

Alternative approach for micropropagation of the date palm c.v. Zaghlool

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ABSTRACT

A procedure for micropropagation of date palm c.v. Zaghlool, through somatic embryogenesis, was developed. Embryonic calli were obtained from shoot tip and primordial leaf explants using MS-medium supplemented with 10 mg/l 2,4-D + 3 mg/l 2iP. High frequency of somatic embryos were proliferated from embryonic calli derived from the two types of explants using MS-medium supplemented with 1 mg/l 2iP. 100% of the somatic embryos had differentiated to plantlets when recultured on hormone free MS-medium. The root system of plantlets had improved and a particular stage of adaptation was achieved by dipping the plantlets in tubes containing tap water. High frequencies of survival rates in free living conditions were obtained after transplanting and acclimatization using pots containing vermiculite and peat-moss(1:1). The SDS-PAGE protein profile analysis was carried out in the different stages of development of date palm plantlets.

INTRODUCTION

Date palm (*Phoenix dactylifera* L) is a dioecious fruit tree that is vegetatively propagated through offshoots. The number of offshoots produced by an individual date palm tree is low (1-20 offshoots in the lifetime). In addition, the number of offshoots established in the soil is low (30-80%) and is cultivar dependent. Therefore, development of micropropagation protocols of date palm is of importance. Several attempts were made based on organogenesis and somatic embryogenesis. Due to the long time requirements for the initiation phase and the low multiplication rate, organogenesis in date palm has a low efficiency (Poulain *et al.*, 1979; Beauchesne, 1982). Somatic embryogenesis has been achieved by several authors (Tisserat, 1982;

1991; Zaid and Tisserat, 1983; Sharma *et al.*, 1984, 1986; Mater, 1986; Bekheet *et al.*, 2001).

The SDS-PAGE protein profiles technique has been used for characterizing and identifying the capacity of embryonic callus and embryo cultures for morphogenesis. (Roberts *et al.*, 1989; Feirer and Simon, 1991; Saker, 1997). Also, characterization of polymorphic high MW glutenin subunits in *Triticum tauschii* by chromatography and electrophoresis were established by Shewry *et al.* (1992) and Gianibelli *et al.* (2001).

The objective of the present study is to develop an alternative method for micropropagation of date palm through indirect somatic embryogenesis, using shoot tip and primordial leaf as explants. Also, attempts to characterize and identify the

different stages of date palm development using SDS-PAGE analysis, were carried out.

MATERIALS AND METHODS

Tissue culture

Offshoots of date palm c.v. Zaghloul separated from adult trees were used as a plant material. The outer leaves were gradually removed till the tender portion was reached. The primordial leaf and apical meristem with sub-apical tissues were excised and kept in anti-oxidant solution (citric acid 150 mg/l). The explants were surface sterilized by immersion for 20 min in a 2.6 % sodium hypochlorite and rinsed three times with sterile distilled water. Shoot tips in 1.5 cm and pieces of internal leaves were cultured on MS-medium (Murashige and Skoog, 1962) supplemented with the following combinations of growth regulators: 1) 10 mg/l dichlorophenoxy acetic acid (2,4-D) + 3 mg/l N-(2-furanylmethyl)-purine-6-amino (Kinetin). 2) 10 mg/l 2,4-D + 3 mg/l α,α dimethyl amino purine (2iP). 3) 10 mg/l 2,4-D+3 mg/l 6-benzylamino purine (BA). The cultures were then incubated in dark conditions. After three subcultures on the same fresh media (five weeks interval), the percentage of callus induction, callus fresh and dry weight, dry matter contents and percentage of embryogenic callus were recorded.

To investigate the effect of supplementation of the culture medium by phytohormones on somatic embryogenesis, nodular and embryogenic cultures derived from the two types of explants (shoot tip and primordial leaf) were subcultured on the following media: 1) MS- hormone free, 2) MS + 1 mg/l 2iP and 3) MS + 1 mg/l BA. Number of embryo/culture, embryo fresh and dry weight and dry matter content were registered after five weeks of subculturing. Also,

differentiation of the obtained somatic embryos were examined. Percentage of embryo germination, plantlet fresh and dry weight and dry matter content were recorded after five weeks of culturing the embryos on the following media: 1) MS-hormone free; 2) MS+1mg/l 2iP and 3) MS + 1mg/l BA

To improve the root formation, the obtained plantlets were transferred into glass tubes containing only tap water. The cultures were then incubated at room temperature for four weeks. Plantlets with well developed root system were taken and washed with current tap water, then disinfected by immersion in a fungicide i.e. benlate solution (1g/l) for 20 min. Plantlets were then transplanted into pots contained vermiculite and peatmoss (1:1). The pots were covered with polyethylene bags and irrigated one time a week. The covers bags were gradually removed and then completely removed after five weeks of transplanting.

All culture media were solidified with 0.7 % agar and adjusted to pH 5.8 before autoclaving at 1.5 Kg/Cm² for 20 min. Cultures were incubated at 25 \pm 2°C in the dark conditions for callus induction and under 16 hr. photoperiod provided by white fluorescent lights for other treatments. Data were analyzed by L.S.D. according to Snedecor and Cochran (1967).

Electrophoresis for SDS-PAGE analysis

One gram of each embryonic callus, somatic embryos as well as in vitro derived plantlet leaves was taken from either shoot tip or primordial leaf explants. The collected materials were then extracted in NA-phosphate buffer, pH 6.8. Electrophoresis was performed according the method described by Laemmli (1970). The acrylamide percentage was 10% in the separating gel and 3% in the stacking gel. Protein samples (20-30 μ g) in a total volume of 16 μ l (8 μ l of sample in extraction buffer + 8 μ l denaturing buffer) were denatured for 3

min in a boiling water bath, cooled, and then centrifuged (15 µl was applied). EC mini gel unit of electrophoretic separation was used for 4 hr at 60 Volt. After electrophoresis the gel was stained with Coomassie brilliant blue (R-250) for over night. Then, the gel was destained 3 times with a methanol solution. The molecular weight of the polypeptide bands were calculated from a calibration curve of a low molecular weight marker kit of *Pharmacia* (Uppsala, Sweden).

RESULTS

Callus proliferation

In this experiment, the effect of three combinations of growth regulators on callus

induction from the two types of explants (shoot tip and primordial leaf) was investigated. Results presented in Table (1) show that the highest percentage of callus induction and embryonic callus were obtained with MS-medium containing 10 mg/l 2,4-D + 3 mg/l 2iP. Also, the best results of callus growth as fresh and dry weight and dry matter content were recorded with the same previous medium. Here it is important to note that the potential of callus induction and callus growth dynamics of shoot tip callus were higher than those of the primordial leaf explant (Table 1 and Fig. 1-A).

Table (1): Effect of different combinations of growth regulators on callus proliferation from shoot-tip and primordial leaf explants of date palm , *c.v. Zaghloul*.

Culture media	Callus induction(%)		Callus fresh weight (g)		Callus dry weight (g)		Dry matter content (%)		Embryonic callus (%)	
	Shoot tip	Primordial leaf	Shoot tip	Primordial leaf	Shoot tip	Primordial leaf	Shoot tip	Primordial leaf	Shoot tip	Primordial leaf
MS1	35	24	0.39	0.27	0.015	0.008	6.32	6.13	35	28
MS2	92	79	0.75	0.56	0.066	0.041	8.63	7.25	69	54
MS3	74	65	0.54	0.48	0.045	0.034	8.12	7.05	62	42
L.S.D _{0.05}	9.63		0.03		0.002		1.23		6.32	

Each treatment is the average of 15 replicates

Initial weight = 250 mg/gar

MS1= MS + 10 mg/l 2,4-D

MS2= MS + 10 mg/l 2,4-D + 3 mg/l 2iP

MS3= MS + 10 mg/l 2,4-D + 3 mg/l BA

Somatic embryogenesis

The influence of phytohormones on somatic embryo formation from embryogenic calli derived from the two types of explants are presented in Table (2) and Fig. (1-B), The results obtained reveal that the highest frequency of somatic embryos as well as the

highest values of their growth dynamics were noticed when hormone-free medium was used. Embryogenic callus derived from shoot tip explants gave the highest values of somatic embryogenesis and growth, compared with those derived from leaf primordial.

Table (2): Effect of growth regulators on somatic embryogenesis from calli of shoot-tip and primordial leaf explants of date palm, c.v. Zaghlool.

Culture Media	No. of embryos/culture		Embryos fresh weight (g)		Embryo dry weight (g)		Dry matter content (%)	
	Shoot tip	Primordial leaf	Shoot tip	Primordial leaf	Shoot tip	Primordial leaf	Shoot tip	Primordial leaf
MS1	24	18	0.175	0.112	0.008	0.007	4.520	6.120
MS2	75	64	0.435	0.325	0.032	0.024	7.630	7.120
MS3	54	48	0.323	0.236	0.025	0.017	7.450	6.950
LSD _{0.05}	4.32		0.053		0.002		1.420	

Each treatment is the average of 15 replicates

MS1= MS + 1 mg/l 2,4-D

MS2= MS + 1 mg/l 2iP

MS3= MS + 1 mg/l BA

1

2

1

2



A



B

Fig. (1): A) Callus proliferation from shoot-tip (1) and primordial leaf (2) explants cultured on MS-medium supplemented with 10 mg/l 2,4-D+ 3 mg/l 2iP. B) Somatic embryogenesis from shoot-tip (1) and primordial leaf (2) derived callus on MS-hormone-free medium.

Somatic embryo development

The effect of two different types of growth regulators in culture medium on differentiation of somatic embryos into plantlets were tested. Data in Table (3) and Fig. (2-B) show that the best results of embryo

germination (100%) were obtained with hormone-free MS-medium. Growth of the developed plantlets showed significant differences between somatic embryos derived from shoot tip and primordial leaf in their differentiation and development.

Table (3). Differentiation of somatic embryos of date palm affected by the type of culture media

Media	Embryos germination (%)		Fresh mass (g)		Dry mass (g)		Dry matter content (%)	
	Sh.t. derived S.E	Pr.l. derived S.E	D. Shoots (1)	D. Shoots (2)	D. Shoots (1)	D. Shoots (2)	D. Shoots (1)	D. Shoots (2)
MS1	100	100	9.32	7.35	0.85	0.83	9.15	8.59
MS2	94	93	6.34	5.45	0.54	0.53	8.63	7.85
MS3	46	40	2.11	1.12	0.18	0.08	7.94	2.74
LSD _{0.05}	1.32		0.83		0.02		0.54	

Each treatment is the average of 15 replicates

MS1= MS hormone-free

MS2= MS + 1 mg/l 2iP

MS3= MS + 1 mg/l BA

D.Shoots (1)= Differentiated shoots from shoot tip explants derive somatic embryos (S.E).

D.Shoots (2)= Differentiated shoots from primordial leaf explants derive somatic embryos (S.E).

1

2



A



B

Fig. (2): A) Plantlets formation from somatic embryos derived from shoot-tip (1) and (2) leaf primordial. B) Plantlets with good root system after four weeks from transferring in tubes containing only tap water.

Improvement of root formation and acclimatization

The roots of plantlets proliferated from somatic embryos were weak thin, and rarely survive when transferred into the soil. To solve this problem, plantlets were transferred into glass tubes containing tap water before acclimatization. New roots began to appear within two weeks. After four weeks, vigorous

and healthy root system was formed (Fig. 2-B).

High frequencies of survival rates were obtained after five weeks of transplanting the plantlets (with well developed root system) to pots containing vermiculite and peat-moss 1:1 (Fig. 3).



Fig. (3): Adapted plantlets after six months of transplanting in pots containing vermiculite and peat-moss (1:1).

SDS-PAGE protein patterns

The of SDS-PAGE protein patterns as biochemical markers to distinguish the differences among the maintained cultures of embryonic calli, embryos and leaflets of *in vitro* regenerated plantlets are illustrate in Fig. (4). They demonstrate three polypeptide bands (52.8, 55.6 and 61 kDa) newly expressed in different cultures derived from either shoot tip or primordial leaf. Moreover, these are four newly polypeptide bands (28,38,79.5 and 88.6 kDa) expressed in both regenerated leaflets of derived plantlets from the two types of explants.

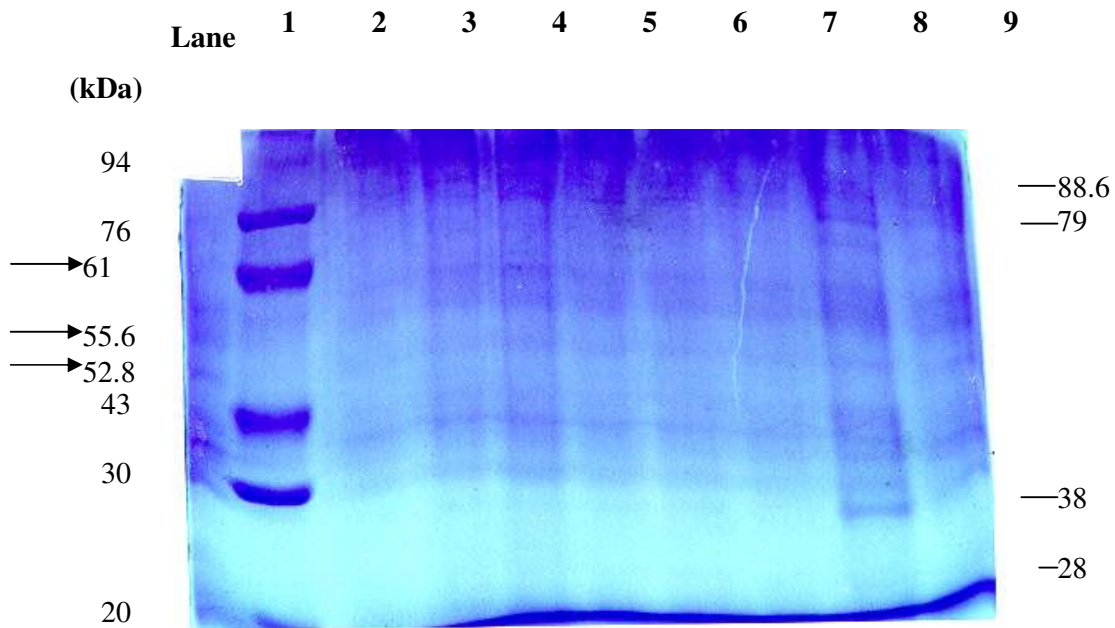


Fig. (4): Protein profiles of different culture types of date palm c.v. Zaghloom. Low molecular weight standard marker (lane 1) embryonic callus derived from shoot tip (lane 2) or primordial leaf (lane 3); somatic embryos derived from shoot tip (lane 4) or primordial leaf (lane 5); *In vitro* leaflets derived plantlets from shoot tip (lane 6) or primordial leaf (lane 7) and *in vivo* leaflets derived plantlets from shoot tip (lane 8) or primordial leaf (lane 9).

DISCUSSION

From the present results, it is clear that calli were obtained from the two types of explants. However, shoot tip explants gave the highest values of callus frequency compared with the primordial leaf. This may be due to its meristematic tissues. In this respect, shoot tip explant of date palm has been successfully used for callus induction by several authors (Tisserat, 1979; Zaid and Tisserat, 1983; Sharma *et al.*, 1984; El-Kazzaz and El-Bahr, 2001). Concerning the effect of phytohormones on callus proliferation, data revealed that addition of 2,4-D at 10 mg/l enhanced callus induction from the two types of explants. These results are in line with those obtained by several researchers (Mater, 1986, 1987; Mattar, 1986; Azra *et al.* 1997; Madhuri *et al.*; 1998). They obtained nodular and embryogenic callus cultures of date palm by culturing shoot tips, leaf sheaths and meristematic tissues on medium containing 2,4-D.

The date palm was found to be capable of plantlet regeneration from several tissues tested. In this study the best results of embryogenesis of the two types of embryogenic calli were obtained by culturing on hormone-free medium. These embryos were recultured on the same medium for germination and subsequent growth and development to plantlets. The results are in accordance with those reported by many authors (Sharma *et al.*, 1984; Kackar *et al.*, 1989; Vermendi and Navarro, 1996; Bekheet *et al.*, 2001). They mentioned that somatic embryos of date palm could be obtained in a large number using medium without plant growth regulators. Madhuri *et al.* (1998) used medium containing adenine sulfate for further development of embryos to plantlets and kept them under high light intensity (6000 lux).

One of the most difficult steps in tissue cultures of date palm is the transplanting of plantlets from the aseptic cultures to the free environmental conditions. In our study, a high frequency of survival rate was obtained. The successful transplanting may be due to the strong and the healthy root system formed during the pre-adaptation stage. In this connection, Ziv (1979) pointed out the critical effect of the root system on the subsequent acclimatization.

Some investigations were carried out to study the relationship between the biochemical status of the *in vitro* cultures and their ability for regeneration (Gyorgyey *et al.*, 1991; Egertsdotter *et al.*, 1993). On the other hand Feirer and Simon (1991) reported that SDS-PAGE protein profiles were not sufficient to distinguish between calli and embryos formation. In this investigation, the variations in SDS-PAGE protein patterns revealed that the novel expression of medium molecular weight (52.8, 55.6 and 61 kDa) proteins may be due to the development of derived undifferentiated calli stage to embryonic calli stage. However, the lower molecular weight proteins (28 and 38 kDa) and the newly embryos molecular weight proteins (79.5 and 88.6 kDa) may be attributed to the successful development to plantlets. Similarly, Gyorgyey *et al.* (1991) reported that the novel (18 kDa) protein was detected in the embryogenic stage of alfalfa. Also, in embryonic cultures of carrot, a novel protein bands (54, 47, 38, 32, and 10 kDa) have been detected by Hendriks and Varies (1995). Similarly, Saker (1995; 1997) has detected (110 and 85 kDa) proteins in embryos of pea derived from immature cotyledon explants and (112, 58 and 30 kDa) proteins in *in vitro* regenerated plantlets of onion through somatic embryogenesis.

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المخلص العربي

تقنية بديلة لإكثار نخيل البلح صنف زغلول باستخدام زراعة الأنسجة

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في هذا البحث تم تطوير طريقة للإكثار المعلمي الدقيق لنخيل البلح "صنف زغلول" من خلال الأجنة الجسدية، حيث تم الحصول على الكالوس الجنيني من البادنتين النباتيين (explants) من القمة النامية والورقة الأولية بزراعتها على بيئة MS مضافا إليها 10 ملليجرام/ ليتر 2,4-D + 3 ملليجرام/ ليتر 2iP . تم الحصول على معدل مرتفع من الأجنة الجسدية وذلك بزراعة الكالوس الجنيني الناتج من البادنتين النباتيين على بيئة MS مضافا إليها 1 ملليجرام/ ليتر 2iP . وتطورت الأجنة الجسدية إلى نباتات صغيرة بنسبة 100% و ذلك بإعادة زراعة الأجنة على بيئة الأساس MS (خالية من الهرمونات) ، تم تحسين المجموع الجذري للنباتات (مرحلة أقلمة أولية) بزراعة النباتات في أنابيب تحتوي على الماء، تم الحصول على معدل مرتفع من النباتات التي نمت بنجاح في البيئة الطبيعية بعد زراعتها للأقلمة في أصص تحتوي على فرميكيولايت وبيت موس بنسبة 1:1 ، في البحث درس أيضا تحليل البروتين SDS-PAGE في المراحل المختلفة لتطور نمو نخيل البلح .

