Minufiya J. Agric. Res. Vol.37 No. 3: 579 - 589 (2012) **"http://www.mujar.net"**

EFFECT OF DIFFERENT MEDIUM FORMULATIONS ON SOMATIC EMBRYOGENSIS DEVELOPMENT OF DATE PALM (*Phoenix dactylifera* L.) cv. KHALAS

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(Received: Feb. 7 , 2012)

ABSTRACT: *Composition of tissue culture is arbitrary selected among various media formulations differing in basal salt strengths to provide essential nutrients for date palm (Phoenix dactylifera L.) cv. Khalas propagated via somatic embryogenesis technique in order to improve plant regeneration. This study examined the efficiency of MS, B5, N.N and WPM media formulations at different strengths (full, half and quarter) on callus growth& somatic embryogenesis formation, germination and root formation stages. Evidence suggests that, the optimum medium formulation varied depends on culture stage. The best callus growth was achieved using B5 medium at half- strength. An optimal medium for callus growth was not necessarily the best for somatic embryogenesis. Therefore, the WPM medium at full strength gave the highest number of somatic embryos. In vitro propagation of date palm by using somatic embryogenesis protocol, often associated with the problem of hyperhydric embryo formation. Evidence suggests that, B5 medium was the optimum medium that minimized the number of hyperhydric embryos when used at quarter strength. Full strength N.N salt medium gave the highest significant shoot formation number. This study provides important information to secondary embryo formation, it showed that B5 medium at full strength was the most suitable medium for date palm secondary embryo formation. While, during rooting stage N.N medium was optimum medium that maximized root number/ plantlet, WPM medium induced the longest plantlet followed by B5, N.N and MS media.. The concentration of inorganic salts plays an important role in root induction as that reduction of N.N salts strength to quarter strength of the original concentration stimulated root formation in date palm tissue culture.*

***Key words**: Phoenix dactylifera L., tissue culture, somatic embryogensis, MS, B5, N.N, WPM, vitrification (hyperhydricity)*

INTRODUCTION

The date palm [*Phoenix dactylifera* L. (2n=36)], is one of the most economically important fruit tree in the desert areas of the Middle East and in North-Africa.(Othmani *et al.,* 2009) . About 90% of the total world production is produced from this region (Aslam and Khan 2009). *In vitro* micropropagation is increasingly becoming an attractive alternative for large-scale propagation of date palm. *In vitro* plant regeneration of date palm occurs through organogenesis and somatic embryogenesis depending on genotype and hormonal manipulations. Somatic embryogenesis from shoot tip derived callus has been viewed as the most appealing process for date palm regeneration (Al-Khayri, 2003). However, several problems still need to be solved and are currently under study, such as the abnormal differentiation of somatic embryo, the proliferation of endophytic bacteria within in vitro cultured material and the occurrence of somaclonal variants in regenerated offspring (Fki *et al.,* 2011). Fortunately, experiments with a number of species have demonstrated that the morphological aspects of vitrification can be controlled by various means including, optimization of growth regulators, increasing agar concentration, reducing ammonium nitrate in culture medium, improving gas exchange and controlling culture environmental. Nevertheless, studies related to mineral nutrients have been limited and often focused on growth responses thus overlooking their role as morphogenic elicitors (Ramage and Williams, 2002).

Comparative studies of various culture medium formulations on different *in vitro* culture stages of date palm are scarce. So far, two studies have been carried out on basal salt formulation of date palm tissue culture medium (Abo El-Nil, 1989). Previous studies have shown that maturation of somatic embryo, germination, *in vitro* rooting and plant establishment can be influenced by various *in vitro* factors [Ibrahim (1999), Al-Khayri, (2003), Hassan *et al.,* (2008), Fki *et al.,* (2011) and Al-Khayri, (2011)].

This study was done to better understand how various medium formulations [MS, B5, N.N WPM] at different strengths (full, half and quarter) influence callus growth, somatic embryo formation & germination, hyperhydricity and rooting of date palm cv. Khalas through somatic embryogenesis technique.

MATERIALS AND METHODS

This work was conducted at Central Laboratory for Date Palm Researches and Development during the period from 2008 to 2010.

Plant material and culture conditions:

Strongly and healthy offshoots were carefully separated from adult date palm trees *(Phoenix dactylifera* L.) cv. Khalas grown at a private farm located in Egypt. The shoot tips were carefully removed and cleaned with soap and running tap water for one hour and soaked in antioxidant solution (150 mg /l ascorbic acid and 100 mg/l citric acid) and surface sterilized sequentially in 0.05 % $HgCl_2$ for 3 min. and thoroughly washed with sterilized distilled water for three times. After that additional leaf primordial were removed from shoot tips and then immersed in 50 % commercial Clorox (5.25 % sodium hypochlorite NaOCl) for 20 min. with rotary agitation and then washed with sterilized distilled water for three times . All surface sterilized solution contained one drop of tween 20 per 100 ml solution used as surfactant. Shoot tip 5-10mm in length, composed of the apical meristem and 5-7 leaf primordial were cut longitudinally at four pieces and cultured in Murashige and Skoog medium supplemented with 10mg/l di-chlorophenoxy acetic acid (2,4- D) + 3 mg /l iso- pentenyl adenine (2iP) + 3 g/l activated charcoal (AC) according to the methods described by Tisserat , 1984). Cultures were incubated at day and night temperature of 27 ±2ºC under complete darkness for eight months with sub culturing to fresh medium of the same composition every two months to form callus.

Friable callus produced from the previous stage was divided into pieces of approximately (1 x 1 cm).

Callus growth and embryo formation.

Cultured on various media formulation (MS Murashige and Skoog (1962)., B5, N.N and WPM) Table (1) at different strengths (full, half and quarter) for 12 weeks (6weeks interval). All formula strengths were supplemented with 1 mg/l NAA +0.05 mg/l BA+0.5 mg/l ABA +1 g/l AC+40 sucrose+100 mg/l glutamine and incubated under total darkness. After this period number of total somatic embryos (normal and vitrified) was recorded. Also, callus growth value which estimated visually according to Pottino (1981) was recorded as follows.

1- Negative result 2- Below average result
3- Average result 4- Good result
5- Excellent result

Embryo growth and development stage (germination stage):

A small cluster containing 3 - 4 embryos, formed previously in different formula strength media were transferred to the same formula strengths supplemented with 200 mg /l glutamine + 170 mg / l NaH_2PO_4, (0.2 mg/l NAA , 0.2 mg/l 2iP and 0.4 mg/l BA) with 0.2mg/l activated charcoal to study their effect on number of germinated embryos (shoots) and secondary embryos production after 9 weeks (3 weeks interval). All cultured jars were incubated at 27±2°C in light provided by white fluorescent tubes giving of about 2000 lux for 16 hrs per day. Each treatment contained 5 replicates and each replicate contained one cluster.

Rooting formation stage:

In this stage, an individual healthy shoot of about 5 cm in length with 2 - 3 leaves resulted from previous stage of this study were used as plant materials. Shoot explants were cultured on different formula strengths to study how they affected root formation number and plantlet length during root stage. All formula strengths media were supplemented with 0.4 mg/l thiamine-HCl + 2.0 mg/l glycine + 30 g/l sucrose + 6 g/l agar +0.1 mg/l paclobutrazole+ 0.2 mg/l NAA.

All culture media of each treatment were distributed into culture tubes (25ml on each one) and each tube containing one shoot and incubated at 27±2°C for 3 months (through two recultures) in growth room under 16 hrs illumination of 4000 Lux white fluorescent lamps.

Statistical analysis:

Data obtained were subjected to the analysis of variances of randomized complete design as recommended by Snedecor and Cochran (1980). LSD at 5% level of significance was used to compare between means according to Waller and Duncan (1969).

Table (1). The chemical composition (mg /L) of the various basal salt media.

Constituents (mg/L)	MS	NN	B5	WPM
NH_4NO_3	1650	720	-	400
KNO_3	1900	950	2500	-
$CaCl_2.2H_2O$	440	220	150	96
$MgSO_4.7H_2O$	370	185	250	370
KH_2PO_4	170	68	-	170
$MnSO_4.4H_2O$	22.30	25	-	22.3
$ZnSO_4.4H_2O$	8.60	10	2.0	8.6
H_3BO_3	6.20	10	3.0	6.20
KI	0.83	-	0.75	
$NaMoO_4.2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	-	0.025	-
Na_2EDTA	37.25	37.25	37.25	37.25
$FeSO_4.7H_2O$	27.85	27.85	27.85	27.85
$Ca(NO3)_2.4H2O$	-	-	-	556
K_2so_4	-	-	-	990
$MnSO_4.H2O$	-	-	10	-
$NaH_2PO4.H2O$	-	-	150	-
$(NH_4)_2SO_4$	-	-	134	-
Nicotinic acid	0.5	5.0	1.0	-
Pyridoxine-HCl	0.5	0.5	1.-0	-
Thiamine-HCl	0.1	0.5	10.0	-
Glycine	2.0	2.0	-	-
Myo-inositol	100.0	100	100	-

RESALUTS

Effect of different medium formulations on:-

Callus and Embryo formation stage.

Callus growth. Callus growth was significant affected by media formula and salts strength as indicated in Table (2) and Fig. (1a). Our results observed that it is evident that B5 and WPM media produced the highest significant callus growth value with insignificant difference in-between. On the other hand, both MS and N.N media proved to be unsuitable media for date palm callus growth. Regarding the effect of salt strength on callus growth it is cleared that quarter strength gave the highest callus growth among various strength. Half strength B5 medium gave the superior effective on date palm callus growth followed by WPM medium at quarter strength.

Number of somatic embryos. Data in Table (3) and Fig.(1b) have shown that WPM medium resulted in highest number of total embryos compared to other media, and that full strength media salt was superior to half- or quarter strength salt. In general, data observed that the full-strength WPM medium was suitable medium to produce the highest number of total embryos, while this number decreased as the lowest when quarter strength B5 medium was used.

Embryo growth and development stage (germination stage):

Shoot number Data in Table (4) and Fig.(1c) clear evident that N.N medium showed the highest significant number of shoot formation followed by MS, B5 and WPM media in a descending order with significant difference in- between except those between B5 and WPM media. The maximum significant shoot formation number was observed from embryos cultured on full strength of media salt. Data also indicated that full strength N.N salt medium gave the highest significant shoot formation number, followed by used strength MS salt medium. On the other side, quarter strength of either MS or WPM salt medium gave the lowest significant shoot formation number.

Table (2): Effect of type of media and concentration of salts strength on callus growth of date palm cultured after 12 weeks.

Salts strength	Type of media				Average
	MS	N.N	B.5	WPM	
Full strength	2.67	2.33	3.67	3.33	3.00
Half -strength	3.33	3.00	5.00	4.33	3.92
Quarter strength	3.67	4.00	4.33	4.67	4.17
Average	3.22	3.11	4.33	4.11	
New L.S.D. at 0.05%	Type of Media 0.59		Salt Strength 0.51	Interaction 1.04	

Table (3): Effect of type of media and concentration of salts strength on number of somatic embryos of date palm cultured after 12 weeks.

Salts strength	Type of media				Average
	MS	N.N	B.5	WPM	
Full strength	11.00	12.67	14.67	17.67	14.00
Half- strength	11.34	10.33	8.00	12.67	10.83
Quarter strength	6.00	7.00	4.00	11.66	7.83
Average	9.78	9.00	8.89	14.00	
New L.S.D. at 0.05%	Type of Media 0.72		Salt Strength 0.63	Interaction 1.13	

Table (4): Effect of type of media and concentration of salts strength on number of shoots (embryo germination) of date palm cultured 12 weeks at embryogenesis stage.

Salts strength	Type of media				Average
	MS	N.N	B.5	WPM	
Full strength	12.00	14.00	8.00	7.00	10.25
Half strength	5.0	11.33	4.00	4.00	6.08
Quarter strength	2.67	9.00	4.00	3.00	4.66
Average	6.55	11.44	5.33	4.67	
New L.S.D. at 0.05%	Type of Media 0.821		Salt Strength 0.648		Interaction 0.939

Secondary embryo formation. Data in Table (5) and Fig.(1b) showed that WPM and B5 media gave the highest significant average number of secondary embryo/embryo (7.67and 7.56, respectively) with in-significant difference in-between. As the same manner the full salt strength of media gave the highest significant average number of secondary embryo/ embryo (11.17). In this investigation it is clear that full strength B5 medium was the most suitable medium for date palm secondary embryo formation as gave 15 secondary embryo/ embryo.

Number of hyprhydric embryos. Data in Table (6) and Fig. (1d) revealed hyperhydric embryos formed through germination stage as affected by different media formulations at various strengths. The vitrified date palm tissues were hyperhydric, translucent; and were visually glassy yellow light-brown in color. Data concluded that B5 medium was that optimum medium that minimizes this phenomenon, while WPM maximizing number of vitrified formed embryos. Vitrified embryos were reduced significantly to minimum value by reducing salt strength from full, half to quarter strength.

Root formation stage.

Plantlet length. Concerning the effect of culture media type, data tabulated in Table (7) Fig.(1e) is clear observed that WPM media produced the longest plantlet length (8.89 cm) followed by B5,N.N and MS (7.89,7.44 and 7.13 cm, respectively) in a descending order with insignificant difference among them. The length of plantlet was affected by salts strength of the media type. It is clear that increasing the salt strength resulted in significant increase in the length of plantlets. The longest plantlets (9.93 cm) were obtained at full strength of salts media, whereas the shortest plantlets (5.92 cm) were obtained at quarter strength of media salts media. The mentioned results clearly indicated that B5 medium at full strength was the most suitable medium for produced the longest plantlet of date palm cv. Khalas.

Root formation. Concerning the effect of culture media type, regardless the concentration of salt strength on average roots number/ plantlet, data in Table (8) Fig.(1e) is clear that N.N medium produced the highest significant average roots number/ plantlet (4.99). On the other hand, WPM medium proved to be unsuitable medium for root formation of date palm explants, since it gave the lowest number of roots/ plantlet (2.77). About the effect of medium salt strength, data showed that quarter salt strength gave the highest significant average root number/ explants (5.24) compared among other strength.

Regarding the effect of interaction of various media type and their salts strength data revealed that N.N medium at quarter salts strength was most suitable medium for root formation of date palm explants.

Table (5): Effect of type of media and concentration of salts strength on number of secondary embryo/ explant of date palm after 12 weeks at embryogenesis stage.

Salts strength	Type of media				Average
	MS	N.N	B.5	WPM	
Full strength	9.67	8.00	15.00	12.00	11.17
Half strength	5.00	9.00	4.00	8.00	6.50
Quarter strength	3.00	2.33	3.67	3.00	3.00
Average	5.89	6.44	7.56	7.67	
New L.S.D. at 0.05%	Type of Media 0.942		Salt Strength 0.834		Interaction 1.299

Table (6): Effect of type of media and concentration of salts strength on number of vitrified embryos of date palm culture after 12 weeks.

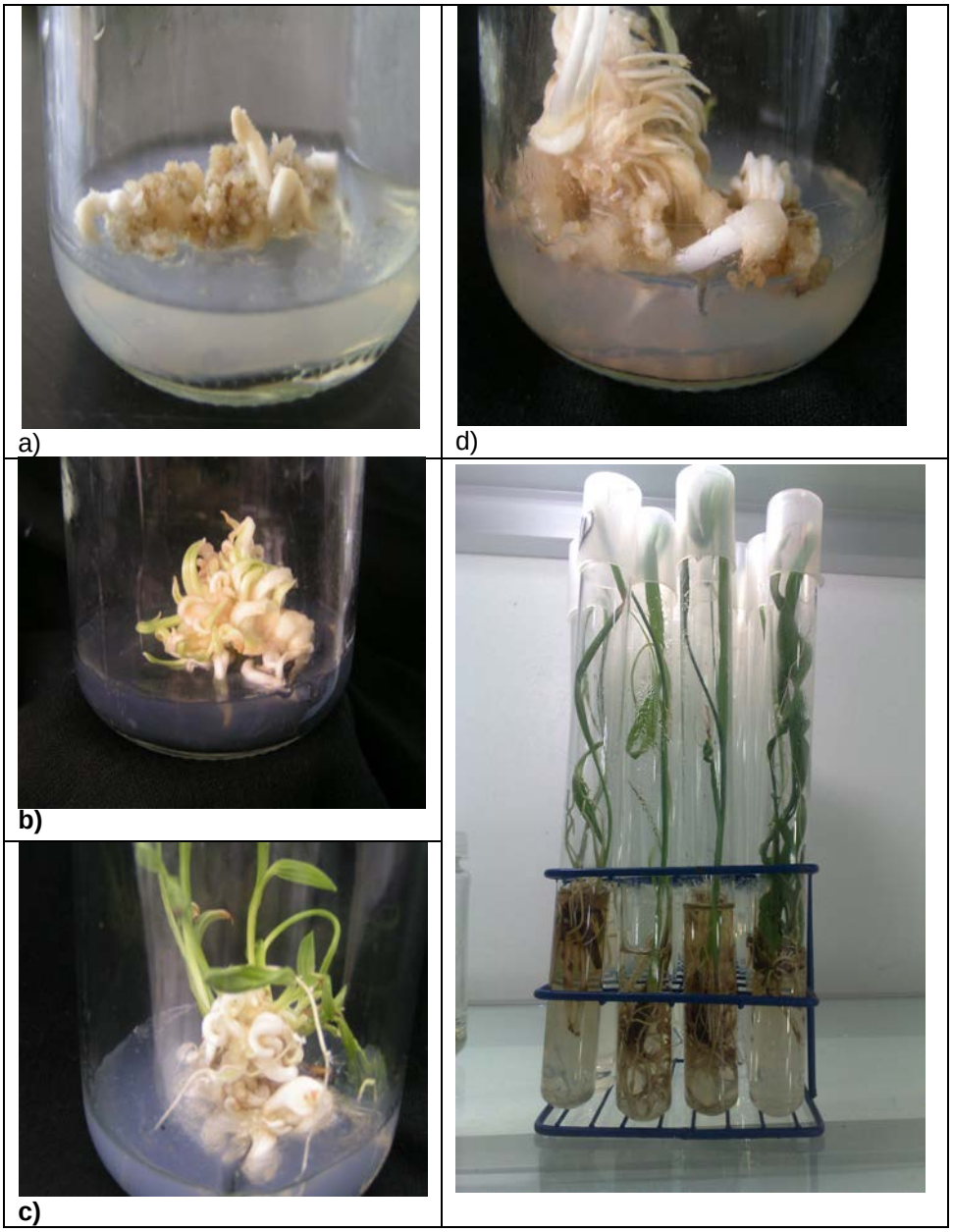
Salts strength	Type of media				
	hyprhydric embryos				
	MS	N.N	B.5	WPM	Average
Full strength	8.67	8.67	4.00	12.00	8.33
Half- strength	7.67	8.00	1.00	9.67	6.58
Quarter strength	4.67	5.67	0.67	10.33	5.33
Average	7.00	7.44	1.89	10.67	
New L.S.D. at 0.05%	Type of Media 0.696		Salt Strength 0.603		Interaction 1.20

Table (7): Effect of type of media and concentration of salts strength on plantlet length (cm) of date palm after 12 weeks during root formation stage.

Salts strength	Type of media				
	MS	N.N	B.5	WPM	Average
Full strength	8.40	8.00	12.33	11.00	9.93
Half strength	6.67	9.00	5.33	9.66	7.67
Quarter strength	6.33	5.33	6.0	6.00	5.92
Average	7.13	7.44	7.89	8.89	
New L.S.D. at 0.05%	Type of Media 0.89		Salt Strength 0.77		Interaction 1.53

Table (8): Effect of type of media and concentration of salts strength on number of roots/ plantlet of date palm after 12 weeks during root formation stage.

Salts strength	Type of media				Average
	MS	N.N	B.5	WPM	
Full strength	3.0	4.66	1.66	1.33	2.66
Half strength	3.33	4.33	3.66	3.00	3.58
Quarter strength	5.33	6.00	5.66	4.00	5.24
Average	3.89	4.99	3.66	2.77	
New L.S.D. at 0.05%	Type of Media 0.62		Salt Strength 0.53		Interaction 1.06



Fig(1): Stages of date palm development *in vitro* a) callus growth, b)secondry somatic embryos, c) germinated embryo showing shoot and root initiation d) hyperhydric embryos e)rooted plantlet ready for transplanting .

DISSICUSSION

Callus growth. The limited success and slow progress in date palm micopropagation technology development may be attributed to the serious deficiency in basic information on tissue and cell response to various media formulation and additives. The formation, devolvement and germination of somatic embryos are controlled by basal salt composition among other known tissue culture factors. Protocols of somatic embryogenesis often use only one medium formulation during the entire process, even though this formulation may not be optimal

for the various stages of the micropropagation process. A complete nutrient medium contains defined amounts of minerals, in the form of inorganic salts, essential for *in vitro* growth and development. Nevertheless, studies related to mineral nutrients have been limited and often focused on growth responses thus overlooking their role as morphogenic elicitors (Ramage and Williams, 2002). A wide range of formulations of macro-and micro-salt mixtures have been arbitrarily selected as a basal nutrient medium in different plant species. Comparative studies of various culture medium formulations on different *in vitro* culture stages of date palm are scarce.

Our results show that best basal medium for embryo germination also differ from that required for shoot growth and root formation through somatic embryogenesis stages. These results were in line with those reported by Al-Khayri (2011), showed that the optimal salt formulation for a particular culture stage may not be the best for the other culture stages. In his study has shown that media formulations that were observed to be most suitable for callus growth of date palm do not necessarily coincide with optimal formulations for somatic embryogenesis. This illustrates that various culture stages may be affected differently by the formulation of the basal media. This was also demonstrated by Capuana *et al.,* (2007) in common ash (*Fraxinus excelsior* L.) where the best proliferation of embryogenic tissue was obtained when the material was subcultured on MS medium; whereas, WPM medium appeared to be more conducive to faster embryo maturation. In the present study, WPM and B5 formulations enhanced callus growth to maximum value while, N.N minimize callus growth.MS gave moderate value of callus growth. Al-Khayri (2011) found that in date palm cv. Khusab, white and WPM media ranked first, SH and MS media ranked second and N.N ranked third in the case of callus growth. Abo El Nil (1989) reported that SH and B5 media promote best callus induction in date palm than MS and other tested media.

Results under discussion observed that WPM was the most effective formulation to enhance number of formed embryos during callus stage followed by MS, N.N while, B5 gave the lowest number. Al-Khayri (2011) revealed that the highest regeneration percentage in cv. Berny occurred on WPM medium followed by MS, N.N while, the lowest regeneration was noticed with other media.

Our results showed that increasing salts strength among tested media increasing callus growth. Enhanced callus growth of date palm cv. Khalas (Gadalla, 2007) and mango (Litz *et al.,* 1983) by using half strength MS compared with double and full strength. Modified MS medium at half strength was more effective than full strength for culture initiation and maintenance.

Ellis and Bilderback (1984) hypothesized the reduction in total nitrogen in the half strength medium enhanced calogensis.

Embryo germination stage. During this stage WPM and B5 were most suitable form producing secondary embryo number followed by N.N while, MS medium produced the lowest number embryo. Al-Khayri (2003) mentioned that the optimum treatment that maximized the percentage of complete plant formation (86%) consisted of half-strength MS medium containing 0.2 to 0.4 mg l–1 IBA. Somatic embryos that formed only shoots ranged from 2 to 26%. Taha *et al.,* (2007) found that 3/4 MS-salt strength medium gave the highest percentage of shoot formation 95 %, 90 % and 83 % derived from shoot tip explants of Sakkoty ,Malkaby and Bartamoda date palm cultivars . Al-Khayri (2011) revealed that MS medium is the most commonly used for date palm formulations are occasionally used. For instance, B5 medium basal salt (Gamborg *et al.,* 1968) was employed by Sharma *et al.,* (1986) for establishment of suspension culture.

Rooting stage. In the present study decreasing the salt strength from full to quarter increased root formation. Also, N.N

formulation which containing half major salts of MS was the most effective formula to promote root formation number. Low levels of macronutrients are beneficial for root formation in many plants. While, Ibrahim (1999) reported that the concentration of inorganic salts plays an important role in root induction as that reduction of MS salts strength to ¾ of the original concentration stimulated root formation in date palm tissue culture. Hassan *et al.,* (2008) found that ¾ MS salt strength increased the number of roots, plantlet growth vigor, and plantlet length and plantlet thickness of date palm. Blazich (1988) suggested that mineral nutrients affected root imitation. Hilae and Te-chato (2005) found that low strength media promoted root formation of oil palm.

Plantlet length in this study was increased with increasing salt strength. Hilae and Te-chato (2005) reported that size of oil palm shoots ranged from 0.5-0.8 cm in height depending on strength of the medium and a 1/5 strength medium gave the smallest shoot.

Finally it is clear from results here while decreasing strength of the culture media increased both callus growth and rooting formation, decreased somatic embryo formation, germination and hyperhydricity phenomena. In this respect Pinto *et al.,* (2008) reported that MS was more efficient for the germination stage as compared to B5 medium.

During germination stage it is obviously that B5 medium lowered and minimized hyperhydricity phenomena while, WPM maximized it; decreasing salt strength also maximized this phenomena. In this respect DeWald *et al.,* (1989) reported that modified medium was better for production of morphological normal somatic embryos of mango than MS or WPM media.

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تأثير تركيب البيئات المختلفة علي تكشف الأجنة الجسدية لصنف نخيل البلح (خلاص)

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الملخص العربي

تم إختيار بعض البيئات المختلفة في تركيبها وبقوي مختلفة لتوفير المغذيات الضرورية لنخيل البلح صنف خلاص خلال المراحل المختلفة لإكثار النخيل . وتضمنت الدراسة تقييم بيئات WPM, NN , B5 , MS بقوي (كاملة ، نصف القوة ، ربع القوة) .

وقد أوضحت النتائج أن الإستجابات للبيئات المختلفة تختلف بإختلاف المرحلة .

ففي مرحلة تكون الكالس أدي إستخدام B5 بنصف القوة إلي زيادة نمو الكالس ، بينما أدي إستخدام WPM للحصول علي عدد من الأجنة الأولية .

و أدي إستخدام بيئة B5 للحصول علي أعلي معدل للأجنة الثانوية وأقل عدد من الأجنة الغير طبيعية في مرحلة التضاعف والإنبات ، بينما وجد أن NN الأكثر ملائمة لزيادة الأفرع .

وقد أدي إستخدام NN في مرحلة التجذير بربع القوي إلي زيادة تكون الجذور .

وقد أدي إستخدام WPM لزيادة طول الجذور .