DIRECT SOMATIC EMBRYOGENESIS OF DATE PALM (*PHOENIX DACTYLIFERA* L.) BY OSMOTIC STRESS

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Abstract

The present investigation aiming at induces somatic embryogenesis directly by osmotic stress of sucrose to shoot tip Malakaby cv. The explants cultured on MS basal medium supplemented with 10 mg/l 2,4–D + 3 mg/l 2ip + 1.5 g/l activated charcoal (AC) and various concentrations of sucrose (30, 50, 70, 90, 110 and 130 g/l). All explants were incubated in darkness at 27 C° ± 2 for 6 months. The percentage of somatic embryogenesis and the percentage of callus formation were recorded as measurement during the incubation period and after 45 days from transfer the explants to fresh growth medium (The basal medium included 10.0 mg/l 2.4-D + 3.0 mg/l 2ip). Few explants produced direct somatic embryos induced directly without an intervening callus phase on shoot tip explants which cultured on 90 and 110 g/l sucrose. Therefore, the high concentrations of sucrose 110.0 and 130.0 g/l caused thick root and globular structures formation. Data indicated that increasing the osmotic stress as a consequence of increasing sucrose in the initial starting medium of date palm induced the direct somatic embryos along with proline accumulation.

Key words: Osmotic stress – *Phoenix dactylifera* L. - sucrose - somatic embryogenesis.

INTRODUCTION

Sucrose is generally used as the major source of carbon and energy in tissue culture medium. Other sugars including mannitol, maltos and sorbitol have been also used, often with sucrose (Kishor and Reddy 1986, Swedlund and Locy 1993, Okamoto *et al.* 1996, Laxmi and Reddy 1997). In addition for being major carbon source for *in vitro* growth, sorbitol and sucrose act as osmotic agents that may introduce osmotic stress above certain concentrations. It was reported that the treatment with high concentrations of mannitol was effective to induce somatic embryogenesis directly of date palm (Zaid *et al.* 2006). Studies on the mechanism of osmotic adjustment in plants are limited by the fact that whole plants contain mostly non-growth cell which makes characterizing biochemical processes in growing cell in response to osmotic changes difficult (Turner and Jones 1980). The use of culture plant cell provides mean to overcome this difficulty since it allows careful measurements of growth in response to various osmotic changes in the environment (Bressan *et al.* 1982). Somatic embryogenesis can be induced in a number of dicotyledonous and monocotyledonous

species by transferring explants from auxin containing medium to auxin free medium (Conger, *et al.* 1983). Somatic embryogenesis in *Haelianthus annuus* and *carica papaya* was stimulated by culturing their explants on medium containing 2,4-D and a high level of sucrose (6 – 12 %) (Finer, 1987 and Litz, 1986) and that adventitious shoot formation in tobacco callus required osmotic stress (Brown *et al.*, 1979). Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of possible means for overcoming osmotic stress caused by the loss of water (Al-Baharany 1994, Shankhadhar *et al.* 2000). This study investigated the influence of osmotic stress, induced by sucrose supplied to the culture medium at various concentration, on callus growth, somatic embryogenesis and accumulation of proline.

MATERIALS AND METHODS

This study was carried out at the Central Laboratory for Date Palm Research and Development Center and Horticulture Research Institute, ARC, Egypt, throughout the period from 2005 to 2007.

Plant material:

The clonal materials was obtained from adult date palm trees *Phoenix dactylifera* L. .cv. Malakaby grown at the Aswan Governorate. Shoot tip explants were surface sterilized with a sodium hypochlorite solution (available chlorine 2.5%) for 30 min. After then, all explants were thoroughly washed with sterile distilled water.

Culture media:

The explants were cultured on 3/4 modified Murashige and Skoog (MS) basal nutrient medium (1962) supplemented with 170 mg/l NaH_2PO_4, 200 mg/l KH_2PO_4, 200 mg/l glutamine, 40 mg/l adenine sulfate, 0.4 mg/l thiamine HCl, 1.5 g/l activated charcoal, 30 g/l sucrose, 6 g/l agar and 10 mg/l 2,4-D + 3 mg/l 2ip (Tisserat 1982).

The effect of sucrose on somatic embryogenesis induction

The explants were treated with various concentrations of sucrose (30, 50, 70, 90, 110 and 130 g/l). The pH was adjusted to 5.8 ± 1 prior to the addition of agar and then 35 ml of medium was dispensed into small jar (150 ml). The jars were autoclaved at 121°C and 1.1 Kg/cm² for 20 min. The cultures were maintained at the growth room under full darkness at 27±2 °C. The experiment was conducted with 9 replicates for each treatment and repeated twice. The shoot tip explants were cultured on different concentration for 6 months. The percentage of somatic embryogenesis and the percentage of callus formation were recorded as measurement during osmotic stress of sucrose and after 45 day from transfer the explants to growth medium (10 mg/l 2,4–D + 3 mg/l 2ip + 1.5 g/l activated charcoal).

Prolin assessment:

Prolin assessment was carried out according to the method recorded by Bates *et al.*, (1973). Half gram of fresh sample was homogenized in sulphosalicylic acid (3 %w/v), and the homogenate filtered through Wt. No. 1 filter paper. Then, the volume was made up to 10 ml with sulphosalicylic acid. Two ml of filtrate was reacted with 2 ml of acid-ninhydrin (1.25 g ninhydrin was dissolved in 30 ml acetic acid glacial in addition to 20 ml phosphoric acid 6 M) and 2 ml acetic acid glacial for 1 hour in a test tube placed in water-bath at 100 °C. The reaction mixture was extracted with 4 ml of toluene, and the absorbance noted at 520 nm. The amount of proline was calculated from a standard curve.

Statistical analysis

Data were subjected to analysis of variance and means were compared, using L.S.D at 5% significance (Snedecor and Cochran, 1972).

RESULTS

Data displayed in Table 1 represented the results of shoot tip explants cultured on MS basal medium containing 10.0 mg/l 2,4–D + 3.0 mg/l 2ip and different concentrations of sucrose (30.0, 50.0, 90.0, 110.0, 130.0 g/l) for 6 months. Data showed that increasing sucrose concentration in the nutrient medium decreased compact callus percentage from 55.55 to 00.00 %. However, this type of callus did not differentiate into shoot or embryo but, after 2 – 3 subcultures from transfer this callus (compact callus) on the fresh growth medium (10.0 mg/l 2,4–D + 3.0 mg/l 2ip) developed to embryogenic callus. the explants with small primary calli were transferred to medium containing 0.5 mg/l ʹ.BA + 0.1 mg/l Kin the for further development (Zaid, *et al.*, 2003). As well as, the culture on this medium triggered somatic embryogenesis and somatic embryo developed from meristematic centers originated on nodular tissue.

Table 1. Effect of the stress by sucrose concentrations on callus and somatic embryos formation percentages in *Phoenix dactylifera* L. cv. Malakaby

Sucrose (g/l)	Callus (%)	Somatic embryo (%)	Nature of callus or/and somatic embryo
30.0	55.55a	00.00c	Compact
50.0	33.33b	00.00c	Compact
70.0	22.22c	00.00c	Compact
90.0	00.00d	33.33a	White mature embryo
110.0	00.00d	11.11b	White mature embryo
130.0	00.00d	00.00c	-
Mean (A)	18.51	7.40	

On the other hand, the high concentration of sucrose (90, 110, 130 g/l) prohibited callus formation process. But, encouragement somatic embryogenesis

directly without intervening callus. Thus after transfer the shoot tip explants to the same medium without different concentration of sucrose as a causative osmotic stress, white mature embryos (somatic embryo) appeared directly at the cut-end of explants without visible callus (Figure 1).

These somatic embryos couldn't be able to develop further on high sucrose medium, but upon transferring them to medium with 30.0 g/l sucrose and 0.05 mg/l BA + 0.1 mg/l NAA (Gabr and Tisserat, 1985), they developed into distinct and intact plantlets.
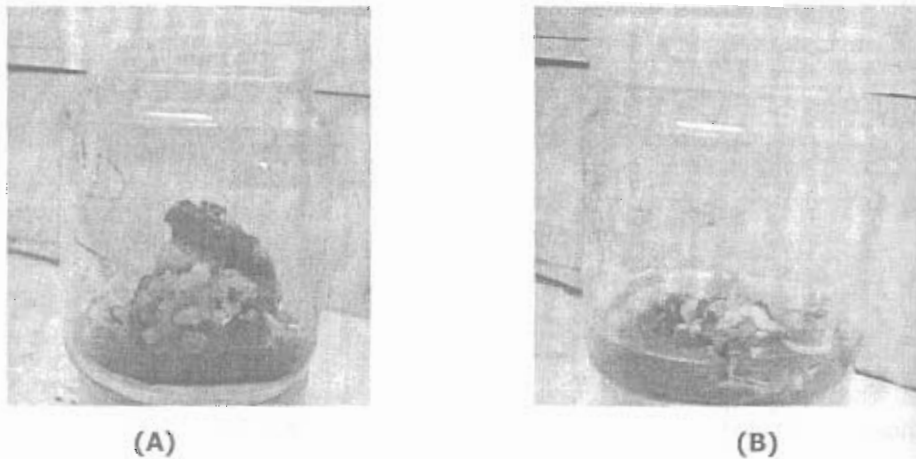

(A)   (B)

Figure1. Somatic embryogenesis induced by osmotic stress in date palm.

A: Mass of somatic embryo formed at the cut end of the shoot tip segments without visible callus formation. B: somatic embryos formed directly on the surface of shoot tip

Data in Table 1 shows the results of an experiment where average number of somatic embryo was 33.33 and 11.11 embryo/explant when the shoot tip explants cultured on 90.0 and 110.0 g/l sucrose, respectively. Thus, addition 90.0 g/l sucrose to culture medium date palm gave the best significant results compared with other treatment for somatic embryogenesis directly.

Moreover, addition 30.0 or 50.0 g/l sucrose to culture medium did not produce any abnormal organs. But addition 70.0 or 90.0 g/l formed on the surface of explants hairy thin adventitious roots, as shown in Table 2. it is observed that, thin root formation average of 70.0 g/l treatment was lower than the average of 90.0 g/l treatment.

While the high concentration of sucrose (110.0 and 130.0 g/l) formed thick root and globular structures. These thick short abnormal roots which had been observed on the cultured explants could not resume their development and turned to very brown (Table 2 and Figure 2).

Table 2. Effect of sucrose stress on the type of differentiated organs of shoot tip explants of *Phoenix dactylifera* L. cv. Malakaby

Sucrose (g/l)	Type of differentiated organ (shoot – root – embryo)
30.0	Non
50.0	Non
70.0	Hairy thin adventitious root
90.0	Hairy thin adventitious root
110.0	Thick root, structure globular
130.0	Thick root, structure globular

Figure 2. Thick short abnormal roots formed on shoot tip explants when cultured on high concentration of sucrose.

Proline content:

Figure 3 showed that increasing osmotic stress as a consequence of increasing sucrose, accompanied with proline accumulation. Therefore, the highest concentration of proline 1.773 (ng) was obtained on 70.0 g/l sucrose. It was noticed that the proline level decreased at 90.0 and 130.0 g/l sucrose concentration as a response to it is consumption in cells during various growth stages, this results agreement with Al-Khayri and Al-Bahrany, 2002, 2004. The lowest proline concentration 0.033 (ng) was found in cultures subjected to the lowest osmotic level which were grown on 90.0 g/l sucrose .
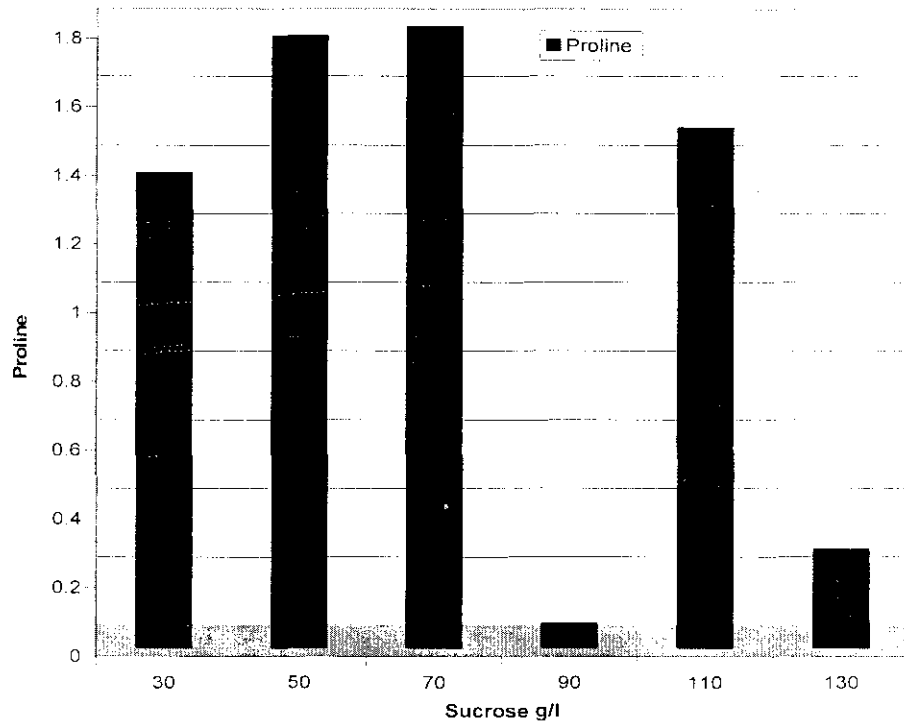
Figure 3. The effect of sucrose concentration on proline accumulation in date palm
(Malakbi c.v.) direct somatic embryogenesis culture medium.

Thus, increasing sucrose concentration further up enhanced the accumulation
of proline in response to obtain higher osmotic stress. This suggests that, sucrose
contributed the osmotic stress and in turns the enhanced accumulation of prolin in an
additive manner.

It can be concluded that making stress on the cultured explants of date palm
(shoot tips) by increasing the concentration of sucrose into the starting nutrient
medium renders some cells to produce direct organs. Mostly, these organs were roots
and exhibited the genetic background of the date palm explants which have wide
extent to produce direct root formation. A few of initial explants exposed ability to
produce direct somatic embryos. However, these somatic embryos have had quick and
vigorous growth in comparison with the somatic embryos produced from callus. As
well as, the osmotic stress by sucrose was associated with proline accumulation.
Further work is required to increase the percentage of direct somatic embryos form
the initial explants of date palm without or with minimal an intermediate callus phase.

DISCUSSION

The results presented in this report show that somatic embryogenesis in date palm could be induced by osmotic stress, although the development of embryos to plantlets was suppressed under high osmotic condition, but upon transferring them to medium with 30.0 g/l sucrose, they developed into plantlets. these results agreement with Kamada, et al., 1993. It has been reported that osmotic stress caused an increase of the endogenous level of abscisic acid in several plant species. When apical meristems cultured on hormone-free medium with 0.7 M sucrose were transferred to hormone- free medium with 0.1 M sucrose, the first leaves were slightly developed and numerous somatic embryos were formed on surface of the leaves without visible callus formation 4 weeks after the transfer (Kamada, et al., 1989). It was also reported that the treatment with osmotic stress (90.0 to 110.0 g/l) induced somatic embryogenesis in date palm explants shoot tip. The treatment with high concentration of mannitol was effective for somatic embryogenesis directly. Shoot tip and sub-shoot tip explants gave the highest percentage of direct somatic embryogenesis, no callus formation was shown (Zaid, et al., 2006). Besides these facts, it is also Known that 2,4 – D is one of the most effective compounds to induce somatic embryogenesis in many plants species and an inducer of stress proteins (Czarnecka, et al., 1984). Thus, it seems likely that different physiological stresses trigger the induction of sômatic embryogenesis> On the other hand, somatic embryogenesis in *Zea mays, Carica papaya, Helianthus annuus* can be induced by the treatment with auxin under high osmotic conditions (Finer, 1987, Litz, 1986). Accumulation of endogenous free proline in plant tissues exposed to osmotic stress has been documented in various *in vitro* culture (Hasegawa, et al., 1984, Handa, et al., 1986, Santos-Diaz and Ochoa-Alejo 1994, Al-Khayri and Al-Bahrany, 2002). Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of the possible means for overcoming osmotic stress caused by the loss of water (Al-Bahrany, 1994). The resultant increase in osmotic stress enhanced praline accumulation that continued to rise reaching its maximum on a treatment representing the greatest osmotic stress induced by the highest sugar (sucrose) concentrations. It appears that application of sucrose induced direct formation of somatic embryos from epidermal cell without the formation of visible calli as osmotic stress.

AKNOLEDGEMENT

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إستحداث تخلق أجنة جسمية مباشر بزيادة الأجهاد الأسموزى فى نخيل التمر

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الكلمات الدالة: الأجهاد الأسموزى – السكروز – نشؤ الأجنة الجسمية – نخيل التمر

تهدف هذه الدراسة إلى الإنتاج المباشر للأجنة الجسمية بواسطة الإجهاد الأسموزى وذلك بزراعة القمم النامية لنباتات نخيل البلح صنف ملكابى على بيئة موراشجى وسكوج ١٩٦٢ ومضاف أليها ١٠ ملجم/لتر D – 2,4 , ملجم/لتر 2iP و ١,٥ حم/لتر فحم نباتى منشط بالإضافة إلى التركيزات المختلفة من السكروز (٣٠، ٧٠.، ٩٠.، ١١٠،٠،١٣٠،٠. جم/لتر) وحضنت العزلات النباتية فى الإظلام التام على درجة حرارة ٢٧±°م لمدة ٦ اشهر. سجلت النسبة المئوية للأجنة الجسمية المتكونة مباشرة وكذلك النسبة المئوية للكالس بعد ٤٥ يوم من نقلها و زراعتها على البيئة الأساسية والمحتوية على ٣٠ جم/لتر سكروز. كون عدد قليل من العزلات (القمم النامية) أجنة جسمية مباشرة بدون ظهور للكالس وذلك على التركيزات العالية من السكروز (٩٠، ١١٠ ج/لتر). بينما التركيزات الأكثر ارتفاعا (١٣٠,٠ حم/لتر) كونت جذور سميكة غير طبيعية وكذلك تراكيب كروية عير محددة. بينت النتائج أيضا زيادة محتوى البرولين فى الخلية بزيادة تركيز السكروز.