IN VITRO DIRECT AND INDIRECT PROPAGATION OF BLACKBERRY (RUBUS SP.)

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ABSTRACT

In vitro direct and indirect propagation of blackberry (Rubus sp.) were studied. Blackberry explants are easily oxidized, so the uses of the running tap water for washing about one hour before sterilization allowed avoiding the oxidation. The disinfection process was represented using different concentrations of Clorox (10, 15, 20%) with exposure times (5 and 10 min.), the best treatment being achieved by the concentration of (10% for 5 min.) for shoot tips and (5% for 5min) for leaves disinfectant. A normal growth and development occurred only in the BA of the multiplication, but at Kin or TDZ decreased in the shoots growth was induced. Maximum volume of callus induction and callus % were produced at TDZ or 2mg/l NAA or 2,4-D each alone from leaf explants. Addition of 2.0mg/l TDZ into media increased the callus induction from *in vitro* leaves than *ex vitro* leaves and having the best response. The highest responses of organogenesis obtained from *In-vitro* leaves than *ex vitro* leaves. The increased rooting of shoots appeared when MS reduced to ½ salts strength and produced highest leaves number, plant length (cm) and roots number. While, MS reduced to ¼ salts strength resulted in tallest of roots (2.72 cm). The rooting of plants derived from ½ MS salt strength medium give highest leaves number, plant length, roots number and roots length than other strengths of MS after two months from acclimatization in greenhouses.

Key words: Acclimatization, callus, *in vitro*, multiplication, organogenesis.

INTRODUCTION

Blackberry belongs to the *Rosaceae* family and is a shrubby tree with erect, semi erect or creep grown habit, and most cultivars have thorny stems. Propagation of blackberry by hardwood or stem cuttings has been poor and variable **(Bray *et al*., 2003).** Tip layering propagation requires a sizeable planting for the layering bed, few tips are available per plant. Softwood cuttings root readily, but require considerably more care for successful plant production **(Broomrand Zimmerman, 1978)**. Successful application of these methods of vegetative propagation is limited to certain extent **(Najaf-Abadi and Hamidoghli, 2009).** Berry fruits are an economically important crop in many countries. Interest in berry fruits has recently increased because they are good sources of health -promoting vitamins, anti -oxidants and many nutrients **(Song and Sing, 2004).** Economical important of *in vitro* propagation has been recorded in great number of blackberry cultivars **(Meng*etal.,* 2004)**. Tissue culture is applied to many nursery crops including red raspberries and other Rubus species. Shoot culture, a form of plant tissue culture, is an important technique for uniform mass production (**Mehlenbacher, 2008).** *In vitro* culture becomes important because the increasing demand worldwide for these fruit. In recent years, *in vitro* multiplication becomes very important since the plant material obtained through this technique high quality and safety compared to the traditional method. Furthermore, this methodology will facilitate the multiplication of some plants with desirable agronomic traits that today are only found in small towns and have a great potential for research purposes and for small farms production (**Jadan *et al.,* 2015**). These experiments were

carried out aiming to direct and in direct propagation, rooting and acclimatization of Blackberry.

MATERIALS AND METHODS.

This study was carried out during 2011-2015 at the Laboratory of Germplasm conservation and gene transfer. Stock plant materials were obtained from Genetic Engineering and Biotechnology Research Institute (GEBRI) farm, University of Sadat City Egypt.

Sterilization.

Shoot tips and leaves sterilization

Explants of Blackberry (*Rubus sp.*) were washed thoroughly under running tap water for one hour, then surface sterilized with commercial disinfectant Clorox (5.25 % NaOCl) solution using different concentration, 10, 15, 20 % (v/v) for (5 and 10 minutes with shoot tips) and (5, 10 and 15 minutes with leaves) followed by rinsing three times with sterile distilled water to remove traces of Clorox then 0.1% (m/v) mercuric chloride for 5 minutes followed by rinsing three times with sterile distilled water to remove traces of mercuric chloride under a laminar airflow cabinet then inoculated in free-growth regulators MS salt medium **(Murashige and Skoog, 1962)** supplemented with 3% sucrose and 7g/l agar, pH was adjusted at 5.7. All cultures were kept at a temperature of 25±2 °C under 16 h photoperiod at 3000lux light intensity. Sterilization had been detected as disinfectant of explants.

Multiplication. The shoots resulting after the initiation phase were passed to the multiplication phase. Shoots were multiplied on MS medium supplemented with 0, 0.5, 1, 1.5 and 2 mg/l 6-benzylaminopurine ((BA), (Kin) Kinetin or Thidiazuron (N-phenyl- N^0-1,2,3-thiadiazol-5yl-urea (TDZ) each alone. The cultures were kept in a growth chamber at 25 ± 2ºC, with 16 hours photoperiod and 3000 lux of light intensity. Data were recorded after 6 weeks as shoots number, leaves number and shoots length (cm).

Callus induction.

Effect of Auxins (IBA, NAA and 2,4-D) and cytokinins (BA and TDZ) on callus induction of blackberry.

For callus induction from *in vitro a*nd *ex vitro* leaves were cultured on MS medium supplemented with different concentration of IBA, NAA and 2,4-D (0.0, 0.5, 1.0 and 2.0 mg/l) and combination with 2mg/l BA or TDZ. Data were taken after 6 weeks as explants able to induction of callus% and volume of callus according to the method described by (**Pottino, 1981)** as follows:

Negative results (−) 1 Below average results (+) 2

Good results (+++) 4 Very good result (++++) 5.

Effect of TDZ at different concentrations on callus induction and differentiation

The experiment was carried out to compare the effect of the different concentration of TDZ on the *in vitro and ex vitro* leaves **on** callus induction. Young, fully expanded leaves from upper thirds of *in vitro* propagated shoots were used as initial explants and the same from the (*ex vitro* leaves after sterilization) were cultured on MS medium contained 0.0, 1.0, 1.5, 2.0 and 2.5 mg/l TDZ. Data were recorded after 6 weeks. Callus formation was estimated as explants able to induction of callus% and differentiation of shoots from callus.

Rooting. Shoots were obtained from multiplication used for *in vitro* rooting. Explants were cultured on different strengths of MS medium (full, ¾, ½ and ¼**)** supplemented with 2mg/l indole-3-butyric acid (IBA), 30g/l sucrose**.** Data were recorded after six weeks as a number of roots, root length (cm), number of leaves and plant length (cm).

Acclimatization. Plants excessed from rooting were transferred to plastic pots (6cm) containing a mixture of sterilized peat moss and perlite (1:1) and covered with lids. The plantlets were gradually exposed to normal greenhouse conditions. Survival of plants is recorded as roots number, roots length (cm), leaves number and plant length (cm) after two months.

Statistical analyses. The randomized factorial design was used and data were subjected to analysis of variance. Separation of means among

treatments was determined using LSD test at 5% **(Steel and Torrie, 1980).**

RESULTS AND DISCUSSION

Effect of Clorox at different concentrations and exposure time on disinfection of shoots tips and leaves sterilization.

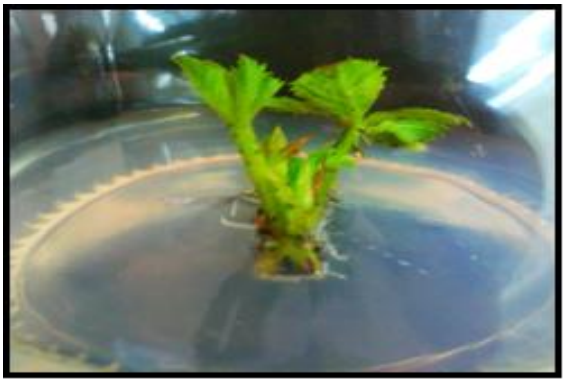
Table (1) and fig. (1) showed that disinfectant concentrations on contamination, indicate that, using colorox at 15% gave the highest survival of disinfectant (14.67), with no significantly different between other treatments. Concerning the main effect of sterilization time, shoot tips remain in disinfectant with colorox at 5min gave high survival of disinfectant appearance (14.33) than 10 min. Data of interaction indicated that, maximum explant were appearance clean (17.67) by using Clorox at 10% for 5 min than other treatments.

The effect of clorox concentrations and exposure times on disinfectant and sterilization of leaves were tested (Table 2) and (Fig. 2). Maximum disinfectant of leaves obtained at a concentration 10% of Clorox than other concentrations of clorox. Concerning the main effect of sterilization time, Blackberry explants are easily oxidized, so the uses of the running tap water for explants washing for one hour before sterilization allowed avoiding the oxidation. The effects of Clorox concentrations and sterilization time on disinfection of shoot tips were studied.

leaves remain in disinfectant at 5min than 10 and 15 min. Data of interaction indicated that, the highest explant were appearance clean by using clorox at 5% for 5 and 10 min (19.00 and 19.33, respectively) than other treatments. These results lead to concede the best treatment for blackberry shoot tips sterilization is using clorox at 5% for 5 and 10 min. considering both contamination appearance and appearance clean. While, using clorox at 15% for 5min best treatment for blackberry leaves disinfectant. From our results showed that contamination and viability of the explants depend on the concentration of Clorox used. **Isac *et al.,* 2014** found that the shoots were surface sterilized in 70% ethanol for 10 minutes followed by 20 min. in sodium hypochlorite (2% available chlorine) were successful and contaminants were infrequent.

Table (1): Effect of Clorox at different concentrations and exposure time on disinfection of shoots tips.

Clorox Concentration %		Disinfection time (min)		
		5	10	Mean
10		10.33	16.67	**13.5**
15		17.67	11.67	**14.67**
20		15.00	11.67	**13.35**
Mean		14.33	13.67	
L S D at 5%	**A**	**2.213**		
	B	**1.807**		
	AxB	**3.129**		



Fig(1):Sterilization and development of shoot tips.

Table (2): Effect of Clorox at different concentrations and exposure time on disinfection of leaves.

Clorox concentration %		Disinfection time (min)			
		5	**10**	**15**	**Means**
10		19.33	19.00	17.33	**18.56**
15		16.33	13.33	11.33	**13.67**
20		15.33	12.33	10.67	**12.78**
Mean		**17.00**	**14.89**	**13.11**	
L S D at 5%	**A**	**0.8844**			
	B	**0.8844**			
	AxB	**1.532**			

Fig (2): Sterilization of leaf

Effect of cytokinins (BA, Kin and TDZ) and concentration on growth and development of blackberry *in vitro*.

I*n vitro* multiplication of plants the use of cytokinins in the multiplication medium is very important, this hormone enhancing cell division and *in vitro* proliferation of shoot tips. Irrespective of the culture media used, BA produced maximum of shoots than Kin or TDZ. Even at low doses of BA, Kin or TDZ medium amount of shoots was induced, while doses such as 1.5 mg/l BA generated high

shoots formation than other treatments as shown in Table (3) and Fig. (3). Using the BA increased the leaves number than Kin or TDZ. Explants cultured on MS medium supplemented with 1.0mg/lBA showed evident faster growth of the leaves number. The leaves were grew less vigorous at 0.5 or 2.0mg/l Kin than other treatments. The multiplication stage was characterized by an approximate six weeks of shoot formation. A normal development occurred only in the BA of the multiplication cycle, but at Kin or TDZ decreased in the shoots growth was induced. Our results are agree with those, **Isac *et al.*, 2014** found that cytokinins like BAP is very effective in promoting direct or indirect shoot initiation of blackberry**. Fira *et al.,* 2014** reported that the optimal cytokinin proved to be BAP at 0.5mg/l concentration for cultivars 'Chester Thornless' and 'Loch Ness', 0.3 mg/l for 'Navaho ' of blackberry. **Debnath 2014** reported that shoot elongation was best in a medium containing 4.4 µM of 6-benzyladenine (BA) for rasberry. **Amalia *et al.,* 2014** found that (BA) resulted the highest shoot initiation rate in three cultivars of rasberry. The increased number of internodes and higher plants were achieved using the concentrations of 2 mg/l BAP **(Jadán *et al*., 2015).**

Table (3): Effect of cytokinins BA, Kin and TDZ on growth and development of blackberry.

Cytokinin type	**Shoots number**					
	Cytokinin con. mg/l					
	0.0	**0. 5**	**1.0**	**1. 5**	**2.00**	**Means**
BA	4.00	7.00	13.60	15.00	14.00	**10.73**
Kin	4.00	3.80	4.40	3.80	4.20	**4.04**
TDZ	4.00	4.40	4.00	3.80	3.40	**3.92**
Means	**4.00**	**5.06**	**7.33**	**7.53**	**7.20**	
L S D at 5% A	**1.041**					
B	**1.345**					
AxB	**2.329**					
Leaves no.						
	0.0	**0. 5**	**1.0**	**1. 5**	**2.00**	**Means**
BA	25.60	21.60	28.00	23.00	22.20	**24.08**
Kin	25.60	21.20	18.00	22.60	15.60	**20.60**
TDZ	25.60	16.40	25.60	18.20	23.00	**21.76**
Means	**25.60**	**19.73**	**23.86**	**21.26**	**20.26**	
L S D at 5% A	**3.113**					
B	**4.019**					
AxB	**6.962**					
Shoot length (cm)						
	0.0	**0. 5**	**1.0**	**1. 5**	**2.00**	**Means**
BA	4.40	3.40	3.18	2.70	2.60	3.26
Kin	4.40	5.06	4.70	5.17	4.43	4.75
TDZ	4.40	3.93	4.00	4.59	4.20	4.22
Means	**4.40**	**4.13**	**3.96**	**4.15**	**3.76**	
L S D at 5% A	**0.4466**					
B	**0.5766**					
AxB	**0.9987**					

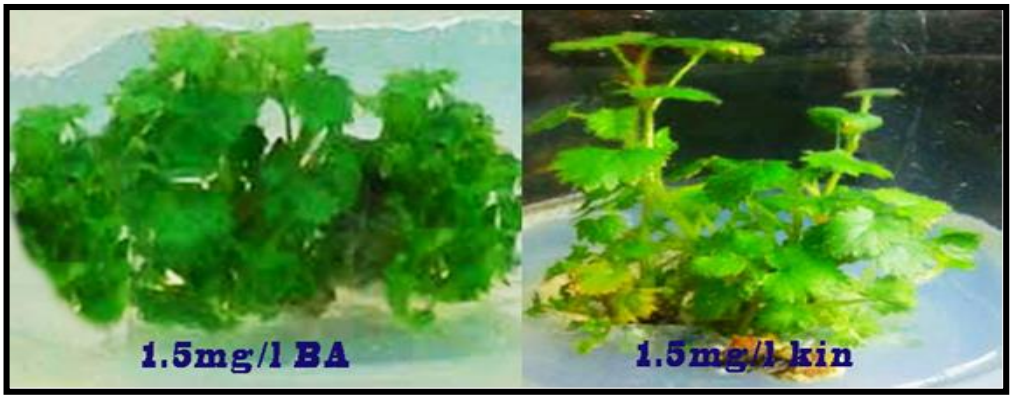


Fig. (3): Development and proliferation of blackberry shoots on MS medium and 1.5mg/l BA.

Effect of Auxins (IBA, NAA and 2,4-D) and cytokinins (BA and TDZ) on callus induction of blackberry.

Callus was initiated mainly in media with (IBA, NAA and 2,4-D) combination with BA or TDZ. Callus initiation from leaves was observed at various concentrations of (IBA, NAA and 2,4-D) at 0.5,1.0 and 2.0mg/L. Optimum concentrations of 2,4-D for callus induction % and volume of callus was observed with 2 mg/L (Fig. 4). The addition of cytokinin, TDZ to the culture media containing different (IBA, NAA and 2,4-D) levels stimulate greatest callus induction % and volume of callus than BA as shown in Table (4). The use of the TDZ alone or 2mg/l NAA or 2,4-D each alone were effective in initiating callus % and volume of callus from leaf explants. TDZ is a high promoted to caulogenesis in the leaves than other treatments in this study. The best callus medium for induction of raspberry cell suspension appeared to be the one supplemented with 2,4,D (1.0 mg/l) **(Dziadczyk, *et al.*, 2013).** The highest callus induction of strawberry (90%) was produced with anther in medium contained 2,4,D (2.0 mg/l) + IAA (1.0 mg/l) + BA(0.4 mg/l) **(Nguyen, *et al.*,2015).**

Table (4): Effect of Auxins concentrations (IBA, IAA and NAA) and (BA or TDZ) on callus formation of blackberry leaves.

Auxins types	Conc. (mg/l)	Callus formation					
		Volume			Percentage		
		BA	TDZ	Means	BA	TDZ	Means
IBA	0	1.25	3.5	2.37	16.25	70	43.12
	0.5	2.5	2.00	2.25	45.00	42.5	43.75
	1.0	1.25	2.25	1.75	12.50	30.25	21.37
	2.0	1.5	2.25	1.87	21.25	38.75	30.00
NAA	0	1.25	3.5	2.37	16.25	70.00	43.12
	0.5	1.75	1.75	1.75	28.75	30.00	29.37
	1.0	2.75	2.5	2.62	54.00	51.25	52.62
	2.0	1.25	3.75	2.12	15.5	59.75	37.62
2,4-D	0	1.25	3.5	2.37	16.25	70.00	42.00
	0.5	1.75	2.25	2.00	27.5	42.5	35.00
	1.0	2	2.25	2.12	31.25	32.5	31.87
	2.0	3.25	3.25	3.25	71. 5	62.5	67.00
Means		1.83	2.64		29.47	50.00	
LSD at 5 %	A	0.8064			21.59		
	B	0.3292			8.814		
	AB	1.140			30.53		

Effect of TDZ concentration on callus induction and differentiation callus from leaves *in-vitro* and *ex-vitro* of blackberry.

The callus induction from *in vitro and ex vitro* leaves of blackberry can be easily achieved by using different concentrations of TDZ. Callus volume and callus % were increased by using TDZ at (2mg/l) than other treatments as shown in Table (5). Maximum volume of callus produced and callud % were obtained from *in vitro* leaves (3.0) than *ex vitro* leaves (1.40). Addition of 1.5 and 2.0mg/l TDZ into media increases the amount of formed calli from *in vitro* leaves than *ex vitro* leaves. Using TDZ did not reduce the callus amount while increased the callus formation (Fig. 5). Such influence of concentrations of TDZ on caulogenesis was also observed in other reporters **(Vescan, *et al.*, 2012)** reported that for callus of blackberry the one produced on T2 (0.2

mg/l TDZ+ 0.1mg/l IBA) and T3(1.0 mg/l TDZ+ 0.1mg/l IBA) media showed the best general condition, these media being recommended for further experiments**. Debnath, 2014** found that leaf of rasberry produced larger callus with increasing levels of TDZ up to 9.0 µM. TDZ is its often-inhibitory effects on shoot elongation. **Yucesan *et al.,* 2015** reported that low concentrations of TDZ have been found to be using for proliferation of Gooseberry, whereas higher concentrations induce callus in woody plants.

Differentiation or (Organogenesis from callus): Table (5) and

Fig. (5) maximum callus differentiation and organogenesis formation (2.70) in MS medium supplemented with TDZ at 2.0mg\l than other concentrations. Also the highest responses of differentiation to produced shoots from callus and differentiation % obtained from *in vitro* leaves (2.12) than *ex vitro* leaves (1.97). TDZ was significantly more effective to inducing adventitious organogenesis from *in vitro* leaves at 2mg/l than other treatments. Adding TDZ into regeneration medium formation of callus mass of light green. The results indicate that the addition of TDZ to the culture medium, acts positively on the regeneration from callus. Results obtained for best concentration of TDZ are similar to those obtained in other studies. **Lazić and Ružić 2007** found that the highest regeneration from callus was obtained on medium supplemented with TDZ alone (41.66%) of blackberry leaves**. Husaini and Abdin 2007** reported that shoot organogenesis is best when leaf explants are cultured on lower concentration of TDZ (9.08µM), whereas somatic embryogenesis is best when leaf explants are cultured on relatively higher concentration of TDZ (18.16µM) of strawberry.

Fig (4); Callus induction from in vitro leavers

Table (5): Effect of TDZ concentration on callus induction and differentiation callus from leaves *in vitro* and *ex vitro* of blackberry.

TDZ conc. (mg)	Callus formation						Differentiation of callus					
	Volume of callus / leaf			Callus %			No. of shoot /callus/ leaf			Differentiation %		
	Ex-vitro	*In-vitro*	Means	*Ex-vitro*	*In-vitro*	Means	*Ex-vitro*	*In-vitro*	Means	*Ex-vitro*	*In-vitro*	Means
Control	1	1	**1.00**	10.00	11.66	**10.83**	0.01	0.01	**0.01**	13.33	16.66	**15.00**
1.0 TDZ	1	3	**2.00**	6.66	58.33	**32.50**	0.46	1.40	**0.93**	17.00	22.00	**19.50**
1.5 TDZ	2	3	**2.50**	20.00	68.33	**44.17**	0.46	2.60	**1.53**	21.00	63.33	**42.16**
2.0 TDZ	2	4	**3.00**	25.00	80.00	**52.50**	0.80	4.60	**2.70**	20.00	86.66	**53.33**
2.5 TDZ	1	4	**2.50**	8.33	83.33	**45.83**	1.20	2.00	**1.60**	17.83	47.16	**32.83**
Means	**1.40**	**3.00**		**13.99**	**60.33**		**1.97**	**2.12**		**17.83**	**47.16**	
LSD at 5 % A	**0.5435**			**5.76**			**0.5621**			**4.66**		
B	**0.8593**			**9.11**			**0.8888**			**6.60**		
AB	**1.215**			**12.90**			**1.257**		**0.5621**	**9.33**		

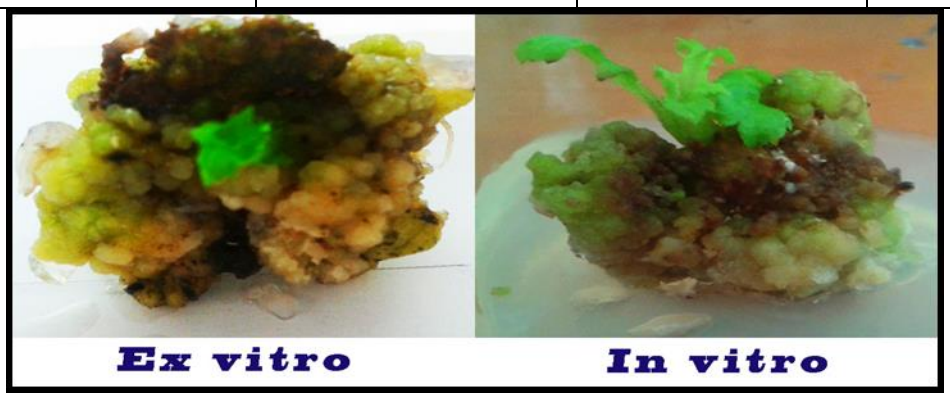


Fig. (5): Development of organogenesis from 1.5 mg/l TDZ on *ex vitro and in vitro* leaves of blackberry *in vitro*.

Effect of MS salt strengths on *in vitro* rooting and adaptation in greenhouse of blackberry.

The success of root formation from Blackberry *(Rubus sp.)* explants lead to study more factors that may affect *in vitro* growth of Blackberry *(Rubus sp.).* Nutrient salt strength is possessed to be important factor affecting *in vitro* growth. In this study MS medium were used with different strengths (1, ¾, ½, ¼ MS). At rooting of shoots indicated that the MS reduced to ½ mineral salts was produced highest, leaves number, plant length (cm) and roots number. While, MS reduced to ¼ mineral salts resulted in tallest of roots (2.722 cm) Table (6) and Fig. (6).

Table (6): Effect of MS salt strengths on *in vitro* rooting of blackberry.

MS salt strengths (g)	Leaves no.	Plant length (cm)	Roots no.	Roots length (cm)
Full	4.230 B	3.646 B	0.9320 C	0.7500 B
¾	6.730 AB	6.074 A	5.160 AB	1.882 AB
½	8.090 A	6.444 A	5.970 A	2.280 AB
¼	4.224 B	4.434 B	3.680 B	2.722 A
LSD at 5%	**2.575**	**1.512**	**2.226**	**1.558**

Fig. (6): Development of rooting on ½ MS medium .

This result agrees with a number of published papers on rooting of blackberry **(Ruzic *et al.,* (2009).** *In vitro* rooting from raspberries reveals that the MS medium with half the minerals and supplemented with auxin is essential for the strengthening of rooting **(Isac, 2009). Karim, *et al*., 2015** reported that 86% of the shoots of strawberry were induced to develop root in ½MS rooting medium without PGR. The rooted of strawberry were gradually acclimatized and transferred to the *ex vitro* condition for field evaluation. 25% MS strength medium without NAA gave the highest percentage of rooted shoots and greatest number of primary and secondary roots (**Ceretta *et al.,* 2000)**.

At the acclimatization, the results proved that the survival of blackberry plantlets at acclimatization stage was highly affected by MS salt strengths that affected on root formation at rooting stage. The produced highest leaves number, plant length, roots number and roots length were obviously obtained with plantlets derived from rooting ½ MS medium than other strengths of MS media Table (7) and Fig.(7). This result was in accordance with those obtained by **Badr-Elden *et al*., 2012** reported that, maximum rooted shoots of watermelon produced on half strength MS medium 0.5mgL^{-1} IBA were transferred to pots and acclimatized in green-house with a survival rate of 80%. **Muhsen *et al*., 2013** found that 0.5g/L^{-1} NPK and half strength of

Hoagland solution increase the acclimatized of Date Palm plantlets 80%, and increasing leave chlorophyll content of the date palm. **Bhandari *et al*., 2013** reported that maximum roots of plantlets when transferred to ¼ MS strength medium having 3% sucrose devoid of PGR for seven days and transferred to polybags containing a mixture of soil: sand: FYM manure (1:1:1) and kept for two weeks in mist-chamber under controlled condition (temp.25°C ± 2°C), humidity 65% ± 5%.

Table (7): Effect of *in vitro* MS salt strengths on growth and development of blackberry in greenhouses after two months.

MS salt strengths (g)	Leaves no.	Plant length (cm)	Root no.	Root length (cm)	Survival %
Full	7.66	5.00	8.600	4.66	63.33
¾	9.33	7.33	9.300	7.00	66.67
½	14.33	10.00	11.100	8.67	98.33
¼	**6.66**	**8.33**	8.400	6.00	76.67
LSD at 5 %	**0.5987**	**1.111**	**1.718**	**1.111**	**7.488**

Fig. (7): Adaptation of blackberry plants derived from *in vitro* ½MS rooting medium in greenhouse

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