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INDUCTION OF DIRECT SOMATIC EMBRYOS BY USING COMBINATIONS OF AUXINS, CYTOKININS AND ABA FROM SHOOT TIP EXPLANTS IN DATE PALM SIWY CULTIVAR.

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ABSTRACT

The aim of this study was to appreciate the effect of combinations of auxins, cytokinins and abscisic acid (ABA) on induction of direct somatic embryos of date palm (*Phoenix dactylifera* L.) cv. Siwy. The best combination for induction of somatic embryogenesis which contains 0.25 mg/l 2, 4- dichlorophenoxy acetic acid (2, 4-D), 0.25 mg/l α-Naphthalene acetic acid (NAA), 0.1 mg/l 6-(γ,γ-dimethylallylamino) purine (2iP), 0.1 mg/l 6-benzyl aminopurine (BAP). Germination of somatic embryos enhanced clearly when culture medium was contained the different concentrations of (ABA) in combination with both NAA and indole-3-butyric acid (IBA), each at concentration of 0.1 mg/l. The highest percentage of germination of direct somatic embryogenesis was obtained when culture medium was contained 0.1 mg/l NAA, 0.1 mg/l IBA and 0.4 mg/l ABA.

Key words: Abscisic acid (ABA), auxins, cytokinins, Date palms, direct somatic embryo, shoot tip.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is considered one of the most important commercial crops in North Africa, and the Middle East. The entire tree of date palm is utilized to provide food, shelter, fiber, furniture and many other products. Moreover, date palm tree successfully tolerates extremely adverse environmental conditions, including drought, high

temperature and salinity, which are the peculiar criteria of desert lands **(Chao and Krueger, 2007).** Propagation by both offshoots and tissue culture generally results in true-to-type plants, but some off-type with abnormal phenotypes have developed in tissue culture plants. These abnormalities may be due to somaclonal variation **(McCubbin *et al.*, 2004).**

Othmani *et al.* (2009) found that direct somatic embryos were formed on the base of young leaf explants when cultured on MS medium enriched with 10 mg/l 2, 4-D. **Sidky and Zaid (2011)** observed direct somatic embryo from the surface of explants without visible callus when treating shoot tip explants with thidiazuron (TDZ) alone at concentration 20 mg/l or with 2, 4-D (10 mg/l TDZ,10 mg/l 2,4-D). The world demand for date palm *in vitro*-plants has increased in recent decades. The use of all available techniques of rapid multiplication to fulfill this demand is an objective of great interest. Actually, there are few laboratories that use this technique to produce date palm *in vitro*-plants at the commercial level. Growth and morphogenesis are brought about by transfer of tissues to a specific inductive media where plant growth regulators play a significant role in determining their developmental direction **(Abhaman, 2011).** The aim of this study was to appreciate the effect of combinations of auxins, cytokinins and abscisic acid (ABA) on induction and germination of direct somatic embryos of date palm (*Phoenix dactylifera* L.) cv. Siwy.

MATERIALS AND METHODS

This work was carried out during the period 2012-2018 in the Central Laboratory of Date Palm Researches and Development (CLDRD), Agriculture Research Center (ARC), Giza, Egypt, and Plant Physiology Section, Faculty of Agriculture, Cairo University, Egypt.

The aim of this investigation was to study the influence of different calture conditions and chemical factors, i.e. auxins and cytokinins on the initiation, formation, of direct somatic embryos of semi dry cultivar (Siwy, shoot tip and leaf primordia explants) of date palm (*Phoenix dactylifera* L.).

Therefore, the experiments of the present work were divided into two parts.

1**-** Establishment of shoot tip and leaf primordia explants.

2- Induction of direct somatic embryos.

a- Plant material

The propagation process was started with the selection of healthy off-shoots from mother palm trees of semi dry cultivar, i.e. Siwy from Umm khenan, El hawamdeya Giza, Egypt. Shoot tips and leaf primordia explants were obtained from off-shoots (2-3 years old, 6-8 kg). The apical regions are surrounded by many layers of tough and fibrous leaf bases, which were removed with machete or hack-saw and knife. When the softer core tissues have been exposed, the young leaves primordia bases still surrounded the entire meristem palm heart, the shoot tip explant was soaked in running tap water for 1-2 minutes and incubated in an antioxidant solution containing 150 mg/l citric acid and 100 mg/l ascorbic acid.

b- Disinfection

Explants were surface sterilized under aseptic conditions by using 70 % ethanol for 1 min. One outer leaves were removed, then transferred to surface sterilization by soaking in 20 % of commercial Clorox® (5.25 % sodium hypochlorite) (1.05 % sodium hypochlorite per liter) supplemented with two drops of Tween-20 for 15 min under vacuum pump (Fig.A), thoroughly washed with sterilized distilled water and then the outer soft leaves were removed. The leaves primordia were taken to obtain the apical meristem with their 4–6 leaf primordia left. Then put in 0.1% mercuric chloride for 20 min (Fig. B), explants were washed with sterilized distilled water for two times and stored in an antioxidant solution of 150 mg/l citric acid and 100 mg/l ascorbic acid (Fig. C), the sterilized explants were cultured on control medium under aseptic conditions. After 4 weeks, explants succeeded free-contamination cultured for different treatments in the further experiments.

Fig. (A)Vacuum pump Fig. (B) shoot tip explant Fig. (c) explants in antioxidant

c- Culture medium

MS basal medium **(Murashige and Skooge, 1962)** was used through this study. However, MS medium was modified by the addition of biotin 0.2 mg/l, and adenine sulfate 40 mg/l, 30 g/l sucrose was added and the acidity of the final medium was adjusted to pH 5.7 ± 0.1 prior to adding the agar (6 g/l) to solidify culture medium. Each culture jar (250 ml) was supplied with 45 ml of MS culture medium and the jars were capped with polypropylene closures and autoclaved at 121 °C at 1.05 kg/cm^2 for 20 min. Cultured jars were incubated at 27±1°C in darkness for 24 weeks. Cultures were transferred to fresh medium every 6-8 weeks.

In this studied the visual characteristics at the end of each studied stage were recorded from all studied treatments during both parts are visually scored according to scale proposed by **Bottino (1981)** as follows:

Number	Results
1	Low results (+)
2	Average results (++)
3	Good results (+++)
4	Very good results (++++)

d- Establishment stage for direct embryo induction

The sterilized shoot-tip and leaf primordial explants were cultured on MS basal medium supplemented with, 170 mg/l $NaH_2PO_4.2H_2O$, 200 mg/l glutamine, and 0.25 g/l activated charcoal. Twelve treatments (S1-S12) of hormonal combinations as well as control were studied. Each culture medium treatment contained two auxins, i.e. 2, 4-D and NAA and two cytokinins, i.e. 2ip and BAP. Four concentrations of either 2, 4-D or NAA, i.e. 0.25, 0.5, 1.0 and 2 mg/l were applied. Meanwhile, three concentration of either 2ip or BAP, i.e. 0.1, 0.5 and 2.5 mg/l were added to the culture medium as tabulated in **Table (1).**

Table (1) Different concentrations of auxins (2, 4-D and NAA) and cytokinins (2ip and BAP) in the induction of direct embryo experiment of date palm explant (Siwy cv.), after three subculture (24 weeks).

Treatments	mg/l				swelling	Browning	Embryo
	2,4-D	NAA	2ip	BAP			
control	0.01	0.01	0.01	0.01	1.33	2.67 ab	1.00 d
S1	0.25	0.25	0.1	0.1	1.67	1.33 b	3.33 a
S2	0.25	0.25	0.5	0.5	2.00	1.33 b	2.00 b-d
S3	0.25	0.25	2.5	2.5	2.00	1.67 b	1.67 b-d
S4	0.5	0.5	0.1	0.1	2.33	1.67 b	2.67 ab
S5	0.5	0.5	0.5	0.5	2.00	1.67 b	2.33 a-c
S6	0.5	0.5	2.5	2.5	2.33	1.33 b	2.00 b-d
S7	1.0	1.0	0.1	0.1	1.33	1.67 b	1.67 b-d
S8	1.0	1.0	0.5	0.5	1.33	2.00 ab	1.33 cd
S9	1.0	1.0	2.5	2.5	1.33	2.00 ab	1.00 d
S10	2.0	2.0	0.1	0.1	1.67	2.33 ab	1.00 d
S11	2.0	2.0	0.5	0.5	1.33	2.67 ab	1.00 d
S12	2.0	2.0	2.5	2.5	2.33	3.33 a	1.00 d
					N.S	1.455	1.054

L.S.D. (0.05)

RESULTS AND DISCUSSION

Direct somatic embryogenesis from shoot tip explants is requiring specific balance of different kind of auxin and cytokinin. Data presented in **Table (1)** showed that the highest significant value of somatic embryos initiation directly from shoot tip explants is (3.33) (Fig. E), data clearly indicated that the best combination from plant growth regulators was obtained when 2, 4-D and NAA at 0.25 mg/l and 2ip, BAP at 0.1 mg/l. These data are insure the theory that direct embryo require balance ratio between auxins / cytokinins.

Data presented in **Table (1)** showed that no significant effect of different concentrations of auxins and cytokinins on degree of swelling, finally, degree of browning has a significant increasing effect and reach to high value (3.33) when the different kinds of auxins (2,4-D and NAA) added to culture media at concentration 2.0 mg/l for each and cytokinins (2ip and BAP) added at concentration 2.5 mg/l .These data insure the

concept of wounding during preparation and culturing the isolated shoot tip explants on culture media that contained high concentrations of artificial auxin and cytokinins represent a clearly oxidative stress. Oxidative stress triggers the phenols exudates, as a result of formation of quinones and finally may cause death of explant.

Fig. (D). Swelling stage

Fig. (E). Induction of direct embryos

Data presented in **Table (2)** showed the effect of plant growth regulators on formation and development of direct somatic embryogenesis when the cultured explants were transferred for another 24 weeks (totally 48 weeks from establishment stage). In this stage, NOA was used instead of NAA, direct somatic embryogenesis increased significantly (3.667) when cultured explants were placed on medium supplemented with 2, 4-D and NOA at 0.25 mg/l for each of them and 2iP, BAP at 0.1 mg/l **(Fig. F).** Increasing the concentrations of auxins (2, 4-D, NOA) to 1 mg/l and cytokinins (2ip, BAP) to 2.5 mg/l caused significantly decreased the formation of direct somatic embryos (1). Also, the same trend was obtained when auxins and cytokinins were added to the culture medium at 2 mg/l and 0.5 mg/l, respectively. Our obtained data showed that the direct somatic embryos could be induced at low levels of auxins and cytokinins. Also, balance between auxins/ cytokinins play an important role for inducing direct somatic embryos. Callus formation was noticed also in this stage, when the auxins (2, 4-D & NOA) were added to the culture media at 1 mg/l and cytokinins (2iP & BAP) at 0.1 mg/l, the callus formation value was increased (3.00). Degree of vitrification and browning were estimated during the present experiment

both of them recorded their highest significant degrees (3.33 and 2.66, respectively) when concentrations of auxins reach up to 2 mg/l and cytokinins reach up to 2.5 mg/l. These results agree with observations which clearly recorded using high levels of PGR induced oxidative stress as well as vitrification. Oxidation of phenols to quinones may result reduce in lignification of cell wall and increasing vitrification.

Table (2): Different concentrations of auxins (NOA and NAA) and cytokinins (2ip and BAP) in the formation of direct embryo experiment of date palm explant (Siwy cv.), after three subculture (48 weeks).

Treatments	mg/l				vitrification	Browning	Callus	D.Embryo
	2,4-D	NOA	2ip	BAP				
control	0.01	0.01	0.01	0.01	1.00 d	2.33 ab	2.00 ab	1.33 de
F1	0.25	0.25	0.1	0.1	1.00 d	1.67 bc	1.33 b	3.67 a
F2	0.25	0.25	0.5	0.5	1.33 cd	1.33 c	1.33 b	3.00 ab
F3	0.25	0.25	2.5	2.5	2.00 bc	1.00 c	1.33 b	2.67 a-c
F4	0.5	0.5	0.1	0.1	1.00 d	1.33 c	1.67 b	2.33 b-d
F5	0.5	0.5	0.5	0.5	1.00 d	1.33 c	2.00 ab	2.00 b-e
F6	0.5	0.5	2.5	2.5	1.67 b-d	1.67 bc	2.33 ab	1.67 c-e
F7	1.0	1.0	0.1	0.1	1.00 d	1.67 bc	3.00 a	1.00 e
F8	1.0	1.0	0.5	0.5	1.33 cd	1.67 bc	2.00 ab	1.67 c-e
F9	1.0	1.0	2.5	2.5	2.33 b	1.67 bc	1.33 b	1.00 e
F10	2.0	2.0	0.1	0.1	1.67 b-d	1.33 c	1.67 b	1.33 de
F11	2.0	2.0	0.5	0.5	2.00 bc	1.67 bc	1.67 b	1.33 de
F12	2.0	2.0	2.5	2.5	3.33 a	2.67 a	2.33 ab	1.00 e
					0.9607	**0.997**	**1.184**	**1.015**

L.S.D. (0.05)

Data presented in **Table (3)** showed that used auxins (IBA and NAA), and ABA for germination of somatic embryos. The results of this experiment demonstrate that direct somatic embryos (DSE) fully developed when culture medium contain auxins (IBA & NAA) at 0.1 mg/l and ABA at 0.4 mg/l **(Fig. G).** This may refer to the pivotal role of ABA in improving the quality and maturation of somatic embryos. It well known that ABA inhibits precocious germination of (DSE) but enhancing

the development of somatic embryos. The higher concentration of ABA (0.9 and 1.0 mg/l) reduced the maturation of somatic embryos. These results are in accordance with those obtain by **Saftner and Wyse (1984); Salajova *et al.* (1999); Corredoira (2003).** Somatic embryos maturation and germination depends on several factors and plantlet regeneration from immature somatic embryos is a result of the interaction of ABA to culture media greatly improved somatic embryo maturation and germination at present ABA is widely used to stimulate the maturation process ABA may involve in regulation of carbohydrate metabolism. ABA may enhance embryogenesis by promoting sucrose uptake from the medium, protein synthesis and protein accumulation patterns.

Fernando and Gamage (2000) obtained a large number of somatic embryos developed on media containing ABA (2.5 - 5 µM) formed normal shoot and complete plants as compared to those produced in the media with low levels of 2, 4-D, different concentrations of ABA have been used to different plant tissues and have been shown to be able to induce gene expression and protein synthesis.

Table (3) Different concentrations of abscisic acid (ABA) in combination with both NAA and IBA, each at concentration of 0.1 mg/l on germination of embryos of date palm explant (Siwy cv.), after two subculture.

Treatments	mg/l			vitrification	Browning	embryo
	NAA	IBA	ABA			
control	0.1	0.1	0.00	1.33 c	2.67 a	1.33 cd
M1			0.1	1.00 c	2.33 ab	1.67 cd
M2			0.2	1.33 c	1.33 bc	3.00 ab
M3			0.3	1.33 c	1.00 c	3.33 ab
M4			0.4	1.00 c	1.33 bc	3.67 a
M5			0.5	1.00 c	1.33 bc	2.33 bc
M6			0.6	1.67 bc	2.33 ab	2.33 bc
M7			0.7	1.00 c	2.00 a-c	1.67 cd
M8			0.8	1.33 c	2.00 a-c	1.33 cd
M9			0.9	2.33 ab	1.67 a-c	1.00 d
M10			1	2.67 a	2.00 a-c	1.00 d
				0.9422	1.201	1.16

L.S.D. (0.05)

Fig. **(F) Multiplication of direct somatic embryos**

Fig. **(G) Germination of embryo**

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استحداث الاجنة الجسدية المباشرة باستخدام التفاعلات بين الاوكسينات والسيتوكينينات وحمض الابسيسك من القمة النامية لنخيل البلح صنف سيوي

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تهدف هذه الدراسة لمعرفة تأثير بعض الأكسينات والسيتوكنينات وحمض الابسيسك علي استحداث الاجنة الجسدية المباشرة من القمم النامية لنخيل البلح صنف سيوي (من الاصناف النصف جافة). تشير النتائج الي ان افضل التداخلات لاستحداث الاجنة التي احتوت علي 0.25 مليجرام /لتر 2,4 – داي كلوروفينوكسي اسيتك اسيد بالاضافة الي 0.25 مليجرام /لتر الفا- نفثالين اسيتك اسيد و تركيزات 0.1 مليجرام /لتر 6جاما داي ميثيل امينوبيورين و 0.1 مليجرام/لتر بنزيل امينو بيورين هذا بالاضافة الي التركيزات المختلفة من حمض الابسيسك (0.1 الي 1مليجرام /لتر) بالتداخل مع 0.1 مليجرام/لتر لكلا من الفا- نفثالين اسيتك اسيد ومركب اندول 3 بيوتريك اسيد علي نمو وتطور الاجنة وكانت افضل النتائج لنسبة الاجنة النامية عندما احتوي الوسط الغذائي علي 0.1 مليجرام / لتر الفا- نفثالين اسيتك اسيد و 0.1 مليجرام /لتر اندول 3 بيوتريك اسيد بالاضافة الي 0.4 مليجرام / لتر حمض الابسيسك.

الكلمات الدالة : نخيل البلح ، حمض الابسيسك ، القمة النامية ، الاجنة الجسدية المباشرة ، الاوكسينات و السيتوكينينات