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In Vitro Conservation of Date Palm Embryos under Slow-Growth Conditions With Osmotic Agent and Abscisic Acid

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Abstract : Retardation of plant *in vitro* growth is usually targeted for conservation of genotypes, saving labors input, remaining germplasm readily available for regeneration. Few researches on germplasm conservation of date palm *(Phoenix dactylifera* L.) somatic embryos were established. Slow-growth *in vitro* storage is one of the conservation techniques used. This investigation aimed to develop an effective and simple protocol with maximum viability for short and medium-term *in vitro* conservation of date palm somatic embryos under dark and cold storage (18° C). High levels of sucrose seemed to be important for conserving date palm *in vitro* cultures as it prolonged conserving period to 10 months without any subcultures. In addition, inclusion of ABA in conserving media for 6, 8 and 10 months without subculturing, increased recovery percentage significantly compared with control medium. Moreover, the highest significant embryo number was achieved after storage for eight months with sucrose and ABA together in conserving media at 0.3 M and 2.0 mg/l, respectively**.** It is the first report of slow growth technique for date palm embryos as we know.
Key words: abscisic acid, conservation, date palm, *in vitro*, slow growth, sucrose.

Introduction

In vitro propagation and conservation usually contribute to the maintenance of natural populations through the reintroduction of preserved materials to the original habitat[1]. Micropropagation technique provides a schedule for the aggregate, preservation, registration, distribution, exchange of germplasm information and technology at the international level. *In vitro* conservation techniques, cryopreservation, cryo-storage or cold storage, are excellent system for genetic resources conservation of forest trees and horticultural crops[2]**.** Furthermore, in vitro plantlets are often a preferred form of plant distribution, as they are more likely to be free of insect-pests and many of the disease problems in the field [3-7]. They are available at any time of the year for distribution[8-12]. However, the maintenance of large collections in conventional *in vitro* systems, which involve subculturing at regular intervals, exposes the cultures to risks of contamination and somaclonal variation[13,14].

A wide range of *in vitro* conservation tools are available for conserving plant biodiversity[15,16]**.** Methods used for *in vitro* germplasm storage must meet two basic goals; minimal maintenance for an extended period and stability of the genetic properties of the stored material[17,18]**.** There are two approaches to the vegetatively propagated germplasm storage; either to reduce the growth rate of plants using minimal growth procedure or to stop the growth compeletly. Fortunately, several ways have been found to suppress the rate of growth of cultured material [19]**.**The methods employed are different depending on the storage duration requested. The main

objective of the minimal growth procedure is to extend the subculturing interval from the normal period (2 to 6 weeks) to much longer period (3 to 12 months) [20]**.** Suppression of growth can be achieved by various modifications of the physical and/or chemical tissue-culture environment. It can be conducted by using reduced temperature, low light intensities or darkness, medium alterations such as decreasing the supply of inorganic nutrients, addition of osmotic agents in conserving medium or using growth inhibitors [21-24]**.**

Slow growth conservation leads to conserve plant genotypes, remain germplasm readily available for regeneration, multiplication and distribution and reduce the number of subcultures and that leads to significant savings in labors input. However, minimal growth technique, for much extended periods, might be constrained by the risk of somaclonal variations which might appear due to the variation in the estimated amount of nuclear DNA [25].

Few number of researches on germplasm conservation of date palm *(Phoenix dactylifera* L.) somatic embryos by *in vitro* slow-growth storage were found [26]**.** It was reported that late globular and early torpedo stage date palm embryos can continue their normal growth and development after cryopreservation [27]**.**

This work describes the results of a study implemented to develop an effective and simple protocol with maximum viability for short and medium-term *in vitro* conservation of date palm somatic embryos under minimal growth conditions.

Materials and Methods

Plant disinfectant

Young off-shoots of Sewy cultivar, 2-4 years old; 5-7 kg in weight and 50-70 cm in length were used as source of explant materials. The selected off-shoots were transferred to the laboratory and prepared by removing the adventitious roots, fibrous and outer leaves until the white soft leaves were visible. The shoot tip explants were surface sterilized, firstly by immersion for 25 min in 60% v/v Clorox (5.25% sodium hypochlorite) + 2 drops of Tween 20, and then rinsing twice with sterilized distilled water. Secondly by immersion in 0.1% mercuric chloride for 15 min and washing three times with sterilized distilled water [28].

Initiation of callus cultures

Sterilized shoot-tips were sectioned and initially cultured on a nutrient medium consists of MS basal medium[29] with 40 mg/l adenine-sulfate, 3.0 mg/l 2-isopentenyl adenine (2iP), 10.0 mg/l 2,4- dichlorophenoxy acetic acid (2,4-D), 40 g/l sucrose, 2.0 g/l activated charcoal and 6.0 g/l agar. The pH was adjusted to 5.8 ± 0.1 prior to the addition of agar. Medium (15 ml) was dispensed into 25 × 100 mm test tubes capped with polypropylene closure and autoclaved at 121°C and 15 Ibs /in^2 for 20 min. Test tubes were incubated at total darkness under 27±1°C for 9 months with subculturing to fresh medium every six weeks to produce white soft callus.

Embryogenic callus formation

Soft callus was subcultured for three months (one month interval) to fresh MS medium modified as half strength NH_4NO_3, three-fourths strength of other major nutrients, full supplements of minor salts with reduction of 2,4-D to 5.0 mg/l in order to form embryonic callus.

Date palm somatic embryos induction:

Embryogenic callus was transferred to the same previous containing medium devoid of growth regulators and incubated under 27±2°C at total darkness for two months to form somatic embryogensis. Somatic embryos were subcultured to half strength MS major element medium contained a combination of 0.5 mg/l BA, 0.5 mg/L kinetin, 40 g/l sucrose, 1.0 g/l activated charcoal with 6.0 g/l agar and maintained at 27±2°C under dark condition for three subcultures (three weeks intervals). After this period, sufficient stocks of *in vitro* somatic embryo clusters were produced for the experimental trials to induce slow growth.

Slow growth treatments:

Twelve different treatments were applied to MS medium, supplemented with 0.5 mg/l BA, 0.5 mg/l kinetin and 6.0 g/l agar, i.e. four sucrose concentrations (0.1, 0.2, 0.3 and 0.4 M) combined with three different concentrations of ABA (0, 1.0 and 2.0 mg/l).The growth retardant ABA was filter sterilized and added to the medium after autoclaving and cooling to 40°C. Clusters of somatic embryos (8–10 somatic embryos for each) were used as explants material for conservation. Culture jars of each treatment were incubated at 18°C at low light intensity during all conservation durations. Browning of conserved clusters was estimated visually, according to Pottino[30], under conservation conditions.

Plant recovery after slow-growth storage

After 2, 4, 6, 8 and 10 months, the cultures were transferred to multiplication medium in order to assess their viability after each conservation period .Viability (recovery percentage) was defined as germination of somatic embryos or formation of secondary embryos presented as percentage.

Multiplication and germination rate

After eight months of conservation, embryo clusters were subcultured three times in multiplication medium without addition of AC under normal conditions. The embryo multiplication rate (average number of new formed embryo) and embryo germination rate (average number of shoots) were recorded for each treatment.

Rooting and acclimatization

After determination of plant recovery and multiplication rate, recovered embryos were transferred into bigger jars (375 mL) containing ¾ MS medium supplemented with 0.1 mg/L NAA and 0.05 mg/l BA with 6.0 g/L agar for 8 weeks (4- weeks interval) to complete their growth and form shoots[28]**.** Formed shoots were transferred to ½ MS rooting medium containing 1.0 mg/l NAA for six weeks to form roots. Vigor rooted shoots were subcultured on pre-acclimatization medium dispensed in test tubes 2.5×25 cm and consisted of ½ MS liquid medium supplemented with 15 g/L sucrose, 10 g/L poly ethylene glycol (PEG) for one month, 50 $\mu mol/m^2/s^2$ light intensity. Rooted plantlets were transferred successfully to the greenhouse.

Experimental design and statistical analysis:

Treatments were arranged in factorial complete randomized design, each treatment contains 5 replicate and each replicate involved 15 culture jars and each jar contained one cluster (8 – 10 somatic embryos). Data for recovery after slow growth storage under different treatments were analyzed using LSD according to method described by Snedecor and Cochran[31].

Results

Effect of conservation on browning

Data in Fig. (1) show the browning degree as affected by sucrose and ABA during conservation period. Browning was perceived as a problem, but frequent subculturing after conservation period was effective in overcoming the problem. In this study, cultures conserved on 0.4 M sucrose exhibited a significant higher browning degree, while the lowest significant one occurred with 0.1M. Referring to ABA effect under slow growth conditions, data revealed that cultures conserved on the absence of ABA recorded the lowest significant value. Interaction reflected that, the phenomenon was significantly greatest on 0.4 M sucrose with 2.0 mg/L ABA combination.

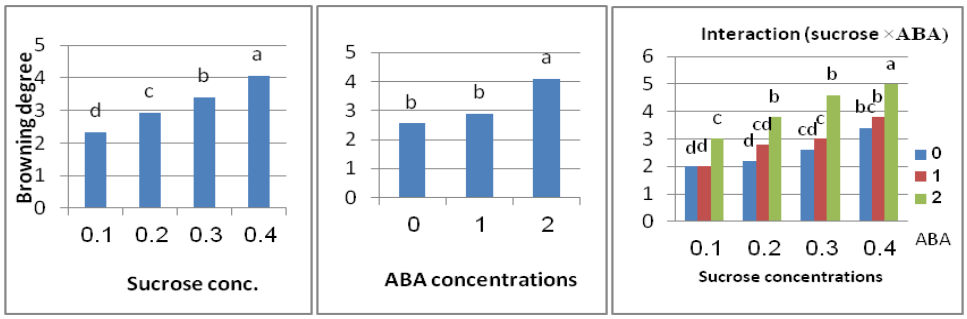

Fig. (1): Effect of sucrose and ABA concentrations on date palm somatic embryos browning degree incubated at 18°C conservation period

Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 2 and 4 months, incubated at 18°C

All conserved clusters in different treatments under investigation remained fully viable after 2 months duration (untabulated data). Data in Fig. (2) indicate the effect of sucrose and ABA concentrations on recovery percentage, after four months conservation, showing that the addition of sucrose at 0.2, 0.3 or 0.4 M in conserving media showed 100% recovery percentage of somatic embryos clusters, while the lowest concentration resulted the lowest percentage (74.09). ABA concentrations did not affect significantly the recovery % while, interaction showed 100% recovery in most of combinations used compared with the control.

Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 6 months

It is clearly from data in Fig. (3) that the highest significant percentage was recorded using 0.3 M sucrose followed by 0.2 and 0.4 without significant differences while, only 40.75 % of conserved clusters remained viable at 0.1 M sucrose. Respecting to ABA effects, it is obvious that the inclusion of ABA in conserving media increased recovery percentage significantly compared with control medium. Interaction in this respect showed that a significant increase was achieved with increasing ABA concentration from 0.0 to 2.0 mg/l in conserving media containing 0.1, 0.2 or 0.3 M sucrose. As increasing ABA to 2.0 mg/l in conserving medium containing 0.1 M sucrose raised the recovery percentage significantly compared with other concentrations of ABA while, a reverse result was obtained with 0.4 M sucrose.

Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 8 months

Data presented in Fig. (4) show pronounced effect of sucrose and ABA on recovery percentage after eight-months-storage. Increasing sucrose concentration from 0.1 to 0.3 M increased significantly recovery percentage from 29.64 to 81.5 %, while increasing sucrose to 0.4 M decreased the percentage. Referring to ABA, data showed a significant effect of the highest concentration compared with others. With respect to the interaction, data revealed that the addition of 0.3 M sucrose in combination with 2.0 mg/L ABA extended conservation period to 8 months with 100% recovery, while the lowest concentration of sucrose (0.1 M) combined with 0 or 1.0 mg/l reduced percentages to the lowest significant values (11.11 and 22.22 %, respectively).

Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 10 months

Data in Fig. (5) show that regardless ABA, sucrose at 0.3 M showed the highest significant value of recovery percentage. Meanwhile, 0.1 M was not ideal for long preservation period. Referring to ABA factor, data reflected that higher concentration of ABA was more suitable to prolong conservation period to 10 months with a significant higher percentage compared with other concentrations. The interaction showed that date palm somatic embryo clusters remained fully viable (100%) after 10 months in conserving medium containing 0.3 M sucrose combined with 2.0 mg/L ABA. However, somatic embryo clusters failed completely to survive in 0.1 M sucrose combined with 0.0 or 1.0 mg/L ABA.

Effect of conservation for eight months with sucrose and ABA on average embryo number after plant recovery

Fig. (6) reveals that date palm somatic embryo clusters growth was successfully recovered after conservation for eight months at 18°C and darkness with different sucrose and ABA combinations and retrieved the ability to multiply. Among sucrose concentrations, number of new formed somatic embryos varied significantly. Results indicated that sucrose at 0.3 and 0.4 M had a positive significant effect on somatic embryo numbers which reduced to the lowest significant value with lower concentrations. In respect to ABA factor, data show that clusters conserved on 2.0 mg/l had the greatest multiplication rate followed significantly by those conserved at 1.0 mg/l. In the absence of ABA, multiplication rate reduced to the lowest significant value. The interaction was highly significant as it was observed that, clusters incubated on medium containing 0.3 M sucrose combined with 2.0 mg/l ABA gave the highest embryo number. However, clusters in this treatment multiplied very slowly at the first; no embryo multiplication occurred during the first and second subcultures while, at the third subculture, the highest significant embryo number (87) was resulted under normal growth conditions. Otherwise, those conserved on medium containing lower concentrations of sucrose and ABA multiplied rapidly with lower significant values.

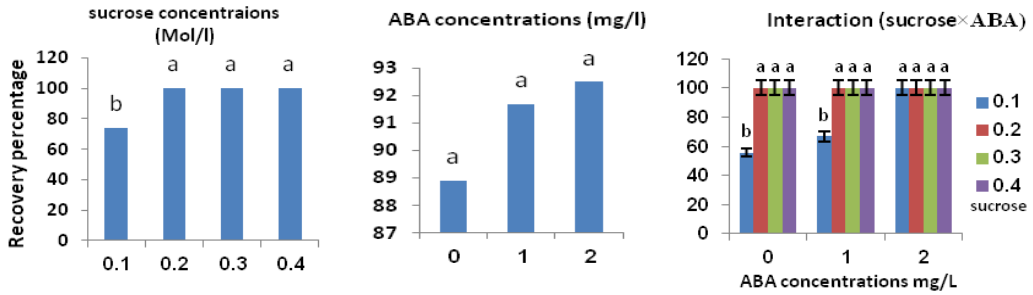

Fig. (2): Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 4 months incubated at 18°C

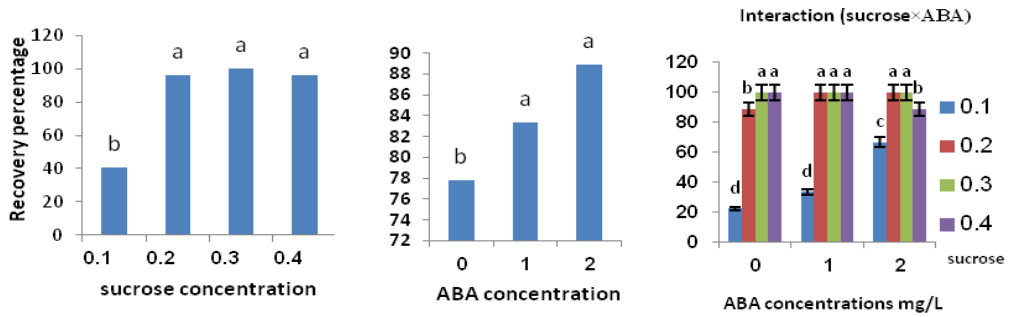

Fig. (3): Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 6 months incubated at 18°C

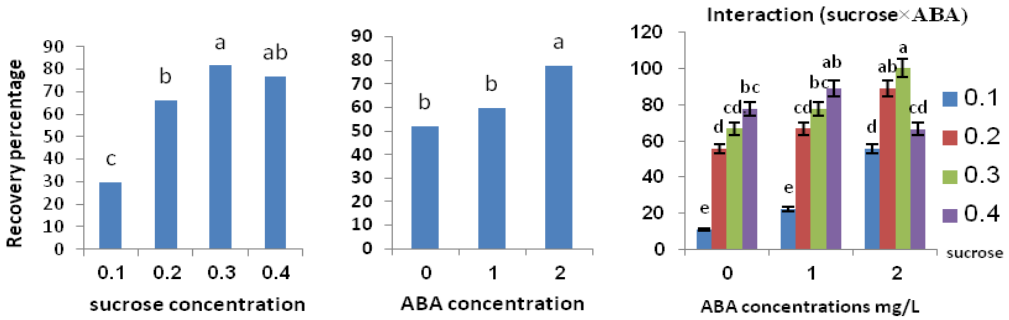

Fig. (4): Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 8 months incubated at 18°C

Effect of conservation for eight months with sucrose and ABA concentrations on germinated embryo number after plant recovery

Analysis of the results in Fig. (7) shows the effect of sucrose and ABA in conservation on subsequent germination rate at normal conditions. It indicated that concentration of both factors as well as various combinations played key roles in growth response of previously conserved cultures. Comparison of growth pattern between various sucrose concentrations indicated that lower concentration exhibited higher significant number of germinated embryos (with low multiplication as mentioned before). This number significantly decreased gradually with increasing concentrations. In respect to ABA, data showed that, clusters conserved on 1.0 mg/l ABA supplemented medium generated the highest significant number of germinated embryos while, no significant differences could be observed with other concentrations. Interaction revealed that clusters conserved on 0.1 M sucrose in combination with 2.0 or 1.0 mg/l ABA regenerated the highest significant numbers of germinated embryos without significant difference. While those conserved on 0.4 M in combination with 2.0 or 1.0 mg/l ABA recorded the fewest significant numbers (See Fig. 8 for all stages).

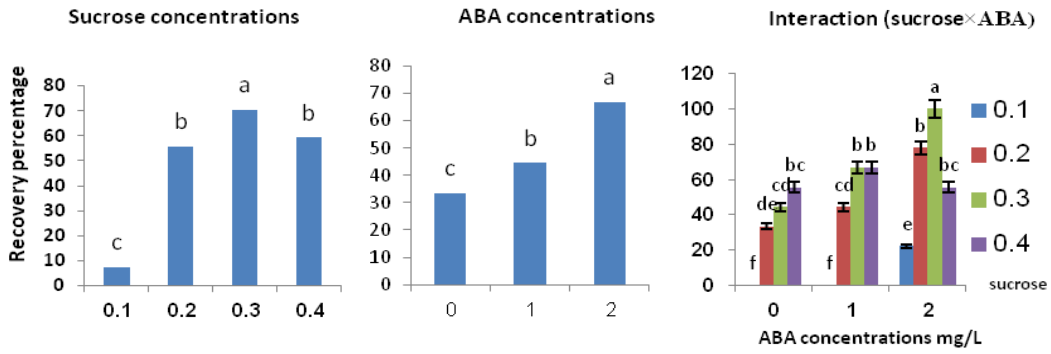

Fig. (5): Effect of sucrose and ABA concentrations on recovery percentage of date palm somatic embryos after 10 months incubated at 18°C

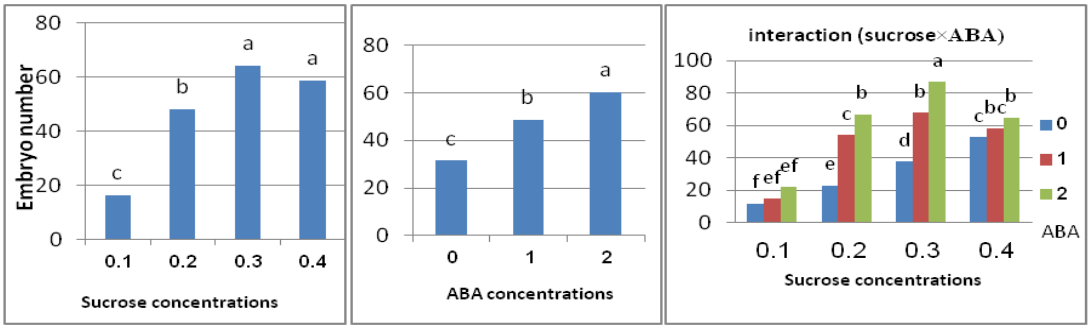

Fig. (6): Effect of conservation for eight months with sucrose and ABA on average embryo number after plant recovery

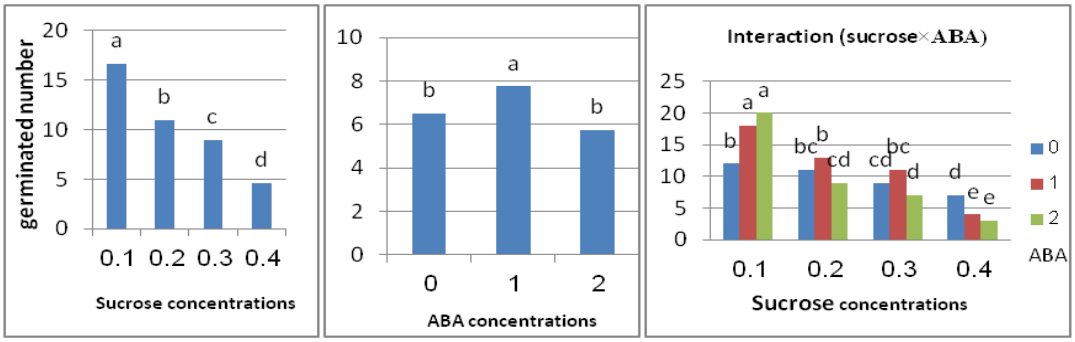

Fig. (7): Effect of conservation for eight months with sucrose and ABA concentrations on germinated embryo number after plant recovery

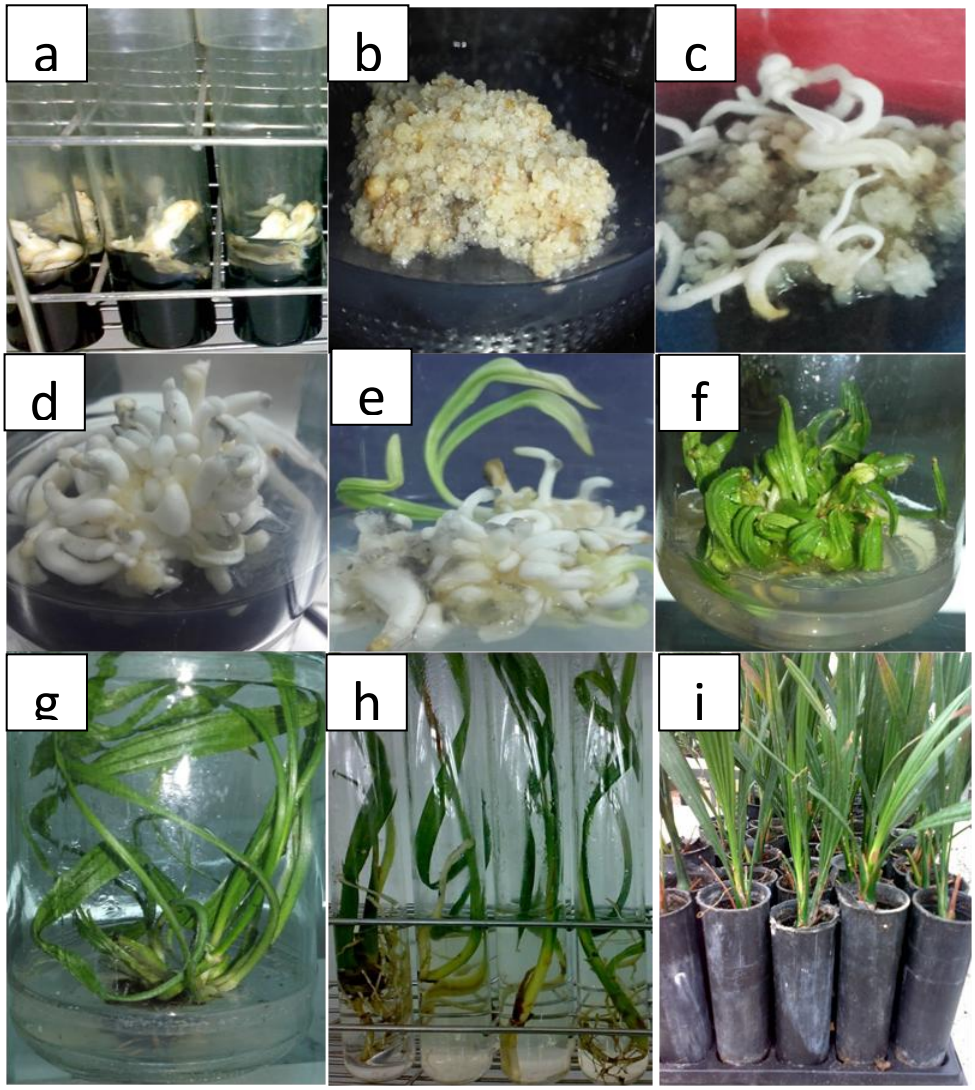

Fig. (8): Stages of micropropagation and conservation of date palm somatic embryos a) Shoot tip explants, b) Embryogenic callus formation, c) Somatic embryo formation, d) Clusters of somatic embryo (conservation materials), e) Somatic embryo in recovery medium after eight-months-conservation, f) germinated embryos, g) Rooted shoots in solid rooting medium, h) pre-acclimatized shoots in liquid medium and i) plantlets under greenhouse conditions.

Table (1): Murashige and Skoog media composition for micropropagation of date palm *phoenix dactylifera* (all amounts are given in mg/l)

Media composition	Callus formation	Emryogenic callus formation	Somatic embryo multiplication	Rooting medium
Macro element				
NH4NO3	**1650**	**850**	**850**	**850**
KNO3	**1900**	**1425**	**950**	**950**
CaCl**2** × 2H2O	**440**	**330**	**220**	**220**
MgSO4 × 7H2O	**370**	**277.5**	**185**	**185**
KH2PO4	**170**	**127.5**	**85**	**85**
Minor element				
FeSO4 × 7H2O	**27.8**	**27.8**	**27.8**	**27.8**
Na2EDTA	**7.3**	**37.3**	**37.3**	**37.3**
H3BO3	**6.2**	**6.2**	**6.2**	**6.2**
MnSO4.4H2O	**22.3**	**22.3**	**22.3**	**22.3**
ZnSO4. 4H2O	**8.6**	**8.6**	**8.6**	**8.6**
KI	**0.83**	**0.83**	**0.83**	**0.83**
Na2MoO4.2H2O.	**0.25**	**0.25**	**0.25**	**0.25**
CoCl2 .6H2O	**0.025**	**0.025**	**0.025**	**0.025**
CuSO4 × 5H2O	**0.025**	**0.025**	**0.025**	**0.025**
Organic constituents				
Sucrose	**30,000**	**45,000**	**30,000**	**30,000**
Glycine	**2**	**2**	**2**	**2**
Myo-inositol	**100**	**100**	**100**	**100**
Nicotinic acid	**0.5**	**0.5**	**0.5**	**0.5**
Pyridoxine-HCl	**0.5**	**0.5**	**0.5**	**0.5**
Thiamine-HCl	**0.1**	**0.1**	**0.1**	**0.1**
Activated	**1500**	**1500**	**1000**	
charcoal	**100**	**100**	**100**	
L glutamine	**40**	**40**	**40**	
adenine-sulfate				
Growth regulators				
2,4-D	**10**	**5**	**-**	
2iP	**3**	**3**	**-**	
BA	**-**	**-**	**0.5**	
Ki	**-**	**-**	**0.5**	
NAA	-	-	-	0.1
Solidifying agent				
Agar	**6000**	**6000**	**6000**	**6000**

Discussion

Some factors affected the *in vitro* conservation of date palm. Sucrose seemed to be an important material for conserving date palm *in vitro* cultures as it prolonged conserving period to 10 months without any subcultures. The highest significant % was recorded using sucrose at 0.3 M. Tarmizi *et al.* [32] assured that sucrose concentrations at 0.3, 0.5 and 0.7 M decreased growth, increased subcultures' intervals of oil palm polyembryonic cultures.

Our results appointed that the inclusion of ABA in conserving media for 6, 8 and 10 months without subculturing, increased recovery % significantly compared with control medium. Similarly, Naidu and Sreenath

[33] conserved immature zygotic embryos of *Coffea arabica* L. cv. Cauvery on MS medium supplemented with abscisic acid at 0, 0.4, 3.8, 18.9, 37.8 and 75.6 µM, L-cystein hydrochloride at 50 mg/L and sucrose at 3%. After six months of storage, the recovery rate remained high for all ABA treatments. In addition, Suksa-Ard *et al.* [34] reported that ABA seemed to be the most suitable growth retardant for tolerance of the *Carica papaya* shoot explants to low temperature. The regrowth of shoots was still satisfactory even after 12 months on medium containing 5.0 µM ABA. Moreover, the promising conservation conditions for mid-long term preservation of lily germplasm resources was at -2°C with 1/4 MS and 3.0 mg/L ABA [35]. Furthermore, *in vitro* conservation of bitter almond (*Prunus dulcis* L.) microshoots were studied. Increasing sucrose (0.09 to 0.35 M), sorbitol, mannitol (0.1 to 0.4 M) or ABA (0.0 to 11.4 µM) reduced growth significantly and extended the subculture interval to 4 months when cultures were kept at room temperature [36].

Our results assured that the inclusion of sucrose and ABA together in conserving media was very effective for date palm *in vitro* conservation as well as growth recovery. Similarly, in the treatments with 0.5 mg/L ABA and 6.0% sucrose, in sweet potato UNPRG-13 genotype, the shoot elongation and the recovery rate were higher than the control treatment [37].

However, it was observed that the longer the durations the less the recovery percentage appeared. This decrease was more pronounced with low concentrations of sucrose and ABA. Similar results were appeared with [33].

We can conclude that date palm embryo cultures could be conserved successfully in medium with combination of sucrose and ABA as they slowed down the growth. This decreasing in growth might be due to proline accumulation [38] caused from moisture stress condition [39] as the feasibility of preservation method was preliminarily validated at the protein level [40]. Some chemical treatments (i.e. ABA) may help to slow down the growth of *in vitro* cultures by acting directly to induce organ dormancy, reduce cellular metabolism or prevent cell nuclear division, making cells less susceptible to cold. This may enable cultures to be stored for longer periods at low temperature [41].

We observed a positive response of date palm embryo culture to the two retardant agents which was manifested as increasing embryo number besides decreasing the germinated number. This indicates a higher ability of embryo culture to multiply. All *in vitro* plantlets resulted from conservation were transferred to greenhouse and successfully acclimatized.

Conclusion

This investigation presented an effective and simple protocol with maximum viability for short and medium-term *in vitro* conservation of date palm somatic embryos under dark and cold storage (18° C) using higher levels of sucrose and ABA.

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