus (Figure 7a) was transferred to the studied regeneration media, it is worth to mention that more than 30 media were used for regeneration (results not shown) and only friable nodular-green callus succeeded to form shoots, where compact and creamy callus failed to initiate shoots. Callus were cut in pieces of 1 cm and sub-cultured on regeneration media. At the first 21 days, no regeneration response was recorded on all media and tomato varieties tested. Than callus were transferred to new fresh media. It should be mentioned that media excluded from further work were the media led to root formation from callus (Figure 7c). Regeneration response firstly noticed as green spots develop in advance to shoots (Figure 7, b,d). There was a significant positive effect of both tomato variety and media composition on regeneration capacity. On average, medium R5 (containing 2 mg/L BAP+0.5 mg/L IAA) produced maximum shoot regeneration (78.71%) regardless of tomato variety as detailed in Table 4, with a maximum mean number of shoots (4.53 shoots) of length 0.645cm as shown in Table 6 and Table 7. Though, it took a longer time for regeneration (16.29 days) preceded by R2 containing 2mg/L

BAP+0.5mg/L NAA (14.33days) as shown in Table 5. On the other hand, results shown in Table 4 revealed significant differences among tomato varieties where the highest regeneration percentage was (50.38%) recorded in line Daher-Aljabal which exceeded significantly other studied varieties, followed by Brieh (36.72%), Baskanta (36.37%) and Daraa (32.45%), with mean shoots number (3.39,1.74, 1.44 and 0.94 shoots) in Daher-Aljabal, Brieh, Baskanta and Daraa, respectively as indicated in Table 6. Additionally, the best mean shoot length was obtained in Brieh (0.37 cm) followed by Baskanta (0.34cm), Daher-Aljabal (0.32 cm) and Daraa (0.25 cm) as shown in Table 7. While, the days for regeneration were the least in Brieh (8.61 days) while callus of variety Baskanta and Daher-Aljabal needed more time to regenerate (13.03 and 14.65 days, respectively).

Nevertheless, each tomato variety response was different according to the regeneration medium. So that, the optimum medium for efficient *in vitro* shoot regeneration was R5 which contain 2mg/L BAP+0.2 mg/L IAA (86.21%) in Brieh, R10 that contain 2mg/L BAP+0.2 mg/L IAA(90%) in Baskanta, R2 containing 2mg/L BAP+0.5mg/L NAA (93.1%) in Daher-Aljabal and R10 that contain 2mg/L BAP+0.2 mg/L IAA (82.25%) in Daraa.

The maximum number of shoot per callus (4.44) was produced at 2mg/L BAP+0.5 mg/L followed by 1mg/L zeatin (3.3). Concerning the shoot length, results in Table 6 cleared that media composition had a significant effect on mean shoot length,

Among studied tomato varieties, A maximum number of shoots per callus (3.39) was observed in Daher-Aljabal which exceeded significantly other varieties. Followed by Brieh (1.76), and Baskanta (1.49) while the lowest number of shoots/callus was produced in Daraa (0.93).

On the other hand, the optimum medium for maximum shoot number/ callus was R5 in all tomato varieties which were (6.29, 4.32, 4.28 and 2.89 shoot/callus) in Daher-Aljabal, Brieh, Baskant, and Daraa, respectively.

No significant difference was recorded among the studied varieties concerning the regenerated shoot length as summarized in Table7. The mean shoot length were 0.37, 0.34, 0.32 and 0.24 cm in Brieh, Baskanta, Daher-Aljabal and Daraa, respectively.

Shoot length was maximum (0.827 cm), in Daher-Aljabal on R1 (1BAPmg/L+0.2mg/LNAA) and R2 (2BAPmg/L+0.5mg/LNAA), while in Brieh (0.8cm) on R4 (1mg/LBAP+0.2mg/LIAA), in Baskanta (0.98 cm) on R10 (2mg/LBAP+0.2mg/LIAA) and in Daraa (0.81 cm) on R5 (2mg/LBAP+0.5mg/LIAA). It was noticed in this experiment that callus cultured on media R6 containing 2mg/L Kin+0.2 mg/L NAA and R7 supplemented with 2 mg/L Kin+0.5 mg/L NAA, failed to initiate shoots in all tomato varieties under experiment conditions while, combing Kin with IAA (R2 and R3)gave better results concerning regeneration efficiency in Daher-Aljabal and Brieh only.

Table4. Effect of media composition on regeneration percentage (%) of four local tomato varieties

Media composition	Regeneration percentage%				Mean of treatment
	Tomato variety				
	Daher-Aljabal	Brieh	Baskanta	Daraa	
R0	0 e	0 e	0 d	0 e	**0e**
R1	31.58 d	18.47e	32.33 c	14.63 d	**24.25d**
R2	**93.1 a**	50 c	25 c	0 e	**42.03c**
R3	80.33 ab	25 de	0 d	0 e	**26.33d**
R4	**90 a**	30.24 d	40 c	40 c	**50.06b**
R5	66.2 bc	**86.21 a**	**85 a**	**77.43 a**	**78.71 a**
R6	0 e	0 e	0 d	0 e	**0e**
R7	0 e	0 e	0 d	0 e	**0e**
R8	66.67 bc	72.82 b	81.47 a	60.09 b	**70.26a**
R9	60 bc	80.69 ab	57.67 b	**75 a**	**68.34a**
R10	66.67 bc	50 c	**90 a**	**82.25 a**	**72.23a**
R11	50 cd	27.17 de	25 c	40 c	**35.54cd**
Mean of variety	**50.38A**	**36.72B**	**36.37BC**	**32.45C**	
$LSD_{0.01}$ (medium composition)		**7.415**			
$LSD_{0.01}$ (line)		**4.281**			
$LSD_{0.01}$ (line*medium)		**14.83**			

Values in the table are the mean of three replications. Means were separated with Duncan's.
The different letters in column or row indicate significant difference among treatments ($p \leq 0.01$)

Table5. Effect of media composition on days for the regeneration of four local tomato varieties

Media composition	Days for regeneration				Mean
	Tomato variety				
	Daher-Aljabal	Brieh	Baskanta	Daraa	
R0	0	0	0	0	**0**
R1	22.2 ab	18 bc	15.5 a	19.66 c	**18.84 d**
R2	15.33 a	0	25 d	17 bc	**14.33 a**
R3	16.5 ab	0	0	16.5 ab	**16.5 bc**
R4	16.5 ab	13.3 bc	27 d	13.3 ab	**7.52 cd**
R5	20.12 bc	13 bc	21.2 c	10.8 a	**16.29bc**
R6	0	0	0	0	**0**
R7	0	0	0	0	**0**
R8	21 cd	19.83 c	15.68 a	13.37 a	**17.47 cd**
R9	21 cd	15.43 bc	18.53 b	19.33 c	**18.57 d**
R10	18.67 abc	10.83 a	16.5 ab	15.4 ab	**15.35 ab**
R11	24.5 a	13 bc	17ab	16.5 ab	**17.75 cd**
Mean	**14.65 C**	**8.61 A**	**13.03 BC**	**11.82 B**	
$LSD_{0.01}$ (medium composition)			**1.683**		
$LSD_{0.01}$(line)			**0.972**		
$LSD_{0.01}$(line*medium)			**3.366**		

Values in the table are the mean of three replications. Means were separated with Duncan's.
The different letters in column or row indicate a significant difference among treatments ($p \leq 0.01$) p.s. Days for regeneration were recorded after the first sub culture of callus on regeneration medium which was 30 days.

Table6. Effect of media composition on the mean number of regenerated shoots (shoot/callus) of four local tomato varieties

Media composition	Mean shoots number (shoots/callus)				Mean
	Tomato variety				
	Daher-Aljabal	Brieh	Baskanta	Daraa	
R0	0 d	0 e	0 e	0 e	**e0**
R1	1.39 cd	0.75 de	0.83 de	1.39 cd	**1.09 d**
R2	**6.5 a**	2.5 bc	1 d	0 e	**2.5 c**
R3	2.167 c	2.25 bc	0 e	0 e	**1.10 d**
R4	5.03 ab	2 bc	2.06 c 7	0.6 e	**2.417 c**
R5	**6.29 a**	**4.32a**	**4.28 a**	**2.89 a**	**4.44 a**
R6	0 d	0 e	e0	0 e	**0**
R7	0 d	0 e	0 e	0 e	**0**
R8	**5.743 a**	2.67 bc	3.37 b	1.43 cd	**3.30a b**
R9	**5.2 ab**	1.6 cd	2.67 bc	1.56 c	**2.76 c**
R10	4.87 ab	1.83 cd	3.23 b	1 de	**2.73 c**
R11	3.5 bc	3 b	0.5 de	2.4 b	**2.35 c**
Mean	**3.39 A**	**1.74 B**	**1.44 B**	**0.94 C**	
$LSD_{0.01}$ (medium composition)			**0.546**		
$LSD_{0.01}$ (line)			**0.315**		
$LSD_{0.01}$ (line*medium)			**1.092**		

Values in the table are the mean of three replications. Means were separated with Duncan's.
The different letters in column or row indicate a significant difference among treatments ($p \leq 0.01$)

Table7. Effect of the media composition on mean shoots length (cm) of four local tomato varieties

Media composition	Mean shoot length (cm)				Mean
	Daher-Aljabal	Brieh	Baskanta	Daraa	
R0	0	0b	0c	0c	**0d**
R1	**0.827 a**	0.6 9 ab	0.54 abc	0.4 bc	**0.61 a**
R2	**0.827 a**	0.69 ab	0.54 abc	0.4 bc	**0.61 a**
R2	0.19 abc	0.24 ab	0.47 abc	0 c	**0.22 cd**
R3	0.1 bc	0.27 ab	0 c	0 c	**0.09 cd**
R4	0.47 abc 7	**0. 8 a**	0.2 bc	0.26 bc	**0.43 bc**
R5	0.32 abc	**0.71 a**	0.78 ab	**0.81 a**	**0.66 a**
R6	0 c	0 b	0 c	0 c	**0 d**
R7	0 c	0 b	0 c	0 c	**0 d**
R8	0.75 ab	0.57 a	0.50 abc	0.48 ab	**0.57 ab**
R9	0.54 abc	0.29 a	0.45 abc	0.5 ab	**0.45 ab**
R10	0.52 abc	0.68 a	**0.98 a**	0.44ab	**0.65 a**
R11	0.1abc	0.23 ab	0.1c	0.1 bc	**0.13 cd**
Mean	0.323 A	0.373A	0.342 A	0.249 A	
$LSD_{0.01}$ (medium composition)			**0.1855**		
$LSD_{0.01}$ (line)			**0.1071**		
$LSD_{0.01}$ (line*medium)			**0.371**		

Values in the table are the mean of three replications. Means were separated with Duncan's.
The different letters in column or row indicate a significant difference among treatments ($p \leq 0.01$)

Rooting and acclimatization

Regenerated shoots were excised aseptically from callus (Figure 7e,f) and cultured on multiplication medium containing 1 mg/L BAP to obtain enough shoots, then transferred to rooting medium.

Regenerated shoots (Figure 7g) were successfully rooted at 1mg/L IBA (Figure 7h) and well developed rooted plantlets were washed gently to remove agar (Figure 7i). then successfully acclimated in plastic pots filled with perlite:peatmoss (Figure 7j). All plants (100%) survived after acclimatization in the greenhouse. Plantlets were grown vigorously and no morphological abnormality was observed

One of the most important steps in tissue culture is the hardening of plantlets to free living conditions. This stage involves the transfer of plantlets from aseptic conditions to greenhouse and ultimately to the final location (environment).

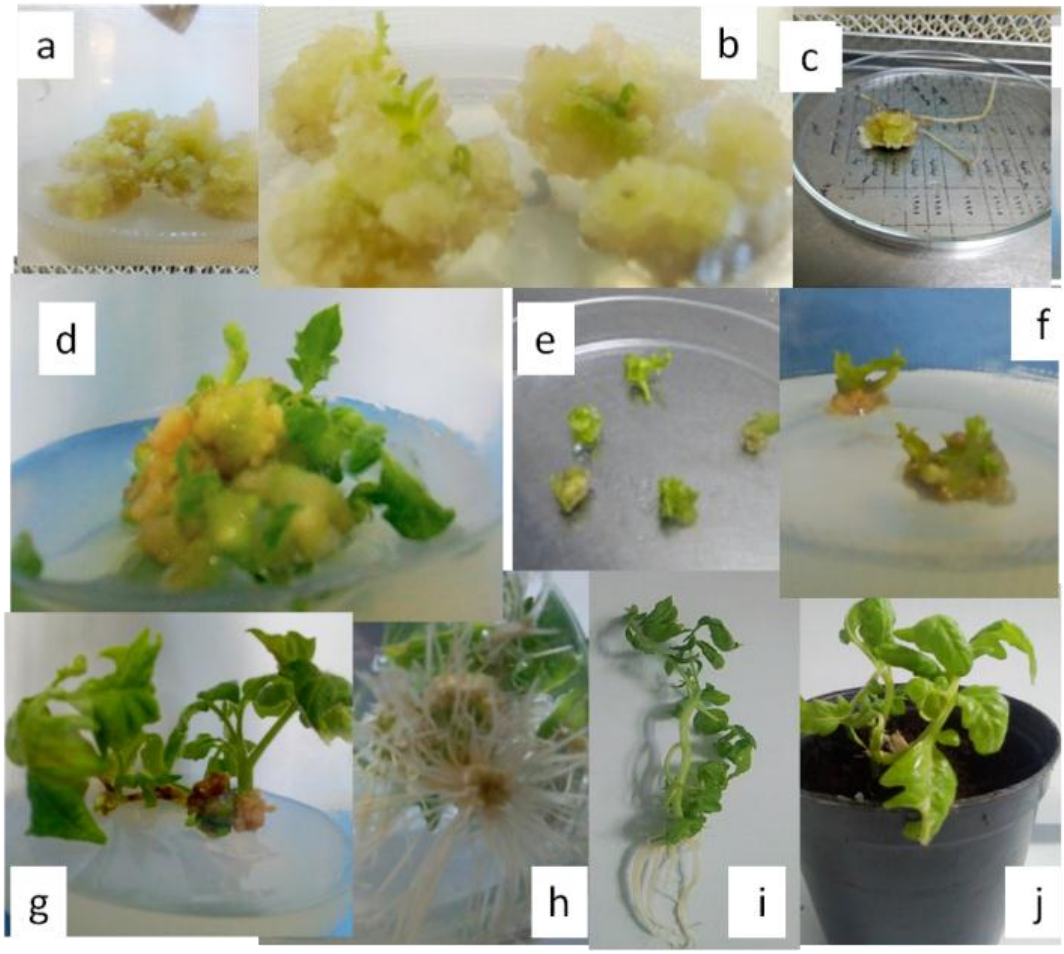

Figure 7: a. Healthy callus. b. shoot formation. c. roots from callus witch excluded. d. shoots formation from root crown explants on R5 media. e. regenerated shoots were cut aseptically to remove callus. f. then cultured on multiplication media. g. growth of regenerated shoots. h. rooting of regenerated shoot. i. rooted plantlet were removed from the media and washed to remove agar. j. acclimatization of rooted plantlets.

Discussion

Choosing the appropriate explants is an important determinant factor in tissue culture responses (Takashina et al., 1998) along with media composition and genotype. In our experiment best callus induction responses were observed for crown explants which were used for the first time in this report, where many previous studies used root explants rather than crowns. Furthermore, when cotyledon used as explants they gave maximum callus induction frequency compared to hypocotyl on the same medium. Our results agree with those obtained by Rashid et al., (2012) and Abdel-Raheem et al., (2007) who found that cotyledon explants were better than hypocotyls explants for callus formation. On contrast, Chaudhry et al., (2007) reported that the best explants for callogenesis were hypocotyls. Additionally, Durrani et al., (2017) found also that hypocotyl explants gave higher callus induction frequency than cotyledon explants depending on the variety. Also our findings disagree with those obtained by Gerszberg et al., (2016) which reported that hypocotyls produced much more callus than cotyledons. In fact differences in tissue culture responses and organogenesis depend on the different potential of explants for internal hormones as mentioned by Jha et al., (2007). Our findings presented 2,4-D as the best effective auxin used for callus induction compared to NAA and IAA, this is due to the reason that (2,4-D) is much more stable and less inactivated during culture processes than the other Auxins (Kareem and Karrar, 2018*)*. Generally, 2,4-D is the most used regulator *in vitro* for callus induction in many plant species (Pal et al., 2007). Our results proved that this fact depends on explants type. 2,4-D failed to induce callus from crown explants but it was the best hormone used to form callus from cotyledons and hypocotyls which is in variety to many of the previous studies which used 2,4-D for callus formation (Jan et al., 2015; Mamun et al., 2004; Baskaran et al., 2006), but disagreed with Durrani et al., 2017 who reported that 2,4-D was not found to be effective for callus induction in tomato Regarding crown explants, the best response in terms of callus induction was in media supplemented with BAP and NAA. Similarly, combination of higher cytokinin with relatively lower auxin was responsible for higher percentage of the callus induction (Hille et al., 1989) and it is expected given that auxins and cytokinins are paramount in promoting both cell division and cell growth, with auxins being necessary for both parameters any cytokinins being crucial in cell division. Callus induction is the product of both cell division and growth (Irene et al., 2019). Furthermore, they showed that cytokinins facilitated the effect of auxins in callus induction (Soheilikhah et al., 2013). *In vitro* morphogenic potential is largely dependent on genotypic differences. The response to callogenesis was found highly dependent on genotype and was notably affected by the reproductive background mode (self-pollination) of the cultivars (Saeed et al., 2019) These results are also confirmed by Lu et al., (1997) who observed that different genotypes differ significantly in callus induction and regeneration.

Nature and texture of callus are changed according to use of auxin and cytokinin with different concentration (Ghasempour et al, 2012). The nodular-green and friable callus was obtained from crown explants which is the best callus texture for efficient regeneration and this in agreement to Saeed et al., (2019) who mentioned that the most suitable treatment is that leading to soft, fleshy green callus that quickly regenerates (Saeed et al., 2019) The characteristics of the callus (weight, color and texture) depended on the genotype of the tomato, the culture conditions and medium composition (Rzepka-Plevneš et al., 2006).

Maximum callus growth on medium containing BAP+NAA might be due to the availability of auxin in optimum concentration for activation of expansion enzyme resulting in loosening of explant cell wall

leading to increase in initial growth of explant. Early callus initiation might be due to the early biochemical conversion of NAA to functional form (Kumar et al., 2017). Our results found that differences in callus growth were highly depending on explants type rather than genotype when crown used as explants where callus size and weight were superior compared to those obtained by cotyledons and hypocotyls. This may be an important factor for enhancing the rate of cell division resulting in more fresh weight of callus (Karim and Kayum, 2007). Maximum callus fresh weight in our study was 2.12 g in Brieh variety when crown used as explants on 2 mg/L BAP+0.2 mg/L NAA while, in previous studies, the maximum callus fresh weight (1.9 g) was obtained by Papry et al., (2016) on media containing 3 mg/L BAP + 0.25 mg/L NAA produced by leaf explants. The highest mean callus fresh weight (0.33 g/explant) was obtained in hypocotyl explants with 2.0mgl-1 2, 4-D and 0.1mgl-1 NAA followed by hypocotyl explant cultured with 1.5mgl-1 2, 4-D. While in our research, callus weight from hypocotyls explants was 0.46g on 1.75mg/L 2,4-D. It is maybe due to the genotype effect. Root explants did not produce callus with used 2, 4-D concentration. Therefore, it can be concluded that hypocotyl is the best explant for all 2,4-D concentrations (Manawadu, et al., 2014). The root crown section where the phloem and xylem crosses, and where most of the fluid translocation of the plant is concentrated in a particularly narrow space. The root crown section where most of the plant fluids stream in a narrow section, may, under certain conditions, be the bottleneck of the plant development while controlling up and down flow of water, nutrients, assimilated products and hormones (Schwarz, 1972), It is the point at which the root and stem of a plant meet and the primary vascular anatomy changes from that of a stem to that of a root (Brown, 2000). In this experiment combination of BAP and IAA found to be the most effective composition for effective shoot regeneration rather than using BAP alone or in a combination of NAA. Our results substantiate previous findings in the Previous work of Rashid, et al., (2012) who reported that for regeneration, MS medium supplemented with IAA (1.0 mg/l) and BAP (2.0 mg/l) was proved to be optimal. Additionally, Shah et al., (2015) found that maximum shoot regeneration was recorded on MS medium containing 3mg/L BAP+0.1 mg/L IAA However, lower IAA concentration was shown to be effective for plant regeneration in our experiment On contrast, Kumar et al., (2017) found that MS medium supplemented with3.0 mg/l NAA+ 1.0 mg/l BAP was most suitable for callus regeneration. This finding could be attributed to the mode of action of BAP on stimulating both cell division and promote axillary shoots in plant tissue culture as reported by George et al., (2008). Significant differences in genotypes responses recorded in this research maybe due to the difference of their uptake, transportation, metabolism and interrelate with endogenously produced cytokinins of explants (Shah et al., 2015). This is in good agreement with previous reports of Ishag, et al., 2009; Irene et al., 2019; Liza et al., 2013).

Conclusions

An efficient protocol for callus induction and regeneration was optimized for some local tomato varieties which have a desirable production characteristics. Traditional ways for callus induction were by using different explants rather than root crown, This paper has proved the high efficiency of root crown explants for both callus induction and shoot regeneration in studied tomato varieties. The results provided further evidence on explants type, PGRs sort and concentrations in media and genotype effect on callus induction and regeneration of tomato. We are currently in the process of *in vitro* selection of a drought tolerant callus of tomato in order to induce them in breeding programs of tomato. These *in vitro* standardized protocols can further be used for raising true to type plants, somaclonal variation and

cell selection studies. To the best of our knowledge, this is the first report on the callus induction and regeneration from root crown in tomato. Traditional ways for callus induction were by using different explants rather than root crown.

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