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# INFLUENCE OF NUTRIENT, ABSCISIC ACID AND CARBOHYDRATE SUPPLY ON BROWNING PHENOMENA (*Phenol composition*) OF DATE PALM SOMATIC EMBRYOS THROUGH CONSERVATION PERIOD

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## ABSTRACT

Metabolic sugar (sucrose, glucose and fructose) and nutrients (MS formula) were found to affect polyphenol accumulation in date palm somatic embryos through conservation period (10 month) incubated under total darkness at 18°C, while alcoholic sugars (polyols) sorbitol and mannitol had no effect on this respect. Increasing the sugar content and reducing the nutrients content of culture media both enhanced the content of the phenolic substances. Also raising the concentration of ABA in conservation medium from 3.7 to 30 µM in conserved media induced excessive accumulation of phenolic compounds. Healthy and succeeded conserved embryos exhibited good growth during *in vitro* and *ex vitro* stages.

## INTRODUCTION

Date palm *Phoenix dactylifera L.* is one of the most important fruit tree crop planted in the Middle East and Arabian lands. Conventional vegetative propagation , using offshoot , is very slow and laborious .Recently, many attempts were reported on date palm propagation through tissue culture techniques (Abo El-Nil , 1986 ;Abo-El-Soud ,1999 and Gaddalla ,2003 ) .

Fruit tree germplasm is being conserved mainly in the form of a field genebank , which requires much labor, money and land . In addition , this germplasm is apt to be lost by attack from pathogens , pests and environmental disaster (Ko *et al.*, 1991). Very little research has so far been carried out in the germplasm storage of dates. Tissue culture and cryopreservation techniques may prove to be the most effective. There have been published reports on the use of cryopreservation for storing pollen grains, somatic embryos , callus and meristems (Watanabe and Pehu ,1997). There is no information on conservation of date palm except Hassan (2002) who established new protocol to improve the production and development of somatic embryos of date palm and to conserve somatic embryos under minimal growth condition .

During the course of growth and development *in vitro*, plant tissues not only depleted the nutrients that are furnished in the medium, but also release substances that can accumulate in the cultures. These substances such as phenols, may have profound physiological effects on the cultured tissues. Browning of the tissue and the adjacent medium is assumed to be due to the oxidation of polyphenols and formation of quinines which are highly reactive and toxic to the tissue (Maier and Metzlier, 1965). Date palm, *Phoenix dactylifera* L., tissue cultures like those of many plants have been commonly

observed to release discolouring substances into the medium, which inhibit their own growth (Zaid, 1984). The phenol synthesis of plant tissues strongly depends on environmental conditions (Feucht and Shmid, 1988; Bauer *et al.*, 1989; Bauer and Treutter, 1990).

There is no available literature on browning phenomenon during storage period of date palm somatic embryos and the role of nutrient media and their additives on this phenomenon.

This work aimed to study the effect of nutrient supply, carbohydrate content and absicisic acid in culture media on phenolic compounds accumulation through conserved period (10 months) of date palm somatic embryos

## MATERIALS AND METHODS

**Plant materials:-**

Healthy offshoots from mother date palm trees cv. Samani ranging from 5 – 7 kg in weight and about 50 – 70 cm in length, grown in the field of Agricultural Ministry at Rashid, Egypt were used as a source of plant tissue culture material. This experimental work was performed in the Central Laboratory for Research and Development of Date Palm during the period from 1999 to 2002.

**Sterilization of plant materials:**

Shoot-tip explants of date palm containing the apical meristem surrounded by leaf primordial proved to be difficult to free from contaminants. For this reason, shoot-tip explants were washed in running tap water for one hour and subsequently soaked onto antioxidant solution consists of ascorbic acid (100 mg/l) and citric acid (150 mg/l) for one hour to minimize the accumulation of phenols or browning degree of explants. Washed explants were firstly sterilized by immersion in 70% ethanol for 5 seconds then followed by immersion in 0.1% mercuric chloride (MC) for 5 min. and thoroughly washed with sterilized distilled water for one-time. After that additional leaf primordial were removed from sterilized explants and these explants secondly were sterilized in 50% (v/v) Clorox (5.25% w/v sodium hypochlorite NaOCl) plus 0.1% Tween-20 for 25 min. and rinsed three times with sterilized distilled water. Injured leaf primordia were carefully removed. Shoot-tips with 4 -6 leaf primordia were sliced longitudinally into 4 sections and cultured onto culture medium.

**Culture medium:**

Shoot-tip sections were cultured on Murashige and Skoog (1962) medium supplemented with 10 mg / l 2,4 dichloro phenoxy acetic acid (2,4-D) and 3 mg/l iso-pentenyl adenine (2iP) solidified by adding 6 g/l agar. Also, activated charcoal was added ( 3 g/ l ) to minimize browning phenomenon of explants. The pH of culture medium was adjusted to 5.7±0.1 with HCl andKOH prior the addition of agar. Culture medium was autoclaved at 121°C and 1.5 lbs/in$^2$ for 20 min.

**Culture conditions:**

Cultured explants were incubated under total darkenss at 27±2°C and transferred to fresh medium every 6 weeks for at least 9 months in order to obtain embryogenic callus. Embryogenic callus was transferred to fresh medium of the same previous containing medium without addition of growth regulators and incubated at 27±2°C and light intensity (1000 – 1500 lux) for 16 hrs to produce somatic embryos which were used as a tool for conservation experiments.

**Experiment 1: Variation of nutrients and sugar supply:-**

Clumps consists of 10-12 somatic embryos of date palm cv. Samani were conserved for at least 10 months on the following treatments (9 jar each), the nutrients were varied from ¼ strength of MS formula (¼ MS), ½ strength (½ MS) and full strength (MS). Each strength was supplemented with various sugars (sucrose, glucose, fructose, mannitol and sorbitol) at 0.1, 0.3, 0.5 and 0.7 M.

**Experiment 2: Variation of ABA and sugar supply:-**

Clumps consists of 10-12 somatic embryos were conserved on MS medium supplemented with different sugar (sucrose, glucose, fructose, mannitol and sorbitol) at 0.1, 0.3, 0.5 and 0.7 M. Each concentration from different sugar was supplemented with abscisic acid (ABA) at 3.7, 7.5, 15 and 30 μM.

All conserved embryos were incubated at 18°C under total darkness and after conservation period, browning accumulation of every treatment were visually recorded as scores (1=No browning, 2= light browning, 3= moderate browning and 4=severe browning) as described by Pottino (1981). After conservation period, healthy succeeded embryos were transferred to multiplication medium ( MS + 0.5 mg / l benzyl adenine (BA+) 0.5 mg / l kinetin + 30 g / l sucrose + 6 g / l agar ) and subseguently to rooting medium (½ MS +0.1mg / l Naphthalene acetic acid (NAA) ) to complete the growth and development during *in vitro* date palm stages and to *ex vitro* planting medium ( peatmoss , vermeculite and sand at equal / volume ) under uncontrolled green house and photographed

**Statistical analysis :**

Experiments were arranged in factorial completely randomized design. Means were compared using L.S.D. values at 5 % according to method described by Snedecor and Cochran (1998)

## RESULTS AND DISCUSSION

**Effect of sugar and MS salt strength :**

Effect of sugar type at different concentrations and MS salt strength on browning degree of embryos conserved for at least 10 months were Presented in Table (1). It is evident from the data that type of sugar affected significantly the browning degree. Fructose supplemented medium caused

the highest significant value of browning degree (3.58). No significant differences were observed between sucrose or glucose supplemented media (2.42 and 2.25),respectively. Browning degree did not appear on medium supplemented with mannitol and also sorbitol, which produced the lowest significant value of browning degree (1.42) Fig. (1)

**Table (1): Effect of sugar( type and concentrations) and M`S salt strengths on browning phenomena through conservation periods.**

| Type of sugar (A) | Con. (M)B | MS salt strengths (C) | | | |
|---|---|---|---|---|---|
| | | MS | ½ MS | ¼ MS | Mean |
| Sucrose | 0.1 M | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.3 M | 1.00 | 1.00 | 4.00 | 2.00 |
| | 0.5 M | 2.00 | 3.00 | 4.00 | 3.00 |
| | 0.7 M | 3.00 | 4.00 | 4.00 | 3.67 |
| | | **1.75** | **2.25** | **3.25** | **2.42** |
| Glucose | 0.1 M | 1.00 | 3.00 | 3.00 | 2.33 |
| | 0.3 M | 2.00 | 1.00 | 3.00 | 2.00 |
| | 0.5 M | 2.00 | 2.00 | 3.00 | 2.33 |
| | 0.7 M | 2.00 | 3.00 | 2.00 | 2.33 |
| | | **1.75** | **2.25** | **2.75** | **2.25** |
| Fructose | 0.1 M | 2.00 | 3.00 | 3.00 | 2.67 |
| | 0.3 M | 3.00 | 3.00 | 4.00 | 3.33 |
| | 0.5 M | 3.00 | 4.00 | 5.00 | 4.00 |
| | 0.7 M | 4.00 | 4.00 | 5.00 | 4.33 |
| | | **3.00** | **3.50** | **4.25** | **3.58** |
| Mannitol | 0.1 M | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.3 M | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.5 M | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.7 M | 1.00 | 1.00 | 1.00 | 1.00 |
| | | **1.00** | **1.00** | **1.00** | **1.00** |
| Sorbitol | 0.1 M | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.3 M | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.5 M | 1.00 | 2.00 | 2.00 | 1.67 |
| | 0.7 M | 2.00 | 2.00 | 2.00 | 2.00 |
| | | **1.25** | **1.50** | **1.50** | **1.42** |
| **Means of MS** | | **1.75** | **2.10** | **2.55** | |
| **Means of sugar conc.** | | **1.60** | **1.87** | **2.40** | **2.67** |

**L.S.D. Separted at 0.05**

**A = 0.295 B = 0.264 C = 0.229**

**AB = 0.590 AC = 0.540 BC = 0.457 ABC = 1.022**

Regarding to the effect of sugar concentrations, data clearly show that decreasing the concentrations of sugars in conserving media gradually decreased significantly the browning degree from 2.67 to 1.60.

Referring the effect of different concentration of MS salt strengths, data in Table (1) clearly show that MS salt strength in conserving media affected significantly the browning degree of conserved embryos (explants). Raising strength from quarter to full strength decreased significantly the browning degree from 2.55 to 1.75.

The obtained results are in line with those reported by Larronde *et al.* (1998) who examined the effect of sugars (glucose, fructose or sucrose) and sugar derivatives (sorbitol or mannitol) on growth and polyphenol accumulation of grape cells grown in IMS medium. They found that the dry weight increase was notably higher in media with the three metabolic sugars, but not with polyols, which were not taken up by the grape cells. Only metabolic sugars (0.15 or 0.20 M) affected the accumulation of anthocyanin and stilbenes in these cells. By contrast, the two non-assimilable sugars, mannitol and sorbitol did not stimulate polyphenol accumulation of grape cells. Hence they recorded that a close correlation has been found between anthocyanin accumulation and the level of intracellular hexoses.

In this respect Treutter *et al.* (1985); Treutter and Feucht (1988) recorded that increasing the sucrose content of MS-medium enhanced the content of phenolic compounds in cherries. Also, this findings was reported in cell culture of strawberry by Mori and Sakurai (1994).

The same Table reflected that decreasing the concentration of sugar in conserving media gradually decreased significantly the browning degree. This result was in line with findings of Endrich *et al.* (2000) who reported that increasing the sucrose and reducing the macro-nutrient content of culture media of apple resulted in enhancing the content of phenolic substances.

Also, data in the same Table clearly revealed that, raising strength of MS medium from quarter to full strength decreased significantly the browning degree. Similarly Endrich *et al.* (2000) found that, the content of all individual phenolic compounds decreased with increasing concentration of macronutrient in culture medium of grape up to the treatment 2 MS.

Data in Table (2) revealed that the browning of date palm embryo through conservation period was affected by type of sugar, concentrations of sugar and different concentration of ABA.

It appears from this Table that embryos conserved on fructose supplemented medium gave the highest significant value of browning degree (4.05), followed by embryos conserved on glucose or sucrose supplemented media without significant difference (3.05 and 2.90). On the other hand, embryos conserved on mannitol or sorbitol produced the lowest significant values of browning degree (1.00 and 1.30, respectively).

Respecting to the effect of different sugar concentrations means value revealed that raising the concentrations of tested sugar in culture media gradually from 0.1 to 0.7 M raising significantly the browning degree from 1.76 to 3.96.

According to the effect of different concentrations of ABA on browning degree data clearly show that embryos conserved on media with adding 30 or 15 μM ABA produced the highest significant values of browning degree without significant differences (2.95 and 2.85, respectively). These values reduced significantly to 2.45 and 2.30 when embryos conserved on media supplemented with 7.5 or 3.7 μM ABA, respectively while control medium without adding ABA at any concentrations produced the lowest significant values of browning degree (1.75).

**Table (2): Effect of sugar( type and concentrations) and ABA on browning phenomena through conservation periods.**

| Type of sugar (A) | Con. (M)B | ABA concentrations( μM) C | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.0 | 3.7 | 7.5 | 15 | 30 | Mean |
| Sucrose | 0.1 M | 1.00 | 1.00 | 1.00 | 2.00 | 2.00 | 1.40 |
| | 0.3 M | 2.00 | 2.00 | 2.00 | 3.00 | 4.00 | 2.60 |
| | 0.5 M | 3.00 | 3.00 | 3.00 | 4.00 | 5.00 | 3.60 |
| | 0.7 M | 3.00 | 4.00 | 3.00 | 5.00 | 5.00 | 4.00 |
| | | **2.25** | **2.50** | **2.25** | **3.50** | **4.00** | **2.90** |
| Glucose | 0.1 M | 2.00 | 2.00 | 3.00 | 3.00 | 3.00 | 2.60 |
| | 0.3 M | 1.00 | 3.00 | 3.00 | 3.00 | 3.00 | 2.60 |
| | 0.5 M | 2.00 | 3.00 | 3.00 | 3.00 | 4.00 | 3.00 |
| | 0.7 M | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| | | **2.25** | **3.00** | **3.25** | **3.25** | **3.50** | **3.05** |
| Fructose | 0.1 M | 1.00 | 2.00 | 3.00 | 4.00 | 4.00 | 2.80 |
| | 0.3 M | 2.00 | 4.00 | 5.00 | 5.00 | 5.00 | 4.20 |
| | 0.5 M | 3.00 | 5.00 | 5.00 | 5.00 | 5.00 | 4.60 |
| | 0.7 M | 3.00 | 5.00 | 5.00 | 5.00 | 5.00 | 4.60 |
| | | **2.25** | **4.00** | **4.50** | **4.75** | **4.75** | **4.05** |
| Mannitol | 0.1 M | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.3 M | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.5 M | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.7 M | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| | | **1.00** | **1.00** | **1.00** | **1.00** | **1.00** | **1.00** |
| Sorbitol | 0.1 M | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| | 0.3 M | 1.00 | 1.00 | 1.00 | 1.00 | 2.00 | 1.20 |
| | 0.5 M | 1.00 | 1.00 | 1.00 | 2.00 | 2.00 | 1.40 |
| | 0.7 M | 1.00 | 1.00 | 2.00 | 2.00 | 2.00 | 1.60 |
| | | **1.00** | **1.00** | **1.25** | **1.50** | **1.75** | **1.30** |
| **Means of MS** | | **1.75** | **2.30** | **2.45** | **2.85** | **2.95** | |
| **Means of sugar conc.** | | | **1.76** | **2.32** | **2.72** | **3.96** | |

**L.S.D. Separted at 0.05**
**A = 0.311 B = 0.197 C = 0.221**
**AB = 0.441 AC = 0.493 BC = NS ABC=NS**

Our presented data show that embryos conserved on fructose supplemented medium gave the highest significant value of browning degree. These results agree with that reported by Al-Dawayati (2000) who recorded that the highest significant value of developed callus browning of date palm was achieved with addition of fructose to culture medium than glucose or sucrose.

Data also show that embryos conserved on mannitol or sorbitol produced the lowest significant values of browning degree. This observation confirmed with the explanation by Takeuchi *et al.* (1994) who mentioned that the level of expression of the chalcone synthase gene coincides with intracellular levels of sugars, in particular sucrose and glucose, but no response is observed with mannitol.

According to the effect of sugar concentrations means values revealed that raising the concentrations of tested sugar in culture media from 0.1to 0.7 M increase significantly the browning degree of conserved embryos.


**Fig. (1):Effect of different sugars on browning phenomena of date palm somatic embryos through conservation period.**

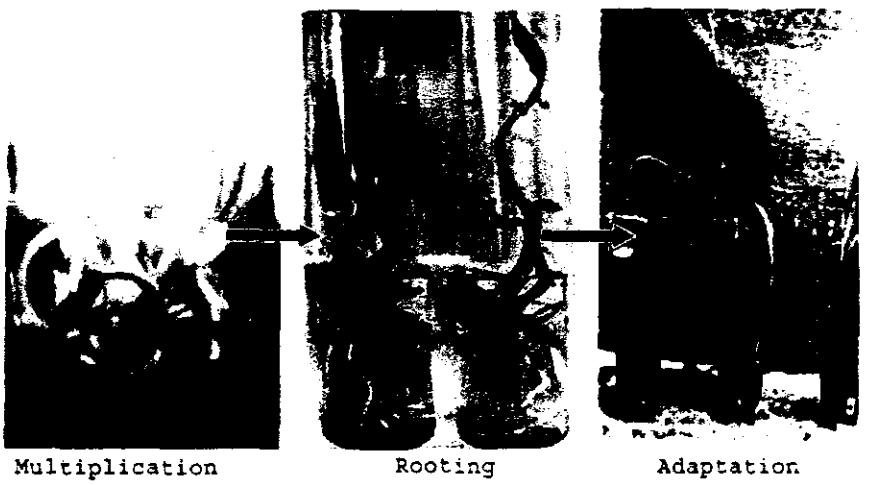


**Fig.(2): Conserved somatic embryos after transfer to multiplication , rooting and adaptation stages**

This result was associated with that reported by Al-Dawayati (2000) who mentioned that raising various kinds of sugar concentrations in culture media markedly increase the browning degree values of date palm developed callus.

Also, Do and Cormier (1990) concluded that the high osmotic potential of the culture medium, resulted from high sucrose content, increased anthocyanin accumulation in the cultured grape cells. However, the growth of the cells was repressed.

In this concern, Panis *et al.* (1996) observed that the media containing higher sucrose levels as a pretreatment before cryopreservation in liquid nitrogen considerably retarded the growth of proliferating meristems of banana. Also, increasing the sucrose level lowered the survival rate and a concomitant blackening of tissue. At 0.6 M sucrose, only 0.17% of the inoculated buds display growth, while the remainder become black and die. At 0.75 M, no buds re-grow and all become brown. They assumed that the osmotic shock at 0.75 M was too high to induce blackening, which is a normal reaction of the living tissues exposed to stress.

Our results showed that the succeeded conserved embryos exhibited good growth when transferred to multiplication, rooting media during *in vitro* stage and *ex vitro* planting medium under green house conditions (Table 2).

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# تأثير إضافة المغذيات وحامض الأبسيسيك والسكريات على ظاهرة التلون البني(تكوين المواد الفينولية ) في الأجنة الخضرية لنخيل البلحأثناء مدة التخزين

عبد الحنيم سيف الدين على

المعمل المركزي للأبحاث وتطوير البلح — مركز البحوث الزراعية

أجرى هذا البحث في المعمل المركزي للأبحاث وتطوير نخيل البلح أثناء الفترة من ١٩٩٩ حتى ٢٠٠٠م بهدف دراسة تأثير بعض مكونات البيئة المغذية (قوى الأملاح — السكريات وحمض الأبسيسيك ) على ظاهرة التلون البني (تكوين المواد الفينولية ) على الأجنة الخضرية لنخيل البلح صنف سماني أثناء التخزين لمدة ١٠ شهور ثم أثناء مراحل النمو في المعمل والصوبة .

أوضحت الدراسة أن السكريات البنائية (سكروز — جلوكوز —فركتوز) وكذلك مغذيات بيئة موراشيجى وسكوج تؤثر على تراكم البولي فينول في الأجنة الخضرية لنخيل البلح أثناء الحفظ لمدة ١٠ شهور والتي تم تحضينها على درجة ١٨م تحت ظروف الإظلام الكامل بينما لم يلاحظ اى تراكم لهذه المواد الفينولية باستخدام السكريات الكحولية (السوربيتول — المانيتول).

أدى زيادة تركيز السكريات في بيئة الحفظ مع تقليل قوة الأملاح المستخدمة الى زيادة المحتوى من المواد الفينولية .

وجد أيضا أنه بزيادة تركيز حامض الابسيسيك في بيئة الحفظ من ٣,٧ إلى ٣٠ ميكرومول زاد من تراكم المواد الفينولية . ولقد نمت النباتات الناتجة من حفظ الأجنة الخضرية لمدة ١٠ شهور بنجاح في المعمل والصوبة .