



Academy of Scientific Research & Technology and
National Research Center, Egypt
Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb



ORIGINAL ARTICLE

In vitro conservation of embryogenic cultures of date palm using osmotic mediated growth agents



M.K. El-Bahr^a, A. Abd EL-Hamid^b, M.A. Matter^{a,*}, A. Shaltout^b, S.A. Bekheet^a,
A.A. El-Ashry^a

^a Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Centre, 33 El Bohouth St. (Former El-Tahrir St.), Dokki, Giza P.O. 12622, Egypt

^b Department of Horticulture, Ain-Shams Univ., Cairo, Egypt

Received 9 June 2016; revised 30 July 2016; accepted 21 August 2016

Available online 3 September 2016

KEYWORDS

Date palm;
In vitro preservation;
Mannitol;
Sorbitol;
Sucrose

Abstract This study was carried out to investigate the effect of mannitol, sorbitol and sucrose as osmotic agents on *in vitro* conservation of embryogenic cultures of date palm (*Phoenix dactylifera*, L.) Bartamoda and Sakkoty cultivars. Embryogenic cultures was obtained using MS medium supplemented with 10 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 3 mg/l isopentenyl adenine (2iP). Among the three types of osmotic substances used for slow growth conservation, sucrose at all concentrations gave the highest percentage of survival with Sakkoty cultivar. However, addition of 40 g/l or 60 g/l mannitol and 20 g/l sorbitol showed the highest percentage of survival percentage with Bartamoda cultivar. The different sucrose concentrations caused higher numbers of germinated embryos of the two cultivars compared with mannitol or sorbitol. Also, the number of germinated embryos was increased with increasing the storage periods till the ninth month. Genetic stability was determined using random amplified polymorphic DNA (RAPD) analysis. There were no clear genetic differences between the two osmotic agents used for preservation. The preserved cultures of Sakkoty cultivar gave the high percent of similarity while Bartamoda cultivar gave low percent of similarity. From the obtained results we can recommend using 40 g/l mannitol or 20 g/l sorbitol for *in vitro* preservation of Bartamoda cultivar of date palm and 20 g/l of sucrose for Sakkoty cultivar.

© 2016 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The date palm (*Phoenix dactylifera*, L.), is a standout among the most monetarily imperative natural product crop developed in Middle East and North Africa [1]. The fruit is highly

nutritious and rich as a source of sugar, minerals, and vitamins and it is considered the most important economical product of date palm tree. Furthermore, all the plant parts of the date palm tree have integrated in traditional or industrial applications [2]. Date palms are propagated sexually via seeds and vegetatively via offshoots. Vegetative propagation method is limited by both the numbers of offshoots produced from a superior selected plant and the development of useful offshoots

* Corresponding author.

Peer review under responsibility of National Research Center, Egypt.

<http://dx.doi.org/10.1016/j.jgeb.2016.08.004>

1687-157X © 2016 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

from a single plant, which occurs only during the juvenile phase of the palm's life [3].

At the present, the most widely recognized technique used to conserve date palm germplasm is as whole plant in field [4]. While *in situ* conservation is essential to maintain the evolution of the species and allow new diversity to be created through natural selection processes, it presents many disadvantages for conservation. The collections are exposed to natural disasters and attacks by pests and pathogens. In addition, distribution and exchange from field genebank are difficult because of the vegetative nature of the material and the greater risk of disease transfer, also, it needs great areas and cost labor. The most limiting factor is that the trees lose their abilities to give new off shoots after about 15–20 years of culturing which may lead to genetic erosion. *In vitro* methods have benefits and allow the successful preservation of date palm germplasm. The plant material is not exposed to different destructive factors and can be illimitably multiplied all year round.

Plant tissue culture and molecular biology techniques are of great interest for collecting, characterization, multiplication and storage of date palm germplasm. *In vitro* conservation involves the maintenance of explants in a sterile, pathogen-free environment. Miniaturization of explants allows reduction in space requirements and consequently labor cost for the maintenance of germplasm collections [5]. There are two main methods of *in vitro* conservation of plant germplasm. First is by reducing growth which was achieved by modifying the culture medium or low temperature incubation [6]. Second is cryopreservation which is understood as storage between -79 and -196 °C, the low extreme being the temperature of liquid nitrogen. *In vitro* storage under slow growth conditions delays the necessity for subculturing and consequently allows efficient utilization of labor year round [7]. Therefore, tissue culture considered an alternative method for date palm conservation that can eliminate the obstacles of field conservation.

The addition of osmotica such as mannitol, sucrose and sorbitol to the culture media has been proved to be efficient in reducing growth and increasing the storage life of many *in vitro* grown tissues of different plant species [8]. Healthy shoot bud cultures of date palm were obtained after 6 months of storage on a medium containing sorbitol. This period was extended for 9 months in the case of callus cultures [9]. Bekheet [10] mentioned that the presence of mannitol or sorbitol in culture medium had a retardant effect on the growth and development of globe artichoke cultures. In this respect, preservation of wild shih microshoots on a medium supplemented with sucrose, mannitol or sorbitol under light at 25 °C was able to inhibit the growth rate and maintain explant quality up to 12 weeks [11]. This study was planned to develop an applicable method for *in vitro* conservation of two Egyptian date palm cultivars i.e., Sakkoty and Bartamoda by adding mannitol, sorbitol and sucrose into the culture medium.

2. Materials and methods

2.1. Plant materials and sterilization

Offshoots about 10–15 kg of date palm cvs. Bartamoda and Sakkoty were detached from the adult females grown in Aswan governorate and used as plant materials. The outer

leaves were removed with the hard bottom and fibrous sheaths belled off. Then the internal leaves were gradually removed and shoot apices in length (5 cm) were taken and kept in an antioxidant solution (100 mg/l ascorbic acid + 150 mg/l citric acid) to avoid explants browning. For disinfection, shoot tips were immersed in 70% ethanol for 1 min followed by 50% of sodium hypochlorite solution for 30 min and then explants were rinsed three times by sterile distilled water.

2.2. Explanting and culture media

Shoot tips were trimmed to about 1 cm in length and they were excised with part of sub-meristematic tissues then aseptically cultured on MS medium [12] supplemented with 2 mg/l dimethyl amino-purine (2iP), 1 mg/l naphthalene acetic acid (NAA). Culture medium was solidified by 7 g agar, the pH was adjusted to 5.8 using 0.1 N of either KOH or HCl then autoclaved at 121 °C and at a pressure of 1.2 kg cm² for 20 min. Cultures were incubated in a growth chamber at 25 ± 2 °C under darkness conditions and recultured on the same fresh medium for three times (six weeks intervals). Calli were transferred to MS medium supplemented with 10 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 3 mg/l 2iP for six weeks and incubated at normal growth conditions (25 ± 2 °C under 16 h light (2000 Lux) and 8 h dark) for induction of embryogenic cultures (Fig. 1).

2.3. *In vitro* conservation using osmotic active agents

For evaluating the role of mannitol, sorbitol and sucrose as osmotic agents on conservation of embryogenic cultures of the two date palm cvs. Bartamoda and Sakkoty, equal pieces (250 mg) of embryogenic cultures of each cultivar were transferred to maintenance medium (MS + 2.5 mg/l kin + 0.5 mg/l 2,4-D) supplemented with 20, 40 or 60 g/l of mannitol, sorbitol or sucrose and cultures were incubated at 24 ± 2 °C at dark conditions. Survival percentage was recorded after 3, 6, 9 and 12 months of storage. The number of germinated embryos was also recorded. Each treatment consisted of three replicates (each replicate was represented by three culture jars).

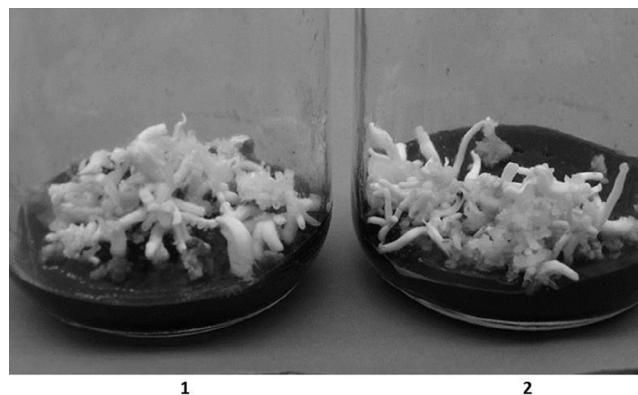


Figure 1 Embryogenic cultures of Bartamoda (1) and Sakkoty (2) cultivars proliferated on MS medium supplemented with 10 mg/l 2,4-D and 3 mg/l 2iP.

3. RAPD analysis

3.1. DNA extraction

CTAB extraction buffer

CTAB (2%)
EDTA (10 mM)
Tris-HCl (50 mM), pH 8.0
Polyvinylpyrrolidone (PVP, 1%)
LiCl (4 M)

DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) method of Doyle [13] as follows:

1. Frozen plant material (100–300 mg) was ground and homogenized with one volume extraction buffer (300–400 μ l).
2. The homogenate was incubated at 65 °C for 10–15 min, during the incubation time the homogenate was mixed 2–3 times by inverting the tube.
3. Afterward one volume (700 μ l) of chloroform–iso amyl alcohol solution (24:1) was added and carefully mixed.
4. After centrifugation (4 min) at maximum speed (14,000 rpm) in a bench-top centrifuge, the supernatant was taken up and extracted again with chloroform–iso amyl alcohol solution (24:1) and centrifuged.
5. The supernatant was transferred to fresh 1.5 ml tubes and extraction with chloroform–iso amyl alcohol was repeated until clarification of the supernatant has taken place.
6. Subsequently, the DNA was precipitated by adding 50 μ l of 7.5 M Ammonium Acetate followed by adding 3 volumes of ice cold absolute ethanol then carefully mixed 2–3 times and centrifuged for 10 min.
7. The pellets were washed with ethanol (70%) to remove any salts then resuspended in (double distilled water) D.D. H₂O.

3.2. Polymerase chain reaction (PCR)

PCR amplification was performed in 0.1 ml reaction mixture containing 20 ng template DNA, 0.5 unit Taq polymerase (Promega, USA), 200 μ M each of dATP, dCTP, dGTP, dTTP, 10 pmol random primers as shown

| Primer name | Primer sequence 5'–3' | GC % | T _m (melting temp.) °C |
|-------------|-----------------------|------|-----------------------------------|
| A6 | CCCTACCGAC | 70 | 34 |
| A10 | TCGTTCCGCA | 60 | 32 |
| A11 | TGGCGACCTG | 70 | 34 |
| A12 | GAGGCGTCGG | 80 | 36 |
| A13 | CACCTTCC | 60 | 32 |

and appropriate amplification buffer. The mixture was assembled on ice, overlaid with a drop of mineral oil. Amplification was performed for 44 cycles, using UNO thermalcycler of Biometra (Germany) as follows: one cycle at 92 °C for 2 min then 44 cycles at 92 °C for 30 s, 36 °C for 30 s and 72 °C for 2 min (for denaturation, annealing and extension, respectively).

Reaction mixture was finally incubated at 72 °C for 10 min and further 10 min at 62 °C. The amplification products were analyzed by electrophoresis in 2% agarose in TAE (Tris-acetate EDTA) buffer, in presence of DNA Ladder (100–3000 bp) and stained with ethidium bromide (0.2 μ g/mL) and photographed under UV light.

4. Statistical analysis

All experiments were arranged in a completely randomized design. Data were analyzed using a two way analysis of variance with interaction and means was separated using Duncan's multiple range test at 5% level test [14].

5. Results and discussions

5.1. Effect of mannitol, sorbitol and sucrose on survival percentages

This study was conducted to investigate the role of mannitol, sorbitol and sucrose as osmotic agents on survival of embryogenic cultures of the two date palm cvs. Bartamoda and Sakkoty. Generally, it was found that the survival percentage of Bartamoda cultivar was decreased as the storage period increased (Table 1). The survival percentage was significantly higher in the first three months of storage than the other three storage periods. Data obtained revealed that supplementation of culture medium with 40 or 60 g/l mannitol and 20 g/l sorbitol was the most suitable treatment for the Bartamoda cultivar comparing with the other mannitol and sorbitol concentrations without significant differences among the three treatments. At these levels of mannitol and sorbitol, Bartamoda cultures showed the highest percentages (66.67%) of survival after the first three months. However, survival percentages were decreased during the followed storage periods. It reached 33.33%, 22.22% and 33.33%, respectively at the end of the storage periods. Otherwise, sucrose gave a relatively lower percentage during the different storage periods compared with mannitol or sorbitol.

Data of survival percentages of embryogenic cultures of date palm cv. Sakkoty during the different storage periods on mannitol, sorbitol and sucrose containing medium are presented in Table 2. It is clear that using sucrose as an osmotic agent significantly increased the survival percentages compared with mannitol or sorbitol. Using different sucrose concentration (20, 40 and 60 g/l) increased the survival percentage of Sakkoty cultivar which recorded 88.88%, 77.77% and 77.77%, respectively after the first storage period. However, there was no significant difference among the three different concentrations of sucrose. It was found that the survival percentage was decreased as the storage period increased. The survival percentage was significantly higher in the first three months of storage than the other storage periods.

Osmotic agents act as a growth retardants by causing osmotic stress to the material under conservation. When added to the culture medium, these carbohydrates reduce the hydric potential and restrict the water availability to the explants [15]. Osmotic agents go about as development retardants by bringing about osmotic anxiety to the material under protection [16]. Osmoticum such as mannitol or sorbitol reduce

Table 1 Effect of mannitol, sorbitol and sucrose on survival percentage of date palm cv. Bartamoda embryogenic cultures during three, six, nine, and twelve months of storage.

| Treatments | Survival (%) | | | | Means |
|-----------------|------------------------|----------|----------|----------|---------|
| | Storage period (month) | | | | |
| | 3 | 6 | 9 | 12 | |
| 20 g/l mannitol | 33.33 b | 22.22 c | 0.00 d | 0.00 d | 13.88 B |
| 40 g/l mannitol | 66.66 a | 44.44 ab | 44.44 ab | 33.33 b | 47.21 A |
| 60 g/l mannitol | 66.67 a | 33.33 b | 33.33 b | 22.22 c | 38.88 A |
| 20 g/l sorbitol | 66.66 a | 44.44 b | 33.33 b | 33.33 b | 44.44 A |
| 40 g/l sorbitol | 44.44 ab | 22.22 c | 11.11 c | 11.11 c | 22.22 B |
| 60 g/l sorbitol | 33.33 b | 11.11 c | 0.00 d | 0.00 d | 11.11 B |
| 20 g/l sucrose | 33.33 b | 11.11 c | 11.11 c | 11.11c | 16.66 B |
| 40 g/l sucrose | 55.55 a | 11.11 c | 0.00 d | 0.00d | 16.66 B |
| 60 g/l sucrose | 44.44 ab | 22.22 b | 11.11 c | 0.00 d | 19.44 B |
| Means | 49.33 A' | 24.68 B' | 16.04 B' | 12.34 B' | |

Implies with the same letter(s) are not altogether varied at 5% level.

Table 2 Effect of mannitol, sorbitol and sucrose on survival percentage of date palm cv. Sakkoty embryogenic cultures during three, six, nine, and twelve months of storage.

| Treatments | Survival (%) | | | | Means |
|-----------------|------------------------|----------|----------|----------|---------|
| | Storage period (month) | | | | |
| | 3 | 6 | 9 | 12 | |
| 20 g/l mannitol | 33.33 c | 11.11 d | 11.11 d | 0.00 e | 13.88 B |
| 40 g/l mannitol | 33.33 c | 22.22 c | 0.00 e | 0.00 e | 13.88 B |
| 60 g/l mannitol | 33.33 c | 11.11 d | 0.00 e | 0.00 e | 11.11 B |
| 20 g/l sorbitol | 33.33 c | 11.11 d | 0.00 e | 0.00 e | 11.11 B |
| 40 g/l sorbitol | 22.22 c | 11.11 d | 0.00 e | 0.00 e | 8.33 B |
| 60 g/l sorbitol | 33.33 c | 22.22 c | 11.11 d | 0.00 e | 16.66 B |
| 20 g/l sucrose | 88.88 a | 77.77 a | 66.66 ab | 66.66 ab | 74.99 A |
| 40 g/l sucrose | 77.77 a | 66.66 ab | 66.66 ab | 55.55 b | 66.66 A |
| 60 g/l sucrose | 77.77 a | 77.77 a | 66.66 ab | 55.55 b | 69.43 A |
| Means | 48.14 A' | 34.56 B' | 24.68 C' | 19.75 C' | |

Implies with the same letter(s) are not altogether varied at 5% level.

Table 3 Effect of mannitol, sorbitol and sucrose on the number of germinated embryos of date palm cv. Bartamoda after three, six, nine, and twelve months of storage.

| Treatments | Average of germinated embryos/culture | | | | Means |
|-----------------|---------------------------------------|---------|---------|---------|---------|
| | Storage period (month) | | | | |
| | 3 | 6 | 9 | 12 | |
| 20 g/l mannitol | 2.66 e | 4.16 d | 5.16 bc | 5.33 bc | 4.32 B |
| 40 g/l mannitol | 3.11 d | 5.00 c | 6.55 b | 6.66 b | 5.33 A |
| 60 g/l mannitol | 2.50 e | 4.00 d | 5.50 bc | 5.66 bc | 4.41 B |
| 20 g/l sorbitol | 2.22 e | 4.66 cd | 6.33 b | 6.44 b | 4.91 AB |
| 40 g/l sorbitol | 2.16 e | 5.00 c | 6.00 b | 6.00 b | 4.79 B |
| 60 g/l sorbitol | 0.77 g | 1.66 f | 2.22 e | 2.22 e | 1.71 C |
| 20 g/l sucrose | 3.16 d | 5.40 bc | 7.66 a | 7.66 a | 5.79 A |
| 40 g/l sucrose | 3.58 d | 5.81 bc | 8.00 a | 8.11 a | 6.37 A |
| 60 g/l sucrose | 3.33 d | 5.50 bc | 7.44 a | 7.44 a | 5.92 A |
| Means | 2.61 C' | 4.59 B' | 6.10 A' | 6.17 A' | |

Implies with the same letter(s) are not altogether varied at 5% level.

Table 4 Effect of mannitol, sorbitol and sucrose on the number of germinated embryos of date palm cv. Sakkoty after three, six, nine and twelve months of storage.

| Treatments | Average of germinated embryos/culture | | | | Means |
|-----------------|---------------------------------------|---------|---------|---------|--------|
| | Storage period (month) | | | | |
| | 3 | 6 | 9 | 12 | |
| 20 g/l mannitol | 1.66 f | 3.22 d | 4.00 cd | 4.00 cd | 3.22 B |
| 40 g/l mannitol | 2.00 ef | 3.00 d | 3.33 d | 3.33 d | 2.91 B |
| 60 g/l mannitol | 1.00 f | 1.85 f | 2.57 e | 2.57 e | 1.99 C |
| 20 g/l sorbitol | 3.33 d | 5.55 bc | 6.77 ab | 6.77 ab | 5.60 A |
| 40 g/l sorbitol | 1.87 f | 3.12 d | 4.25 c | 4.25 c | 3.37 B |
| 60 g/l sorbitol | 0.75 g | 1.75 f | 2.25 e | 2.25 e | 1.75 C |
| 20 g/l sucrose | 3.75 d | 4.50 c | 7.50 a | 7.50 a | 5.81 A |
| 40 g/l sucrose | 3.60 d | 7.00 ab | 8.12 a | 8.12 a | 6.71 A |
| 60 g/l sucrose | 3.60 d | 6.00 b | 7.20 a | 7.20 a | 6.00 A |
| Means | 2.40 C' | 3.99 B' | 5.11 A' | 5.11 A' | |

Implies with the same letter(s) are not altogether varied at 5% level.

mineral uptake by cells through differences in osmotic pressures thereby retarding plant growth [17]. In this context, mannitol, sucrose and sorbitol were reported as osmotic agents to lengthen the storage life of the *in vitro* grown tissues [18]. Our results revealed that providing the conservation media with 20 g/l sorbitol or 40 and 60 g/l mannitol gave the highest survival percentages of Bartamoda embryogenic cultures. However, using the three different sucrose concentrations (20, 40 and 60 g/l) gave the highest survival percentage of the embryogenic cultures of Sakkoty. The obtained results are in agreement with those reported by [19]. They mentioned that healthy shoot buds of date palm were obtained after 6 months of storage on a medium containing sorbitol and this period was extended for 9 months in case of callus cultures. On the other hand [20], stated that all shoot tip explants of Zaghoul cultivar conserved at 5 °C for six months or at 15 °C for 12 months on medium supplemented with 0.3 M sucrose or sorbitol were able to survive. As for Gundila cultivar, all callus explants conserved on media supplemented with sucrose were able to survive and the survival percentage reached 100% while using sorbitol gave a survival percentage of 88.88%. A similar result was reported by Sarkar [21] reported that 20 or 40 g/l mannitol in combination with sucrose could enhance the survival of *in vitro* conservation of potato. For *in vitro* conservation of Rose Colored Leadwort, it was found that mannitol at concentrations of 40 and 60 mg/l had tended to reduce the number of shoots per plantlet [22].

5.2. Effect of mannitol, sorbitol and sucrose on embryos germination

The effect of mannitol, sorbitol and sucrose were studied in respect to germination of preserved embryos of Bartamoda and Sakkoty cultivars of date palm. The influence of the three osmotic agents added to the culture medium at three (20, 40 and 60 g/l) concentrations on the average number of germinated embryos/culture of the two date palm cultivars was recorded after three, six, nine and twelve months of storage. Data in Table 3 showed the average number of germinated embryos/culture of Bartamoda cultivar. It was found that among the three different osmotic agents, the different sucrose

concentrations obviously gave the highest numbers of germinated embryos/culture, without a significant difference among them. Also, there was no significant difference with 40 g/l mannitol and 20 g/l sorbitol. The generated data reveal that the lowest number of germinated embryos/culture was recorded with 40 g/l sorbitol. On the other hand the number of germinated embryos/culture was increased with increasing the storage periods. The lowest number of germinated embryos/culture was recorded at the first three months then the number of germinated embryos/culture was increased with increasing the storage periods till the ninth month significantly. It can be noticed that at the twelfth month the number of germinated embryos/culture increased insignificantly.

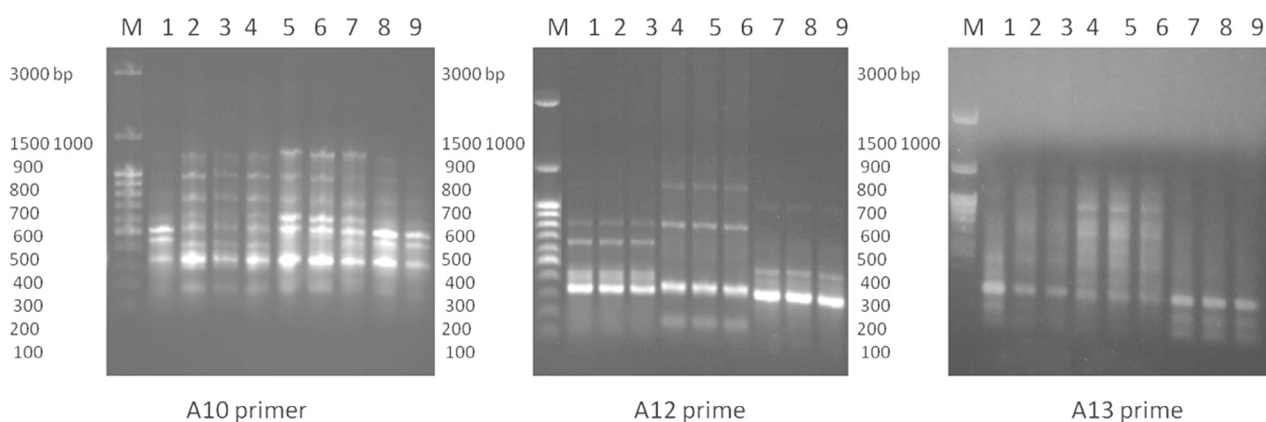
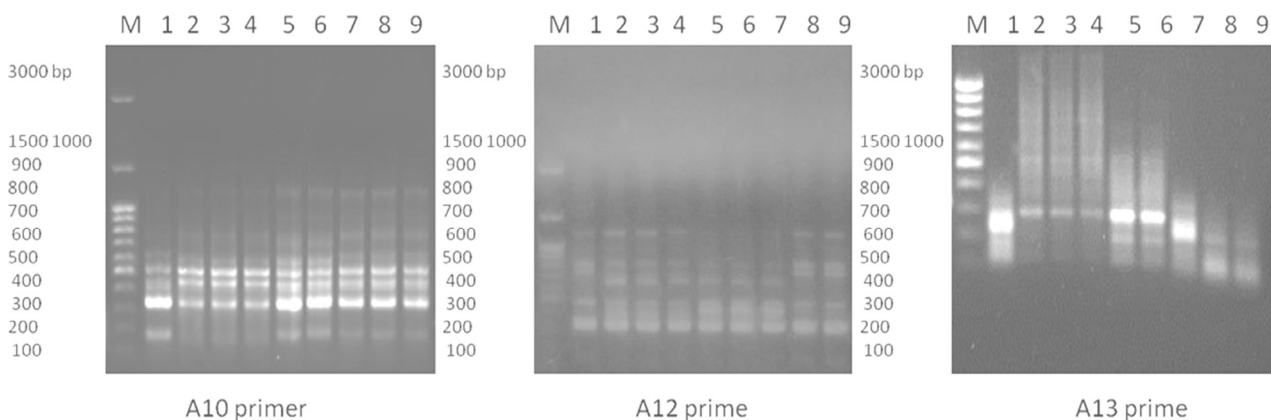
Data in Table 4 showed the average number of germinated embryos/culture in Sakkoty culture. Among the three different osmotic agents, sucrose concentrations gave the highest numbers of germinated embryos/culture, without significant difference among them. Also, there was no significant difference with 20 g/l sorbitol. The data pointed out that the lowest number of germinated embryos/culture was recorded with 60 g/l mannitol and 60 g/l sorbitol. It was found that the number of germinated embryos/culture was increased with increasing the storage periods. Regarding storage periods, the lowest number of germinated embryos/culture were recorded at the first three months. Then the number of germinated embryos/culture was gradually increased with increasing the storage periods till the ninth month significantly. At the twelfth month the number of germinated embryos/culture increased insignificantly.

As for the effect of sugars on embryos germination, our results revealed that supplementing different sucrose concentrations to preservation medium gave the highest number of germinated embryos/culture of the two cultivars. The regrowth ability was lost with the highest osmotic agent concentration, in particularly sorbitol. These results are in line with those reported by [20] in their study on *in vitro* somatic embryos maturation of date palm. They mentioned that 40 g/l sucrose were added to MS solid medium supplemented with 0.1 NAA mg/l + 0.2 mg/l BA + 0.2 mg/l kin, 200 mg/l KH₂PO₄ for embryo germination and development of plantlets, while, the lowest number of germinated embryos were obtained with 60 g/l sorbitol. Such results were opposite of those reported by

Table 5 Total number of bands, polymorphic bands and percentage of polymorphism as revealed by RAPD markers among the different *in vitro* preservation treatments using different osmotic agents (mannitol, sorbitol and sucrose) of the two cultivars used.

| Cultivars | Primer code | Sequence 5'–3' | Total No. of bands | Polymorphic bands | Polymorphism % |
|----------------|-------------|----------------|--------------------|-------------------|----------------|
| Bartamoda | A10 | TCGTTCCGC | 8 | 3 | 37.5 |
| | A12 | GAGGCGTCGG | 8 | 7 | 87.5 |
| | A13 | CACCTTCCC | 6 | 3 | 50 |
| | | | 22 | 13 | 59.09 |
| Sakkoty | A10 | TCGTTCCGC | 8 | 1 | 12.5 |
| | A12 | GAGGCGTCGG | 6 | 1 | 16.6 |
| | A13 | CACCTTCCC | 5 | 2 | 40 |
| Over all total | | | 19 | 4 | 21.05 |

T, C, G and A refer to Thyamine, Cytosine, Guanine and Adenine, respectively.

**Figure 2** Agarose gel electrophoresis of randomly primed DNA amplifications of different *in vitro* preservation treatments of Bartamoda cultivar. M refers to DNA marker given in bp. Lane 1 indicates preservation using 20 g/l mannitol. Lane 2 indicates preservation using 40 g/l mannitol. Lane 3 indicates preservation using 60 g/l mannitol. Lane 4 indicates preservation using 20 g/l sorbitol. Lane 5 indicates preservation using 40 g/l sorbitol. Lane 6 indicates preservation using 60 g/l sorbitol. Lane 7 indicates preservation using 20 g/l sucrose. Lane 8 indicates preservation using 40 g/l sucrose. Lane 9 indicates preservation using 60 g/l sucrose.**Figure 3** Agarose gel electrophoresis of randomly primed DNA amplifications of different *in vitro* preservation treatments of Sakkoty cultivar. M refers to DNA marker given in bp. Lane 1 indicates preservation using 20 g/l mannitol. Lane 2 indicates preservation using 40 g/l mannitol. Lane 3 indicates preservation using 60 g/l mannitol. Lane 4 indicates preservation using 20 g/l sorbitol. Lane 5 indicates preservation using 40 g/l sorbitol. Lane 6 indicates preservation using 60 g/l sorbitol. Lane 7 indicates preservation using 20 g/l sucrose. Lane 8 indicates preservation using 40 g/l sucrose. Lane 9 indicates preservation using 60 g/l sucrose.

El-Dawayati [19] who found that sorbitol gave the highest number of mature somatic embryos followed by those produced from callus explants conserved on medium supplemented with sucrose. However, Mazriland Meziani [23] stated that embryogenic callus induction and somatic embryo formation in date palm is influenced by different parameters such as genotype, explant type, induction period and plant growth regulators added into the culture medium. Bekheet [10] mentioned that the presence of mannitol or sorbitol in culture medium at 40 g/l for each had a retardant effect on the growth and development of globe artichoke cultures. However, African violet microshoots complete loss of regrowth occurred when concentrations of mannitol exceeded 0.33 M for 6 weeks or 0.16 M for 12 weeks of preservation period [24]. In preservation of Orchid *Epidendrum chlorocorymbos* by slow growth it was found that shoots were subsequently recovered, multiplied and rooted on MS medium with sucrose 3% without the addition of growth regulators [25]. On contrast, in their study on *in vitro* preservation of strawberry, Hassan and Bekheet [26] mentioned that sorbitol containing medium registered the best results of plantlet recovery comparing with sucrose.

5.3. Random amplified polymorphic DNA (RAPD) analysis

DNA of date palm tissues were extracted and amplified by polymerase chain reaction (PCR) using three random oligonucleotide primers. Amplification products were separated by agarose gel electrophoresis to reveal band polymorphism. Data presented in Table 5 and shown in (Figs. 2 and 3) reveal the RAPD analysis for *in vitro* preservation treatments using mannitol, sorbitol and sucrose as osmotic agents for the two date palm cultivars using three primers, A10, A12 and A13. The used primers produced 22 bands with 13 polymorphic bands for Bartamoda cultivar. Primers A10 and A13 gave the same number of polymorphic bands (3 polymorphic bands) and primer A12 gave 7 polymorphic bands. The percentage of polymorphism recorded for all primers was 59.09. However, for the Sakkoty cultivar, the primers produced 19 bands with four polymorphic bands, the largest number of amplified bands was recorded with primer A10 (8 bands) while, the lowest number of amplified bands were observed with primer A13 (5 bands). However, the average percentage of polymorphism recorded for all three primer was 21.5. It is quite clear that, the overall total polymorphism for Bartamoda (59.09) was higher than polymorphism for Sakkoty (21.05). The nearness and nonattendance of polymorphic bands among *in vitro* preservation treatments of the two date palm cultivars appeared. For Bartamoda, the three primers used produced 13 amplification products. A10 primer gave 3 polymorphic bands (200, 1000 and 1200 bp) among all treatments but, it was monomorphic for both mannitol and sorbitol treatments. A12 primer gave 7 polymorphic bands (100, 400, 500, 600, 800, 1000 and 1200 bp) among all treatments but, 1200 bp band was monomorphic for each mannitol and sorbitol treatments, whereas, the rest of polymorphic bands were monomorphic for each treatment alone. Primer A13, gave 3 polymorphic bands (400, 600 and 800 bp), each band was monomorphic for each treatment alone but polymorphic for all treatment together. Concerning Sakkoty, three primers gave four amplification products (500, 800 and 900 bp) that were polymorphic

among all treatments. From the acquired results in this study, it could be reasoned that, RAPD examination of *in vitro* conservation treatments of the two date palm cultivars displayed a hereditary varieties. The Bartamoda cultivar gave the low percent of similarity (40.91) and the Sakkoty cultivar gave the high percent of similarity (78.95). This variety might be because the somaclonal variations happened amid refined explants and multiplication of callus tissues with a high variety in cell division or separation under *in vitro* conditions or as a result of *in vitro* preservation for long period.

Clonal fidelity is a major consideration in micropropagation and preservation using *in vitro* tissue culture methods [27,28]. In this respect, RAPD markers are used for varietal identification, evaluation of date palms and for studying the genetic stability of *in vitro* grown cultures [29]. In the present study, the effect of *in vitro* storage using osmoticum on genetic stability of Bartamoda and Sakkoty cultivars of date palm was assessed by RAPD analysis. The obtained bands and the analysis indicate that conservation of embryogenic cultures of Sakkoty 12 months by slow growth method induced by mannitol, sorbitol and sucrose showed high genetic stability at the end of the storage period, while Bartamoda cultivar indicates to low percent of genetic similarity base on the used primers. This variation may be due to the somaclonal variations occurred during culturing of explants in non-normal conditions and proliferation of callus tissues with high variation in cell division or differentiation under *in vitro* conditions. Our results are in agreement with Sales et al. [30] who studied cryopreservation of *Digitalis obscura* L. selected genotypes by encapsulation – dehydration and analyzed the produced shoots by RAPD analysis and found that the band patterns were different between the original parent plant. The shoots were grown *in vitro* especially after prolonged subcultures while, after two subcultures were higher (98.6–99.5%) when band patterns from subcultured shoots were compared to those obtained from their respective control or frozen progenies indicating that cryopreservation ensure genetic stability of selected genotypes. In this context, Kovalchuk et al. [31] in a preliminary RAPD investigation found no huge contrasts between apple germplasm plants put away for 39 months and non stored controls. Furthermore, it was mentioned that the *in vitro* preservation of potato using tissue culture medium supplemented with the development retardant mannitol causes morphological changes in the proliferated material [32]. In contrast, number of studies indicate that plants recovered from *in vitro* cold storage or slow growth have no genetic alteration [33,34].

References

- [1] B. Sghaier-Hammami, L. Valledor, N. Drira, J.V. Jorin-Novo, *Proteomics* 9 (2009) 2543–2554.
- [2] W.H. Barreveld, *Date Palm Products*, FAO AgrServBul No.101, Food Agr Org of the United Nations, Rome, 1993, p. 216.
- [3] K.J. Kunert, M. Baaziz, C.A. Cullis, *Emirates J. Agric. Sci.* 15 (2001) 1–16.
- [4] E. Bettencourt, T. Hazekamp, M.C. Perry, *Directory of Germplasm Collections. 6.1. Tropical and Subtropical Fruits and Tree Nuts*, IBPGR, Rome, 1992.
- [5] F. Engelmann, *In vitro conservation methods*, in: B.V. Ford-Lloyd, J.H. Newbury, J.A. Callow (Eds.), *Biotechnology and*

- Plant Genetic Resources: Conservation and Use, CABI, Wallingford, pp. 119–162.
- [6] L.A. Withers, Biotechnology and plant genetic resources conservation, in: R.S. Paroda, R.K. Arora (Eds.), *Plant Genetic Resources, Conservation and Management Concepts Application*, International Board for Plant Genetic Resources, New Delhi, 1991, pp. 273–297.
- [7] V. Gollagunta, W. Jeffery, J. Rieck, N. Ragapakse, *Plant Cell Tissue Organ Cult.* 80 (2005) 191–199.
- [8] R.A. Shibli, M.A.L. Smith, L.A. Spomer, *J. Plant Nutr.* 15 (1992) 1373–1381.
- [9] S.A. Bekheet, H.S. Taha, M.M. Saker, *Biol. Plant.* 45 (2001) 121–124.
- [10] S.A. Bekheet, *Plant Tissue Cult. Biotechnol.* 17 (2007) 1–9.
- [11] S.A. Sharaf, R.A. Shibli, M.A. Kasrawi, S.H. Baghdadi, *J. Food Agric. Environ.* 10 (2012) 1359–1364.
- [12] T. Murashige, F. Skoog, *Physiol. Plant.* 15 (1962) 473–479.
- [13] J.J. Doyle, J.L. Doyle, *Foucs* 12 (1990) 13–15.
- [14] G.W. Snedecor, W.G. Cochran, *Statistical Methods*, seventh ed., The Iowa State Univ. Press, Ames, Iowa, USA, 1982.
- [15] G.R. Fortes, J.E.S. Pereira, *Rev. Cient. Rural* 6 (2001) 181–186.
- [16] R.D. Shibli, M.A. Shatnawi, W.S. Subaih, R.F. Viera, M.M. Ajlouni, *World J. Agric. Sci.* 2 (2006) 372–382.
- [17] J.H. Dodds, W. Roberts, *Experiments in Plant Tissue Culture*, Second ed., Cambridge University Press, Cambridge, UK, 1985.
- [18] M.R. Thompson, T. Douglas, J. Obatasamoto, T.A. Thrope, *Physiol. Plant.* 67 (1986) 365–369.
- [19] M.M.A. El-Dawayati, *Using Tissue Culture Technology to Storage some Plant Tissues of Date Palm* (Ph.D. thesis), Faculty of Agriculture, Cairo University, 2008.
- [20] R.A. Sidky, E.G. Gadalla, *Arab J. Biotechnol.* 16 (2013) 119–130.
- [21] D. Sarkar, P.S. Naik, *Euphytica* 102 (1998) 275–280.
- [22] J. Kasetsart, *Nat. Sci.* 38 (2004) 97–102.
- [23] M.A. Mazrii, R. Meziani, *Cell Dev. Biol.* 4 (2015) 160.
- [24] A.D. Moges, N.S. Karam, R.A. Shibli, *Adv. Hortic. Sci.* 17 (2003) 223–230.
- [25] G. Lopez-Puc, *Trop. Subtrop. Agroecosyst.* 16 (2013) 61–68.
- [26] N.A. Hassan, S.A. Bekheet, *Res. J. Agric. Biol. Sci.* 4 (2008) 505–511.
- [27] M. Martins, D. Sarmento, M.M. Oliveira, *Plant Cell Rep.* 23 (2004) 492–496.
- [28] C.C. Giri, B. Shyamkumar, C. Anjaneyulu, *Trees* 18 (2004) 115–135.
- [29] A. Ben-Abdallah, K. Stiti, P. Lepoivre, P.D. Jardin, *Cah. Agric.* 9 (2000) 103–107.
- [30] E. Sales, S.G. Nebauer, I. Arrillaga, J. Segura, *Planta Med.* 67 (2001) 833–838.
- [31] I. Kovalchuk, Y. Lyudvikova, M. Volgina, B.M. Reed, *Plant Cell Tissue Organ Cult.* 96 (2008) 127–136.
- [32] K. Harding, *Plant Cell Tissue Organ Cult.* 37 (2004) 31–38.
- [33] Y.J. Hao, X.P. Wen, X.X. Deng, *J. Plant Physiol.* 161 (2004) 479–484.
- [34] B. Renau-Morata, I. Arrillaga, J. Segura, *Plant Cell Rep.* 25 (2006) 636–642.