IN VITRO MID-TERM STORAGE OF DATE PALM GERMPLASM BY SLOW GROWTH AND ENCAPSULATED SOMATIC EMBRYOS

SHAWKY A. BEKHEET

Plant Biotechnology Dept., National Research Center, Dokki 12622, Cairo

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Abstract

Several applicable methods for *in vitro* mid-term storage of date palm cultures as a commercially vegetatively propagated crop were developed. Somatic embryos and undifferentiated cultures were successfully stored for 12 months at 5 °C in the dark with relatively high percentage (50 %) of cultures remained viable without serious signs of senenscence. Adding 40 mg/l of both mannitol or sorbitol to storage medium as osmotic stress agents was examined for storing by slowing down growth. Storage at cold and dark conditions was more effective compared to slow stressed growth approach. Storing differentiated cultures registered higher viability compared with undifferentiated cultures. Another successful system for preservation was realized via artificial seeds. Somatic embryos proliferated directly from shoot-tip cultures in different maturation stage were encapsulated in 3 % of sodium alginate and stored for 12 months at 5 °C and normal growth conditions. The highest conversion percentage (75 %) was registered with the storage of encapsulated late cotyledon stage of embryos at 5 °C. Genetic stability of different types of preserved tissues was observed through RAPD analysis. The amplification products of primers used indicated the genetic similarity of preserved tissue cultures with their source. The developed plantlets were successfully adapted to the free living conditions after phase of simple acclimatization procedures. The regenerated plants showed no morphological differences from those grown *in vivo*.

Key words: Date palm, slow growth, osmotic agent, encapsulation, somatic embryos.

INTRODUCTION

Because of the fact that sexual propagation produces high level of heterogeneity, date palm conventionally propagated vegetatively by offshoots grown beside the mothers. The disadvantages of this type of propagation are: the low rate of multiplication and transmision of diseases. Thus, date palm germplasm cannot be effectively stored using conventional means. Considering limitations of the two methods of propagation, tissue culture propagation offers an alternative method of producing large, homogeneous and disease-free population of plants and can allow the *in vitro* storing of selected genotypes. *In vitro* storage offers several advantages over maintained field collections because of spatial and maintenance requiremennts are minimal. Moreover, propagation potential of cultures is greater and genetic

erosion, pests and diseases are avoided. There are two methods for storage *in vitro* of plant cultures, by slowing down or suspending their growth. Slow growth is achieved for short and mid-term storage by modifying the culturre medium or reducing temperature requirements (Withers, 1991). Morover, synthetic seed production is an applied technology which capitalizes on the capcity for rapid multiplication and preservation via somatic embryogenesis. Artificially encapsulated somatic embryos,shoot-tips or other tissues can be sown under *in vitro* or *ex vitro* conditions producing uniform clones.

This paper describes a method for preservation of date palm tissue cultures for mid-term period through *in vitro* slow growth and preservation of encapsulted somatic embryos.

MATERIALS AND METHODS

1- Establishment of tissue cultures

Offshoots of date palm (*Phoenix dactylifera* L.) c.v. Zaghlool were used.The outer leaves were removed and the shoot apices with small part of sbmeristematic tissues were taken and sterlized using 70 % ethanol for 1 min and 2.5 % sodium hypochlorite for 20 min. Explants were then rinsed three times with sterile distilled water. External leaves were removed and shoot-tips were excised and cultured on MS (Murashige and Skoog, 1962) medium supplemented with 10 mg/l dichlorophenoxyacetic acid (2,4-D) + 3 mg/l dimethylaminopurie (2ip). Media were solidfied with 0.7 % agar and adjusted to pH 5.8 before autoclaving at 121 °C and 1.5 Ib/M^2 for 25 min. Cultures were normally incubated at 25 °C and 16 hr photoperiod. The embryonic cultures were obtained after three subculturing on same fresh medium. Somatic embryos were proliferated in different maturation stages using MS-hormone free medium (Fig 1-A).

2- Low temperature storage

For cold storage of date palm tissue cultures, undifferentiated cultures and somatic embryos were transfferred into jars (80 × 40 mm) conatining hormone free medium and then incubated at 5 °C in complete dark. Survival and healthy cultures percentages were recorded for ten replicates after 3, 6, 9 and 12 months of storage.

3- Osmotic stress storage:

To evaluate the role of mannitol and sorbitol as osmotic agents in storage of date palm tissue cultures, 40 g/l of both mannitol and sorbitol were added seperately to storage media. Survival and healthy cultures percentages were calculated from ten replicates after 3, 6, 9 and 12 months of storage on normal growth conditions.

4- Encapsulation and storage

Somatic embryos in four maturation stages i.e globular, torpedo, cotyledon and late cotyledon were taken and dried in a laminar flow bench and then mixed with gel of 3 % sodium alginate prepared in distilled water. An antibiotic mixture contained rifampicin (60 mg) , cefatoxine (250 mg) and tetracycline HCL was used to avoid contamination. The embryos were placed into calcium chloride solution (2.5 %) for 30 min and then stored at 5 and 25 ^{o}C. After 12 months, the encapsulated embryos were taken and sown in distilled water and then cultured on MS-hormone-free medium.The percentage of viabilty and conversion to plantlets were recorded after four weeks of culturing.

6-Randomly Amplified Polymorphic DNA(RAPD) analysis

DNA isolation was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1987). Half gram of fresh samples of shoot buds developed from the three types of storage cultures and the *in vivo* growing cultures were ground to powder in liquid nitrogen with a prechilled pestle and mortar, suspended in 5 ml preheated CTAB buffer, and incubated at 65°C for 1 hour with occasional shaking. The suspension was then mixed with 1/3 volume of chloroform, mixed gently, centrifuged and the upper phase was transferred to a new sterilized tube. Extraction was repeated with an equal volume of chloroform. The aqueous layer was transferred to a new tube, 2/3 volume of isopropanol was added and nucleic acids were either spooled using a Pasteur pipette or sedimentated by centrifugation. The pellet was washed carefully twice with 70% ethanol, dried at room temperature and resuspended in 0.5 ml TE buffer. The enzyme, RNAse A (20 µg) was added to the resuspended mixture to dugest any contaminating RNA and the tube was incubated at 37 °C for 30 min. To remove the enzyme and other contaminating protein, phenol/chloroform extraction was performed. The polymerase chain reaction (PCR) mixture (25 ul) consisted of 0.8 units of Taq DNA polymerase, 25 pmol dNTPs, and 25 pmol of random primer, and 50 ng of genomic DNA. The reaction mixture was placed on a DNA thermal cycler. The PCR programme included an initial denaturation step at 94°C for 2 mins followed by 45 cycles with 94°C for 1 min for DNA denaturation, annealing as mentioned with each primer, extension at 72°C for 30 seconds and final extension at 72 °C for 10 minutes were carried out. The amplified DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. Three 10-mer primers (Operon technologies Inc., Alameda, California) randomly selected were used in RAPD analysis (Table 1). A 100 bp DNA ladder (Promga) was used as a Marker with molecular size of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. The amplified pattern was visualized on a UV transilluminator and photographed.

7- Plantlets development and acclamatization

Shoots were proliferated from preserved cultures using phytohormone-free medium. The shoots were elongated and rooted *in vitro* using medium contained 1 mg/l indole-3-acetic acid (IAA) and 0.2 % activated charcoal. Healthy plantlets were washed with tap water and disinfected by soaking in benlate solution (1g /l) for 20 min. Then plantlets were transplanted into plastic pots contained peatmoss and vermiculite (1:1). The pots were covered with clear polyethylene bags which were sprayed with water to maintain a high relative humidity. Gradually, humidity was reduced and covers were completely removed within four weeks of transplanting.

RESULTS AND DISCUSSION

1- Storage by slow growth

Table (2) shows the percentages of survival cultures of the stored somatic embryos and undifferentiated date palm cultures. Generally, the storage at low temperature and dark conditions was more effective compared with osmotic stress medium caused by addition of mannitol and sorbitol. Hundred percentage of survival of somatic embryos cultures were observed after three months of storage at low temperature or osmotic stress medium. Also, the highest percentages of survival of undifferentiated cultures were observed after three months of storage. The survival percentages were decreased as storage duration increased. It is important here to notice that 50 % of survival of somatic embryos cultures were registered after twelve months of storage at low temperature. Two fifth of this percentage was vitrified and the rest was healthy and was suitable for recovery and proliferation into new cultures in high frequencies (Fig 2). On the other hand 50 % of undifferentiated cultures suffered mortality and then death after nine months of storage at low temperature and complete dark conditions. In this respect, growth reduction is achieved by modifying the environmental conditions and/or the culture medium. The most widely applied technique is temperature reduction, which can be combined with a decrease in light intensity or culture in dark (Engelmann, 1991). The present results also are in accordance with those reported by Bekheet (2000) in his study on *Asparagus officinalis*. He mentioned that tissue cultures remained viable after eighteen months of storage at 5 °C. Also, 80 % of pear and 85 % of apple shoot bud cultures grown *in vitro* remained alive after eighteen months storage at 4 °C and 8 °C, respectively (Wanas *et al.* 1986, Wanas 1992). In this respect, strawberry (*Fragaria* x *ananassa*) plantlets have been stored at 4 °C in the dark and kept viable for 6 years with the regular addition of a few drops of liquid medium (Mullin and Schlegel, 1976). Moreover,

Dodds (1988) reported that at temperature lower than 3 °C, potato *in vitro* cultures experience frost damage, but 6 °C seem to be well tolerated.

2-Encapsulation

Emryogenic callus was derived from meristematic tissues and all the stages of somatic embryoid formation were identified within five weeks of subculturing. The embryos in four maturation stages i.e. globular, torpedo, cotyledon and late cotyledon were coated with sodium alginate and they were stored at 5 and 25 °C for 12 months. Data presented in Fig. (3) show that, the storage of encapsulated embryos of date palm at 5 °C generally gave the best results of viability and conversion compared with the storage at 25 °C. Data also show that, the highest percentage of viability was recorded with cotyledon stage. However the highest percentage of conversion to plantlets was registered with the late cotyledon stage. These results are agree with those obtained by Bekheet *et al.* (2002). In their study on date palm they reported that ,the highest frequency of conversion to plantlets was obtained when somatic embryos in late cotyledon stage was used for synthetic seeds. The results also are in line with Redenbaugh *et al.* (1986). They mentioned that the late maturation phase of somatic embryos is important for achieving high conversion frequencies and that encapsulation and coating system. The earlier stages of somatic embryos may lack storage proteins or some other compounds required for conversion (Lai *et al.*, 1992). On the other hand, Mycock *et al.* (1997) used late golbular and early torpedo stages for cryoperservation of somatic embryos of date palm. In this respect, McKerise and Bowley (1993) mentioned that, the capability of prolonged storage of encapsulated somatic embryos was achieved when the embryos could be dried to moisture contents less than 20 %.

4. Molecular analysis

RAPD-DNA analysis was used to determine the genetic stability of preserved tiusse cultures of date palm. Three randomly selected primers (K1, K2 and K3) were used in this investigation. Only one of them (K2) did not give reproducible and sufficient amplification products. As shown in Fig. (4), DNA fragments varied in numbers and sizes depending on the primers used. The banding reveals that the three methods of preservation produced identical cultures with primers K1 and K3. It is particularly important to confirm that the preserved cultures of date palm produce plantlets genetically similar to both grown *in vitro* or in free-living conditions. From the obtained results, we can conclude that no genetic variability between differentiated and nodifferentiated cultures of date palm. The present results are in line with those reported by Saker *et al.* (2000). They mentioned that no significant variation observed

of tissue cultures derived plantlets. RAPD analysis showed genetic variation in only 4% of analalyzed plants (70 regenerants) which were incubated for 6-12 months under 25 °C. In this respect, genetic marker analysis has been used to study the degree of genetic change in plants regenerated *in vitro* such as pea (Cecchini *et al.*, 1992), sugarbeet (Sabir *et al.* 1992) and wheat (Brown *et al.* 1993).

Plantlets development and acclamatization

Because the development of a good root system on the *in vitro* grown plantlets of date palm is considered one of the most important factor affecting acclimatization to *ex vitro* environment, shoots derived from cryopreserved cultures were transferred into charcoal containing medium for *in vitro* elongation and rooting. The plantlets with healthy root system were successfully transplanted to free-living conditions within short period of acclimatization. In this respect, Tisserat (1984) reported that high survival rate (nearly 100 %) could be obtained when date palm plantlets with 2-3 foliar leaves and of shoot length greater than 10 cm (with a well-developed adventitious root system) were transplanted in pots containing a mixture of peatmoss and vermiculite.

Table 1. Primers used and their annealing temperatures.

Primer	Sequence 5'- 3'	Annealing Tm °C / Sec
K1	TGGCGACCTG	36
K2	GAGGCGTCGC	
K3	CCCTACCGAC	

Table 2. Survival percentage of date palm cultures during storage at low temperature of 6 °C or osmotic stress induced by 40 g/l of mannitol or sorbitol.

Storage duration (month)	Survival (%)					
	Somatic embryos			Undifferentiated cultures		
	6 °C	Mannitol	Sorbitol	6 °C	Mannitol	Sorbitol
3	100	100	100	90	80	80
6	80	70	60	60	50	50
9	60	50	50	50	30	30
12	50	30	20	30	20	10

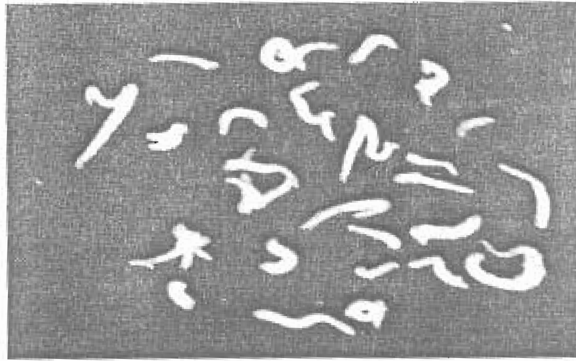

Fig. 1. Somatic embryos of date palm proliferated in different maturation stages using MS-hormone free medium

Fig. 2. Germination of somatic embryos after twelve months of storage at low temperature of 5°C .

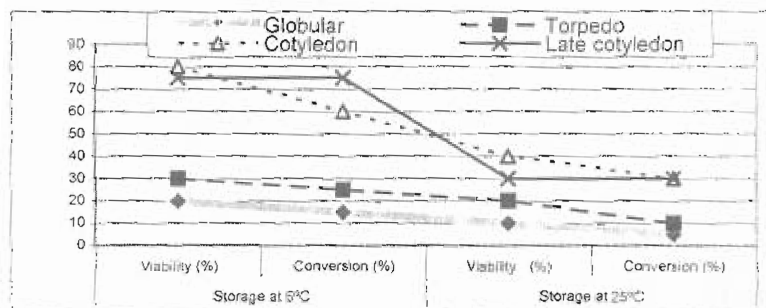

Fig. 3. Effect of maturation stage on recovery of coated somatic embryos of date palm

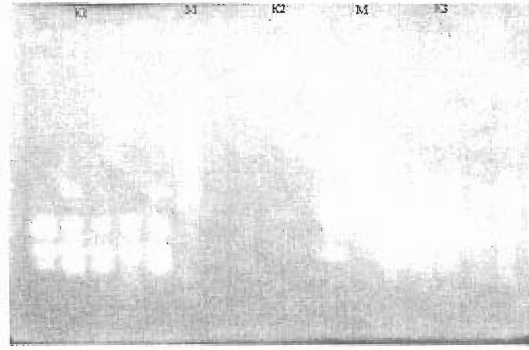

Fig. 4. RAPD profile of culture preserved low temperature (Lane 1), osmotic stress (Lane 2), capsulated somatic embryos (Lane 3), normally *in vitro* grown (Lane 4) and the *in vivo* grown cultures (Lane 5) and the DNA marker (M) from left to right using rabdom primers i.e. K1, K2, and K3.

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تخزين الأصول الوراثية لنخيل البلح لفترات متوسطة فى الأنابيب عن طريق النمو البطىء والأجنة الجسدية المغلفة

شوقى عبد الحميد بخيت

قسم التكنولوجيا الحيوية النباتية- المركز القومي للبحوث ش التحرير- الدقي- القاهرة.

تم تطويرعدة طرق تطبيقية لتخزين نموات نخيل ابلح كمحصول خضرى التكاثرفى الأنابيب لفترات متوسطة. حيث تم حفظ الأجنة الجسدية المخلقة معمليا وكذلك النموات الغير متكشفة لمدة ١٢ شهرا على درجة حرارة ٥ م° فى الاظلام. تحت هذه الظروف، نسبة ٥٠٪ من النموات ظلت حية بدون ظهور علامات الشيخوخة عليها،تم اختبار اضافة ٤٠جم/لترمن المانيتول والسربيتول الى بيئة الزراعة كمواد ذات لها اجهاد اسموزى على التخزين وابطاء النمو. التخزين على درجات حرارة منخفضة مع الاظلام كان اكثر فاعلية بالمقارنة بابطاء النمو الناتج عن الاحهاد الاسموزى للبيئة. أيضا النموات المتكشفة سجلت نسب اعلى من الحيوية بالمقارنة بالنموات غير المتكشفة . من ناحية أخرى تم ترسيخ نظام ناجح لحفظ الأصول الوراثية لنخيل التمر بواسطة البذور الصناعية. حيث تم تغليف الأجنة الجسدية المتكشفة مباشرة من زراعة القمم الساقية بكسولات من مادة ألجينات الصوديوم (٣٪) و خزنت على ظروف الضوء العادية و درجة حرارة ٥ م° لمدة اثنتى عشرة شهرا. أعلى نسبة (٧٥ ٪) لتحول الأجنة المخزنة الى نباتات كان قد سجل مع الطور الفلقى المتأخر لتطور الأجنة. تم دراسة مدى الثبات الوراثى للنموات النسيجية المحفوظة والمخزنة على المستوى الجزيئى (RAPD) . نتائج تحليل التكبير العشوائى لجزيئات الحمض النووى DNA أشارت الى تطابق الزراعات المحفوظة بالنمو البطىء وبالتغليف مع تلك النامية فى الظروف الطبيعية. أحذت النباتات المتطورة من نموات النخيل المحفوظة وتم تجذيرها معمليا ثم اقلمتها للظروف البيئية الطبيعية ولم تسجل أى اختلافات مورفولوجية.