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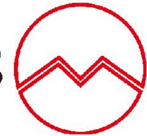

Induction of Direct Somatic Embryogenesis from Mature Female Inflorescences of Date Palm (*Phoenix dactylifera* L.) Hayani cv. *In Vitro*

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Abstract: This study was conducted to examine the induction of direct somatic embryos from mature female inflorescence explants of date palm cv. Hayani. This technique is simple and much faster than the conventional method of using shoot tip explants. To stimulate direct somatic embryos, mature inflorescence explants were cultured on MS medium containing 0.5 mg/l NAA combined with different concentrations of BA and 2ip (0.5, 1.0, 2.0 and 3.0 mg/l), the highest number of direct embryos (9 and 33 embryo/explant) resulted after 8 and 24 weeks in culture, respectively was observed on a ¾ MS medium containing 2.0 BA, 1.0 2ip and 0.5 NAA (mg/l). The formed direct embryos were divided into small clusters and cultured in ¾ MS medium containing two different combinations of plant growth regulators, it is evident from the results that medium supplemented with 3.0 BA, 1.0 2ip and 0.5 NAA (mg/l) was found to be the best in the somatic embryos multiplication (39.0 embryo/explant) with length 0.85 cm. Also, MS basal medium supplemented with 2.0 BA, 0.5 2ip and 0.5 NOA (mg/l) was the most effective combination for maximum embryos proliferation which recorded the highest significant number of embryos (42.0 embryo/explant) with the longest length of embryos (0.88 cm). The success of regeneration pathway depends on the optimum combination between BA and TDZ. MS medium supplemented with 3.0 BA and 0.5 TDZ (mg/l) possessed the best results for both of shoots number (32.0 shoot/explant) and shoots length (6.3 cm). GA_3 added to the medium promoted shoot elongation, shoots elongation were achieved on ¾ MS supplemented with 0.5 mg/l BA, 1.0 mg/l NAA and 0.5 mg/l GA_3. Well-developed shoots were cultured for rooting in ½ MS medium supplemented with 1.0 mg/l NAA. Plantlets with well-developed roots were successfully hardened in the greenhouse. Total soluble sugars, total indoles and phenols were determined. Finally, the inflorescence explants proved to be a promising alternative explant source for micro propagation of date palm Hayani cv.
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Key words: *In vitro*, date palm, mature female inflorescence, direct organogenesis and somatic embryos.

1. Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious, monocotyledon plant species belonging to the family Arecaceae. Date palm is a multipurpose tree having fresh fruit and food processing, medicinal, ornamental and landscape purposes. It is one of the oldest fruit crops mainly cultivated in North Africa and Middle East countries **(Chao and Krueger, 2007)**.

The Date palm can be propagated naturally through seeds or offshoots and by plant tissue culture artificially. Seed propagated palms do not bear true to type fruit due to high levels of heterozygosity and require up to 7 years before fruiting **(Othmani *et al.*, 2009a)**. While, the use of offshoots for commercial propagation usually produce limited number of offshoots during the lifespan of a tree and often a source of spreading diseases in case the offshoots are taken from infected trees thus, *in vitro* micro propagation is the preferred mean to produce vast number of true-to-type plants and free from pests **(Al-Khalifah and Askari, 2011; Jatoi *et al.*, 2015; Al-Khayri and Naik, 2017)**.

Micro propagation of date palm from offshoot tip explants requires a high auxin medium causing many technical problems such as endogenous bacterial contamination, browning, somaclonal variation and long-term duration approximately 3 years **(Tisserat, 1984; Zaid and Tisserat, 1983)**.

Alternatively, female inflorescence tissue represents an abundant and successful source of explants for Arecaceae family species especially date palm **(Loutfi and Chlyah, 1998 and Zayed, 2011)**. However, excision of immature female inflorescence may damage the growing tip of the mother tree due to curious isolation of the spathe. Mature inflorescences are protected by sheaths which prevent infestation by fungi and bacteria and avoid chemical damage from sterilization solution and isolated easily without

harming the vegetative tissue of the mother plant, to produce ideal mature inflorescence explants.

The large flower spikes of the Arecaceae family that reach 30–40 cm in length are an inexpensive source of explants. Moreover, the mature female inflorescence remains healthy longer due to the surrounding protective spathe. Mature flower tissues have zones of meristematic cells at the base of the sepals and petals that have the capability to stimulate vegetative growth **(Kriaa *et al.,* 2012; Zayed and Abdelbar, 2015; Zayed *et al.,* 2016)**.

Inflorescence explants have many advantages of used shoot tip explants for date palm micro propagation such as: no or less bacterial contamination, no browning, short production cycle and possibility to produce rare male and elite female cultivars of date palm in case of no offshoots availability **(Bhaskaran and Smith, 1992; Zaid *et al*., 2007; Abul-Soad and Mahdi, 2010; Jatoi, 2013)**. Subsequently the high probability of inflorescence explants to produce direct **(Abul-Soad *et al*., 2004)** and indirect shoot formation of date palm **(Drira and Al-Sha'ary, 1993; Abul-Soad *et al*., 2005; Sidky *et al*., 2007)** were investigated with variable success.

To avoid the risk of somaclonal variation, the use of minimal concentrations of plant growth regulators in the culture medium is recommended **(Cohen *et al.,* 2004)**. In fact plant growth regulators have a major influence on tissue culture success which involved the regulation of cell division, tissue and organ differentiation **(Jennifer *et al.,* 2010)**. Exogenous auxins and cytokinins are the main plant growth regulators (PGRs) which, regulate organ regeneration and the concentration ratio between these hormones is critical for determine specific organogenesis processes **(F´eher *et al*., 2003)**, while endogenous hormone metabolism play a key role in somatic embryogenesis in different plant species **(Feher, 2006)**. Cytokinin promotes plant growth and has anti-aging potential also protective effects in plants **(Marcu, 2005)**.

Date palm is mainly *in vitro* multiplied through somatic embryogenesis **(Rashid and Quraishi, 1994; Fki *et al*., 2003; Al-Khateeb, 2008a; Othmani *et al*., 2009b)**. Most of the protocols for somatic embryogenesis of date palm made the use of high concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) in media which is known to be associated with genetic instability in regenerated plants. Furthermore, callogenesis is prerequisite for somatic embryogenesis in date palm **(Gueye *et al*., 2009)** which enhances the possibility of producing off type plants **(Saker *et al.*, 2006)**. Conversely, micro propagation through direct organogenesis lacking callus phase, has the advantage of producing highly identical plants in their vegetative characteristics, with the mother plant although there are reports on direct organogenesis of date palm **(Hussain *et al*., 1995; Bekheet and Saker; 1998; Al-Khateeb, 2008b)**. Moreover, the applied technique of floral buds through direct somatic embryogenesis produced very low percentage of somaclonal variations after open field fruiting and in some varieties with no variations at all **(Mirani *et al*., 2019)**. Direct somatic embryogenesis was obtained from inflorescences explants of areca nut which is ideal because it allows the production of plants without a callus phase leading to somaclonal variation and hence useful for efficient genetic transformation **(Radha *et al*., 2006)**.

Accordingly, the main aim of this study was to determine the best combination of plant growth regulators and culture growth conditions for stimulating the initiation and multiplication of somatic embryos directly from mature female inflorescence (without callus formation) and then to proliferate these embryos to shoots and roots.

2. Materials and Methods

This study was conducted in the Central Lab of Date Palm for Researches and Development - Agricultural Research Center, Egypt during the period from 2016 to 2018.

2.1 Plant preparation and sterilization

Mature inflorescences of Hayani cv. (the average spathe length 25 - 43 cm) were collected just before they split open from mother trees grown in Al-Mansoria, Giza and transferred directly to the laboratory, as shown in **Fig. 1**. Spaths were rinsed under running tap water and liquid soup for half an hour. Surface sterilization in laminar air flow started by the outer surface of the protective spathes was cleaned with cotton and sprayed with 70% (v/v) ethanol. The external hairs of each entire spathe were then removed using a flame applied for a few seconds. Then, the spathes were dipped in 50% Clorox (sodium hypochlorite NaOCl at 5.25%) containing 2 drops of between 20 for 20 minutes, as shown in **Fig. 2**. After that the sterilized female spaths were opened and the spiklets were isolated and sterilized by immersion in 0.1% mercuric chloride ($HgCl_2$) solution for 5 minutes. Sterilized spiklets were then rinsed with sterilized distilled water three times; inflorescence explants were divided longitudinally into 2–3 segments for use as explants, each piece containing 4-5 flowers then 2 or 3 spiklets were cultured in each medium jar.

2.2 Establishment medium

The basal cultured medium consists of MS medium and vitamins **(Murashige and Skoog, 1962)** supplemented with (mg/l): 0.5 pyridoxine-HCL; 0.5 nicotinic acid; 1.0 thiamine-HCL; 2.0 glycine; 2.0 biotin; 100 myo-inositol; 200 glutamine; 40 mg/l adenine sulphate; 170 mg/l $NaH_2PO_4.2H_2O$; Ca-

pantothenate (0.2 g/l); 0.5 activated charcoal (AC); 50.0 g/l sucrose and solidified with 6.0 g/l agar. Adjust pH medium at 5.7 ± 0.1 by using KOH or HCl diluted solutions before the addition of agar. The media were dispensed into culture small jars (150 ml) at 40 ml/jar and capped with polypropylene closures. Medium was then autoclaved for 20 minutes at 1.5 kg/cm^2 and 121°C.

Fig. 1. Mature inflorescences of Hayani cv.

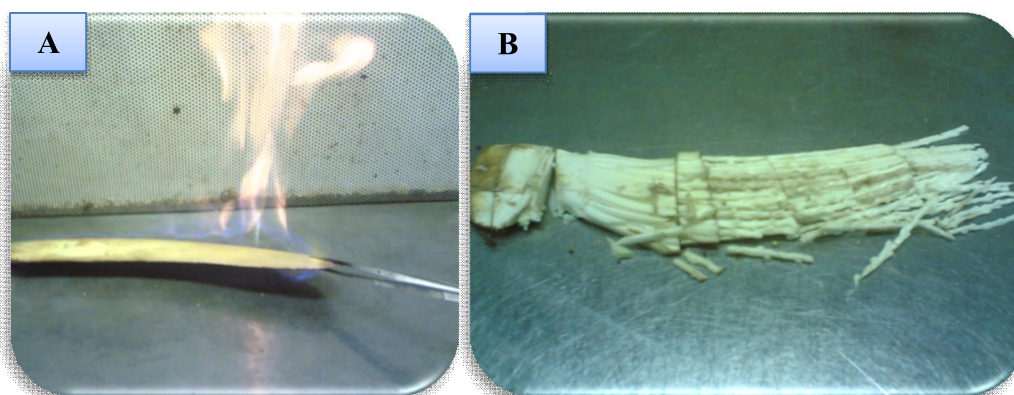

Fig. 2. Preparation of inflorescence explants: A, the external hairs of spathe were removed using a flame; B, the spiklets were isolated and sterilized

2.3 Direct inflorescence proliferation

In initiation stage, the plant growth regulators were added to the basal medium to study the effect of 0.5 mg/l Naphthalene acetic acid (NAA) combined with different concentrations of Benzyl Adenine (BA) and 2-Isopentenyl adenine (2ip) (0.5, 1.0, 2.0 and 3.0 mg/l) on date palm direct organogenesis, the control treatment was free of plant growth regulators. The cultures were incubated in growth room under total darkness to reduce phenolic secretion from the explants at 25 ± 2 °C and the explants were re-cultured to the same fresh media for 3 subcultures, each subculture was 8 weeks. Different morphological observations were recorded at initial culture time and after 8 (first subculture) and 24 weeks (third subculture). Each treatment contained 10 replicates and each replicate contained 2-3 explants. The following data were recorded; swelling, direct organogenesis % and number of direct somatic embryos formation.

2.4 Somatic embryos multiplication

The formed direct embryos were divided into small clusters, each containing not less than 8 embryos after that cultured on ¾ MS medium containing two different combinations of plant growth regulators, in the first combination different concentrations of BA at (3.0, 4.0 and 5.0 mg/l) with added 1.0 2ip and 0.5 NAA (mg/l) were used. In the second combination 2.0

BA, 0.5 2ip (mg/l) and different concentration of 2-naphthoxyacetic acid (NOA) at (0.5, 1.0 and 2.0 mg/l) were used. There were ten replicates for each treatment. Clusters of somatic embryos were continued in previous media for 12 weeks with regular transfer to fresh media with the same supplements every 6 weeks. At the end of the second re-culture; number of embryos, length of embryos (cm) and number of germinated embryos were recorded.

2.5 Embryos regeneration and maturation

The composition of the plant growth regulators is a great importance in the regeneration and germination of somatic embryos. The globular embryos were transferred to ¾ MS medium supplemented with 40 g/l sucrose, 3.0 mg/l BA and different concentrations of Thidiazuron (TDZ) (0.5, 1.0 and 2.0 mg/l). The explants were incubated at 27 ± 1 °C at a light intensity of 1000 lux for 16 h/day. The number of shoots formed, length of shoots (cm) and number of embryos induction were determined after 12 weeks of culturing; each subculture was then conducted every 6 weeks.

2.6 Shoot elongation

The clusters of small shoots (about 2-4 cm length) were used as explant materials. In order to increase the shoot length, shoots were cultured in basal culture medium ¾ MS supplemented with 35 g/l Sucrose; 0.5 mg/l BA, 1.0 mg/l NAA and 0.5 mg/l GA_3 to elongate the obtained shoots **(Al-Mayahi, 2014; Khierallah *et al.*, 2017)**. Explants were cultured for 12 weeks with regular re-culture to fresh culture media with the same components every 6 weeks. All cultures were incubated at 27 ±2 °C under light provided by white fluorescent tubes giving 2000 lux of intensity for 16 hrs/ day.

2.7 *In vitro* shoot rooting

To enhance root formation, the healthy elongated shootlets (6-10 cm) were cultured on ½ MS medium supplemented with 1.0 mg/l NAA, 0.5 g/l activated charcoal and 30 g/l sucrose **(Al-Kaabi *et al*., 2001)**. The cultured jars were incubated at 25 ± 2°C with light intensity 3000 lux for 16h photoperiod in the growth room for 12 weeks and each 6 weeks the shoots were transferred to the same fresh medium.

2.8 Acclimatization

The used acclimatization protocol of date palm was that described by **Taha and Hassan (2014)**. The well *in vitro* rooted and healthy plantlets were carefully removed from jars and the rooted plantlets were washed with running tap water to remove residual medium. Before planting, the plantlets were immersed in fungicide solution (Rizolex-T 50% 2.0 g/l) for 10 minutes. The plants were placed into 250 mm plastic pots containing sterilized soil mixture (peat moss and perlite; 2:1 by volume). Then, the plantlets were covered with polyethylene sheets to create a high relative humidity during initial stages of pre-acclimatization under 28± 2 °C in growth chamber. The polyethylene sheets were gradually opened and removed after eight weeks to allow the plants to develop under microclimate greenhouse conditions. The plants were watered once a week and sprayed with fungicide as needed. The acclimatized plants were transferred to a greenhouse for further hardening at 6 – 8 months before transfer to an open field.

2.9 Biochemical analyses

Two different morphogenesis stages (mature female inflorescences taken after 16 weeks in culture and direct somatic embryos) of date palm cv. Hayani were taken for the determination of chemical contents as (total soluble sugars, total indoles and phenols).

2.9-1 Determination of total soluble sugars

The total soluble sugars were estimated according to the method of **Shales and Schales (1945)**.

2.9-2 Determination of total indoles

The total indoles were determined according to **Larsen *et al.* (1962)**.

2.9-3 Determination of total phenols

Phenols determination was carried out according to **Malik and Singh (1980)**.

2.10 Statistical analysis:

The experimental design was completely randomized with six replicates in each treatment. The best three results from each treatment were statistically analyzed using MSTAT Computer Program. To verify differences among means of various treatments, means were compared using **(Ducan's Multiple Range Test, 1955)**.

3. Results and Discussion

3.1 Direct inflorescence proliferation

Plants can be obtained by micro propagation via three morphogenic pathways, namely: proliferation of buds, in which multiplication occurs by the proliferation and growth of existing meristems in the plant; (direct or indirect adventitious) organogenesis, which lead to the formation of organs from non-meristem tissues; and somatic embryogenesis, which might also occur either directly or indirectly and consists of the regeneration of embryos from somatic cells **(Chawla, 2004)**. Organogenesis has the advantage of using low concentrations of plant growth regulators and avoiding the callus phase. In addition, direct regeneration of vegetative buds minimizes the risk of somaclonal variation among plant regenerates **(Abahmane, 2017)**.

In this stage, plant growth regulators were added to the basal medium to study the effect of 0.5 mg/l NAA combined with different concentrations of BA and 2ip (0.5, 1.0, 2.0 and 3.0 mg/l) on date palm swelling, direct organogenesis % and number of direct

somatic embryos formation. Organogenesis is the process in which the explants make changes that lead to the formation of a unipolar structure (shoot or root primordium) with vascular connections to parent tissues **(Darwesh *et al.*, 2011)**.

Data in **Table (1)** showed that the swelling percentage initiation from inflorescence explants affected significantly by different BA and 2ip concentrations added to culture media. All inflorescence spike explants responded well to the starting nutrient medium, as shown in **Fig. 3**. After 8 weeks in culture, the highest significant swelling percentage achieved was (62.5 %) with 2.0 BA + 1.0 2ip + 0.5 mg/l NAA (mg/l) followed significantly by (41.3 %) with the addition of 3.0 BA, 2.0 2ip and 0.5 NAA (mg/l) to culture media while adding 1.0 BA, 0.5 2ip and 0.5 NAA (mg/l) to culture media and the control treatment caused the lowest swelling percentage (25.8 and 8.2 %, respectively).

After 24 weeks in culture, there was significant difference between different combinations of plant growth regulators and swelling percentage. The treatment 2.0 BA, 1.0 2ip and 0.5 NAA (mg/l) resulted the highest swelling percentage (85.7 %) rather than the treatment 3.0 BA, 2.0 2ip and 0.5 NAA (mg/l) which recorded (60.4 %). However, the control treatment and used 1.0 BA, 0.5 2ip and 0.5 NAA (mg/l) resulted in the lowest swelling percentage (11.6 and 36.3 %, respectively). The obtained results clearly showed that there was a gradual increase in swelling percentage from the first subculture to the third subculture.

There were significant differences obtained among the different combinations of auxins and cytokinins to initiate direct organogenesis from inflorescence explant as shown in **Table (1)**. The explants developed a number of small globular creamy structures after initial swelling (8 weeks through first re-culture) and maturation of initial structures occurred within 16 and 24 weeks in culture through second and third re-cultures. The highest direct organogenesis percentage (33.2 and 78.5 %, respectively) resulted after 8 and 24 weeks from culturing the inflorescence explants on basal medium containing 2.0 BA, 1.0 2ip and 0.5 NAA (mg/l), followed by the treatment 3.0 BA, 2.0 2ip and 0.5 NAA (mg/l) which induce direct organogenesis percentage (20.7 and 45.2 %) after 8 and 24 weeks, respectively. On the other side, the significant lowest percentage of direct organogenesis (12.6 and 22.0 %) were recorded in explants cultured onto media formulations of 1.0 BA, 0.5 2ip and 0.5 NAA (mg/l) after 8 and 24 weeks in culture. However, the control treatment did not have any initiation of organogenesis.

Data in **Fig 3 and 4** indicated that the number of direct somatic embryos which formed directly from inflorescence explants was affected significantly by adding different concentrations of BA and 2ip in culture medium. The treatment 2.0 BA, 1.0 2ip and 0.5 NAA (mg/l) initiated the formation of a significantly highest number of direct embryos (9.0 and 33.0 embryo/explant) after 8 and 24 weeks in culture, respectively. Increasing the concentrations of cytokinins to 3.0 BA and 2.0 2ip (mg/l) combined with 0.5 mg/l NAA reduced the number of direct embryos to (5.0 and 21.0 embryo/explant, respectively) after 8 and 24 weeks in culture. The lowest number of direct embryos (3.0 and 15.0 embryo/explant) was recorded with 1.0 BA, 0.5 2ip and 0.5 NAA (mg/l) after 8 and 24 weeks in culture, respectively.

The findings obtained in the current study induced successfully direct embryos formation using different combinations of auxins and cytokinins from the date palm inflorescence explants. Hence, the basal medium containing 2.0 BA, 1.0 2ip and 0.5 NAA (mg/l) considered an appropriate medium to stimulate direct embryos formation and later multiplication.

Table 1. Effect of different concentrations of BA and 2ip added with 0.5 mg/l NAA on swelling %, direct embryos % and number of direct embryos of date palm inflorescence (Hayani cv.) after 8 and 24 weeks in culture.

Treatment (mg/l)	Swelling %		Direct organogenesis %		Number of direct embryos (embryo/explant)	
	8 weeks	24 weeks	8 weeks	24 weeks	8 weeks	24 weeks
Control	8.2 d	11.6 d	0.0 d	0.0 d	0.0 c	0.0 d
A	25.8 c	36.3 c	12.6 c	22.0 c	3.0 b	15.0 bc
B	62.5 a	85.7 a	33.2 a	78.5 a	9.0 a	33.0 a
C	41.3 b	60.4 b	20.7 b	45.2 b	5.0 b	21.0 b

A: 1.0 BA + 0.5 2ip + 0.5 NAA **B:** 2.0 BA + 1.0 2ip + 0.5 NAA
C: 3.0 BA + 2.0 2ip + 0.5 NAA

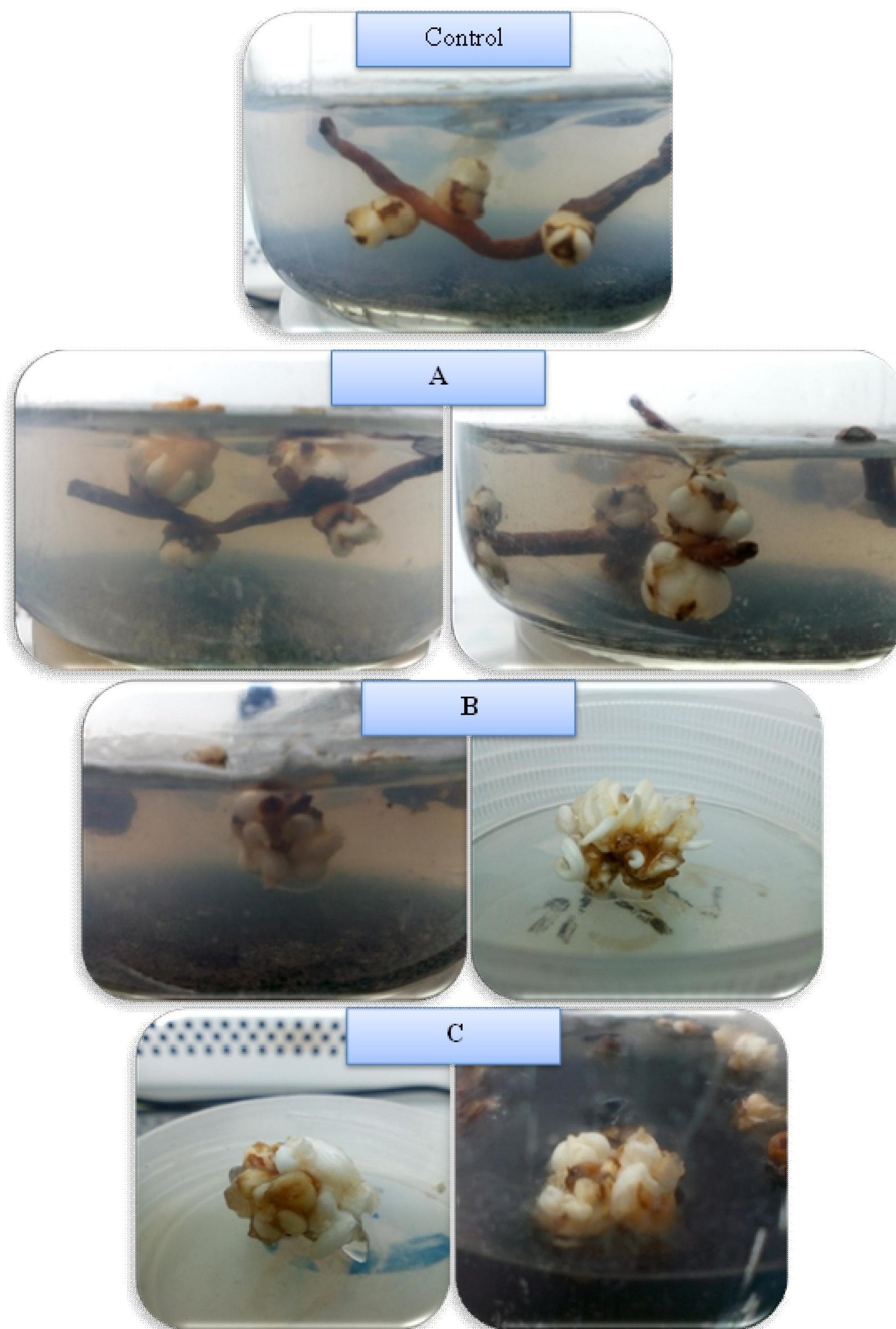

Fig. 3. Direct organogenesis from female inflorescence on medium; A, 1.0 BA + 0.5 2ip + 0.5 NAA; B, 2.0 BA + 1.0 2ip + 0.5 NAA and C, 3.0 BA + 2.0 2ip + 0.5 NAA after 8 weeks in culture

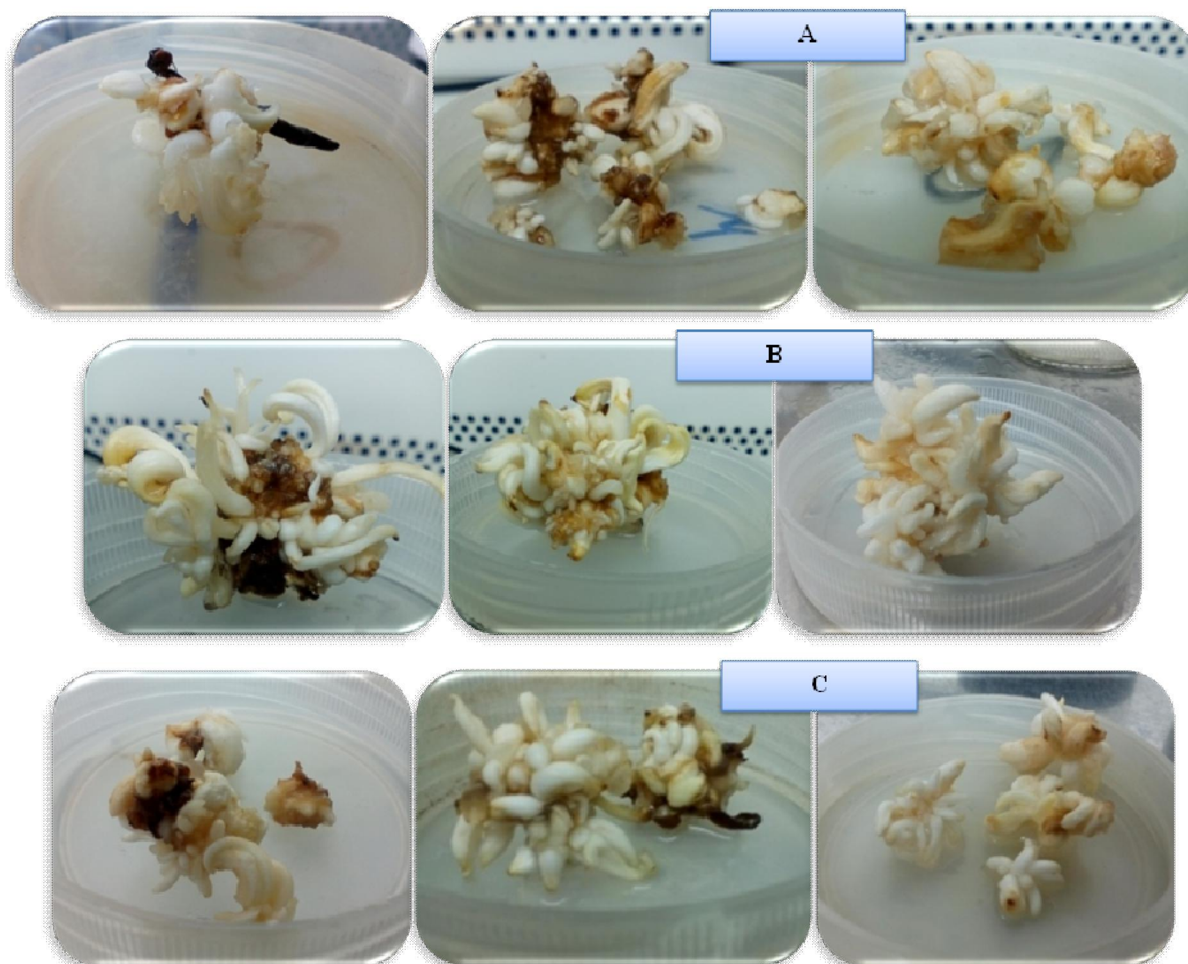

Fig. 4. Direct embryos from female inflorescence on medium; A, 1.0 BA + 0.5 2ip + 0.5 NAA; B, 2.0 BA + 1.0 2ip + 0.5 NAA and C, 3.0 BA + 2.0 2ip + 0.5 NAA after 24 weeks in culture (three re-cultures)

The previous researchers found that date palm organogenesis by direct formation of adventitious buds on the floral explant occurred without the production of an intervening callus. Regeneration through adventitious organogenesis is slower but avoids the risk of somaclonal variation **(Bekheet *et al*., 2001; Taha *et al*., 2001; Al-Khateeb, 2008a)**. Date palm direct organogenesis from floral explants includes the following steps: adventitious bud initiation, shoot bud multiplication, shoot elongation, rooting and plantlet acclimatization **(Abahmane, 2011)**.

Auxin and cytokinin have been found to regulate *in vitro* shoot and root formation in many plant tissues. Different concentrations of auxin and cytokinin were used in initial stage. The selected auxin was NAA because it often appears to be more effective for induction of morphogenetic responses and had greater long-term stability during autoclaving of the medium and during the 8 weeks culture period **(Nissen and Sutter, 1990)**. Cell division seems to be regulated by the joint action of auxin and cytokinins each of which appears to influence phases of cell cycle, where auxin exerts an action on DNA replication, while cytokinin was required only for mitosis **(Pasternak *et al.,* 2000)**. Cytokinin (BA) combined with auxin (NAA) had great effect on the floral reversion process and subsequently on the performance of floral organs to regenerate bulblets, shoots and roots in *in vitro* culture. This was confirmed with the studies of **Ziv and Kipnis (2000); Kumar *et al.* (2006); Werner and Schmulling (2009); Sakakibara (2006) and Bartrina *et al.* (2011)** who demonstrated that these plant growth regulators play an essential role in plant morphogenesis *in vitro*.

Loutfi and Chlyah (1998) used explants from inflorescences of date palm from different female cultivars and they found that, the treatment 0.5 mg/l

NAA, 2 mg/l BA and 1 mg/l 2ip was considered as a better hormone balance for initiation of shoots premordia and the shoot multiplication occurred on the same medium, but plant growth and rooting were only obtained by a reduction in cytokinin concentration or by increasing NAA to 2.0 mg/l in combination with 1.0 mg/l 2ip on 1.0 mg/l BA. **Khierallah and Bader (2007)** found that the type and concentration of cytokinin affected the response percentage as well as the formation of buds. No response was noticed among explants cultured on media free from cytokinin or those supplemented with 0.1 mg/l kinetin. The medium containing 2.0 2ip, 1.0 mg/l BA, 1.0 NAA and 1.0 NOA (mg/l) gave a better result of growth response percentage (80 %) and average bud formation (6.2 bud) in the date palm cultivar Maktoom through direct organogenesis. On the other hand, the superiority of BA over other cytokinins (kinetin and 2ip) for the initiation and development of buds. Direct shoot regeneration for date palm cv. Dhakki was reported by **Khan and Bibi, (2012)** as they cultured shoot tips on MS media supplemented with 1 mg/l NAA, 3 mg/l 2iP and 3 mg/l BA for initiation and developing buds at initiation stage.

These results did not agree with those of **Fayek *et al.*, (2017)** who reported that the swelling percentage of inflorescence explants was affected significantly with different TDZ, BA and NAA concentrations added to culture medium, the highest significant value of swelling (50.2) was produced by culturing inflorescence explants on MS medium supplemented with 0.5 mg/l NAA, followed significantly by (47.2%) swelling of explants cultured on medium supplemented with 1 mg/l BA + 0.5 mg/l NAA, while the lowest significant value of swelling of inflorescence explants (40.6%) was produced on media without TDZ and BA.

3.2 Somatic embryos multiplication

The combination of different concentrations of auxins and cytokinin may have induced a hormonal balance that stimulated the multiplication of the direct somatic embryos. The formed direct embryos were divided into small clusters, each one containing not less than 8 embryos and cultured in ¾ MS medium containing two different combinations of plant growth regulators. Different concentrations of BA at (3.0, 4.0 and 5.0 mg/l) with added 1.0 2ip and 0.5 NAA (mg/l) was used in the first combination, while 2.0 mg/l BA, 0.5 mg/l 2ip and different concentration of NOA at (0.5, 1.0 and 2.0 mg/l) were used in the second combination.

3.2-1 Effect of different concentrations of BA with 1.0 2ip and 0.5 NAA (mg/l)

The treatments 2ip at 1.0 mg/l, NAA at 0.5 mg/l in combination with different concentrations of BA (3.0, 4.0 and 5.0 mg/l) were tried to determine the proliferation of somatic embryos. The combination of 2ip and NAA with different concentrations of BA showed a significant variation in the number of somatic embryos. The treatment of 3.0 BA, 1.0 2ip and 0.5 NAA (mg/l) was found to be the best for the multiplication of somatic embryos resulting in 39.0 embryo/explant with length 0.85 cm followed by the treatment of 4.0 BA, 1.0 2ip and 0.5 NAA (mg/l) resulted in 25.0 embryo/explant with length 0.62 cm. The lowest number of embryos (15.0 embryo/explant) was recorded for 5.0 BA, 1.0 2ip and 0.5 NAA (mg/l) with length 0.54 cm, while the control treatment recorded the worst result, as shown in **Table (2) and Fig. 5**.

Table (2). Effect of different concentrations of BA with 1.0 2ip and 0.5 NAA (mg/l) on stimulated the multiplication of direct somatic embryos of date palm Hayani cv. after 12 weeks in culture.

Treatment	Number of embryos	Length of embryos	Number of germinated embryos
Control	11.0 d	0.40 d	3.0 bc
A	39.0 a	0.85 a	8.0 a
B	25.0 b	0.62 ab	5.0 b
C	15.0 c	0.54 c	3.0 bc

A: 3.0 BA, 1.0 2ip and 0.5 NAA (mg/l)
B: 4.0 BA, 1.0 2ip and 0.5 NAA (mg/l)
C: 5.0 BA, 1.0 2ip and 0.5 NAA (mg/l)

The highest number of germinated embryos (8.0 initiation shoots) was achieved in medium containing 3.0 BA, 1.0 2ip and 0.5 NAA (mg/l), however the other treatments recorded slight germinated embryo (5.0 and 3.0 initiation shoots, respectively).

Several authors have indicated the need to add an auxin and cytokinin combinations to culture medium for a rapid multiplication of embryos of date palm. In fact, exogenous hormones modify the concentrations of the endogenous ones **(Gaspar *et al.*, 2003)**. Other studies also reported that BA and 2ip can multiply date palm tissue *in vitro* **(Bekheet and Saker, 1998; Al-Khateeb *et al*., 2002)**. The induction of an *in vitro* development model, even it is the proliferation of existing buds, the formation of callus or the formation of new buds or roots; depends on the balance between

the concentrations of auxin and cytokinin added to the culture medium **(Ribeiro and Teixeira, 2017)** which shows the optimum combination of plant growth regulators for high multiplication rates. This result is consistent with other results where BA and 2ip have been used in the medium for initiation and multiplication of date palm *in vitro* **(Al-Khateet *et al.*, 2002).** MS medium supplemented with 2 mg/l 2ip and 1 mg/l NAA promotes organogenesis buds in Zaghlool cv. **(Bekheet, 2013)**. The medium containing 2 mg/l 2ip plus 3.0 mg/l BA gave the best results in growth response percentage (80%) and bud formation (4.6 buds) which showed importance of the combination between those two cytokinins in the initiation and development of buds **(Elghayaty *et al.*, 2016)**. The 2ip at a concentration of 5.0 mg/l in combination with 1.5 mg/l 2,4-D and 1.0 mg/l NAA were the most effective combination in stimulating the emergence of lateral buds from inflorescence explants and inflorescence proliferation. Clearly, low auxin concentrations in the presence of cytokinin stimulated the multiplication of adventitious buds **(Al-Najm *et al.*, 2018)**. These results confirmed that, culture media containing low ratios of Auxin/Cytokinins (NAA 0.5 mg/l, BA or 2ip 2.0 mg/l) enhanced floral piece multiplication, mostly petals and usually followed by shoot formation **(Abdelaziz *et al.*, 2019)**.

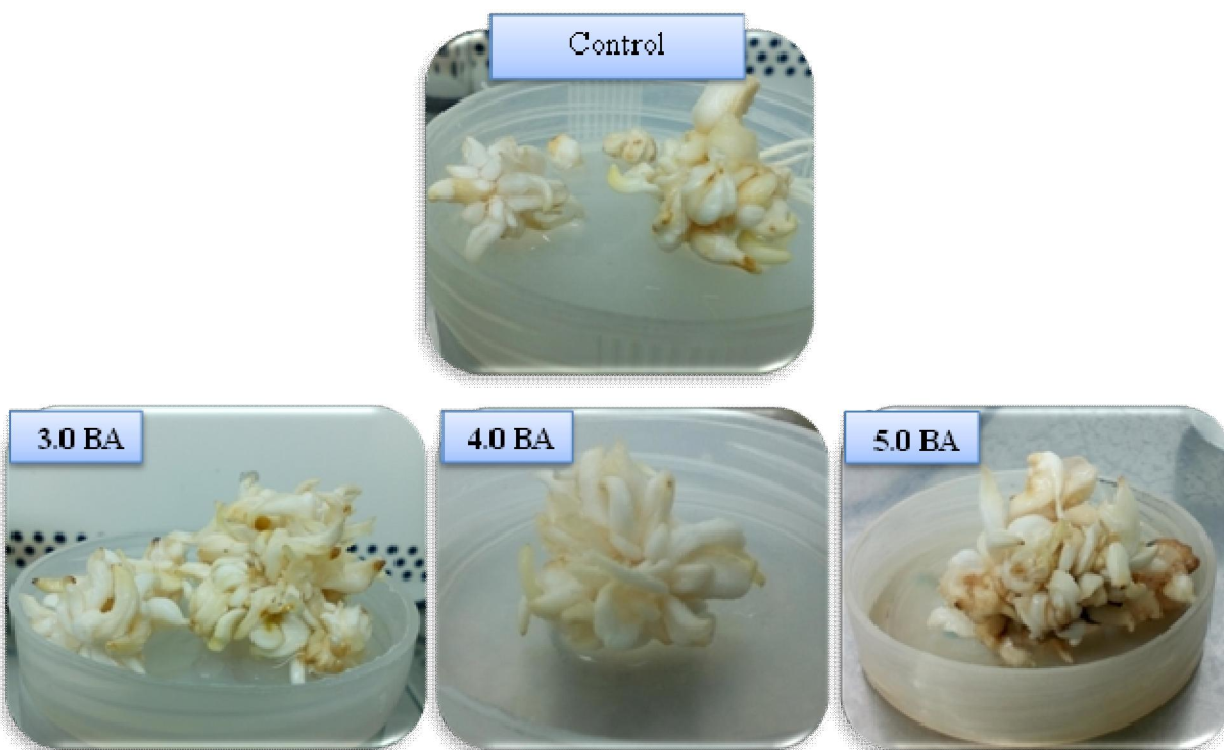

Fig. 5. The combinations of 1.0 2ip and 0.5 NAA (mg/l) with different concentrations of BA (3.0, 4.0 and 5.0 mg/l) showed a significant variation in the number of somatic embryos after 16 weeks in culture

3.2-2 Effect of different concentrations of NOA with 2.0 BA and 0.5 2ip (mg/l)

There was significant difference among different combinations of BA, 2ip and NOA with multiplication of somatic embryos. Data in **Table (3)** showed that different concentrations of NOA (0.5, 1.0 and 2.0 mg/l) and 2.0 mg/l BA with 0.5 mg/l 2ip added to culture media had a significant effect on number of embryos of Hayani cv. It was clearly observed that MS basal medium supplemented with 2.0 BA, 0.5 2ip and 0.5 NOA (mg/l) recorded the highest significant number of embryos (42.0 embryo/explant) with the longest length (0.88 cm), followed by the treatment 2.0 BA, 0.5 2ip and 1.0 NOA (mg/l) which resulted (30.0 embryo/explant) with length (0.78 cm). The lowest number of embryos (18.0 embryo/explant) was achieved in medium 2.0 BA, 0.5 2ip and 2.0 NOA (mg/l) with the lowest length (0.53 cm), as shown in **Fig (6)**.

The combination of BA and 2ip with different concentrations of NOA showed significant differences for the number of germinated embryos. The best result for shoot initiation (11.0 shoots) resulted from the treatment 2.0 BA, 0.5 2ip and 0.5 NOA (mg/l); however, other treatments achieved the lowest number of germinated embryos (5.0 and 4.0 shoots, respectively). It was observed also that small roots appeared in cultures (2.0 roots/explant) with 2.0 BA, 0.5 2ip and 0.5 NOA (mg/l), and (2.0 and 5.0 roots/explant) with others treatments.

These results illustrated the importance of low concentration of NOA (0.5 mg/l) for somatic embryos multiplication with BA at 2.0 and 2ip at 0.5 (mg/l).

Table (3). Effect of different concentrations of NOA with 2.0 BA and 0.5 2ip (mg/l) on multiplication of direct somatic embryos of date palm Hayani cv. after 12 weeks in culture.

Treatment	Number of embryos	Length of embryos	Number of germinated embryos	Roots initiated
Control	11.0 d	0.40 cd	3.0 c	0.0 c
D	42.0 a	0.88 a	11.0 a	2.0 b
E	30.0 b	0.78 ab	5.0 b	2.0 b
F	18.0 c	0.53 c	4.0 b	5.0 a

D: 2.0 BA, 0.5 2ip and 0.5 NOA (mg/l) **E:** 2.0 BA, 0.5 2ip and 1.0 NOA (mg/l)
F: 2.0 BA, 0.5 2ip and 2.0 NOA (mg/l)

Fig. 6. MS basal medium supplemented 2.0 BA, 0.5 2ip and 0.5 NOA (mg/l) recorded the highest significant number of embryos and the best result for germination embryos after 16 weeks in culture

In many species, the addition of auxins and cytokinins to the culture medium is necessary to induce *in vitro* morphogenetic response, for example, somatic embryogenesis, organogenesis or both **(Victor *et al.*, 1999; Fernandez *et al.*, 2000)**. After the differentiation process, three types of cultures were obtained e.g., embryogenic callus, somatic embryos and green shoots. Somatic embryos can be generally divided into two categories either individual somatic embryos or a cluster of embryos (multiple embryos) **(Jatoi *et al.*, 2015)**.

Many researchers have found that different auxin and cytokinin combinations promote embryos multiplication in different date palm cultivars. **Khierallah and Bader (2007)** found that no vegetative buds were regenerated in the medium without cytokinins and they revealed that MS medium supplemented with 1 mg/l NAA, 1 mg/l NOA, 2 mg/l 2iP and 1 mg/l BA stimulated the adventitious buds of date palm Maktoom cv.; and they also mentioned that the date palm cv. Maktoom showed higher shoot-bud multiplication in MS medium with a hormone combination of 1 mg/l NAA, 1 mg/l NOA, 4 mg/l 2iP and 2 mg/l BA. **Hegazy and Aboshama (2010)** reported that date palm axillary bud proliferation of Medjool cv. occurred under dark condition after three subcultures on MS medium supplemented with 2ip (1 mg/l), Kin (1 mg/l), BA (1 mg/l) and NOA (0.5 mg/l). When these shoot buds were transferred under light condition onto the same medium about 55% of them showed direct somatic embryo formation. **Hegazy (2013)** recommended NOA 0.5 mg/l in combination with equal level of BA, Kin and 2iP (1.0 mg/l) for the highest significant values of axillary bud growth (33.33%) cv. Khlass. **Mazri and Meziani (2013)** found that ½ MS medium supplemented with 0.5 mg/l NOA and 0.5 mg/l Kin produced 23.5 shoot buds/explant after 3 months of multiplication in cv.

Najda. **Al-Mayahi (2014)** reported that production of an average of 18.2 buds per culture in cv. Hillawi in MS medium containing 1 mg/l BA and 0.5 mg/l TDZ. **Rad *et al.* (2015)** reported that the presence of cytokinins such as BA and 2iP in culture medium, causing bud regeneration, the highest number of regenerated buds (4.51 buds) from two date palm cultivars, Medjool and Mazafati through direct organogenesis was in the medium containing 1 mg/l 2ip, 1 mg/l BA, 0.5 mg/l NAA and 0.5 mg/l NOA. Bud multiplication of date palm cv. Mejhoul after 3 months of culture on MS medium supplemented with 0.9 µM NOA, 1.1 µM IAA, 1.8 µM kinetin and 1.9 µM 2ip was reported by **Mazri *et al.*, (2018)**. The effects of textures of the multiplication medium were evaluated, the adventitious buds were cultured on ½ MS medium supplemented with 2.4 µM NOA, 2.3 µM Kin and 40 g/l sucrose was the most effective for shoot bud multiplication with 30 shoot buds per explant **(Mazri *et al.*, 2019)**. The plant growth regulators combinations which have been recommended for date palm genotypes (Najda and Mejhoul), have all shown very high multiplication rates, with averages ranging from 26.7 to 28.4 adventitious shoot buds with the four organogenic cultures (A: 0.5 mg/l NOA + 0.5 mg/l Kin; B: 0.2 mg/l NOA + 0.2 mg/l IAA + 0.4 mg/l 2iP + 0.4 mg/l Kin; C: 0.5 mg/l NAA + 0.5 mg/l Kin and D: 0.5 mg/l IBA + 0.6 mg/l BA) **(Saida *et al.*, 2020)**.

3.3 Embryos regeneration and maturation

The clusters of embryos were transferred to ¾ MS medium supplemented with 40 g/l sucrose, 3.0 mg/l BA and different concentrations of Thidiazuron (TDZ) (0.5, 1.0 and 2.0 mg/l). The success of this regeneration pathway depends on the optimum combination between BA and TDZ.

For embryos regeneration and maturation, three different concentrations of TDZ were used (0.5, 1.0 and 2.0 mg/l) with added 3.0 mg/l BA for Hayani date palm cultivar. Highly significant differences were recorded among the three combinations for number of shoots and shoots length (cm). These results showed the importance of using optimum plant growth regulators combinations for success of embryos regeneration, MS medium supplemented with 3.0 BA and 0.5 TDZ (mg/l) possessed the best results for both of number of shoots (32.0 shoot/explant) and shoots length (6.3 cm), followed significantly by the number of shoots formed from embryos (16.0 shoot/explant) with length (6.8 cm) cultured on medium supplemented with 3.0 BA and 1.0 TDZ (mg/l). The embryos cultured on medium supplemented with 3.0 BA and 2.0 TDZ (mg/l) showed significant reducing in the number of shoots (10.0 shoot/explant) with the lowest length (2.3 cm), as shown in **Table (4) and Fig. 7**.

There were significant differences among different concentrations of TDZ with BA and the number of embryos formation. The highest number of embryos (22.0 embryo/explant) was resulted with treatment 3.0 BA and 1.0 TDZ (mg/l), rather than the others treatments.

Table 4. Effect of ¾ MS medium supplemented with combination between 3.0 mg/l BA and different concentrations of Thidiazuron (TDZ) on embryos regeneration and maturation of date palm Hayani cv. after 12 weeks in culture.

Treatments	Number of shoots	Length of shoots (cm)	Number of embryos
Control	9.0 c	1.2 d	5.0 d
A	32.0 a	6.3 ab	10.0 b
B	16.0 b	6.8 a	22.0 a
C	10.0 c	2.3 c	8.0 c

A: 3.0 BA + 0.5 TDZ **B:** 3.0 BA + 1.0 TDZ **C:** 3.0 BA + 2.0 TDZ

Many researchers have found that different concentrations of cytokinins promote embryos regeneration and maturation in different date palm cultivars, although cytokinins are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants **(Madhulatha *et al.*, 2004)**. TDZ is a substituted phenyl urea has been used for rapid plant regeneration of different plant species because it has a cytokinin-like activity **(Malik and Saxena, 1992)**. It works to balance or modify the internal levels of auxins and cytokinins within cells, which it is reflected on the increase in the number of lateral buds and their weight as a result of the increase of lateral growth. TDZ also reduces the disruption of chlorophyll and protein and stimulation the enzymes of photosynthesis, thus increasing the number of buds and shoots length **(Abbasi *et al.*, 2011; Thomas, 2003; Casanova *et al.*, 2004; Al-Mayahi, 2014)**. TDZ was effective to induce *in vitro* morphogenesis in several plants, such as shoot regeneration, proliferation and direct somatic embryogenesis **(Chen and Chang, 2002).** The low concentrations of TDZ induced organogenesis while the high concentrations

shifted the response to somatic embryogenesis **(Chhabra *et al.*, 2008)**. These results are in accordance with the observations of **Kahia *et al.* (2016)** who reported that, cytokinins strongly enhanced induction and regeneration of somatic embryos from leaves derived from *in vitro* germinated seedlings of a *Coffea arabica* especially TDZ. **Al-Mayahi (2014)** demonstrated that the presence of cytokinin is critical for buds induction and differentiation from explants of date palm. The multiplication medium supplemented with 0.5 mg/l TDZ recorded the best response (66.67%), with the highest number of shoots (18.2 shoot) after 24 weeks from culturing. **Al-Mayahi (2015)** mentioned that several concentrations of TDZ (0.0, 0.5 and 1.0 mg/l) were tested in order to investigate their effect on the growth of adventitious buds and their multiplication of date palm cv. Barhee grown *in vitro*. The buds were divided from two - three buds and re-culture in MS media with addition 0.5 mg/l NAA, 0.5 mg/l BA, 0.5 mg/l kin and 0.5 g/l activated charcoal. The adventitious buds derived from different date palm cultivars then exhibited different multiplication ratios possibly because of genetic factors. These findings confirmed the significance of cytokinins and their influence on the initiation of shoot differentiation and the frequency of shoot proliferation **(Al-Najm *et al.*, 2018)**. The treatment with 0.5 mg/l TDZ gave a high number of shoot growths of date palm cv. Barhee reached 37 shoot growth followed by the treatments 1.0 and 0 mg/l **(Al-Asadi *et al.*, 2019)**.

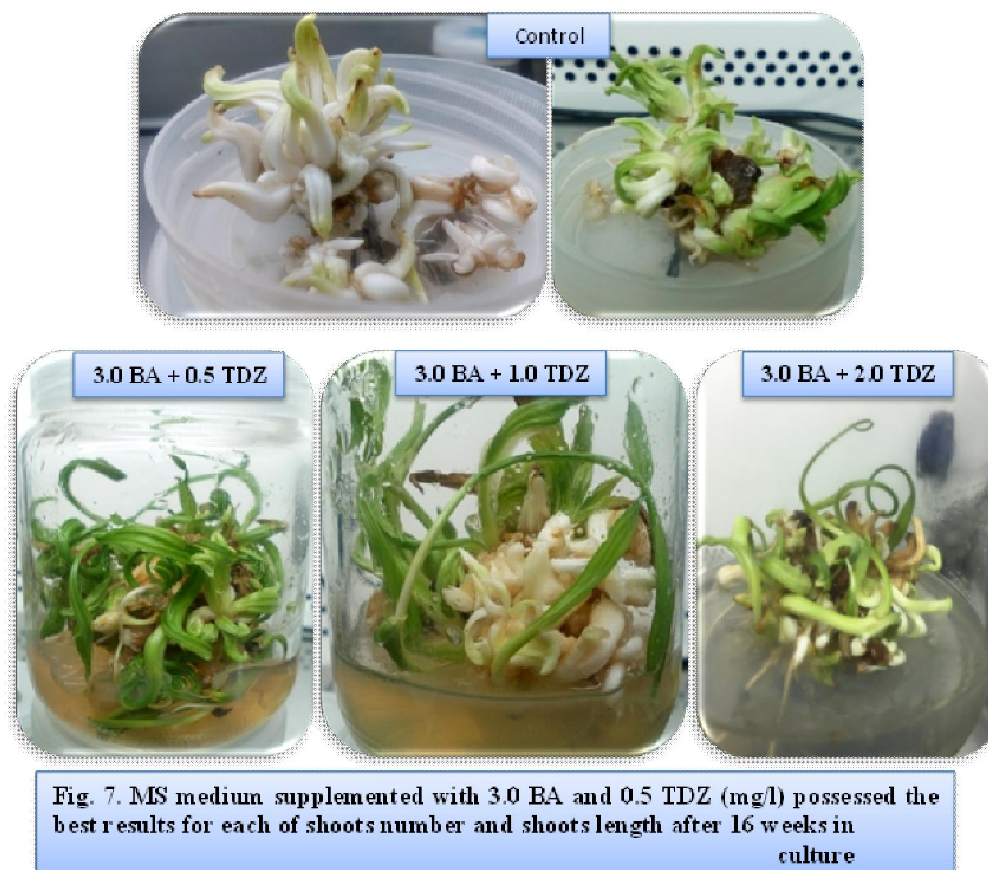

Fig. 7. MS medium supplemented with 3.0 BA and 0.5 TDZ (mg/l) possessed the best results for each of shoots number and shoots length after 16 weeks in culture

These results were not in agreement with **Al-Mayahi (2014)** who reported the production of an average of 18.2 buds per culture in cv. Hillawi, in the MS medium containing 1.0 mg/l BA and 0.5 mg/l TDZ. Interaction revealed that 2.0 mg/l IBA combined with 1.0 mg/l TDZ produced the highest significant percentage of direct shoot buds formation of date palm compared with other combinations **(Hassan *et al.*, 2017)**.

3.4 Shoots elongation

The color of pre-shoots changed from whitish to green due to light after two weeks. Shoots elongation were achieved on basal culture medium ¾ MS supplemented with 35 g/l Sucrose; 0.5 mg/l BA, 1.0 mg/l NAA and 0.5 mg/l GA_3 **(Al-Mayahi, 2014; Khierallah *et al.*, 2017)**. After 12 weeks of culture, the average length of shoots was 12.9 cm, as shown in **Fig. 8**.

Fig. 8. Shoots elongation were achieved on ¾ MS supplemented with 0.5 mg/l BA, 1.0 mg/l NAA and 0.5 mg/l GA_3 after 16 weeks in culture

The results of the previous studies supported the general statement that plant growth regulators promote *in vitro* shoot elongation. Auxins and cytokinins are the most important classes of plant growth regulators **(Machakova *et al.*, 2008)**. Accordingly, their combinations were successfully used to stimulate shoot elongation in many plant species by promoting nuclear division; in addition they promote adventitious root formation **(Perrot-Rechenmann, 2010)**. Also, cytokinins are essential for plant cell division and very effective in promoting shoot bud formation and proliferation **(Van Staden *et al.*, 2008)**. GA_3 is a plant growth regulator that plays various roles, when added to the medium it may promote or inhibit shoot and root formation depending on the species **(Moshkov *et al.*, 2008)**. The gibberellins cause elongation by encouraging sub-apical meristem cell proliferation, elongation and help dissolve polysaccharides into simple sugars that benefit the plant tissue. These findings were consistent with the earlier reports of **Hamid (2001) and Zaid and De Wet (2005)**. In the cv. Najda, shoot elongation was faster in the medium supplemented with hormones when compared to hormone free medium, which also adds high frequency of root formation, the hormone free medium showed wider and green leaves with optimum survival rates **(Mazri and Meziani, 2013)**. The average length of shoots cultured on MS medium containing 1.0 mg/l GA_3 caused maximum values of shoot length (4.5 cm) compared with other treatments **(Elghayaty *et al.*, 2016)**. GA_3 positively affected the elongation of shoots produced in the multiplication stage. Apart from good elongation and strong growth, the average cultivar shoot length was 7.64 cm under 0.5 mg/l GA_3, which was significantly better than other treatments. Shoots length increased with increasing GA_3 concentration in the medium; however, some malformations were noticed at 1.0 mg/l, the average length of shoots was 7.4 cm, but they were thin and difficult to root and transplant in greenhouse **(Al-Najm *et al.*, 2018)**.

3.5 *In vitro* shoot rooting

NAA had a positive effect on the rooting of shoots produced in the elongation stage. The healthy elongated shootlets (6-10 cm) were cultured on ½ MS medium supplemented with 1.0 mg/l NAA, 0.5 g/l activated charcoal and 30 g/l sucrose **(Al-Kaabi *et al.*, 2001).** Therefore, the concentration of NAA at 1.0 mg/l was considered a better treatment with an average 7.0 roots with length 5.2 cm, as shown in **Fig. 9**.

In this context, the role of NAA as an effective auxin in root formation has been established in date palm micro propagation by several researchers. Root formation is a critical stage in date palm micro propagation, as it regulates the subsequent success of production of free-living date palm plants **(Shaheen, 1990)**.

Moreover, the continuous formation of lateral roots is a vital part of establishing a root system and enables plants to react with developmental plasticity to changing soil conditions. Auxins are known to play an active role in root initiation and formation **(IPGSA, 1998)**. The average root length decreased with increased auxin concentrations **(El-Hammady, 1999)**. While, **Al –Kaabi *et al.* (2001)** found that addition of NAA at 1.0 and 2.0 mg/l induced root on the *in vitro* grown date palm. **Khierallah and Bader (2007)** reported that the addition of 0.5 mg/l NAA gave the best results for rooting (average root length 5.4 cm) and that increasing the concentration of auxin did not result in shorter roots. **Jatoi (2013)** reported that rooting was achieved using quarter strength MS medium containing 0.1 mg/l NAA without activated charcoal. **Bekheet (2013)** tested the effect of MS media supplemented with different auxins like IBA,

NAA and IAA at 0.0 and 1.0 mg /l on rooting of date palm and he found that no root formation was observed on MS hormone-free medium. Using NAA was found to be superior over the IBA or NAA for *in vitro* roots formation of date palm cv. Zaghlool. Addition 1.0 mg/l NAA gave the maximum percentage of root formation (85 %) with the highest root number and length. With this treatment, white and strong roots began to appear within three weeks. After five weeks of culturing, vigorous and healthy rooting system was induced. **Meziani *et al.* (2015)** reported cv. Mejhoul shoots grew with average of 13.4 cm and an average of 4.6 roots per shoot with wide and green leaves from 3 months old hormone-free half MS medium. **Abdelaziz *et al.* (2019)** mentioned that highly significant difference between two rooting media was recorded for number of roots/plant and root length (cm). In basal medium supplemented with IBA 2.0 mg/l + BA 2.0 mg/l gave higher response than medium with NAA 2.0 mg/l + BA 2.0 mg/l.

Fig. 9. Rooted shootlets on NAA at 1.0 mg/l after 16 weeks in culture

3.6 Acclimatization

Acclimatization of plantlets is the final step of micro propagation. It was successfully achieved with a high survival rate of 70%, as shown in **Fig. 10**.

Fig. 10. Acclimatization of plantlets

During *in vitro* culture, the closed bottles limit the inflow and outflow of gases and plantlets grow in aseptic conditions under high humidity, low light, warm environment and controlled nutrient. These conditions together with the use of growth regulators and the lower level of water potential result in abnormal plantlets **(Kozai 1991; Kozai and Smith 1995)**. Therefore, during the transferring of plantlets to *in vivo* conditions in the growth chamber or in the field where there is difficult condition including higher light intensity, low relative humidity and higher water potential in the substrate, the plantlets have to correct these abnormality **(Pospíšilová *et al.*, 1999)**.

The pre-acclimatization is an important step to complete micro propagation process. The plantlets grown in lab under optimum conditions (moisture, salts, sucrose and water) were lack cuticle layer in leaves with high transpiration rate. They observed that the presence of plant growth regulator in MS medium increased the cuticle formation in leaves and root thickness and decreased transpiration rate that made a balance between transpiration and salts uptake from nutrient media. They established pre-acclimatization stage by gradual removing caps of culture vessels that resulted in high survival rates after five weeks of transplanting the plantlets **(Gabr and Abd-Alla, 2010)**.

Different survival rates were reported in the literature, depending on the cultivar and the method of acclimatization. In date palm cv. Boufeggous, a survival rate of 60% was reported by **Othmani *et al.* (2009b)** while **Al-Khayri (2010)** observed 72-84% survival rate in cvs. Khasab and Nabout Saif plantlets after *ex vitro* transfer. **Kurup *et al.* (2014)** found

nearly 60% survival rate of cv. Kheneizi when transferred to pots with a peat/vermiculite mixture of 2:1. **Meziani *et al.* (2015)** reported after 3 months of observation in the greenhouse the cv. Mejhoul plantlets showed 88% survival rate. In cv. Najda, a survival rate of 80% was reported by **Mazri *et al.*, (2017)**. A high rate of acclimatization is a good indicator of a successful micro propagation protocol.

The nature of the substrate plays an important role in the success of acclimatization. **Tisserat (1984)** indicated that high survival rate was obtained when date palm plantlets with 2-3 foliar leaves and of shoot length greater than 10 cm (with a well-developed adventitious root system) were transplanted in pots containing a mixture of peat moss and vermiculite. According to **Hegazy (2008)** the advantages of this mixture are the high levels of organic matter in the compost and the high-water retention and aeration attributes of the vermiculite. **Hegazy and Aboshama (2010)** tested the survival of the cultivar Medjool on four different substrates (1:1 compost: sand and peat, perlite or vermiculite). The best survival rate (80 %) was on the compost + perlite substrate. **Mazri and Meziani (2013)** achieved good survival frequency in cv. Najda when a peat-gravel mixture was used as the substrate.

The optimization of the acclimatization process is the most important stage in the date palm micro propagation to complete with a successful protocol. Factors affecting the successful production of free-living date palm, including length of plantlets, strength of root system, humidity conditions, number of leaves and composition of the soil have been reviewed. Successful adaptation of *in vitro* plantlets of date palm cv. Zaghlool was obtained by transplanting well rooted plantlets into pots contained equal volumes of peat moss and vermiculite under high humidity conditions. The high survival may be due to the healthy, well developed root system and the composition of transplanting medium **(Bekheet, 2013)**. The cv. Boufeggous shoots from semi-solid medium, found the highest survival rate up to 92.5% whereas the shoots from stable liquid media culture showed a survival rate of 50% after 3 months in the greenhouse **(Mazri, 2015)**. The plantlets were hardened in a sterilized mixture of peat moss and perlite (1:1) inside a microclimate room. Increasing light intensity enhanced the plantlet photosynthesis and promoted the change from heterotrophic to autotrophic status. The gradual lifting of the plastic covers in the microclimate culture room helped to harden the plantlets and a survival rate of about 80% was achieved. Thus, the evaluated procedures were practically appropriate and effective for micro propagating date palm **(Al-Najm *et al.*, 2018)**. Acclimatization was successfully achieved with a 70% survival rate after transferring the plantlets of date palm cv. Aziza Bouzid to the greenhouse **(Saida *et al.*, 2020)**.

3.7 Biochemical analyses

Two different morphogenesis stages (mature female inflorescences taken after the 16 weeks in culture and direct somatic embryos) of date palm cv. Hayani were taken for the determination of chemical contents as (total soluble sugars, total phenols and indoles).

Table 5. Total sugars, indoles and phenols content in the mature female inflorescence and somatic embryos of date palm Hayani cv. *in vitro*

Morphogenesis stages	Total soluble sugars (mg/g FW)	Indoles (mg/g FW)	Phenols (mg/g FW)
Mature female inflorescence	20.3	1.2	0.6
Somatic embryos	12.8	1.0	0.52

Results in **Table (5)** indicates the content of total soluble sugars in different morphogenesis stages. Mature inflorescence female stage led to significant increase in total soluble sugars (20.3 mg/g FW). However, the content of total sugar was decreased to (12.8 mg/g FW) in the somatic embryos induced directly from inflorescence explant.

The present results also showed that mature female inflorescences contained much high levels of indoles (1.2 mg/g FW) than the direct somatic embryos that contained (1.0 mg/g FW).

The female inflorescences also contained the high level of phenols (0.60 mg/g FW) compared to somatic embryos that contained (0.52 mg/g FW).

These results could simply reflect differences in content of total sugar, phenols and indoles during different morphogenesis stages. Juvenility of the used inflorescence explants have high potential to differentiate quickly into organs. At the initial stages of growth, the explant has masses of meristematic tissue making it easy to express morphogenetic responses **(Abul-Soad *et al.*, 2011)**. In the beginning, most of spikelet florets produced pro-embryonic masses, these small masses multiplied and increased in number and became more organized. The growth and development was so quick as to make the initial explant of the spikelet appear as a swelled aggregate of these shiny globular structures. Also, the shiny

brown color and the maturation of these globular structures increased over time **(Abul-Soad, 2011)**.

Changes in soluble sugar contents in the tissue of hyacinth explants were noted. Explants were cultured onto MS medium containing BA, NAA and/or paclobutrazol as well as varying sugar levels (3% and 6% of sucrose) **(Bach *et al.,* 1992)**. The increased sucrose supply to the meristem precedes the activation of energy consuming processes such as mitotic activation and thus does not result from a higher demand by the meristem. This suggests a message-like role for sucrose **(Bernier *et al*., 1993)**. High levels of carbohydrates in somatic embryos of *Medicago sativa*, at all developmental stages were also due to high concentrations of carbon in the culture medium **(Horbowicz *et al*., 1995). Cailloux *et al*. (1996)** have suggested that carbohydrate accumulation by somatic embryos of *Hevea brasiliensis* was a normal metabolic path when abundant carbon sources are present in the culture medium. **Catarina *et al*. (2003)** who found high level of total sugars in the globular embryoides, this increase could have occurred as a consequence of the uptake and metabolism of the carbohydrates supplied exogenously. The level of total sugars in different morphogenesis stages was studied. Immature inflorescence female stage led to significant increase in total soluble sugars (17.2 mg/g DW) as compared to the other developmental stages. The low significant value of total soluble sugars was showed with mature somatic embryo stages (6.3 mg/g DW) **(Zayed and Abdelbar, 2015)**. On the other hand, at the end of the induction process of the somatic embryogenesis, there was a gradual decrease in total soluble sugar content in the explants tissues **(Bartos *et al.,* 2018)**.

Furthermore, phenols in plant cells may include a participation in auxin catabolism, an ability to modify the free polyamine levels by their coupling and the inhibitory effects of some phenolic acids on cell division **(Cvikrova´ *et al*., 1998)**. The metabolized phenols affect tissue culture systems positively with auxin metabolism (rapid cell division, synthesis of the cell wall and other related components). In addition, especially in direct regeneration studies, the scarred surfaces of explants are important for phenolic aspirate and oxidation. Although the phenols were being metabolized, they affect both regeneration and growth positively **(Ozyigit *et al*., 2007)**. It is well known that pre-embryogenic cells are highly metabolically and actively dividing cells. Once embryogenic cells have been formed, they continue to proliferate forming embryonic mass of cells **(George *et al*., 2008)**.

Mucciarelli *et al.* (2000) reported that many phenolic compounds probably act by raising or lowering the level of indole acetic acid (IAA) through enzymatic reactions. Regarding the indole and phenol concentrations, data revealed that immature female inflorescence was containing (1.0 and 0.4, mg/g FW, respectively). On the other hand, the mature somatic embryos showed reduction in this concern (0.9 and 0.3 mg/g FW, respectively) **(Zayed and Abd Elbar, 2015)**.

Furthermore, there are two opinions about total phenolics and *in vitro* proliferation, some authors described the phenolics being positively related to *in vitro* proliferation; while others say the negative and explained the negative effect resulted with enzymatic discoloration **(Ozyigit, 2008)**. The consequence phenolic bridges are a loss of cell wall extensibility, thus leading to the discontinuation of the cell wall **(Kroon and Williamson, 1999)**. The importance of phenolic substances for normal growth and development was demonstrated in transgenic tobacco plants with inhibited phenolic acid metabolism **(Tamagnone *et al*., 1998)**. Analysis of these transgenic plants revealed that morphological changes (e.g. abnormal leaf development) resulted from the lack of phenolic intermediates. Thus, extremely low as well as high levels of phenolics might be significant factors in limiting growth.

Al-Maarri and Al-Ghamdi (1995) reported that using MS nutrient medium supplemented with 1 mg/l biotin, 200 mg/l glutamine, 1 mg/l Ca pantothenate and 1.5 g/l activated charcoal minimized date palm explant browning. In addition, some antioxidants such as citric acid and ascorbic acid, PVP (polyvinyl pyrolidone) and activated carbon, which are added into medium, can also reduce phenolic oxidation and contribute to regeneration from explants **(Toth *et al*., 1994)**. The treatment which included citric acid (75 mg/l), ascorbic acid (75 mg/l), PVP (1.5 g/l) and Ca-pantothenate (2.5 mg/l) was much effective in reducing the degree of browning in leaf primordial explants of Zaghloul and Barhi cultivars. This was accompanied by an increase in growth value and higher concentration of total soluble phenols **(Hegazy *et al.,* 2006)**.

Abdallah *et al*. (2001) found that, phenols were in large amount in plantlet to form the basic material that will be used during the subsequent secondary wall formation. **Zein Eldin (2010)** found that the high concentration of phenols found in the mature somatic embryos may be used during their conversion to complete plantlets. **Zein Eldin and Ibrahim (2015)** reported that gradual increase in the concentration of phenols was noticed in the subsequent developing stages of somatic embryos (67.77 and 94.7 mg/100 g fw. of phenols).

In conclusion, an efficient and rapid protocol for plant regeneration from mature female inflorescences of date palm Hayani cv. is established. The different balanced combinations of plant growth regulators are considered a very important factor for the success of

date palm micro propagation. Based on the obtained results, the initiation medium containing 2.0 BA, 1.0 2ip and 0.5 NAA (mg /l) was the best medium that recorded the highest induction of direct embryos. The high rates of embryos proliferation were achieved on medium containing 3.0 BA, 1.0 2ip and 0.5 NAA (mg/l) and 2.0 BA, 0.5 2ip and 0.5 NOA (mg/l). In addition, the embryos regeneration medium consists of 3.0 BA and 0.5 TDZ (mg/l) resulted in the best number and shoots length.

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