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MICROPROPAGATION OF ZAGHLOL AND BARHY DATE PALM CULTIVARS USING IMMATURE FEMALE INFLORESCENCE EXPLANTS: EFFECT OF GROWTH REGULATORS BALANCE

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ABSTRACT: The present study aimed to micropropagate two date palm cultivars (Zaghlol and Barhy) using immature female inflorescence and various growth regulators combinations. This experiment was done in Biotechnology Laboratory, Department of Genetics, Faculty of Agriculture Zagazig University, during three consecutive seasons 2017, 2018, and 2019. Three types of culture media were used for initiation stage, results showed that initiation medium1 (1.0 mg /l 1-Naphthaleneacetic acid (NAA) + 0.1 mg Indole-3-acetic acid (IAA) + 1.0 mg /l 2,4-Dichlorophenoxyacetic acid (2, 4-D)) was the best medium that recorded the highest callus formation rate in both Zaghlol and Barhy cultivars. But the Barhy cultivar showed higher response for callus formation than Zaghlol cultivar, especially for number of days to initiate callus (average mean 125.58 days compare to 279 days for Barhy and Zaghlol, respectively. For plant regeneration stage, it was used three plant regeneration media. Results showed that plant regeneration medium 1 (NAA 0.5 mg/l +benzyl adenine (BA) 2.0 mg/l) showed the best value for each of number of leaves (19.35), leaf length (39.75 cm) and number of days to initiate regeneration (19). Two types of rooting media were used for root formation, it was found that rooting medium 1 (Indole-3-butyric acid (IBA) 2.0 mg/l + BA 2.0 mg/l) gave higher response than rooting medium 2 (NAA 2.0 mg/l +BA 2.0 mg/l) for almost measured criteria except root length. Also, using rooting medium 1 decreased the number of days for root formation to 40 days compared to 110.25 days obtained from rooting medium 2.

Key words: Micropropagation, Date palm, immature female inflorescence, growth regulators.

INTRODUCTION

Date Palm (*Phoenix dactylifera* L.) is a dioecious, perennial monocot plant species of the Arecaceae family. It is one of the most important fruit crops cultivated in arid and semi-arid regions. It is distributed throughout the Middle East and North Africa, South Sahel, areas of east and south Africa, Europe and USA (**Jan, 2012**), with approximately is a million trees worldwide (**Al-Khayri *et al*, 2015**).

Date palm can be propagated sexually by seeds or asexually by offshoots. Propagation by seeds cannot be used for the commercial production of elite genotypes due to its heterozygous character (**Tisserat, 1982**), and because of the considerable difference between seedlings and vegetatively propagated plants in terms of production potential, fruit maturation and quality, and harvesting time (**Zaid *et al*., 2011**). While using offshoots for commercial propagation facing limitation, offshoots availability and source of spreading disease in case if the offshoots taken from infected trees.

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Usage offshoots derived explants in tissue culture of date palm have been practicing since decades. After wards, the potential of inflorescence explants have been tested to develop direct (**Abul-Soad *et al.*, 2004**) and indirect somatic embryos of date palm (**Drira and Al Shaary, 1993, Abul-Soad *et al.*, 2005**). Inflorescence explants have many advantages over worldwide frequently used shoot tip explants for date palm micropropagation 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**).

Loutfi and Chlyah (1998) studied the vegetative multiplication of date palms from *in vitro* cultured inflorescence. They found that the 0.5 mg/l NAA, 2 mg/l BA, and 1mg/l 2ip consider as a better hormone balance for initiation of culture shoot multiplication occurred on the same medium, but plant growth and rooting were only obtained by a tenfold reduction in cytokinin concentration or by increase in NAA to 2.0 mg/l in combination with 1.0 mg/l 2ip on 1.0 mg/l BA Histological analysis of cultured explant, revealed that buds originated from petal premordia. Percentages of reactive inflorescence explant in various media different among examined cultivars. The technique offers great promise for vegetative propagation of date palms since several hundred plants could be obtained from a single inflorescence annually.

Many researches were done on micropropagation of date palm at commercial scale using inflorescences (**Drira and Al shaary, 1993; Abahmane, 1998, 2003, 2005a, 2005b, 2010, 2011a, 2011b, 2013; Abul-Soad *et al.*, 2005; Abul-Soad and Mahdi, 2010, Abul-Soad, 2011, 2012; Mazri and Meziani, 2013**).

Therefore, the present study aimed to micropropagate two date palm cultivars with a great commercial importance Zaghlol and Barhy cultivars, using immature female inflorescence: effect of hormone balance.

MATERIALS AND METHODS

Plant Materials

This experiment was carried out in Biotechnology Laboratory, Department of Genetics, Faculty of Agriculture, Zagazig University, Egypt, in three consecutive seasons 2017, 2018, and 2019, to propagate two date palm cultivars by using immature female inflorescence. Two date palm cultivars used in this study were Zaghlol and Barhy. Immature female inflorescences with 25-35 cm length of Zaghlol cultivar were obtained from Kassasen Horticulture Research Institute, in 2017, and in 2018, with 15-20 cm length for Barhy cultivar.

Sterilization and Preparation of Spathe and Immature Inflorescence

First spahtes were washed under running water for 30 min followed by surface sterilization in 60% chlorox (5.25% sodium hypochlorite) for 30 min followed by aseptically washing in sterilized distilled water for 30-60 seconds (Fig. 1) and then the protective sheath was longitudinally cut from the middle like T letter from one side only, Spikelet explants were cut from their base, divided to parts with 2-3 florets and cultured on initiation medium under aseptic conditions.

Media Preparation

Inorganic macronutrient and micronutrient levels used in plant tissue culture media are based on levels established in the plant tissue culture medium developed by **Murashige and Skoog (1962)**. Full strength (4.4g/l) and half strength (2.2g/l) Murashige and Skoog (MS) medium was used in this experiment. 30g/l of sucrose (3%) was added into the medium and the growth hormones have been added as mentioned in Schedule 1 before pH adjustment. The pH of the medium was adjusted before autoclaving to 5.7±0.1 using 0.1 M HCl and 0.1 M NaOH followed by addition of 7 g/l agar. Then the medium was autoclaved at 121ºC for 20 min. A total of 50ml of the sterile medium were poured into sterilized screwed jars in the laminar flow and allowed for solidification to be used.

Culture Conditions

All cultured explants were incubated in a controlled growth room at 27ºC under full darkness. Incubated explants were re-cultured 1-

2 times; every 3-4 weeks on same initiation medium as described in Schedule 1. Well-responded explants were transferred into initiation medium for 1-2 re-cultures. Matured and early-differentiated explants under darkness were shifted onto plant regeneration medium under illumination conditions for 1-2 re-cultures. Subsequently the differentiated cultures were shifted to the multiplication stage or may be the rooting stage directly (rooting medium). Well rooted plantlets were subjected to the *in vitro* hardening by culturing onto low nutrients medium along with more ventilation. Some date palm plantlets were successfully transplanted to the greenhouse.

Data Analysis and Heritability Calculation

The experiment was analyzed as factorial experiment design by using SPSS program to determine the significance of differences between cultivars and media. Heritability in broad sense was calculated from the following equation (**Singh and Chaudhary, 1977**).

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2} \times 100$$

σ_e^2: Error variance

σ_g^2: Genetic variance

RESULTS AND DISCUSSION

Initiation Stage

Highly significant difference were recorded between the three different initiating media for callus induction frequencies, callus weight and number of days to initiate callus and subsequently the importance of hormone combination for success of obtaining induction of callus and embryonic callus (Table 1 and Fig. 2). Im1 medium (1.0 mg /l NAA + 0.1 mg/l IAA + 1.0 mg /l 2, 4-D) was the best medium that recorded the highest callus formation rate in Zaghlol cultivar (Table 2). These results confirmed the importance of 2, 4-D for callus induction and callus embryogenic. This conclusion was confirmed by the results of **Jatoil, (2013)** for days to initiating callus embryonic and of **Othmani *et al.*, (2010)** who obtained data on embryonic callus induction of date palm cv. Boufegous (**Othmani *et al*., 2009**). In date palm cultivars **Hassan and Taha (2012)** observed somatic embryos after 9 months of induction, while **Eshraghi *et al.* (2005)** suggested an induction period of 12 months for cvs. Khanizi and Monadarsive. Their findings were in agreement with our results.

Multiplication (Regeneration) Stage

For plant regeneration and multiplication, three different hormone combinations were used (PRM1, PRM2, and PRM3) for Zaghlol date palm cultivar. Highly significant differences were recorded between the three media for number of leaves, leaf length (cm) and number of days to initiate regeneration (Table 3 and Fig. 3). These results showed the importance of using optimum hormone combinations for success of plant regeneration PRM1 (NAA 0.5 mg/l +BA 2.0 mg/l) medium possessed the best value for each of number of leaves (19.35), leaf length (39.75 cm) and number of days to initiate regeneration (19) (Table 4).

In addition, these result illustrated the importance of low concentration of NAA (0.5 mg/l) for plant regeneration with BA (2.0 mg/l).

Many researches confirmed with the results of the present study. The study of **Othmani *et al*. (2009)** was in consistence with our results in which they indicated that 1.0mg /l NAA was necessary for the germination and conversion of 81% of somatic embryos into plantlets. These results confirmed that, culture media containing low ratios of Auxin/Cytokinins (NAA 0.5mg/l, BA or 2ip 2.0 mg/l) enhance floral piece multiplication, mostly petals and usually followed by shoot formation. Explants showing petal multiplication were about 5% of the total cultured tissue. In contrast, the use of culture media with high auxins/cytokinins ratios: NAA (0.5mg/l), IBA (0.5mg/l) BA (0.1 mg/l) leads to root formation and carpel developments. The percentage of explants showing carpel development was about 30%. This percentage decreased when cytokinins (BA or 2ip) concentrations were augmented (**Abahmane, 2013**). Earlier study by **Drira and Benbadis (1985)** reported that well formed plantlets can be obtained on MS medium supplemented with BA and NAA at 1.0 mg/l each, in the case of immature inflorescences, as well as, **Loutfi nd Chlyah (1988)** used explants from inflorescences of date palm from different female cultivars and they found that, shoot premordia formed mostly on

Schedule 1. Composition of Nutrient media used in various stages of micro propagation

Medium name/Stage	Medium composition
Initiate medium (IM)	
IM1	MS+ 30 g sucrose +6 g agar+ 1 mg/lNAA+0.1mg/l IAA+1mg/l 2,4-D
IM2	MS+ 30 g sucrose +6 g agar+ 1mg/l NAA+0.1mg/l IAA+0.2mg/l 2,4-D
IM3	MS+ 30 g sucrose +6 g agar+ 0.1mg/l IAA+0.5mg/l 2,4D
Plant regeneration medium (PRM)	
PRM1	MS+ 30 g sucrose +6 g agar+ 0.5mg/NAA+2mg/LBA+2 mg/l 2ip
PRM2	MS+ 30 g sucrose +6 g agar+ 0.5mg/l 2,4-D+ 0.5mg/ NAA+2mg/ LBA
PRM3	MS+30 g sucrose +6 g agar+1 mg/l 2,4-D+0.1 mg/NAA+4mg/ LBA + 2mg/l 2ip
Rooting medium (RM)	
RM1	1/2MS+ 30g sucrose +6g agar+ 2mg/LIBA+2mg/l BA
RM2	1/2MS+ 30g sucrose +6g agar+ 2mg/L NAA+2mg/l BA

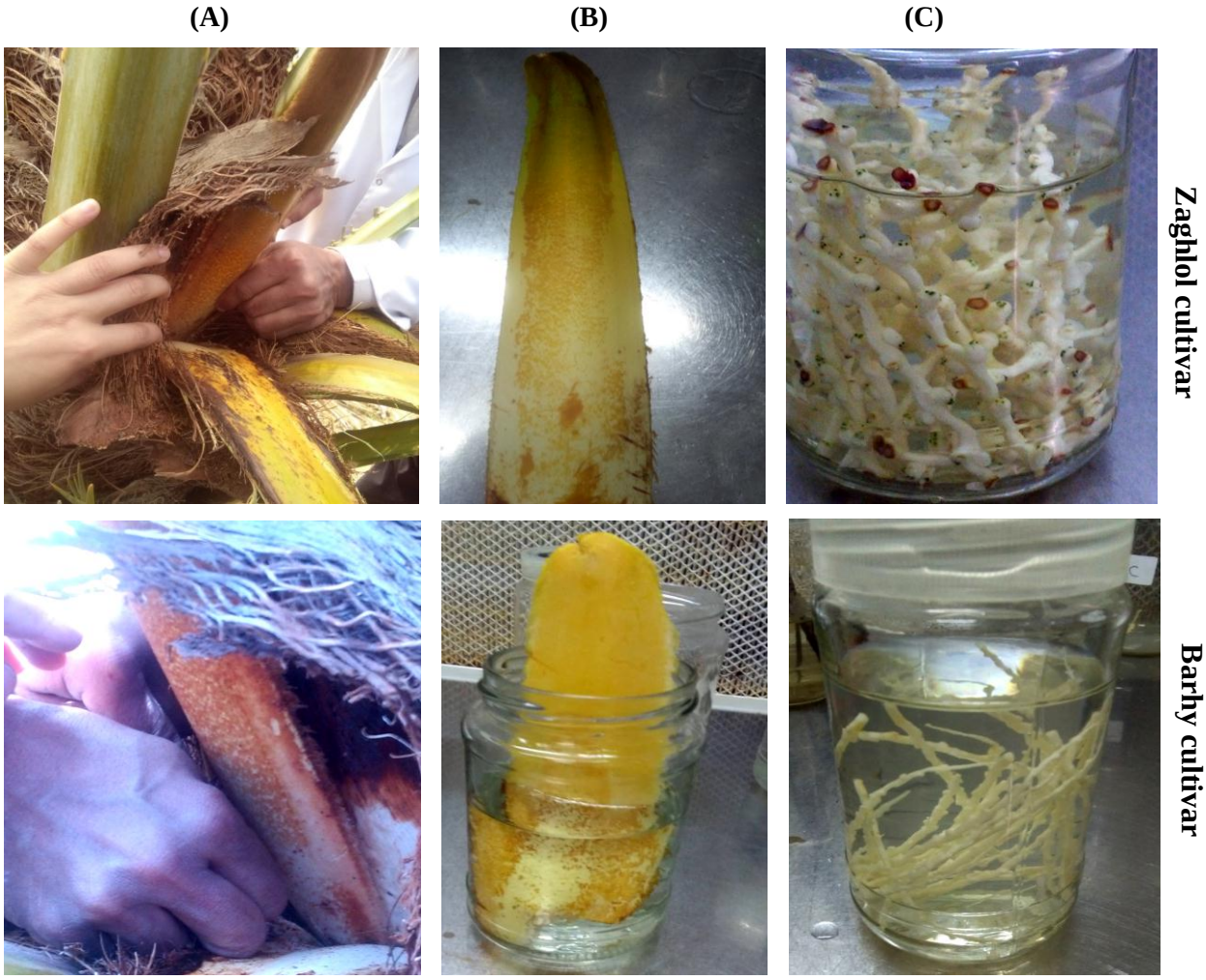

Fig. 1. Plant material used for date palm micropropagation using inflorescence tissue: (A) Spathe harvesting during flowering period (B) Spathe ready to be opened under aseptic conditions. (C) Inflorescence disinfection in sodium hypochlorite solution

Table 1. Means sum of squares (MS) of callus induction frequencies, callus weight (g) and number of days to initiate callus in Zaghlol cultivar

SOV	df	MS		
		Callus induction frequencies (%)	Callus weight (g)	No. of days to initiate callus
Replicates	3	1.30	0.48	16.33
Treatments	2	698.90**	45.80**	3387.5**
Error	6	0.95	2.38	28.58

* =significant at $p < 0.05$, ** = significant at $p < 0.01$, Values are means of four replicates.

Table 2. Average means for callus induction frequencies, callus weight (g) and number of days to initiate callus in Zaghlol cultivar

Treatment	Over all means		
	Callus induction frequencies (%)	Callus weight (g)	No. of days to initiate callus
IM1	36.05	9.43	193.25
IM2	9.87	3.565	339.75
IM3	19.75	2.637	304.50
Mean	21.89	5.21	279.00
LSD 0.05	**1.68**	**2.66**	**9.22**
LSD 0.01	**2.55**	**4.04**	**13.98**

LSD =Least significant deference.

Table 3. Means sum of squares (MS) for number of leaves/plant, leaf length (cm) and number of days to regeneration in Zaghlol cultivar

SOV	df	MS		
		No. of leaves /plant	leaf length (cm)	No. of days to regeneration
Replicates	3	23.80	0.38	0.22
Treatments	2	347.12**	2.56**	150.58**
Error	6	3.39	0.079	1.13

* =significant at $p < 0.05$, ** = significant at $p < 0.01$, Values are means of four replicates.

Table 4. Average means for number of leaves/plant, leaf length (cm) and number of days to regeneration in Zaghlol cultivar

Treatment	Over all means		
	No. of leaves /plant	**leaf length (cm)**	**No. of days to regeneration**
PRM1	19.35	39.75	19.00
PRM2	16.78	29.00	25.75
PRM3	13.23	22.00	31.25
Mean	16.24	48.25	25.33
LSD 0.05	**3.60**	**0.56**	**2.1**
LSD 0.01	**5.5**	**0.85**	**3.2**

LSD =Least significant deference.

0.5 mg/l NAA 2.0 mg/l BA and 1.0 mg/l 2ip and the shoot multiplication occurred on the same medium.

For success and continuous of date palm micropropagation should other factors of interest of such as biotin and thiamine (**Al-Khayri, 2001**), coconut water (**Al-Khayri, 2010**), Yeast extract and casein hydrolysate (**Al-Khayri, 2011**), basal formation of the culture medium, silver nitrate (**Al-Khayri and Al-Bahrany, 2001**).

Rooting Stage

Highly significant difference between two rooting media was recorded for number of roots/ plant, root length (cm) and number of days for root formation (Table 5). RM1 (IBA 2.0mg/l + BA 2.0 mg/l) gave higher response than RM2 medium (NAA 2.0 mg/l +BA 2.0 mg/l) (Table 6 figure 4) for almost criteria except root length. Interest note, number of days for root formation decreased (40 days) for RM1 medium compared to (110.25 days) for RM2. This note confirms that IBA consider a better hormone for root formation. These results are in agreement with many other studies. Good rooting of elongated shoots was obtained on culture media containing BA at mg/l and IBA at 1.0mg/l (**Drira and Benbadis, 1985**). In other study, for shoot rooting (**Bekheet, 2013**) recommended 1.0mg/l NAA, which showed better results than IAA or IBA at the same concentration. **Jatoi, (2013)** reported that rooting was achieved using quarter strength MS medium containing 0.1 mg/l NAA without activated charcoal.

Regarding, Barhy cultivar was studied for callus formation for three criteria and it is found that there was highly significant difference between three media for all three studied criteria as showed with cultivar Zaghlol (Tables 7, 8 and Fig. 5), but the Barhy cultivar showed higher response for callus formation than Zaghlol cultivar, especially for number of days to initiate callus (average mean valued 125.58 days compared to 279 days for Barhy and Zaghlol (respectively). As well as the same results with Zaghlol cultivar for IM1 medium was better than other media.

Results of interaction between genotypes (Zaghlol and Barhy) and hormone balance combinations were shown in Tables 9 and 10 for callus induction frequency, callus weight and number of days to initiate callus. Highly significant differences were recorded between genotypes, media and interaction between genotypes and media for all studied criteria. These results confirmed that interaction between genotypes and different hormone balance combinations is considered a very important factor for success of date palm micropropagation. These results are in harmony with many other studies (**Loutfi and Chlyah, 1998; Abahmane, 2010; Abhmane, 2011b; Abul-Soad, 2012; Abahmane, 2013**).

Table 5. Means of squares (MS) for number of roots/plant, root length (cm) and number of days to initiate roots in Zaghlol cultivar

SOV	df	MS		
		No. of roots/ plant	Root length (cm)	No. of days for root formation
Replicates	3	0.83	0.019	2503.79
Treatments	1	12.50**	3.39**	7381.125**
Error	3	0.75	0.0003	0.125

* =significant at $p < 0.05$, ** = significant at $p < 0.01$.

Table 6. Average means for number of roots/plant, root length (cm) and number of days to initiate roots in Zaghlol cultivar

Treatment	No. of roots /plant	Root length (cm)	No. of days for root formation
RM1	5.25	1.60	40.00
RM2	2.75	2.90	101.25
Mean	4.00	2.25	70.62
LSD 0.05	**1.89**	**0.037**	**0.775**
LSD 0.01	**3.55**	**0.071**	**1.45**

LSD =Least significant deference.

Table 7. Means sum of squares (MS) for callus induction frequencies, callus weight (g) and number of days to initiate callus in Barhy cultivar

SOV	df	MS		
		Callus induction frequencies (%)	Callus weight (g)	No. of days to initiate callus
Replicates	3	24.50	0.66	252.08
Treatments	2	413.50**	3.818**	2075.08**
Error	6	2.57	0.105	0.083

* =significant at $p < 0.05$, ** = significant at $p < 0.01$, Values are means of four replicates.

Table 8. Average means for callus induction frequencies, callus weight (g) and number of days to initiate callus in Barhy cultivar

Treatment	Over all means		
	Callus induction frequencies (%)	Callus weight (g)	No. of days to initiate callus
IM1	63.40	8.52	130.50
IM2	50.17	4.16	100.75
IM3	70.20	5.86	145.50
Mean	61.25	6.18	125.58
LSD 0.05	**2.76**	**0.553**	**0.49**
LSD 0.01	**4.19**	**0.849**	**0.75**

LSD =Least significant deference.

Table 9. Means sum of squares (MS) for callus induction frequencies, callus weight (g) and number of days to initiate callus of two cultivars (Zaghlol and Barhy)

SOV	df	Ms		
		Callus induction frequencies (%)	Callus weight (g)	No. of days to initiate callus
Replicates	3	17.44**	0.25**	184.375**
Treatments	5	2299.91**	29.032**	38490.475**
Genotype (G)	1	28743.32**	130.00**	70763.52**
Media (M)	2	4029.48**	2.83**	19773.25**
G x M	2	13815.25**	906.33**	5576.04**
Erorr	15	3.70	0.23	28.275
H^2 Broad sense		**99.99%**	**99.9%**	**99.3%**

* =significant at p < 0.05, ** = significant at p < 0.01.

Table 10. Means for callus induction frequencies, callus weight (g) and number of days to initiate callus of two cultivars (Zaghlol and Barhy)

Media	Genotype								
	Callus induction frequencies (%)			Callus weight (g)			No. of days to initiate callus		
	Zagloul	Barhy	Mean	Zgloul	Barhy	Mean	Zagloul	Barhy	Mean
M1	36.05	63.25	49.65	9.43	8.52	8.97	193.02	130.5	161.5
M2	9.875	50.175	30.025	3.26	4.16	3.71	339.75	100.7	220.25
M3	19.75	70.20	44.975	2.63	5.86	4.24	304.5	145.5	225
Means	21.89	61.20	41.545	5.11	18.54	11.825	179.16	125.6	202.58
LSD% 0.05		**2.85**			**0.722**			**7.8**	
LSD% 0.01		**3.99**			**0.997**			**10.9**	

LSD =Least significant deference.

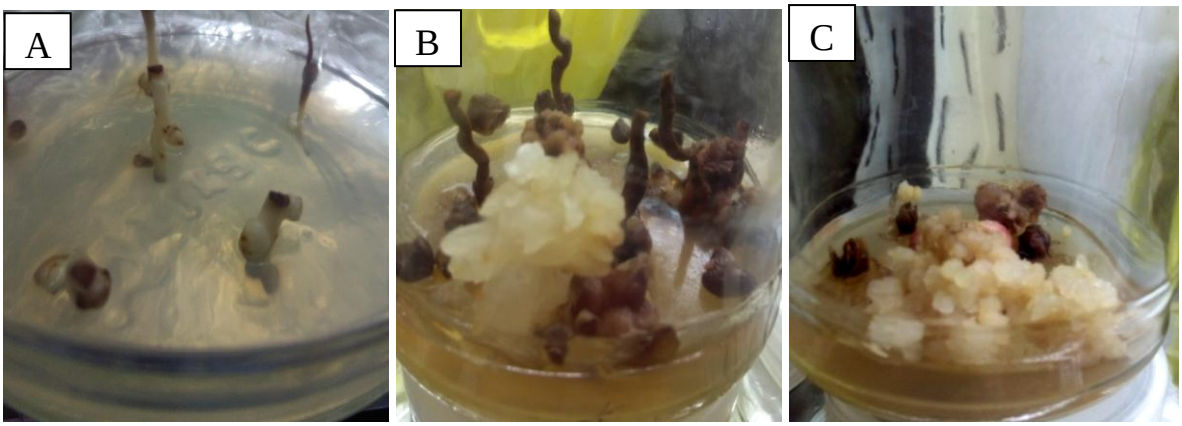

Fig. 2. Different callus formation stages of date palm micropropagation using female inflorescence explants in Zaghlol cultivar. A. Inflorescence spikelets on initiation medium. B, C. Embryogenic callus formation stage

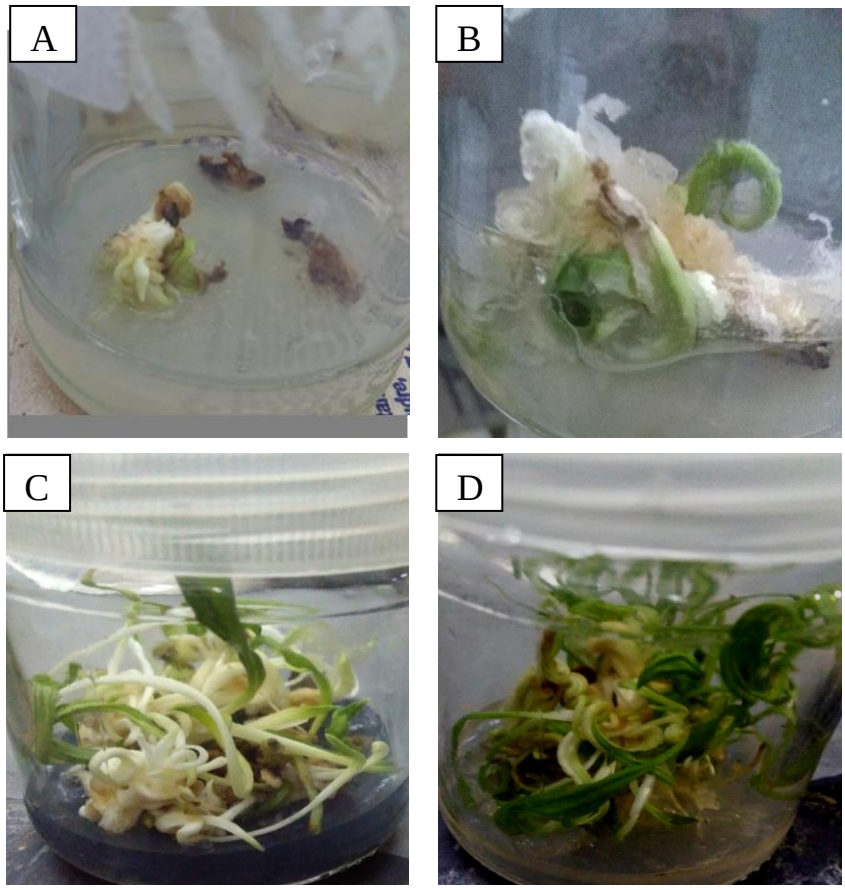

Fig. 3. Different callus formation stages of date palm micropropagation using female inflorescence explants in Zaghlol cultivar. A, B. Shoot proliferation with somatic embryos C, D. Shoots cluster on plant regeneration medium

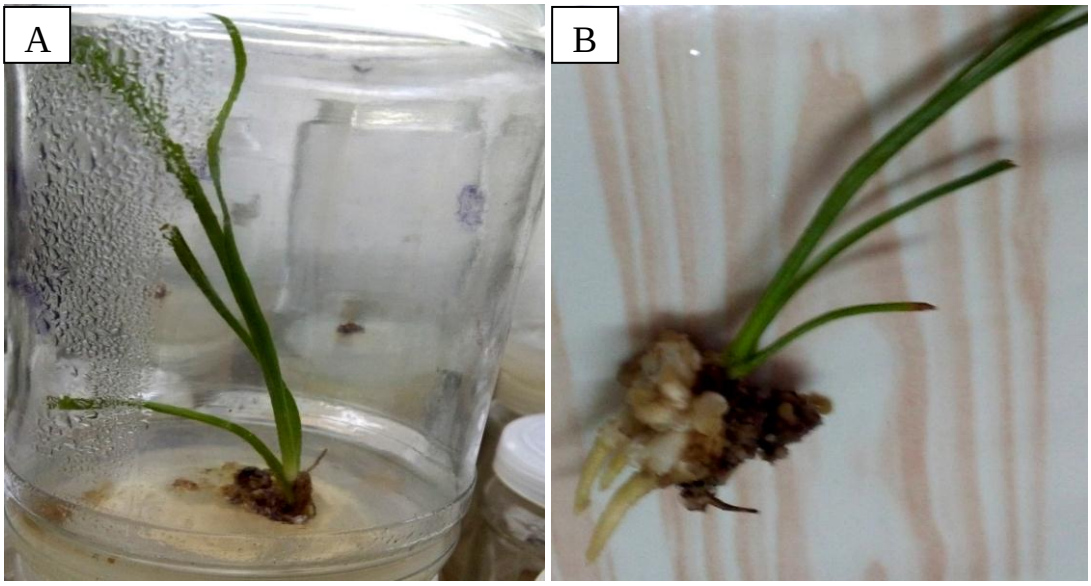

Fig. 4. A. Plantlets on rooting medium. B. Rooted plants in Zaghlol cultivar

Fig. 5. Embryogenic callus formation stage in Barhy cultivar

In addition, Barhy cultivar showed higher response than Zaghlol cultivar for callus formation, which might be due to using of optimum stage of immature inflorescence or because of the different genetic background between the two cultivars.

Despite of the numerous works published on date palm micropropagation, research is still needed to optimize culture conditions for the newly selected genotypes and important cultivars to shorten the time needed to produce plantlets, and to reduce the incidence of physiological disorders.

It is also important to carry out studies related to the application of somatic embryogenesis needed for genetic transformation, synthetic seeds production and cryopreservation of embryogenic culture.

Conclusion

Based on obtained results, it could be concluded that initiation medium1 (1.0 mg /l NAA + 0.1 mg IAA + 1.0 mg /l 2, 4-D) was the best medium that recorded the highest callus formation rate in both Zaghlol and Barhy cultivars. Plant regeneration medium 1 (NAA

0.5 mg/l +BA 2.0 mg/l) possessed the best value for each of number of leaves (19.35), leaf length (39.75 cm) and number of days to initiate regeneration. Rooting medium 1 (IBA 2.0mg/l + BA 2.0 mg/l) gave higher response than rooting medium 2 (NAA 2.0 mg/l +BA 2.0 mg/l). It is important to shed light on interaction between genotypes and different hormone balance combinations as it consider very important factor for success of microprpagation of date palm.

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الإكثار الدقيق لأصناف نخيل البلح (الزغلول والبارحى) باستخدام النورات المؤنثة غير الناضجة : تأثير توازن منظمات النمو

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تهدف الدراسة الحالية الى إكثار صنفين من نخيل البلح (زغلول، بارحى) باستخدام النورات المؤنثة وتوليفات متنوعة من منظمات النمو، هذه الدراسة تمت فى معمل البيوتكنولوجى بقسم الوراثة كلية الزراعة جامعة الزقازيق خلال ثلاث سنوات متتالية ٢٠١٧، ٢٠١٨، ٢٠١٩، تم إستخدام ثلاث بيئات للزراعة فى مرحلة البداية، وأوضحت النتائج أن البيئة الأولى (1.0 mg /l NAA + 0.1 mg IAA + 1.0 mg /l 2, 4-D) كانت أفضل البيئات والتى سجلت أعلى معدل إنتاج للكالس فى كلا الصنفين زغلول ، بارحى، ولكن الصنف بارحى أظهر استجابة أعلى لتكوين الكالس من الصنف زغلول وخاصة فى عدد الأيام اللازمة لتكوين الكالس (١٢٥.٥٨ يوم) مقارنتة ب ٢٧٩ يوم فى حالة الصنف زغلول، لمرحلة إعادة التكشف تم استخدام ثلاث بيئات وأعطت البيئة الأولى (NAA 0.5 mg/l +BA 2.0 mg/l) القيم الأعلى لعدد الأوراق (19.35) وطول الورقة (39.75 cm) وعدد الأيام اللازمة للتكشف (١٩)، فى مرحلة التجذير تم إستخدام نوعين من بيئات التجذير، وجد أن البيئة الأولى (IBA 2.0mg/l + BA 2.0 mg/l) هى الأفضل من البيئة الثانية (NAA 2.0 mg/l +BA 2.0 mg/l) لكل القياسات ماعدا طول الجذر ، أوضحت النتائج أيضا أن استخدام بيئة التجذير الأولى أدى إلى تقليل عدد الأيام اللازمة لتكوين الجذر إلى ٤٠ يوم مقارنة ب ١١٠ يوم فى حالة استخدام بيئة التجذير الثانية.

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