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Induction of direct somatic embryogenesis in date palm (*Phoenix dactylifera* L.) cv. Hellawi

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Abstract. The aim of this study was to evaluate the effect of auxins and cytokinins on direct somatic embryogenesis in date palm (*Phoenix dactylifera* L.) cv. Hellawi. Results revealed that the high rate of direct somatic embryos (25%) were obtained from shoot tip explants cultured on MS medium supplemented with naphthalene acetic acid (NAA) 50 mg/L + 2,4-diclorophenoxy acetic acid (2,4-D) 10 mg/L + 6-ϒ-ϒ-(dimethlally amino)- purine (2ip) 3 mg/L, while shoot tips grown on MS medium containing NAA 50 mg/L + 2,4-D 5 mg/L + 2ip 3 mg/L gave less rate (15%) of induction of direct somatic embryo. The highest frequency of somatic embryo germination (88.88%), roots and shoots numbers and length (2.57, 3.87, 3.25 and 3.53 respectively) were obtained on MS medium containing 6-benzyl amino purine (BAP) 4 mg/L, Kinetin (KN) 4 mg/L and NAA 0.1 mg/L (RM4). Shoots were individualized successfully by using temporary immersion bioreactor (TIB) system. Rooted shoots were acclimatized successfully under green house conditions.

Key Words: *In vitro*, somatic embryo, 2,4-D, NAA, BAP, TDZ, organogenesis, temporary immersion bioreactor (TIB).

Introduction. Plant micropropagation has been greatly helped by development of somatic embryogenesis technique, started more than decades ago by Steward et al (1958).

Researchers believed that auxins such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), Picloram, Dicamba, 2,4,5-tricholorophenoxy acetic acid (2,4,5-T) and endogenous hormone metabolism which is affected by genetic, physiological and environmental cues play a key role in somatic embryogenesis in different plant species (Rao 1996; Dodeman et al 1997; Feher 2006). But, other studies reported the importance of cytokinins in inducing and developing somatic embryos (Chen & Chang 2001; Jiménez 2005).

Micropropagation of date palm through somatic embryogenesis has been succeeded in various cultivars (Taha et al 2003; Eshraghi et al 2005; Aslam & Khan 2009). In date palm, there are two pathways in production of somatic embryos. The first pathway is indirect method which is based on the induction of embryogeneic callus, and the second pathway is direct somatic embryogenesis (somatic embryos without visible callus) (Sudhersan et al 1993; Al-Khayri 2005).

The first occurrence of direct somatic embryogenesis in date palm was observed on leaf of *in vitro* plant by Sudhersan et al (1993). Later, Othmani et al (2009) found that direct somatic embryos were formed on the base of young leaf explants when cultured on MS medium enriched with 10mg/L 2,4-D. Sidky & Zaid (2011) observed direct globular somatic embryo when treating shoot tip explants by thidiazuron (TDZ) alone or in combination with 2,4-D. Whereas Sidky & Eldawyati (2012) were able to induced direct somatic embryogenesis from female inflorescences explants of date palm on MS medium containing 2,4-D 5 mg/L, 2ip 0.5 mg/L and Abscisic acid 1.5 mg/L.

Since, the first successful demonstration of induction of direct somatic embryogenesis made, less studies were conducted to examine the effect of auxins, cytokinins, and explants on induce direct somatic embryogenesis. In this respect the aim

of the present work is to study the effect of various plant growth regulators (PGR) to induce direct somatic embryos in date palm shoot tip explants.

Material and Method

Effect of different PGRs on induction of direct somatic embryogenesis. This research project work was achieved in Date Palm Research Centre, Basra University and Date Palm Tissue Culture Lab., Fadak Company, Basra, Iraq during the period 2012-1014. Date palm offshoots (3-5 years old) cv. Hellawi were obtained from Abul-Khseeb Orchard, Basra. Shoot tip (apical; meristem with some adjoining leaf bases) was taken after carefully removing of all leaves from the offshoot. The excised shoot tip (about 1 cm high and 0.8 cm wide) explants were disinfected by soaked it in 0.1% mercuric chloride for 3-5 minutes, explants then rinsed 5 times with sterilized distal water under aseptic conditions to ensure that no traces of mercuric chloride were left. Shoot tips explants were cut it into 4 segments and each one cultured on MS inorganic salt medium (Murashige & Skoog 1962) supplemented with various combinations of PGRs (Table 1).

Beyond the previously mentioned mineral salt and growth regulators, the following chemicals are routinely added to the culture medium, sodium dehydrogen phosphate dihydrate (170 mg/L), calcium nitrate (1.5 g/L), myo-inositol (100 mg/L), adenine sulphate (40 mg/L), nicotinic acid (1 mg/L), glutamine (200 mg/L), Ca-pantothenate (20 mg/L), thimanie-HCl (0.4 mg/L), pyridoxine-HCl (1 mg/L), biotin (1 mg/L), sucrose (30 g/L) and agar (7 g/L).

Table 1

Media supplemented with various PGRs for evaluating somatic embryo induction

Treatment	*2,4-D (mg)*	*IBA (mg)*	*IAA (mg)*	*NAA (mg)*	*NOA (mg)*	*BAP (mg)*	*2ip (mg)*	*KN (mg)*	*TDZ (mg)*	*AC (g)*
1	0	0	0	0	0	0	0	0	0	0
2	5	5	0	5	0	5	0	5	0	1
3	10	5	0	5	0	5	0	5	0	1
4	10	5	0	5	0	5	0	5	0	1
5	20	5	0	5	0	5	0	5	0	1
6	5	5	0	5	0	10	0	10	0	2
7	5	5	0	5	0	20	0	20	0	2
8	5	5	0	5	0	25	0	25	0	2
9	10	10	0	10	0	5	0	5	0	2
10	20	20	0	20	0	5	0	5	0	2
11	10	10	0	10	0	10	0	10	0	2
12	20	20	0	20	0	20	0	20	0	2
13	0	0	0	5	0	25	0	0	0	2
14	0	0	0	5	0	25	0	25	0	2
15	0	0	0	5	5	5	0	5	0	2
16	0	0	0	10	5	5	0	5	0	2
17	0	0	0	20	5	5	0	5	0	2
18	0	0	0	25	5	5	0	5	0	2
19	0	0	0	50	0	5	0	5	0	2
20	5	0	0	50	0	0	3	0	0	2
21	10	0	0	50	0	0	3	0	0	2
22	5	0	0	50	0	0	3	0	0.1	2
23	10	0	0	50	0	0	3	0	0.1	2

2,4-D - (Diclorophenoxy acetic acid), IBA - (Indole-3-butric acid), IAA - (Indole-3-acetic acid), NAA - (Naphthalene acetic acid), NOA - (β-Naphthoxyacetic acid), BAP - (Benzylamino purine), 2ip - (6-ϒ-ϒ-Dimethylally amino purine), KN - (Kinetin), TDZ - (Thidizuron), AC - (Activated charcoal).

The data with respect to number of culture producing direct somatic embryo were recorded after 3-4 months. The responses were recorded on the basis of percentage (%)

of cultures. For each treatment 5 test tubes or jars were inoculated in three replicates. Cultures were incubated in dark at 27±2°C and recultured to the same medium each 8 weeks interval.

Somatic embryo germination. Proembryos were transferred to MS medium supplemented with various combinations of growth regulators (Table 2) for germination and development of somatic embryos. MS basal medium was modified with adenine sulphate (40 mg/L), glutamine (200 mg/L), biotin (1 mg/L), nicotinic acid (1 mg/L), myo-inositol (100 mg/L), thiamine-HCl (0.4 mg/L), Ca- pantothenate (20 mg/L), pyridoxine-HCl (1 mg/L) sodium dihydrogen phosphate dihydrate (170 mg/L), calcium nitrate (1.5 g/L) and sucrose (30 g/L). The PH of all media mentioned above was adjusted to 5.8 prior to agar addition. Media were dispensed either in glass test tube (250 mm × 25 mm, Borosilicate) with 20 mL or in jar (250 mL) at the rate of 50 mL. Test tubes were then plugged with cotton plugs and covered with aluminum foil. Media autoclaving was done at 121°C and 1.1 Kg/cm^3 for 20 minutes.

Table 2

Media supplemented with various PGR for evaluating somatic embryo germination and development

Treatment no.	*IBA (mg)*	*IAA (mg)*	*NAA (mg)*	*NOA (mg)*	*BAP (mg)*	*KN (mg)*	*AC (mg)*
RM1	-	0.1	0.1	-	-	-	100
RM2	1	-	1	1	0.5	0.5	100
RM3	-	-	0.1	-	0.5	0.5	100
RM4	-	-	0.1	-	4	4	2000

In somatic embryo germination and development stage, test tubes or jars were grown in growth chamber at 27±2°C with photoperiod of 16/8 hour day/dark of 10 µmol m^{-2} s^{-1}. Somatic embryo germination was recorded on the base of percentage (%). Shoots and roots (numbers and length) were submitted to analyze using one-way ANOVA. Also, the mean values of treatments were subjected to Duncan Multiple Range Test (DMRT). Significance was determined at $P<0.05$ (Gomez & Gomez 1976) by using the software SPSS 17th Windows operating system.

Shoot clumps obtained from the last step were transferred to TIB system for shoots elongation and individualization with MS liquid medium enriched with naphthalene acetic acid (NAA) 0.5 mg/L, benzyl amino purine (BAP) 0.1 mg/L and Kinetin (KN) 0.1 mg/L at immersion frequency 4 minute every 8 hours for 16 weeks. Separated shoots are lastly subcultured on a plant rooting medium containing MS basal salts, NAA 0.2 mg/L and KN 0.1 mg/L. In this stage the test tubes or jars were grown in growth chamber at 27±2°C with photoperiod 16/8 hour day/dark of 70 µmol m^{-2} s^{-1}. The developed plantlets were treated with Benlate fungicide and planted on plastic pot containing coco-peat and grown under green house condition.

Results and Discussion

Determination of optimum auxins and cytokinins on direct somatic embryo formation. Table 3, shows different response of shoot tip explants to various combinations and concentrations of auxins and cytokinins. Few explants produced direct somatic embryo without intervening callus phase (Figure 1A) in rate 15, 25, 15 and 25% only on MS medium containing NAA 50 mg/L + 2,4-D 5 mg/L + 2ip 3 mg/L, NAA 50 mg/L + 2,4-D 10 mg/L + 2ip 3 mg/L and medium containing NAA 50 mg/L + 2,4-D 5 mg/L + TDZ 0.1 mg/L, and NAA 50 mg/L + 2,4-D 10 mg/L + TDZ 0.1 mg/L respectively after 16 weeks. In contrast no such response was observed on the other treatments. In similar study Sidky & Zaid (2011) were able to induced somatic embryos in date palm (*Phoenix dactylifera* L.) directly from the surface of explants without visible callus on MS medium enriched with TDZ alone or in combination with 2,4-D. In other study, Abd El Bar & El

Dawayati (2014) found that direct embryogenesis was inducing on *in vitro* young leaf when treated with BAP 2 mg/L, whereas the old leaf treated with same concentration of BAP had no response to induce direct somatic embryo. In current study we also recognized the formation of direct somatic embryos from surface of cotyledon part or first leaf of *in vitro* embryoids on medium containing high cytokinin (Figure 2 A & B), this result was in accordance with Sudhersan et al (1993), who observed the same phenomenon on the leaf lamina of *in vitro* plant when transferred from media containing NAA 1 mg/L, 2ip 3mg/L and Kinetin 3mg/L to hormone free medium.

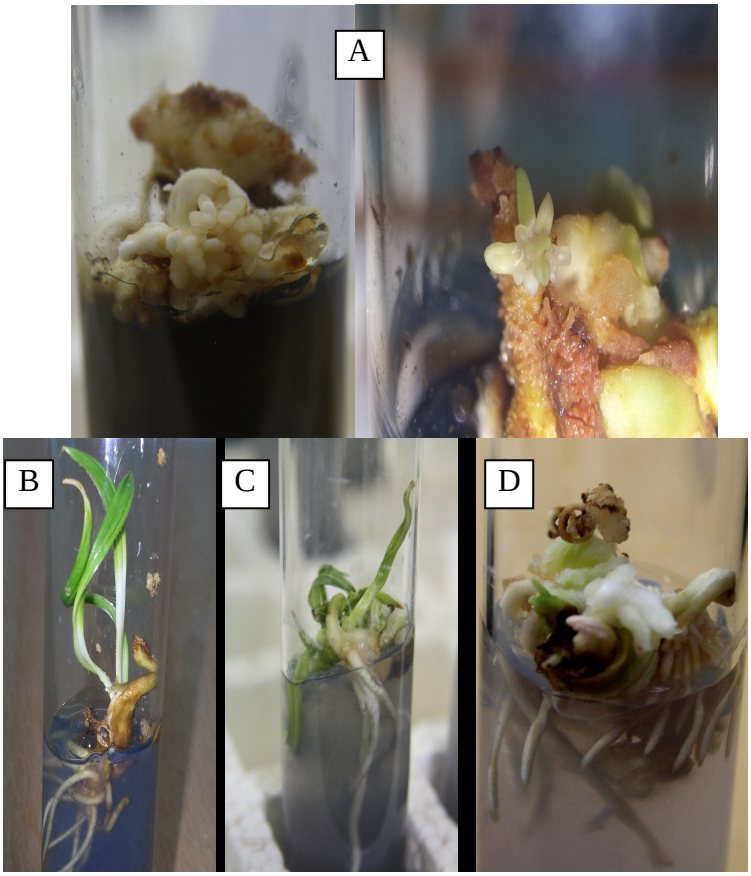

Figure 1. A - Direct somatic embryogenesis from the shoot tip explant; B & C - Germination of somatic embryos on RM3 and RM4 respectively; D - Somatic embryo showing abnormal root formation on RM2.

Many studies mentioned that the totipotency is not inherent property of all plant cells but it can be obtained when the explant is exposed to synthetic auxins like 2,4-D (De Vries et al 1988; Mordhorst et al 1998). Eapen & George (1993) reported that 2,4-D is the most effective auxin for producing high frequency of responding cultures and somatic embryo numbers. Nolan et al (2003) found that the expression gene SERKI is responsible for cell differentiation to somatic embryo, and this gene is induced by auxin and augmented by cytokinin during cell division.

TDZ gained a considerable attention during the past decades, due to its effective role in plant tissue culture system. Originally, TDZ is a synthetic phenylurea-type growth regulator, was considered as a cytokinin inducing responses similar to those caused by natural cytokinins. However, TDZ is able to induce both cytokinin and auxin morphogenic responses (Jones et al 2007; Guo et al 2011). Hutchinson et al (1996) mentioned that TDZ increased the embryogenic response of the explants with normal growth and development, this phenomenon has been observed in many other species. Rougkhla & Jones (1998) suggested that TDZ may induce somatic embryogenesis solely, but it become more effective when applied with an auxin 2,4-D. Victor et al (1999) indicated

that, TDZ effectively induce somatic embryogenesis within a relatively short exposure time, it is possible to speculate a potential dual role for TDZ in the induction of somatic embryogenesis: a cytokinin-like activity that promotes cell division and differentiation and a minor auxin-like activity that seems to be crucial for induction of embryogenic competence. In this study TDZ was not found effective for induction of direct somatic embryogenesis (Table 3), this may be attributed to the low concentration of TDZ used. Thus, the current study is not concurred with that reported by Huetteman & Preece (1993), who mentioned that, low concentration of TDZ more efficient in inducing organogenesis or somatic embryogenesis than other cytokinins particularly in recalcitrant woody species, whereas, high concentrations of TDZ stimulate callus formation. On the contrary, Singh et al (2003) stated that the continuous presence of TDZ at lower concentration (0.05-1 mM) induced shoot formation, while increase the concentration of TDZ (10-20 mM) caused shift in the regeneration pathway to direct somatic embryogenesis. However, the action of TDZ in plant regeneration *in vitro* is not clear.

Table 3

Effect of PGRs on induction of direct somatic embryogenesis

Treatment	*Morphogenetic response from shoot tip explants*					
	Frequency (%) of direct somatic embryos/cultures	*Roots*	*Shoots*	*Callus*	*Vegetative growth*	*Tissue browning*
1	0	0	0	0	0	0
2	0	-	-	+	+	+
3	0	-	-	+	+	+
4	0	-	-	+	+	+
5	0	-	-	+	+	+
6	0	-	-	+	+	+
7	0	-	-	+	+	+
8	0	-	-	+	+	+
9	0	-	-	+	+	+
10	0	-	-	+	+	+
11	0	-	-	+	+	+
12	0	-	-	+	+	+
13	0	-	-	-	+	+
14	0	-	-	-	+	+
15	0	-	-	-	+	+
16	0	-	-	-	+	+
17	0	-	-	-	+	+
18	0	-	-	-	+	+
19	0	+	-	-	-	+
20	15	+	-	-	-	+
21	25	+	-	-	-	+
22	15	+	-	-	-	+
23	25	+	-	-	-	+

Treatment 1 ... 23 – see Table 1.

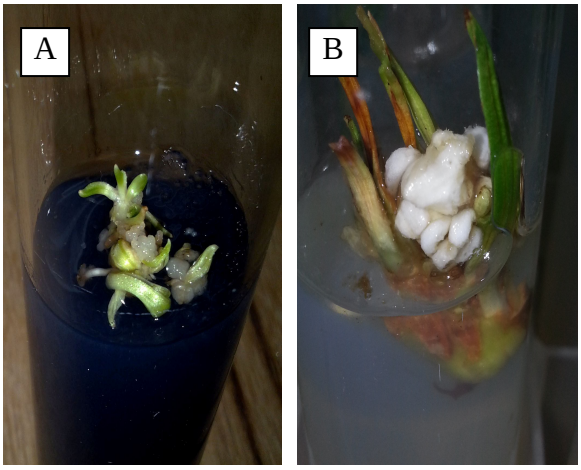

Figure 2. Direct somatic embryogenesis on somatic embryo cotyledons (A) and leaf (B).

Effect of growth regulators on somatic embryos development. The efficiency of growth regulator combinations on somatic embryos development was determined based on the percentage of somatic embryo germination, shoots and roots (numbers and length). High somatic embryos germination was obtained from treatment RM4 (88.88%), other treatments showed reduced germination rate (Table 4 & 5).

Table 4

Responses of somatic embryos on MS medium enriched with different PGR combinations

Treatment	*No. of cultures*	*Embryo germination (%)*	*Morphogenetic response*			
			Roots no. Mean+SD	*Roots length Mean+SD*	*Shoots no. Mean+SD*	*Shoots length Mean+SD*
RM1	16	70.84	0.94 ± 0.68^{a}	1.23 ± 1.14^{a}	2.19 ± 0.83^{a}	1.29 ± 0.86^{a}
RM2	16	75	1.30 ± 0.50^{a}	1.69 ± 1.56^{ab}	2.06 ± 1.34^{a}	1.41 ± 1.29^{a}
RM3	16	66.67	1.81 ± 1.83^{a}	2.78 ± 2.60^{bc}	2.88 ± 1.63^{ab}	1.78 ± 1.03^{a}
RM4	16	88.88	2.57 ± 1.69^{b}	3.87 ± 2.33^{c}	3.25 ± 1.29^{b}	3.53 ± 1.04^{b}

Table 5

Statistics for responses of somatic embryos on MS medium enriched with different PGR combinations

Variances due to	*df*	*Mean square*	*F value*	*P value*
Roots no.	3	10.771	6.206	0.001
Roots length	3	23.526	6.255	0.001
Shoots no.	3	5.104	2.999	0.038
Shoots length	3	17.286	15.152	0.000

Roots and shoots number and length are other factors indicating the effect of PGRs on somatic embryos development and varied depending on type and concentrations of auxin and cytokinin. High root and shoot number and length were observed on the same medium RM4 (2.57, 3.25, 3.87 and 3.53 respectively). However, statistical analysis (ANOVA test and Dunken multiple range) indicated significant differences between RM4 medium and other media (Table 4 & 5), (Figure 1 B & C) and (Figure 3).

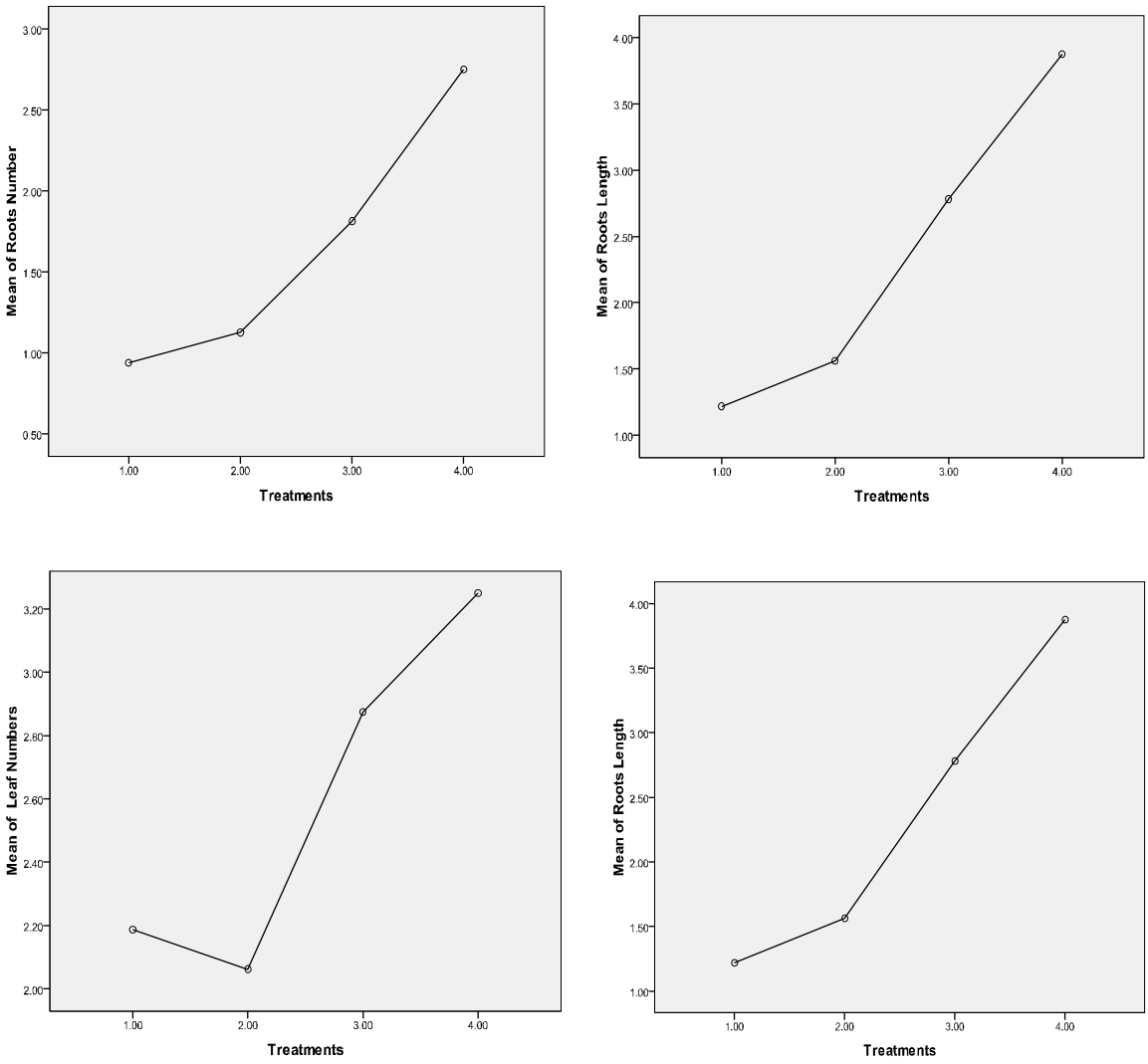

Figure 3. Effect of PGRs on somatic embryo development.

Shoots initiated on medium RM1 and RM2 became brown and degenerated if not transferred to medium RM3 or RM4. In medium containing IBA 1 mg/L (RM2) some embryo cultures shown profuse roots formation (Figure 1D). Shoots obtained were then multiplied on the same medium RM4 under low intensity light. High intensity light caused suppressed the shoot multiplication rate. These results is in line with those obtained by Loutfi & Chlyah (1998), they proposed that best shoot multiplication rate was obtained on medium containing NAA 0.5 mg, 1 or BAP 2 mg/L and 2ip 1 mg/L under low light intensity. Also, Aslam & Khan (2009) got a positive effect of shoot formation on medium fortified with BAP 7.84 µM. Where, Al-Khateeb (2006) demonstrated that in multiplication stage of date palm, low hormone concentrations promoted formation of buds while high concentrations resulted in abnormal growth without any observed of budding or shoot formation. Shoots grown on TIB were successfully individualized after 2 months (Figure 4 A & B). Individual shoots rooted successfully on medium enriched with NAA 0.1 mg/L (Figure 4C). Rooted shoots were successfully transferred to soil (Figure 4D).

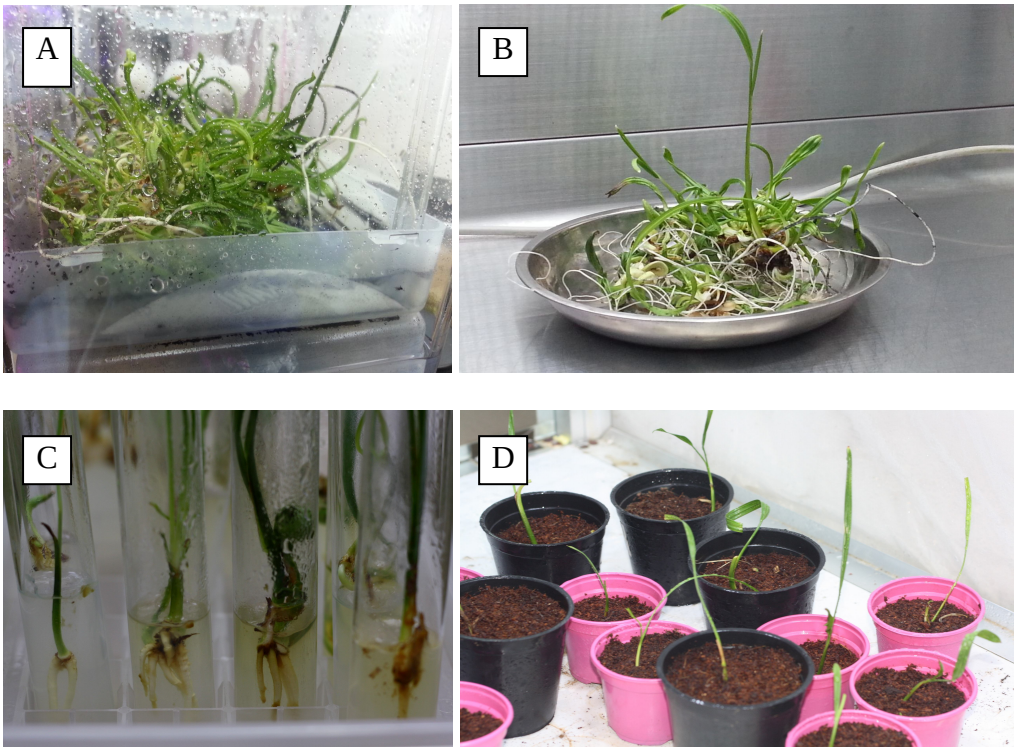

Figure 4. A - Shoots grown in TIB bioreactor for individualization; B- Separated shoots; C - Adventitious roots development on individual shoots grown on rooting medium; D - Plantlet during the acclimatization stage.

Conclusions. Present study has addressed the relation between direct somatic embryogenesis in date palm and various growth regulators. The study highlighted on the auxin 2,4-D as important factor in inducing direct somatic embryogenesis in date palm shoot tip explants (mature tissue). The study also found that high level of cytokinin can induce direct somatic embryogenesis or organogenesis (shoot formation) from younger explants like (*in vitro* somatic embryo cotyledons or first leaf). So, this phenomenon will give a permissive tool for genetic transformation and improvement in date palm (*Phoenix dactylifera* L.).

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