ROLE OF MANNITOL IN INDUCING DIRECT SOMATIC EMBRYOGENSIS OF DATE PALM

*Zeinab E. Zaid, *Mona M. Hassan and *Rehab A. Sidky

*The Central Laboratory for Date Palm Researches and Development, Agriculture Research Center, Cairo, Egypt.

Abstract: This study was carried to discus the effect of mannitol on direct somatic embryogenesis of *Phoenix dactylifere L.* cvs. Malakaby and Zaghlool, using shoot tip, sub-shoot tip and leaf primordia used as a plant material. The explants were cultured on 3/4 MS basal medium supplemented various concentrations of mannitol at (0.0, 5.0, 10.0 and 15.0 g/l) during culture periods (60, 90 and 120 day). The addition of mannitol to culture medium significantly suppressed callus formation compared to the control medium (Mannitol free medium). The treatment with high concentrations 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, but on the other hand, leaf primordial explants failed to form direct somatic embryos. Histologically, somatic embryos derived from common epidermal cells and study showed the, formation of embryogenic cell clumps. Finally it appears that application of mannitol induced direct formation of somatic embryos from epidermal cell without the formation of visible calli as osmatic stress.

Key words: Direct somatic embryogensis, Mannitol, Date palm

Introduction

In vitro cultured plants and tissue need an exogenous supply of carbohydrates as source of carbon. In general, sucrose is the carbohydrate of choice as carbon source for *in vitro* plant culture, probably because it is the major transport-sugar of many plants (Murashige and Skoog, 1962; Thorpe, 1982; Thompson and Thorpe, 1987). However, there are number of species that can grow on carbohydrates different than sucrose (Pua and Chong. 1984; Nadel *et al*., 1989; Vu *et al.*,. 1993). Sugar alcohols primary photosynthetic products that fulfill the functions of reduced carbon translocation and storage in a wide range of plant species (Steinnitz, 1999). In many cases, those alternative carbohydrates are sugar alcohol, such as sorbitol, glycerol or mannitol (Garcia *et al.*, 2002). Mannitol, the most widely distributed sugar alcohol in plant kingdom (Bieleski, 1982), can substitute for sucrose in callus cultures of *Fraxinus americana* (Wolter and Skoog, 1966) and cell suspension of celery (Stoop and Pharr, 1993), both species that produce and translocate mannitol.

Mannitol has been widely used to apply osmotic stress *in vitro* when added to media at high concentrations. Since it was believed to be nearly metabolically inert, the *in vitro* mannitol-dependent phenomena are usually defined predominantly as osmotic effects (Steinitz, 1999). The objective of this study was to investigate the effect of mannitol on direct somatic embryogenesis of date palm.

Materials and Methods

Plant material

Shoot tip were surface sterilized with a sodium hypochlorite solution (available chlorine 0.5%) for 30min. They were then the extensively washed in sterile distilled water. Three explants were used in this investigation:-

1- Shoot tip 5 – 10 mm in length cut longitudinally four pieces (ST).

2- Sub-shoot tip that included the base of leaf primordial with a part of sub lending base (S-ST).

3- Leaf primordia (LP).

Culture media

The three explants for two cultivars Zaghlool and Malakaby were culture on 3/4 Murashige and Skoog basal nutrient medium (1962) 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 as described by (Tisserat 1982).

The effect of mannitol on induction of somatic embryogenesis

The three explants were treated with various concentrations of mannitol (0.0, 5.0, 10.0 and 15.0 g/l) for three culture periods (60, 90 and 120 day). 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 culture were maintained in growth room in total darkness at 27 °C. The experiment was conducted with 10 replicates for each treatment and repeated at least twice. The percentage of somatic embryogenesis and the percentage of callus formation was recorded as measurement

Histology

For histological analysis, specimens 1 cm from globular embryo which formed when the explants was treated with mannitol concentrations, were killed and fixed in F.A.A washed in 50% alcohol, dehydrated in normal butyl and embedded in paraffin wax. Cross sections, 20µ thick were cut, and stained by crystal violet /erythrosine combination and mounted in Canada balsam and then the slides were examined microscopically.

Statistical analysis

Data were subjected to analysis of variance and means were compared, where appropriate, using LSD at 5% significance (Snedecor and Cochran, 1972).

Results and Discussion

The percentage of callus formation:

Shoot tip, sub-shoot tip and leaf primordial explants of date palm cvs. Zaghlool and malakaby which were cultured on MS medium with 0.0, 5.0, 10.0 and 15.0 g/l mannitol to induce direct somatic embryogenesis, it was clearly noticed from Table 1. that addition of mannitol to culture medium significantly suppressed callus formation compared with the control medium (Mannitol-free medium). The best response to callus induce was recorded when the explants were cultured on medium containing 10 mg/l 2,4-D + 3 mg/l 2ip without mannitol (control medium) 3.22%. These results agreed with Tisserat, 1981, 1982; Mater, 1986; Hassan, 2002.The explants could not form callus when were cultured on basal medium containing high concentration of mannitol.

The culture periods significantly affected callus production, it was observed that, after 120 day from culturing the explants on MS basal medium with 10.0 mg/l 2,4-D + 3.0 mg/l 2ip (Mannitol-free medium) gave the highest response for callus production of both genotypes Zaghlool and Malakaby (1.74 and 1.08 respectively). Abo–El Soaud, 1999 declared that, through two subcultures on medium containing high levels of auxins, date palm explants gave rise to yellowish aggregated type of callus tissue.

All types of explants produced callus, but the shoot tips explants were superior in increasing the number of explants which able to produce callus comparing with other explants. The mean value of callus percentage of shoot tips was 1.75, sub–shoot tips was 1.41 and leaf primordial was 0.91. These explants have also been successfully used other authors (Sharma *et al*., 1984; Veramandi and Navarro 1997).

The percentage of direct somatic embryogenesis:

The effects of various concentrations of mannitol on direct somatic embryogenesis shown in Table 2. The treatment with high concentrations of mannitol was effective for somatic embryo formation directly. When the explants (shoot tip and sub-shoot tip) cultured on high concentrations of mannitol medium (10 and 15 g/l), somatic embryos appeared directly on the surface of the explants without visible callus formation. This results agreed with (Kamada *et al*., 1993) who reported that, when the primary culture medium contained 0 .61 M mannitol and 0.09 M sucrose, cotyledon segments and apical tip segments also produced somatic embryos when transferred to hormone

Table(1): Effect of mannitol on callus formation percentage during the three culture periods of three types of explant of *Phoenix dactylifera* L. cvs.Zaghlool and Malakaby.

(A) Mannitol g/l	(B) Culture period (Days)	(C) Type of explant							
		Zaghlool				Malakaby			
		ST	S-ST	LP	Mean	ST	S-ST	LP	Mean
0.0	60	3.0	3.0	2.0	2.66	2.0	1.0	1.0	1.33
	90	4.0	3.0	2.0	3.00	3.0	2.0	1.0	2.00
	120	5.0	4.0	3.0	4.00	3.0	2.0	2.0	2.33
Mean (A)		**4.0**	**3.33**	**2.33**	**3.22a**	**2.66**	**1.66**	**1.33**	**1.88a**
5.0	60	2.0	2.0	0.0	1.33	1.0	1.0	0.0	0.66
	90	3.0	2.0	2.0	2.33	2.0	1.0	0.0	1.00
	120	3.0	3.0	2.0	2.66	2.0	2.0	1.0	1.66
Mean (A)		**2.66**	**2.33**	**1.33**	**2.10b**	**1.66**	**1.33**	**0.33**	**1.10b**
10.0	60	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	90	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0..0
	120	1.0	0.0	0.0	0.33	1.0	0.0	0.0	0.33
Mean (A)		**0.33**	**0.0**	**0.0**	**0.11c**	**0.33**	**0.0**	**0.0**	**0.11c**
15.0	60	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00
	90	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00
	120	0.0	0.0	0.0	0.0	0.	0.0	0.0	0.00
Mean (A)		**0.0**	**0.0**	**0.0**	**0.0d**	**0.0**	**0.0**	**0.0**	**0.0d**
Mean (B)		**0.99c**	**1.33b**	**1.74a**		**0.49c**	**0.75b**	**1.08a**	
Mean (C)		**1.75a**	**1.41b**	**0.91c**		**1.16a**	**0.75b**	**0.41c**	

Mean separation by L.S.D at 0.05

AB	0.05	0.02
AC	0.05	0.02
BC	0.04	0.02
ABC	0.08	0.04

ST Shoot tip

S-ST Sub-shoot tip

LP Leaf primordia

Table(2): Effecet of mannitol induced stress on direct somatic embryogenesis percentage during the three culture periods of three types of explant of *Phoenix dactylifera cvs.*Zaghlool and Malakaby.

(A) Mannitol g/l	(B) Culture period (Days)	(C) Type of explant							
		Zaghlool				Malakaby			
		S.T	S-S.T	LP	Mean	S.T	S-S.T	LP	Mean
0.0	60	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.00
	90	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.00
	120	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.00
Mean (A)		0.0	0.0	0.0	0.0	0.00	0.00	0.00	0.0
5.0	60	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.00
	90	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.00
	120	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.00
Mean (A)		0.0	0.0	0.0	0.0	0.00	0.00	0.00	0.0
10.0	60	2.0	2.0	0.0	1.33	1.0	2.0	0.0	1.00
	90	3.0	3.0	0.0	2.00	1.0	3.0	0.0	1.33
	120	3.0	4.0	0.0	2.33	2.0	3.0	0.0	1.66
Mean (A)		2.66	3.00	0.0	1.88	1.33	2.66	0.00	1.33
15.0	60	2.0	3.0	0.0	1.66	2.0	2.0	0.0	1.33
	90	3.0	4.0	1.0	2.66	2.0	3.0	0.0	1.66
	120	4.0	5.0	1.0	3.33	2.0	4.0	0.0	2.00
Mean (A)		3.00	4.00	0.66	2.55	2.00	3.00	0.00	1.66
Mean (B)		0.74c	1.16b	1.41a		0.58c	0.74b	0.91a	
Mean (C)		1.41b	1.75a	0.16c		0.83b	1.41a	0.0c	

Mean separation by L.S.D at 0.05

AB	0.09	0.02
AC	0.09	0.02
BC	0.08	0.02
ABC	0.16	0.04

ST Shoot tip

S-ST Sub-shoot tip

LP Leaf primordia

– Free MS medium with 0.09 M sucrose. In all cases, somatic embryos were formed directly from the explants without visible callus formation.

Leaf primordial explant could not form somatic embryos directly, especially Malakaby cv. (0.0). The highest significant percentage (1.41 and 1.75 respectively) of direct somatic embryogenesis was achieved when the shoot tip and sub-shoot tips explants were cultured on basal medium supplemented with 15 g/l mannitol after 120 day from culturing for zaghlool cv.

These somatic embryos did not develop further on high mannitol medium, but upon transferring them to medium with 30 g/l sucrose, they developed into plantlets.

Results obtained from Tables 1 and 2 showed that, Zaghloal cv. was superior than malakaby cv.in the mean values of direct somatic embryos induction.

Mass of somatic embryos formed directly on the surface of the explants base (shoot tip and sub-shoot tip). The photograph was taken 120 day after the transfer.

Histology analysis

Histologically study showed that somatic embryos derived from common epidermal cells and differentiated without formation of embryogenic cell clumps, but directly from epidermal cell Fig 1. Application of mannitol induced direct formation of somatic embryos from epidermal cell without the formation of visible calli as osmatic stress.

The results presented in this report show that somatic embryogenesis in date palm could be induced by giving osmotic stress without hormonal treatment, although the development of embryos to plantlets was suppressed under high osmotic conditions. It has been reported that osmotic stress caused an increase of endogenous level of abscisic acid in several plant species (Davies and Jones, 1991; Pierpoint, 1994; Voesenek and Vanderveen, 1994; Yalpani *et al*., 1994). These results show that, somatic embryos did not develop further on high mannitol medium, but upon transferring them to medium with 30 g/l sucrose, they developed into plantlets.

In celery, the anabolic and catabolic pathway of mannitol metabolism have been elucidated (Rumpho *et al*., 1983). Pharr *et al* (1995) suggested that the metabolic use of mannitol provides some energetic advantage to the plants, since the hexose-P generated from mannitol in sink cell is accompanied by the net generation of two ATP_s per mannitol converted.

Therefore, this experiment should provide a novel method for analyzing the induction process from the somatic cell stage to the mature embryo, and may be useful for study the initiation stage of somatic embryogenesis.

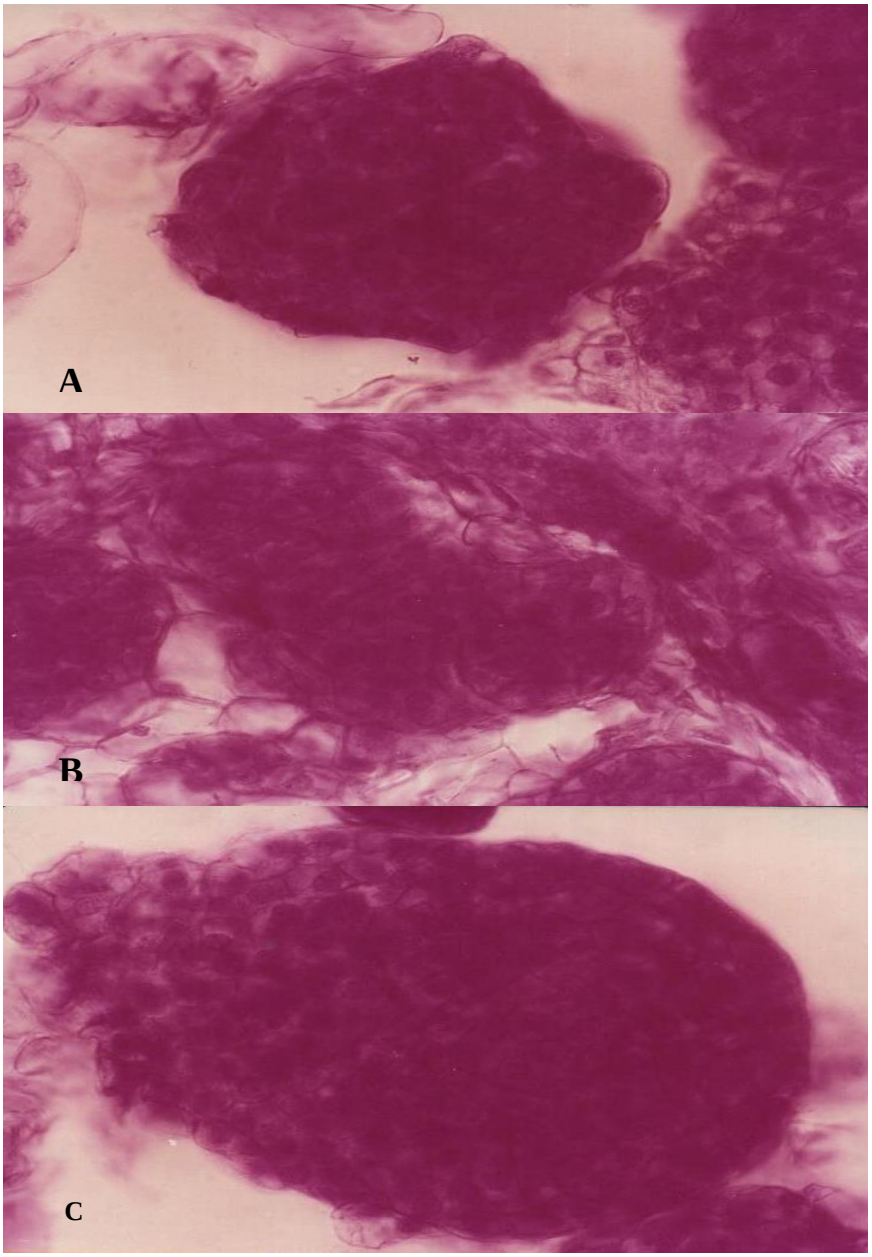

Fig.(1): Different stages of somatic embryos. (A) Globular stage; (B) Heart shaped; (C) Torpedo shaped

Acknowledgement

This study was supported by The Central Laboratory for Date Palm Researches and development. The authors wish to acknowledge of Dr. M.A. Abdel-Baky for the help in the histological examination and also Dr. M.M. El-Dawyaty for helpful discussion.

References

Abo-El-Soaud, A.A. 1999. Studies on date palm propagation through tissue culture. M.Sc. Thesis, Department of Pomology, Faculty of Agriculture, Cairo University, Egypt, pp.62.

Bieleski, R.L. 1982. Sugar alcohols. In: Loewus FA & Tanner W (eds) Encyclopedia of Plant Physiology, New Series. Vol 13 [a] (pp 158 – 192) Springer. Berlin. Heidelberg. New York.

Davies, W. J. & H. G. Jones. 1991. Abscisisc Acid. Bios Scientific, Oxford.

Gareia, J.I., J. Troncoso., R. Sarmiento & A. Troncoso. 2002. Influence of carbon source and concentration on the *in vitro* development of olive zygotic embryos and explants raised from them. Plant Cell, Tissue and Organ Culture, 69: 95 – 100.

Hassan, M.M. 2002. *In vitro* studies on somatic embryogenesis conservation of date palm. Ph.D. Thesis, Department of Pomology, Faculty of Agriculture, Cairo University, Egypt.

Kamada, H., Keiko Ishikawa., Hitoshi Saga and Hiroshi Harada. 1993. Induction of somatic embryogenesis in carrot by osmotic stress. Plant Tissue Culture Letters, 10 (1): 38 – 44.

Mater, A.A. 1986. *In vitro* propagation of (*Phoenix dactylifera* L.). Date Palm J., 4 (2) : 137-152.

Murashige, T. and F.A. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol.Plant., 15: 473-479.

Nadel, BL., A. Altman & M. Ziv. 1989. Regulation of somatic embryogenesis in celery cell suspensions. 1 Promoting effects of mannitol on somatic embryo development. Plant Cell, Tissue and Organ Culture, 18: 181- 189.

Pharr, DM., JMH. Stoop., J.D. Williamson., ME. Studer Feisi., MO. Massel & MA. Conkling. 1995. The dual role of mannitol as osmoproteclanet and photoassimilate in celery. HortScience 30: 1182 – 1188.

Pierpoint, W.S. 1994. Salicylic acid and its derivatives in plants: medicines, metabolites and messenger molecules. Adv. Bot. Res. 20: 165 – 235.

Pua, E.C & C. Chong. 1984. Requirement for sorbitol (D-g:chol) as carbon source for in vitro propagation of Malus. NO. 5. Can. J. Bot. 62: 1545-1549.

Rumpho, ME., GE. Edwards & WH. Loescher 1983. Apathway for photosynthetic carbon flow to mannitol in celery leaves. Plant Physiol. 73: 869 – 873.

Sharma, D.R., S. Dawrara, and J.B. Chowdury.1984. Somatic embryogensis and plant regeneration in date palm (*Phoenix dactylifera* L.) cv. Khadravi through tissue culture. Indian Journal of Experimental Biology, 22, 596-598.

Snedecor, G.W. and W.G. Cochran .1972. Statistical Method 6th. The Iowa State University Press, Ames., Iowa U.S.A., 59P.

Steinitz, B. 1999. Sugar alcohols display nonosmatic role in regulating morphogenesis and metabolism in plants that do not produce polyols as primary photosynthetic products. J. Plant Physiol. 155: 1 – 8.

Stoop, JMH & DM. Pharr. 1993. Effect of different carbon sources on relative growth rate, inertial carbohydrates, and mannitol l-oxidoreductase activity in celery suspension cultures. Plant Physiol. 103: 1001 – 1008.

Thompson, MR &TA. Thorpe. 1987. Metabolic and nonmetabolic role of carbohydrates. In: Bonga J.M & D.J. Durzan (eds) Cell and Tissue Culture in Forestry. Vol. 1 (pp 89 - 112). Martinus Nijhoff. Dordrecht.

Thorpe, TA. 1982. Carbohydrates utilization and metabolism. In Bonga J.M & D.J. Durzan (eds) Cell and Tissue Culture in Forestry. (pp325 – 368). Martinus Nijhoff/Dr W Junk, The Hague.

Tisserat, B. 1981. Production of free - living date palms through tissue culture. Date Palm J. 1: 43–54.

Tisserat, B. 1982. Factors involved in the production of plantlets from date palm callus cultures. Euphytica, 31 (1): 201-214.

Veramendi, J. and L. Navarro. 1996. Influence of physical conditions of nutrient medium and sucrose on somatic embryogensis of date palm". Plant Cell, Tissue and Organ Culture, 40 (2) 159-164.

Voesenek, LACJ & R. Vanderveen 1994. The role of phytohormones in plant stress – too much or too little water. Acta Bot. Neerl. 43: 91 – 127.

Vu, JCV. RP. Neidz & G. Yelonosky 1993. Glycerol stimulation of chlorophyll synthesis, embryogenesis, and carboxylation and sucrose metabolism enzymes in nucellar callus of Hamlin sweet orange. Plant Cell, Tissue and Organ. Cult. 33: 75 –80.

Wolter, K.E & Skoog 1966. Nutritional requirements of Fraxinus callus cultures. Am. J. Bot. 53: 263 – 269.

Yalpani, N. A.J. Enyedi, J. Leon & I. Raskin. 1994. Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta 193: 372 – 376.

دور المانيتول فى إنتاج الأجنة الجسمية مباشرة لنباتات نخيل البلح

* زينب زايد * منى حسن * رحاب صدقي

* المعمل المركزي لأبحاث وتطوير نخيل البلح – مركز البحوث الزراعية - مصر

أجريت هذه الدراسة لبحث تأثير المانيتول على إنتاج الأجنة الجسمية مباشرة لنبات نخيل البلح صنفى ملاكابى وزغلول. استخدمت ثلاثة أجزاء نباتية فى هذه الدراسة هى القمة النامية و تحت القمة النامية و الأوراق الأولية. زرعت الأجزاء النباتية على 4/3 بيئة موراشيجى وسكوج مضاف إليها تركيزات مختلف من المانيتول (5.0 و 10.0 و 15.0 حم/ لتر) خلال ثلاثة دورات للنمو (60 و90 و 120 يوم). وجد أن إضافة المانيتول إلى بيئة الزراعة ثبط معنوياً تكوين الكالس مقارنةً بالبيئة الكنترول الخالية منه. تكونت أجنة جسمية مباشرة عند زراعة الأجزاء النباتية على بيئة تحتوي تركيزات عالية من المانيتول. القمة النامية وتحت القمة النامية أعطت أكبر نسبة مئوية لتكوين الأجنة الجسمية المباشرة بدون تكوين للكالس، بينما فشلت الأوراق الأولية في إنتاج الأجنة الجسمية مباشرةً. هستولوجياً تكونت الأجنة الجسمية من خلايا طبقة البشرة بدون تكوين كالس مرئي تحت ظروف الإجهاد باستخدام السكريات الكحولية (المانيتول).