OPTIMIZATION PROTOCOL FOR *IN VITRO* ROOTING OF DATE PALM (*Phoenix dactylifera* L.)

REHAB A. SIDKY, ZEINAB E. ZAID AND ABDEL – MONEEM EL- BANA

The Central Laboratory for Date Palm Research and Development, ARC, Giza

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Abstract

A successful tissue culture regime for date palm is determined by the ability of producing healthy *in vitro* plantlets capable of surviving for acclimatization stage which normally requires a period of incubation in the laboratory. An improved protocol involving paclobutrazol (PBZ) and polyethylene glycol (PEG) has been developed to achieve rooting improvement. Plantlets of date palm cv. Samani, derived from direct somatic embryogenesis, were investigated in two separate experiments. In the first experiment, Plantlets were cultured on 1/2 MS solid medium containing 0.1 mg/l NAA+ 0.1 mg/l BA + 0.5 g/l activated charcoal as a basal nutrient medium supplemented with PBZ at 2, 4, 6 and 8 mg/l combined with sucrose at 30, 40 and 50 g/l. In the second experiment, explants were cultured on 1/2 MS medium+40 g/l sucrose+0.1 mg/l NAA+ 0.5 g/l activated charcoal supplemented with PEG at 0, 2, 4, 6 and 8 g/l. All cultures were incubated under a 16h photoperiod of 4000 lux light intensity at a temperature of 27± 2 °C. The data revealed that, medium contained 4 mg/l PBZ combined with 40 or 50 g/l sucrose increased the thickness of plantlets, also accelerated the root formation and elongation and promoted secondary roots. Addition of PEG at 6 or 8 g/l to the culture medium enhanced plantlets survival during acclimatization stage. Histological study showed that PEG at high concentrations increased the wax deposition on epidermal layer than control plants.

INTRODUCTION

Date palm is a member of the family Palmae (Arecaceae), with tropical and subtropical habitats. Because most of the arable lands are desert, the date palm is the major plantation crop in it. In Saudi Arabia, for example, date palm trees are grown on about 90% of the cultivated land **.**

Date palm is considered one of the most important commercial crops in the Arab world. An important problem encountered in date palm plantlets production is the rooting of the shoots which able to be successfully transplanting, also there is no detailed information on the optimum conditions or factors affecting rooting in date palm plantlets *in vitro* and their survival when transplanting *ex vitro* (Ibrahim *et al.,* 1999). On the other hand, the process of acclimatization can begin while plantlets are still *in vitro*.

Paclobutrazol is one of the number of triazoles that enhance tolerance to various environmental stresses in several plant species (Fletcher *et al.,* 2000),e.g., the relative water loss from loss detached leaves of *in vitro* grown plantlets can be reduced by application of paclobutrazol , or 6-benzyl-aminopurine(Eliasson *et al*., 1994). Triazoles act as GA-biosynthesis- inhibitors and fungicides by blocking cytochrome P450-mediated oxidation reactions Fletcher *et al.,* 2000). In higher plants the triazoles reduce growth by interfering with the conversion of ent-kourene to ent-kourenoic acid, an early step in GA biosynthesis (Rademacher, 2000). Triazole application also triggers other hormonal changes including an increase in cytokinins, a transient rise in abscisic acid (ABA), and reduction in ethylene levels (Fletcher and Hofstra, 1988).

These studies involved the effect of Paclobutrazol as plant growth retardant to accelerate root formation and increase thickness of plantlet.

Transfer of date palm plantlets derived from *in vitro* cultures to the permanent soil is a critical factor limiting the success of such technique. Shaheen (1990), mentioned that, the factors affecting the successful production of free-living date palm, including length of plantlets, strength of the root system, humidity conditions, number of leaves and composition of the soil media.

PEG has been used as an osmotic to decrease the water potential of culture solutions also, PEG has only recently been used *in vitro*, to reduce desiccation and death *ex vitro* **.** Zaid and Hughes(b) 1995 found that inclusion of polyethylene glycol "PEG" in the culture media of *in vitro* micropropagated palm offshoots prior to transfer to soil enhanced the percentage of survival during the acclimatization stage. They pointed to the possibility of using PEG as an osmotic regulator during *in vitro* acclimatization of plants before transfer to soil.

The objective of the present study was to obtain healthy full plantlets derived via *in vitro* cultures with high potential to success in transplanting to the *ex vitro* conditions for best acclimatization process.

MATERIALS AND METHODS

The experimental work was performed at Central Laboratory for Date Palm. Researches and Development, Agriculture Research Center, Cairo, Egypt.

Plant material and culture conditions:

Shoot tips of date palm (*Phoenix dactylifera* L.) cv. Samani were cultured as described by Sidky (2004). One year old *in vitro* cultured plantlets "which derived from direct somatic embryogenesis" with two expanded leaves and an adequate root system (2 to 3 roots) were used for this study. The cultures were incubated under

a 16-hour at light intensity of 4000 lux with temperature of 27± 2 °C. Each treatment consisted of 9 replicates of one plant per jar for evaluation of two experiments. The experiments were carried out using completely randomized design and treatment was replicated two times. The results were analyzed using analysis of variance and the means compared using L.S.D test at 5% according to Steel and Torrie (1980).

Experiment (1): Effect of sucrose and PBZ:

Plantlets were cultured on 1/2 MS medium (Murashige and Skoog, 1962) supplemented with (in mg 1^{-1}), 0.1 Naphthalene acetic acid (NAA); 0.1 Benzylamino purine (BA) ; 500 activated charcoal (AC) ; 6000.0 agar (phytotechnology) and different combinations of Paclobutrazol (PBZ) at (2.0, 4.0 , 6.0 and 8.0)mg\L with sucrose (30, 40, and 50 g/L) were used as different treatments. Data were determined after 12 weeks (after two recultures) the following data were recorded:

1. Roots number/ plantlet.
2. Roots length/ plantlet (cm).
3. Plantlet length (cm).
4. Thickness of trunk. Data of trunk thickness was expressed visually as scores according to Pottino (1981).

a- Negative results = 1
b- Below average results = 2
c- Average results = 3
d- Good results = 4

Experiment (2): Effect of different concentrations of "PEG" through pre-acclimatization.

Plantlets were cultured for 4 weeks on 1/2 MS medium supplemented with (in mg 1^{-1}): 0.1 Naphthalene acetic acid (NAA); 500 activated charcoal (A.C) ; 6000.0 agar (phytotechnology); 3000.0 sucrose and different concentrations of "PEG" 8000 (molecular weight of 7000- 9000) at (0, 2.0, 4.0 , 6.0 and 8.0 g/l). The following data were recorded:

1. Plant length (cm).
2. Roots number/ plantlet.
3. Roots length/plantlet (cm).
4. Width of leaf.(was expressed visually as scores according to Pottino 1981)

Acclimatization stage:

The plantlets were washed in tap water and transferred to greenhouse for acclimatization on plastic pots (30 cm in diameter) containing mixture of peatmoss,vermiculite and perlite (1:1:1) Each treatment consisted of 9 replicates of one plant per pot, covered with a plastic cap that was gradually opened during the

acclimatization period of 3 months. The survival percentage of plantlets under investigation was recorded.

Histological study:

The wax deposits on leaf surfaces of *in vitro* plantlets were quantified after PEG treatment by the end of the period (4 weeks), taking 1 sample per jar and 9 jar per variant. The paraffin sections of plantlets were prepared essentially according to Johansen (1940). Briefly, samples were fixed with 50% formaldehyde/acetic acid/ethanol/water (1:1:9:9, v/v/v/v) for at least 48 h. After washing with 50% ethanol, the samples were dehydrated gradually in an ethanol-butanol series, and infiltrated with paraffin. Serial longitudinal sections of 12 µm were cut by a rotary microtome. Sections were stained by crystal violet/erythrosine combination and mounted in Canada balsam. Observation was made using a microscope.

RESULTS AND DISCUSSION

Effect of sucrose and PBZ on rooting:

The aim of this study was to obtain the best thickness of trunk, high plant length and good root formation using sucrose combined with PBZ to increase the survival percentage during acclimatization stage.

Sucrose at 30, 40, and 50 g/l were added to MS medium applied to the culture. The results in Table (1) revealed that only the usage of 40 and 50 g/l sucrose significantly increased the root number per plantlet (2.66, 2.44 respectively). Observing trunk thickness, the same effect was obtained, It was increased from (1.74) at 30 g/l to (2.44) by 40 g/l (2.83) using 50 g/l with no significant difference between the two concentrations (40 or 50 g/l).

Regarding root length per plantlet, no significant difference was observed within 30 to 50 g/l sucrose. On the other hand, the application of 40 g/L sucrose gave the highest significant value of plantlet length (6.41 cm), followed by 50 g/l sucrose (5.74 cm). Similar results were reported by Ibrahim *et al.,* (1999) who mentioned that 50 g/l sucrose is considered the optimum concentration. Since energy requirements of *in vitro* plants are substantially covered by the sucrose taken up from the medium. Also, Chenevard *et al.,*(1995) suggested that the assimilates produced by photosynthesis are not sufficient to meet the energy requirements of the root primordial which are very high for the initiation and development of the organ of hybrid Walnut. The role of sucrose in rooting is more closely linked to the energy supplies than to its osmotic properties .

Regarding the influence of PBZ on rooting stage, the purpose of this study improved the trunk thickness of plantlets, accelerated root formation and increased

root branching (secondary roots) for successful transplanting. Thus, using 4 mg/L PBZ was more effective to increase all parameters on rooting stage. On the other hand, the interaction between sucrose and PBZ was significant for plantlet length. Medium contained 40 g/l sucrose and 4 mg/l PBZ stimulated plantlet length. Also, the interaction between 40 g/l sucrose and 4 mg/l PBZ gave the highest mean value of root number, root length and trunk thickness.

The results are in line with Abd El-Baky (2006) who mentioned that growth retardants promoted the initiation of short and strong roots and increased the number of fine roots, Zaid (2003) reported Paclobutrazol encourage the growth and development of adventitious roots and shoots formation and also increased trunk diameter. Furthermore, Vidom and Frez (1995) reported that, row paclobutrazol levels applied to the roots suppressed shoot and root development, but to a different degrees, A much stronger retarding effect was observed on the shoot , with very limited effect of the roots.

Generally, we can conclude that, culturing the plantlets onto nutrient medium containing 40 or 50 g/l sucrose + 4 mg/l PBZ increased trunk thickness, accelerated root formation and increased root branching (secondary roots)(Fig.1). Our observation also indicated that PBZ stimulated root length and increased greenish of plantlets.

Effect of PEG concentration on growth of plantlets through pre-acclimatization:

This is attempting to improve plantlets by application of high molecular mass osmotic, PEG 8000 on the culture media before transplanting to soil, and study this effect on plantlets response through acclimatization.

Table (2) showed that, addition of PEG to rooting medium did not affect plantlet length and as well as no significant difference between PEG at different concentrations was observed. While, number of roots and root length per plantlet (cm) were significantly improved by adding 6 g/l PEG compared with medium without PEG. However, the leaf width of plantlets tend to expansion in wide when PEG concentrations increased, the leaf width significantly increased by adding 6 or 8 g/l PEG on rooting medium (4.00, 3.66 respectively) compared with medium without PEG. This observation is similar to that of Aboul-Soad *et al.*, (2006) who reported that the leaf width tends to expansion in wide during elongation stage.

The effect of PEG on survival percentage of plantlets grown in greenhouse:

After 3 months of acclimatization, the percentage of survival increased gradually by raising PEG concentration compared to medium without PEG (Table 3). The highest survival percentage was observed by 6 or 8 g/l PEG (88.88 %). The ultimate success of plant tissue culture depends on transplanting the plantlets

successfully to soil with a high rate of survival. Ziv (1986) reported that about 50-to 90% of *in vitro* propagated plantlets of many species have been lost at the time of transfer to soil. Nesiem *et al.,* (2006) reported that adding PEG at the concentration of 10 g/l to modified MS medium during the second month of root formation, raised the plantlets survival to about 20-22%. Using PEG as a mean to acclimatize *in vitro* date palm plantlets and to reduce their subsequent desiccation and poor survival after its transfer *ex vitro* .PEG treatment reduced water loss of *in vitro* plantlets to levels similar to those of greenhouse (Zaid and Hughes (b), 1995)

Histological study:

An anatomical study performed on plantlets cultures after 4 weeks on rooting medium containing different concentrations of PEG 8000 revealed that , cultures were treated with PEG concentrations were more deposits of wax on epidermal layer than control plants. Increasing the level of polyethylene glycol concentrations led to increase in wax deposition on epidermal layer (Fig.2). These results are confirmed with (Zaid and Hughes (a), 1995) the increase in wax deposition as a result of polyethylene glycol treatment explains the decreased water loss observed in acclimatized *in vitro* plantlets when transferred *ex vitro*. Meanwhile, Ziv (1986) found that reduction of the relative humidity (RH) in the culture medium through use of desiccants increased wax deposition in carnation.

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Table1. Effect of sucrose and Paclobutrazol on rooting stage of date palm cv. Samani , after two recultures.

(A)	(B) Paclobutrazol concentration (PBZ) mg/l																			
Sucrose	No. of roots					Root length (cm)					Plantlet length (cm)					Thickness of trunk				
g/l	2	4	6	8	mean	2	4	6	8	mean	2	4	6	8	mean	2	4	6	8	mean
30	2.00	2.00	2.00	2.33	2.08	2.50	2.66	2.66	2.33	2.53	5.66	6.00	5.33	3.83	5.20	1.66	1.66	1.66	2.00	1.74
40	2.33	3.33	3.00	2.00	2.66	2.66	3.66	3.00	3.00	3.08	6.33	8.33	6.00	5.00	6.41	2.33	3.66	2.66	3.33	2.44
50	2.33	3.00	2.66	2.00	2.44	2.16	3.16	2.66	2.66	2.66	6.00	7.00	5.33	4.66	5.74	2.00	3.66	2.66	3.00	2.83
mean	2.22	2.77	2.55	2.11		2.44	3.16	2.77	2.66		5.99	7.11	5.55	4.49		1.99	2.99	2.32	2.77	

Mean separation by L.S.D at 0.05

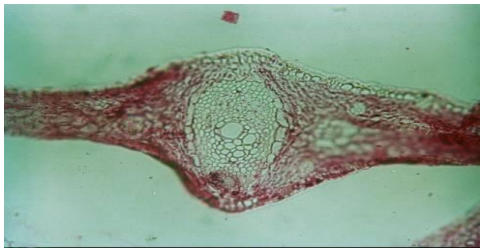
A:	0.35	N.S	0.16	0.43
B:	0.41	0.37	0.97	0.50
AB:	N.S	N.S	0.33	N.S

Table 2. Effect of different concentrations of polyethylene glycol "PEG" on plantlets through pre-acclimatization after 4 weeks.

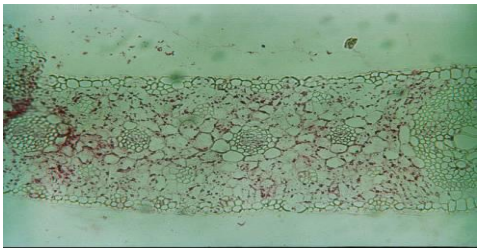
PEG Concentration g/L	Plantlet length (cm)	No. of Roots	Root length (cm)	Width of leaf
0.0	11.33	2.33	3.16	2.00
2.0	11.00	2.66	3.00	2.66
4.0	9.66	3.00	4.00	3.33
6.0	10.66	4.33	5.16	4.00
8.0	10.00	3.33	4.33	3.66
Mean	10.53	3.13	3.93	3.13
Mean separation by L.S.D at 0.05	N.S	1.33	1.23	0.95

Table 3. Effect of different concentration of "PEG" on survival percentage of plantlets grown in greenhouse.

PEG concentration g/l	0.0	2.0	4.0	6.0	8.0
Survival percentage %	55.55	66.66	77.77	88.88	88.88



(A)



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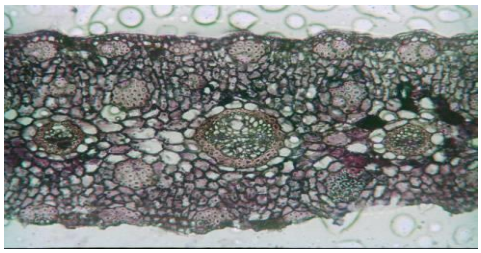

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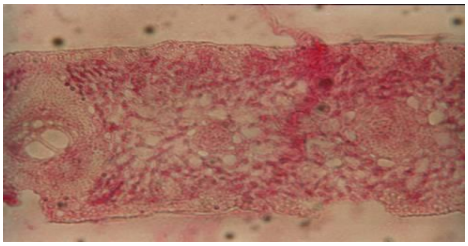

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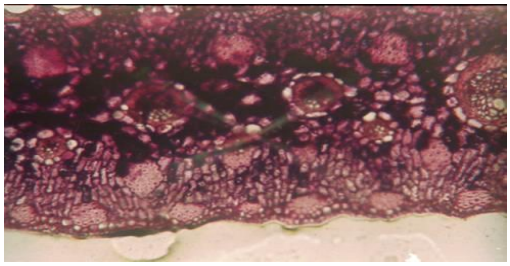

(E)

Fig 1. Anatomical features of leaflets for *in vitro* plantlets treated with different concentration of PEG. A (control), B (2.0g/l PEG), C (4.0g/l PEG), D (6.0g/l PEG), E (8.0g/l PEG).

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بروتوكول مثالى لمرحلة التجذير لنباتات نخيل البلح صنف سماني

رحاب أحمد صدقى، زينب السيد زايد ، عبد المنعم البنا

المعمل المركزى للأبحاث وتطوير نخيل البلح – مركز البحوث الزراعية – جيزة

نجاح إنتاج نباتات نخيل البلح عن طريق زراعة الأنسجة مرتبط بنجاح عملية الأقلمة لهذه النباتات لذا تم عمل بروتوكول باستخدام باكلوبترازول (PBZ) وبولى إثيلين جليكول (PEG) للحصول على أفضل النتائج فى مرحلة التجذير. زرعت النباتات التى تم الحصول عليها عن طريق إنتاج الأجنة الجسمية مباشرة وذلك من خلال تجربتين منفصلتين. التجربة الأولى: زرعت النباتات على نصف قوة أملاح موراشيجى وسكوج مضاف إليها تركيزات من باكلوبترازول (٢.٠، ٤.٠، ٦.٠، ٨.٠ ملجم/لتر) مع تركيزات من السكروز (٣٠، ٤٠،٥٠ جم/لتر). التجربة الثانية: زرعت النباتات على نصف قوة أملاح موراشيجى وسكوج مضاف إليها تركيزات مختلفة من بولى إثيلين جليكول (٠.٠، ٢.٠ ، ٤.٠، ٦.٠، ٨.٠ جم/لتر). سجلت النتائج أن ٤.٠ ملجم/لتر PBZ + ٤٠.٠ أو ٥٠.٠ جم/لتر سكروز أعطت زيادة فى سمك الجذع وكذلك كفاءة فى المجموع الجذرى وتشجيع تكون الجذور الثانوية .أظهر تشريح أوراق نباتات نخيل البلح زيادة فى ترسيب طبقة الشمع عند إضافة بولى إثيلين جليكول بمختلف تركيزاته الى البيئه.