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Somatic Embryogenesis in *Phoenix dactylifera* L: Effect of Exogenous Supply of Sucrose on Proteins, Sugars, Phenolics and Peroxidases Activities During the Embryogenic Cell Suspension Culture

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Abstract: The present study was conducted to determine the effect of three concentrations of usually used (1.5, 3 and 6%) of sucrose on the proliferation of date palm cell suspension culture. The medium containing 3% sucrose, leads to best results than 1.5 and 6% sucrose especially for JHL cultivar. In fact, when sucrose concentration increased from 1.5 to 3%, the embryogenic mass significantly increased from 2.15 to 14.6 g per flask and 8.7 to 10 g per flask, respectively for JHL and BSTN cultivars. The biochemical analysis showed that the maturation medium with 3% sucrose significantly increased the proteins (14.9 mg g^{-1} FW for JHL cultivar and 5 mg g^{-1} FW for BSTN cultivar) and sugars contents (22.5 and 22.7 mg g^{-1} FW, respectively, for JHL and BSTN cultivars) in the embryogenic mass. In addition, as compared to 1.5 and 6%, the use of 3% sucrose enhanced phenolics contents (0.69 mg g^{-1} FW) and peroxidases activities ($2.9.10^3$ n Kat g^{-1}FW) in BSTN cultivar.

Key words: Date palm, peroxidases, phenols, proteins, somatic embryogenesis, sucrose, sugars

INTRODUCTION

Date palm (*Phœnix dactylifera* L.), an economically important commodity, is a monocotyledonous tree widely cultivated in arid regions of the Middle East and North Africa. *In vitro* micro propagation is increasingly becoming an attractive alternative for large-scale propagation of date palm. *In vitro* plant regeneration of date palm occurs through organogenesis and somatic embryogenesis depending on genotype and hormonal manipulation[1-5].

In plant tissue culture, carbohydrates are generally considered as a carbon source needed to sustain growth of tissues in culture. Among the different carbohydrates, sucrose is the most frequently used in the culture medium[6]. In several species, sucrose is the preferred carbohydrate for induction, proliferation and embryos maturation[7,8]. In Alfalfa, embryos fresh weight, embryos maturation, conversion to plantlets and vigour of seedlings increased linearly as sucrose concentration increased from 3 to 5% in the maturation medium[9]. In addition, these authors indicated that embryos matured on a medium containing 5% sucrose exhibited the highest levels of proteins. In cucumber, although similar weights of tissue were produced with sucrose, glucose or fructose in the maintenance medium, embryos produced on sucrose showed a higher germination rate than those produced on fructose[10]. For black spruce, sucrose concentration in the maturation medium decreased from 6% initially to 0.3% at the end of a 6-week maturation period[11]. This decrease in sucrose was correlated with an increase in fructose and glucose leading to an increased osmotic pressure of the medium. However, replacing 6% sucrose by 3% of each glucose and fructose in the maturation medium resulted in a decrease in the number of mature embryos produced. Additionally, these authors showed that mannitol was not suitable to study the effect of the osmotic pressure on maturation.

In the case of date palm, several works have been published describing some culture media for organogenesis or somatic embryogenesis[1-5,12,13]. However, little is known concerning the effect of sucrose on some physiological parameters such as proteins, sugars, phenolics and peroxidases activities during the embryogenic cell suspension culture.

The objective of this study was to determine the effect of exogenous supply of sucrose on proteins, sugars, phenolics and peroxidases activities during the embryogenic cell suspension culture of date palm.

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MATERIALS AND METHODS

Plant material: Shoot tips of two cultivars (JHL and BSTN) of *Phoenix dactylifera* L. were surface sterilized for 20 min with 2% Desogerm, following 20 min immersion in commercial hypochlorite containing 300 mg L^{-1} of potassium permanganate, then rinsed 3 times with sterile distilled water.

Embryogenic callus induction and multiplication: Callus induction was conducted according to[3,4,14]. The explants were placed on callogenesis induction medium containing MS salt and vitamins[15], 30 g L^{-1} sucrose, 150 mg L^{-1} activated charcoal, 6.8 g L^{-1} carrageenan, 5 mg L^{-1} of 6-benzylamino-purine (BAP), 5 mg L^{-1} of dichlorophenoxyacetic acid (2,4-D). After 6 months of culture, the friable calli formed were selected and transferred to medium containing 0.1 mg L^{-1} of BAP and 0.5 mg L^{-1} of 2,4-D[3,5]. Tissues were incubated at 25±2°C in the dark and subcultured to freshly medium every 5 weeks.

Establishment of cellular suspension: The cell suspension method used has been described by Norggard[7], Loutfi[12], Côte *et al.*[16]. The embryogenic callus explants (500 mg) were cut with a sterile scalpel into small pieces as possible and then transferred in 50 ml of liquid medium in 250 ml Erlenmeyers. The content of Erlenmeyers is filtered using a sieve (500 μm diameters); the obtained filtrate is incubated on a rotary shaker (100 rpm) at 25°C in the same light conditions. The liquid culture medium is that MS diluted a half and supplemented with 2,4-D (0.1 mg L^{-1}), BAP (0.5 mg L^{-1}) and sucrose (1.5, 3 or 6%).

Extraction and analysis of proteins: The total soluble proteins were extracted according to the method described by Lecouteux[17]. Fresh plant material (250 mg) was extracted with 2 ml of 0.25 M Phosphate buffer (pH 7) and centrifuged for 3 min at 7000 g. The supernatant was used as the crude proteins extract. The total proteins were measured by spectrophotometer at 595 nm according to the method described by Bradford[18].

Extraction and analysis of sugars: Sugars extraction was carried out according to Booij *et al.*[19]. Briefly, fresh plant material (250 mg) was extracted in 2 ml of 80% aq ethanol. After heating for 30 min at 100°C, the homogenate was centrifuged for 3 min at 7000 g. The supernatant constituted the soluble sugar fraction. Sugars were analysed by spectrophotometer at 485 nm according to Dubois[20].

Extraction and analysis of phenolics: Phenolics compounds were extracted and analysed as described by El Hadrami[3] and Macheix *et al.*[21]. Embryogenic mass was homogenized with 2 ml (80%) methanol at 4°C and centrifuged three times at 7000 g for 3 min, supernatants were recuperated each time. 100 μl of the supernatant was added to Folin-Ciocalteu reagent (250 μl) and sodium carbonate (20%). The mixture was incubated at 40°C for 30 min and the blue colour was determined at 760 nm. The content of soluble phenolics was expressed in mg-equivalent of catechin per g of FW.

Extraction and analysis of peroxidases: Extraction of peroxidases was conducted with Tris-maleate buffer (0.1 M, pH 6.5) containing Triton X-100 (0.1 g L^{-1}). Peroxidase activity was assayed spectrometrically at 470 nm using guaiacol as a substrate. Twenty microlitres of enzyme extract (250 mg FW per 2 ml) was added to 2 ml of reaction mixture consisting of a solution of 0.1 M Tris-maleate buffer (pH 6.5) and 25 mM guaiacol. Reactions were initiated with 20 μl of H_2O_2 (10%) and stopped after 3 min[22].

Statistical analysis: To test the effect of sucrose on somatic embryogenesis in JHL and BSTN cultivars, the results were analysed by variance analysis (ANOVA), followed by Tukey test at p= 0.05 level to compare means.

RESULTS AND DISCUSSION

Varying the carbohydrate treatments in the culture medium had a marked effect on the proliferation of cell suspension culture in date palm. The best result was obtained on the medium containing 3% sucrose especially for JHL cultivar as compared to 1.5 and 6%. In fact, when sucrose concentration increased from 1.5 to 3%, the embryogenic mass significantly ($P<0.05$) increased from 2.15 to 14.6 g and 8.7 to 10 g per flask, respectively for JHL and BSTN cultivars (Table 1). The supply of 3% sucrose in the culture medium may be responsible for the increase of osmotic pressure of medium. In fact, the plasmolysis of cells may stimulate cell division activity and increase

Table 1: Effect of sucrose on the increase of embryogenic mass of JHL and BSTN cultivars after two months of culture

		Sucrose concentration (%)		
	Cultivars	1.5	3	6
Embryogenic mass per flask (g)	JHL	2.15±0.35^{c}	14.6±0.13^{a}	5.77±0.29^{b}
	BSTN	8.7±0.16^{b}	10.7±0.30^{a}	3.3±0.40^{c}

Values are means ±standard error of three replicates from two experiments
For each cultivars, values followed by the same superscript letter(s) are not significantly different at P= 0.05

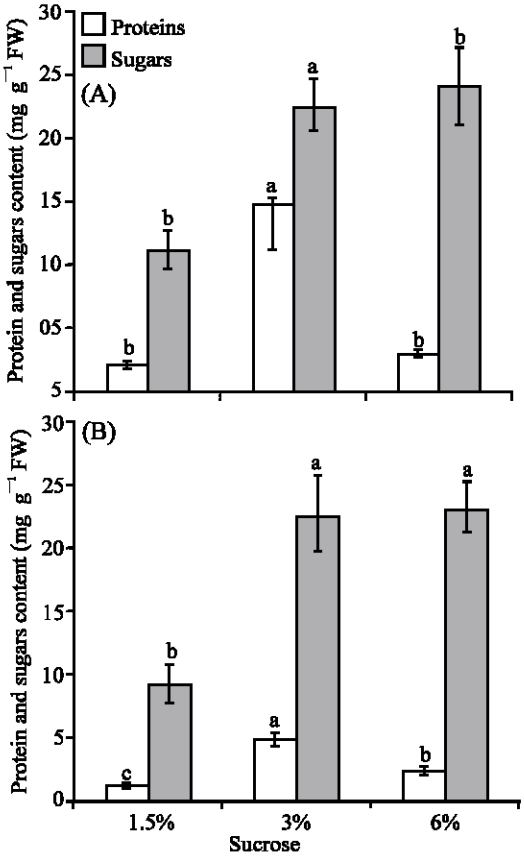

Fig. 1: Effect of sucrose on the accumulation of proteins and sugars in embryogenic mass of JHL (A) and BSTN (B) cultivars after two months of culture. Values are means±standard error of three replicates from two experiments. For each parameter values followed by the same superscript letter are not significantly different at P= 0.05 level

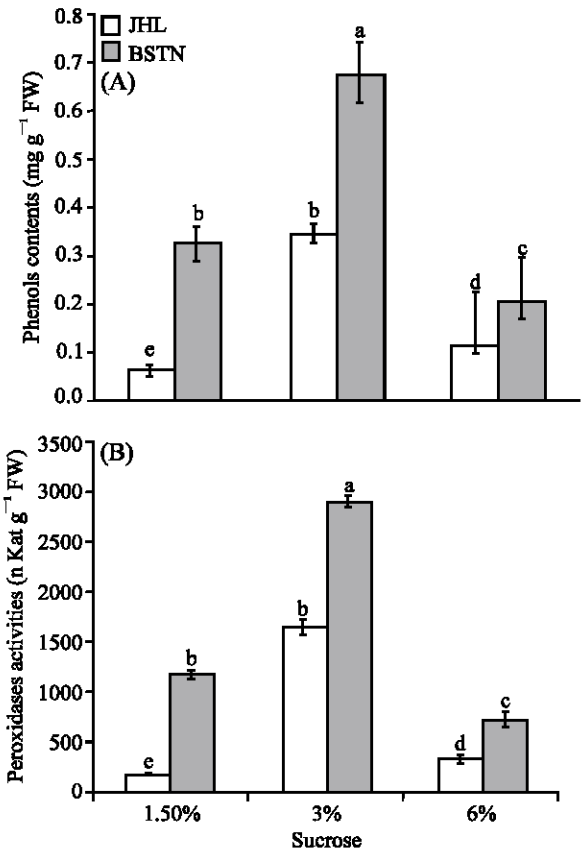

Fig. 2: Effect of sucrose on the accumulation of phenolics (A) and on peroxidases activities (B) in embryogenic mass of JHL and BSTN cultivars after two months of culture. Values are means±standard error of three replicates from two experiments Values followed by the same superscript letter are not significantly different at P= 0.05 level

somatic embryos production[23]. A similar result was obtained in Soybean (*Glycine Max*) using 8.75 or 17.5 mM sucrose. In additional tests using sucrose concentrations of 29.2; 58.4; 87.6; 116.9; 146.1 or 175.3 mM, which correspond, respectively to 1, 2, 3, 4, 5 and 6% sucrose, the resulting biomass increases in the Soybean system were 143.3, 136.9, 100.0, 89.8, 63.6 and 63.1%, respectively, in comparison to what it has been obtained on medium with 3% sucrose[24]. In *Abies nordmanniana,* it was found that maltose was superior to sucrose in terms of the number of somatic embryos formed as well as their germination percentage; the mixture of maltose and sucrose were not found to produce as good results as pure maltose[25].

The biochemical analysis showed that the increase of sucrose concentration from 1.5 to 3% leads to a significant ($P<0.05$) enhancement in soluble proteins (14.9 mg g^{-1} FW for JHL cultivar and 5 mg g^{-1} FW for BSTN cultivar) and sugars content (22.5 and 22.7 mg g^{-1} FW, respectively, for JHL and BSTN cultivars) (Fig. 1). In addition, a higher increase of phenolics (0.69 mg g^{-1}FW) and peroxidases activities (2.9.10^3 n Kat g^{-1}FW) was found in BSTN cultivar (Fig. 2). Recently, Iraqi and Tremblay[6] indicated that embryos matured on 6% sucrose contained significantly more soluble and insoluble proteins than embryos matured on any other treatments. These authors indicated also that a maturation medium of black spruce and white spruce somatic

embryos containing 6% sucrose, gave significantly ($P<0.05$) more embryos than a medium containing 3.16% of each glucose and fructose. In *Abies alba,* it was found that no mature embryos were produced in the presence of 4.1% sucrose, whereas maltose promoted maturation[26]. A similar carbohydrate effect has been found in *Pinus taeda*[27]. Samoylov *et al.*[24] indicated that sucrose promotes faster embryos histo-differentiation and maturation and allows the recovery of up to 50% more mature, cotyledon stage embryos within 3 weeks. According to the above results, it seems that sucrose followed by hydrolysis throughout the maturation period may be a regulator factor for the synthesis of storage proteins and sugars in somatic embryos[28]. These authors indicated that sucrose has been considered as a regulator of storage proteins and sugars genes by enhancing their expression.

On the other hand, when sucrose concentration increased from 1.5 to 3%, a low accumulation of proteins (Fig. 1B) and a higher accumulation of phenolics (Fig. 2A) were observed in BSTN cultivar (susceptible to tissue browning) compared to JHL cultivar (less susceptible to tissue browning). This negative relation between proteins and phenolics contents indicates a competition between the two metabolic ways for phenylalanine and the amino acid precursor for proteins and phenolics compounds[5,29]. In tobacco and alfalfa suspension cultures, the increase in phenylalanine ammonia-lyase (PAL) activity was accompanied by an elevated accumulation of phenolics, which by oxidation by polyphenoloxidases and peroxidases contribute to the phenomenon of tissue browning[30]. These authors indicated that growing and accumulation of phenolics are inversely related. The same situation is here described when JHL cultivar (the fast growing cultivar) is compared to BSTN cultivar (the low growing cultivar).

The stimulation of tissue browning in BSTN cultivar on our maturation medium rich in sucrose could be partly explained by the rapid metabolism of sucrose followed by hypoxia and ethanol accumulation in the cells[7]. This author indicated that sucrose and glucose were toxic to the microspores, whereas maltose allowed some microspores to undergo embryogenesis. Moreover, the effect of maltose on maturation of *Abies nordmanniana* somatic embryos is related to the fact that maltose is taken up directly from the medium, whereas sucrose is hydrolysed into monosacharides[7]. This same author also suggested that sucrose was more rapidly metabolized than maltose leading to hypoxia and ethanol accumulation in cells. Thus, the slow metabolism of maltose would lead to sufficient oxygen being present in the cells to allow survival and subsequent somatic embryo development.

Finally, when sucrose concentration increased from 3 to 6% we have a decrease of embryogenic mass (Table 1), soluble proteins (Fig. 2), phenolics contents and peroxidases activities (Fig. 2). The supply of 6% sucrose may be responsible for a higher osmotic pressure of medium that consequently induced the phenomenon of osmolysis of cells contributing to the stop of cells metabolism and to the suppression of suspension culture growth[31]. These results agree with those described by El Bellaj[5] showing that the increase of sucrose concentration from 3 to 6% suppressed the callogenesis index.

In conclusion, this work shows that the effect of sucrose on cell suspension culture of date palm depends on cultivar. In fact, 3% sucrose could be useful for embryo proliferation and accumulation of the proteins and sugars storage in embryogenic mass especially for JHL cultivar. In addition, as compared to 1.5 and 6%, the use of 3% of sucrose enhanced phenolics contents and peroxidases activities in embryogenic mass of BSTN cultivar.

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