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The Role of Different Osmotic Agents on Embryogenesis and Plant Regeneration in Date Palm cultivar, Khalas

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Original Research Article

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Abstract: *In vitro* embryogenesis and plant regeneration were studied in date palm cultivar, Khalas using three osmotic agents (PEG, Mannitol, and Glucose) and NaCl for five different time periods ranging from 1 to 24 hrs. The highest frequency of somatic embryogenesis was recorded in the medium containing 15% PEG in the MS-culture medium. Similarly, the highest number of plantlets was also obtained on the regenerating medium containing PEG under elevated treatment time periods. Mannitol and glucose showed almost the same response at all treatment levels producing on an average 2 to 3 embryos per culture but did not had any positive effect on plant regeneration. Of all the osmtic agents, PEG proved to be better in producing more callus fresh and dry weights followed by mannitol indicating that at this stage these osmotica worked as non-metabolic osmotic agents. The use of NaCl as an osmotic agent produced the most adverse effects on callus fresh weight and embryogenesis, with no embryos formation at 12 and 24 h treatments. These studies revealed that elevated levels of PEG showed stimulating effects and helped in the production of more callus mass, somatic embryos and plantlets formation compared to other osmotic agents used.

Keywords: Carbon sources, Embryogenesis, Khalas, Mannitol, Osmotic agents, Polyethylene glycol.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a member of the family *Palmace* (*Arecaceae*), inhabiting tropical and sub-tropical habitat. It consits of woody perennial monocots comprising about 200 genera and 2500 species [1]. Date palm is native to almost all the Middle east countires concentrating in Arabic Penunsela and economically the most conspicous commercial crops with average number of trees estimated >62 million [1, 2].

In vitro micropropagation in date palm is carried out by somatic embryogenesis and organogenesis [3]. Somatic embryogenesis involved the regular development of the vegtative embryos from haploid or diploid embryogenic calli without gametogenesis [4, 5]. In this technique callus formation is induced by culturing explants on proper nutritional media supplemented with high auxin concentrations [6]. The callus formation can be increased many folds simultaneously by sub-culturing on proper nutritional media. This method has the characteristic ability of massive plant producion in the shortest possible time periods [2].

On the other hands, in organogenesis plantlets are directly propagated from buds bypassing the callus stage [5]. However compared to embryogenesis, organogenesis results fewer plantlets in relatively longer period of time. Nevertheless, the resulted plantlets resemble the mother plants because these are produced directly from mother tissues [2, 5].

Carbohydrates provide energy and act as a substrate for carbon biosynthesis in the tissue culturing media [7]. Sucrose has been widely used as a rich

carbon source and osmotic agent to induce somatic embryogenesis in various plants [8-11]. It is well documented that the callus formation, somatic embryogenesis, metabolism and shoot proliferation rates improved significantly in a carbohydrate enriched tissue culture media [9, 11-14]. However, the use of high sucrose concentrations (9/L2%) in the nutrient media exerts adverse effects on embryo maturation [15].

Osmotic stress due to depleted water is an important factor in deciding proper embryo development in many plant species [16, 17]. The low molecular weight osmotica are not supposed to be optimal for embryo maturation because these molecules penetrate into the developing cell and disturb the internal osmotic potential, which results into reduced internal osmotic potential. As a result, the osmotic stress is imposed temporarily and interfere with cellular metaboltic acticities [18]. In many studies it has been shown that non-plasmolyzing osmotica such as polyethylene glycol-4000 are able to increase number of embryos, enhance embryo quality and promote embryo maturation in plants [16, 3]. The medium containing these non-plasmolyzing osmotica curbs the water uptake leading to a natural drought stress to the developing embryos and thus, speed up their maturation [17].

The current pace of progress in date palm regeneration and/or transformation systems is very slow due to a longer time period required for callus induction and plantlet regeneration [1]. The optimization of a highly robust, efficient and reproducible *in vitro* regeneration protocol is pre-requisite for functional genomics and transgenic studies in date palm. Moreover, a successful tissue culture technique is also of utmost importance to collect, multiply, characterize and conserve the date palm germplasm [10]. The effect of high molecular weight osmotic agents, such as Polyethylene Glycol (PEG), Mannitol, and high concentrations of glucose and NaCl has not been investigated in date palm before. Moreover, the optimization of time periods under these osmotic agents is still not clear. Keeping in view the importance of these osmotic agents in embryogenesis and plant regeneration, the present studies were therefore, conducted to (i) identify various osmotic agents for enhancing embryogenesis and plant regeneration, and (ii) optimize the duration for the effectiveness on embryos production and plant regeneration in date palm cultivar, Khalas.

MATERIALS AND METHODS

Preparation of plant material

The terminal shoot tips and surrounding leaf primordia excised from offshoots of date palm cultivar 'Khalas' were carefully and immediately put into antioxidant solution containing 150 g/L ascorbic acid and 100 g/L citric acid. The shoot tip were sterilized twice using Benlate fungicide for 20 min followed by multiple washes with sterile water. Then the shoot tips were placed in 20 % chlorox solution with few drops of Tween-20 for at least 20 min. Shoot tips were then washed thrice with sterile water and kept in the antioxidant solution until explants were excised.

Culture media and multiplication stages

Terminal shoot tips and surrounding leaf primordia excised from offshoots were cultured on MS medium [19] salts supplemented with 170 mg/L $NaH_2PO_4.2H_2O$, Inositol 125 mg/L, Glutamine 200 mg/L, Thiamine 5 mg/L, Pyridoxine HCl 1 mg/L, Nicotinic acid 1 mg/L, Glycine 2 mg/L, Sucrose 30 g/L, Activated charcoal 1.5 g/L and purified agar 6.5 g/L. The explants were subsequently transferred to MS-media for culture initiation (100 mg/L 2,4-D and 2iP 3 mg/L), culture swelling (10 mg/L NAA and 6 mg/L 2iP) and formation of embryogenic calli (10 mg/L NAA and 30 mg/L 2iP) for 9, 16 and 3 weeks, respectively. All the incubations were carried out at 25±2°C in a 16h photoperiod provided from cool white florescent lamps

Experimental treatments and design:

The resultant cultures were placed onto agar-solidfied phytohormone-free MS medium containing 0.7 M each of mannitol and glucose, 0.4M NaCl and 15% PEG-8000 for 1, 3, 6, 12 and 24h. The calli were washed with liquid MS medium and were then transferred to hormone free medium supplemented with 30 g/L sucrose for the production of embryos and plantlets. Each treatment was replicated 10 times. Cultures were incubated at 25±2°C in 16 hr of day light supplied by 65/80 Warm White Weisse 3500 fluorescent tubes. Data was recorded for total number of somatic embryos, total number of germinating embryos and fresh and dry weights of the cultures after 16 weeks of culturing.

Statistical Analysis

A randomized complete block design was used according to Gomez and Gomez [20]. The treatment means were compared using Least Significant Difference (LSD) at 5 % level of probability. All statistical analyses were performed using the facility of computer and SAS software package 30 [21].

RESULTS AND DISCUSSION

The effect of four different types of osmotic agents namely the Glucose, Mannitol, PEG-8000 and NaCl on callus growth, embryogenesis and plant regeneration in date palm cultivar, Khalas is presented in Table-1. The results on the main effects of these osmotic agents on fresh and dry weights of calli at five time periods such as 1, 3, 6, 12 and 24 h is presented in Table-1. The data reveal that both the PEG and mannitol significantly enhanced the callus growth obtaining the fresh callus weights of 3.59 and 2.87 g, respectively followed by glucose (2.39 g) and NaCl

(0.447 g) treatment. A similar trend was observed in dry weight at different time periods showing significantly varying effects on callus growth (Table-1). Maximum dry weight (0.86 g) was obtained using PEG compared to the other osmotic agents. However, the callus proliferation showed increases with increasing the time period upto 12 h. At 12 h time period, the maximum fresh weight (3.96 g) and dry weight (0.90 g) of the calli was obtained. On the other hand, a decline in fresh (3.03 g) and dry weights (0.67 g) was noticed at 24 h time period. (Table-1). Thus, 12 h time period treatment showed positive effects on callus growth and development yielding the maximum callus fresh and dry weights compared to 1, 3 and 6 h time periods. However, after 12 h time period, the callus proliferation reduced and produced less fresh and dry weights.

Table-1: Effect of different osmotic agents (/L.73 MPa) on fresh and dry weights of date palm callus cultures

	Treatments	Fresh Weight (g)	Dry Weight (g)
Osmotic Agents	NaCl	0.447 d*	0.105 c*
	PEG-8000	3.591 a	0.858 a
	Glucose	2.397 c	0.533 b
	Manitol	2.870 b	0.803 a
L.S.D 0.05		0.382	0.150
Time (h)	1	1.052 d	0.282 c
	3	1.843 c	0.419 c
	6	1.748 c	0.44 bc
	12	3.959 a	0.899 a
	24	3.030 b	0.670 b
L.S.D 0.05		0.427	0.168

- Means followed by the same letter do not differ significantly at 0.05 level.

The effects of osmotic agents and time periods on embryogenesis is presented in Table-2 and Fig-1. A comparison of all the osmotic agents showed that PEG proved better by producing more embryos per culturefollowed by mannitol than other agents. The PEG treatment at 12 h yielded the maximum number of embryos per culture than other osmotic agent and time period used in this study (Fig-1). Also, Mannitol and glucose produced consistant results in all the time periods except 1 hrs treatment. The NaCl treatment significantly affected the embryogenesis at low time intervals, but after 6-h time period it showed negative effects without producing any embryogenesis (Table-2 and Fig-1).

Table-2: *In Vitro* effect of different osmotic agents (/L.73 MPa) on Embryogenesis in date palm callus cultures of *cv* Khalas.

Time (hrs)	Glucose	Mannitol	NaCl	PEG
1	+	-	+	+
3	++	+++	+++	+
6	+	++	+++	++
12	++	++	-	++++
24	+	++	-	+++

(-) No. embryos, (+) shows the number of embryos produced per culture

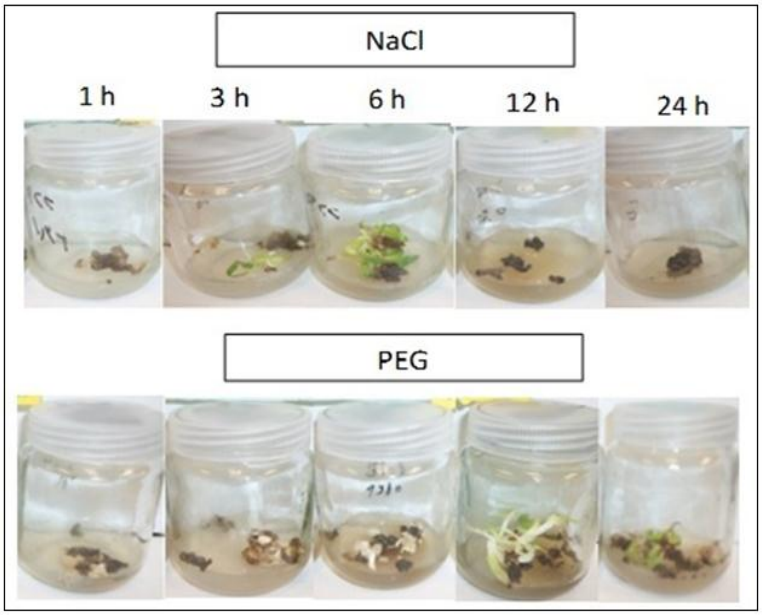

Fig-1: Effects of osmotic shock peroids and type of osmotica (PEG and NaCl) on number of embryos in date palm callus cultures of *cv*. Khalas.

Plant regeneration potential of the osmotic agents under different time periods was also assesed either as carbon source or as an osmotic agent (Table-3). The data revealed that PEG significantly improved the regeneration potential of the cultures when treated for 12 h time period producing 3 plantlets per culture. However, glucose, mannitol and NaCl showed varying effects at different time periods without obtaining any plantlets (Table-3).

The correlation coefficients between osmotic shock peroids and type of osmotica showed that the PEG at 12 hrs time period proved to be the most effective osmotic agent with a coefficient of determination (R^2) value of 0.8439. However, the other osmotic agents showed very poor values of co-efficient of determination to PEG. The correlation coefficient data for PEG indicated that 12 hrs time period is the best for induction of somatic embryos and is recommended in the date palm tissue culture studies (Fig-1).The NaCl osmotic agent showed poor correlation coeffcient with with R^2 value of 0.42. Hence, the NaCl treatment is not recommened for longer period of time due to its toxicity effects (Fig-1).

Table-3: *In Vitro* effect of different osmotic agents (/L.73 MPa) on plant regeneration in date palm callus cultures of cv Khalas.

Time (hrs)	Glucose	Mannitol	NaCl	PEG
1	-	-	-	-
3	+	+	-	-
6	-	+	++	+
12	-	-	-	+++
24	-	-	-	+

(-) No plant formation, (+) Shows the number of plants produced per culture

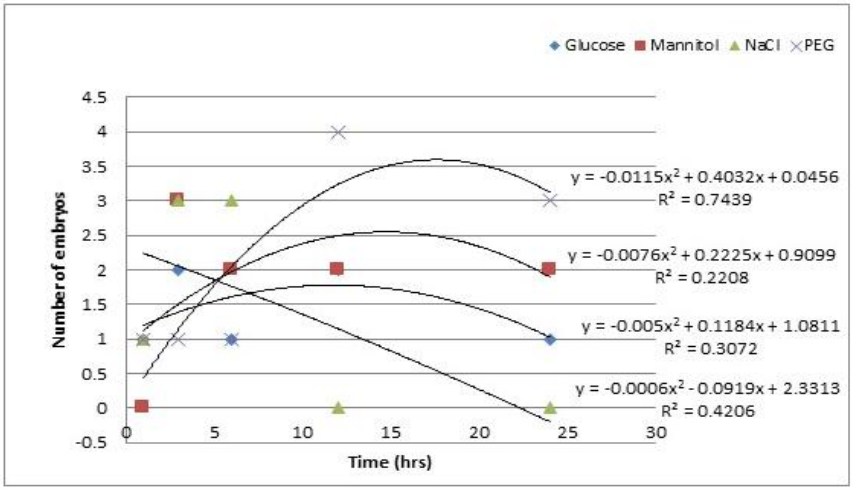

Fig-2: Relationships between osmotic shock peroids and type of osmotica on number of embryos in date palm callus cultures of *cv*. Khalas.

Previously, different carbohydrates and osmotic compounds were used *in vitro* either as a source of energy or as an osmoticum [6].

The results of this study revealed that both the PEG and Mannitol significantly enhanced the callus growth while, the NaCl showed deleterious effects on callus growth. Our results corroborates with those of Alkhateeb [2] who observed a substantially enhanced callus growth in the date palm cultivarSukary when the medium was fortified with PEG along with sucrose. According to some reports, the addition of sucrose in the growth medium may also act as osmotic agent thus inducing stress above the threshold levels and caused growth reduction in callus [11]. However, the accumulation of proline and soluble carobohydrates the increased the callus dry weight during high sucrose levels [18]. With increasing the time period, the callus proliferation was enhanced except with NaCl which was more prounouced with time. This might be due to increasing osmotic stress induced by the osmotic agents rather than the energy source according to the findings of Aazami *et al*., [13] and Amiri and Kazwmitabar [22].

Although in this study, the PEG induced significant affects on the callus growth in date palm cultivar, Khalas which might be due to the species-specific characterisistic. In other studies, the addition of PEG alonghwith sucrose in soybean [23] and flax [8] did not produce significant effects on callus induction and shoot regeration.

The significant effects of PEG on callus growth in this study might be due to the negative osmotic potential (/L.73 MPa) in the culture medium that might have contributed in mainatining turgor in tissues resulting in the absorption of more nutrients causing promoted growth of the cultures. During the maintenance of the turgor, the cells accumulate more sugars and organic solutes which leads to osmotic adjustment and enhanced growth [24].

The use of glucose as an osmotic agent also produced consistant results in all the time periods during this study (Table-2). This may indicates that glucose is metabolized continously irrespective of the time periods improving embryogenesis with no or very low osmotic effect as observed in other osmotic agents. On the other hands, NaCl may produce deliterious effects on the embryo induction and plant regeneration after a certain time period (Tables-2 & 3 and Fig-2) by generating osmotic stress and ionic toxicity. Such effect was also evident from the low coefficient of determination (R2) value of 0.42 (Fig-2) where a rapid decline was observed after 3 hrs of NaCl treatment.

Plant regeneration potential of the osmotic agents under different time periods was higher in PEG and manitol. However, the low or no regeneration in NaCl after prolonged treatment may be due to either the toxic or osmotic effects of the salt in the culture medium. Therefore, the NaCl at low time intervals seems beneficial for regeneration. However, its concentration needs to be optimized as an important osmotic agent in tissue culture studies.

CONCLUSIONS

A number of osmotic agents have been used during different studies in crop plants for inducing water stress, both *in vivo* and *in vitro*. On the basis of findings of this study, it is suggested that PEG has the potential to induce better callus growth, embryos development and plantlet regeneration compared to other osmotic agents in date palm. Hence, the use of PEG is highly recommended in date palm tissue culture for large scale plant production. However, some other important factors such as higher concentrations of PEG and time period optimization need to be explored in future studies for more productive results.

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REFERENCES

1. Jain, S. M., Al-Khayri, J. M., & Johnson, D. V. (Eds.). (2011). *Date palm biotechnology*. Springer Science & Business Media.
2. Alkhateeb, A. A. (2006). Somatic embryogenesis in date palm (Phoenix dactylifera L.) cv. Sukary in response to sucrose and polyethylene glycol. *Biotechnology*, *5*(4), 466-470.
3. Eldin, A. F. Z., & Ibrahim, H. A. (2015). Some biochemical changes and activities of antioxidant enzymes in developing date palm somatic and zygotic embryos in vitro. *Annals of Agricultural Sciences*, *60*(1), 121-130.
4. Quiroz-Figueroa, F. R., Rojas-Herrera, R., Galaz-Avalos, R. M., & Loyola-Vargas, V. M. (2006). Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell, Tissue and Organ Culture*, *86*(3), 285.
5. Ree, J. F., & Guerra, M. P. (2015). Palm (Arecaceae) somatic embryogenesis. *In Vitro Cellular & Developmental Biology-Plant*, *51*(6), 589-602.
6. Holobiuc, I. (2015). Somatic embryogenesis in long-term cultures of Gentiana lutea L. in the presence of osmotic stress. In *The Gentianaceae-Volume 2: Biotechnology and Applications* (pp. 139-161). Springer, Berlin, Heidelberg.
7. Amiri, S., Kazemitabar, S. K., Ranjbar, G. A., & Azadbakht, M. (2011). In vitro propagation and whole plant regeneration from callus in Datura (Datura stramonium. L). *African Journal of Biotechnology*, *10*(3), 442-448.
8. Körbes, A. P., & Droste, A. (2005). Carbon sources and polyethylene glycol on soybean somatic embryo conversion. *Pesquisa Agropecuária Brasileira*, *40*(3), 211-216.
9. Gerdakaneh, M., Mozafari, A. A., Khalighi, A., & Sioseh-Mardah, A. (2009). The effects of carbohydrate source and concentration on somatic embryogenesis of strawberry (Fragaria x ananassa Duch.). *Am-Eurasian J Agric Environ Sci*, *6*(1), 76-80.
10. El-Bahr, M. K., EL-Hamid, A. A., Matter, M. A., Shaltout, A., Bekheet, S. A., & El-Ashry, A. A. (2016). In vitro conservation of embryogenic cultures of date palm using osmotic mediated growth agents. *Journal of Genetic Engineering and Biotechnology*, *14*(2), 363-370.
11. Mishra, V. K., & Singh, R. M. (1990). 3. Sorbitol and Sucrose-Induced Osmotic Stress on Growth of Wheat Callus and Plantlet Regeneration. *Culture*, (28).
12. Wani, S. H., Sofi, P. A., Gosal, S. S., & Singh, N. B. (2010). In vitro screening of rice (Oryza sativa L) callus for drought tolerance. *Communications in Biometry and Crop Science*, *5*(2), 108-115.
13. Aazami, M. A., Torabi, M., & Jalili, E. (2010). In vitro response of promising tomato genotypes for tolerance to osmotic stress. *African Journal of Biotechnology*, *9*(26), 4014-4017.
14. Ghobeishavi, H., Dorani Uliaie, E., Alavikia, S. S., & Valizadeh, M. (2015). Study of factors influencing somatic embryogenesis in rice (Oryza Sativa L.). *International Journal of Advanced Biological and Biomedical Research*, *3*(1), 43-50.
15. Lipavská, H., Svobodová, H., Albrechtová, J., Kumstýřová, L., Vágner, M., & Vondráková, Z. (2000). Carbohydrate status during somatic embryo maturation in Norway spruce. *In Vitro Cellular & Developmental Biology-Plant*, *36*(4), 260-267.
16. Belmonte, M. F., Macey, J., Yeung, E. C., & Stasolla, C. (2005). The effect of osmoticum on ascorbate and glutathione metabolism during white spruce (Picea glauca) somatic embryo development. *Plant Physiology and Biochemistry*, *43*(4), 337-346.
17. Hudec, L., Konrádová, H., Hašková, A., & Lipavská, H. (2016). Norway spruce embryogenesis: changes in carbohydrate profile, structural development and response to polyethylene glycol. *Tree physiology*, *36*(5), 548-561.
18. Javed, F., & Ikram, S. (2008). Effect of sucrose induced osmotic stress on callus growth and biochemical aspects of two wheat genotypes. *Pakistan Journal of Botany*, *40*(4), 1487-1495.
19. Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497.
20. Gomez, K. A., Gomez, K. A., & Gomez, A. A. (1984). *Statistical procedures for agricultural research*. John Wiley & Sons.
21. SAS Institute. (2011). *SAS/IML 9.3 user's guide*. Sas Institute.
22. Amiri, S., & Kazemitabar, S. K. (2011). Enhancement of callus induction and regeneration efficiency from embryo cultures of Datura stramonium by adjusting carbon sources and concentrations. *African Journal of Biotechnology*, *10*(50), 10101-10107.
23. Chen, Y., & Dribnenki, P. (2004). Effect of medium osmotic potential on callus induction and shoot regeneration in flax anther culture. *Plant cell reports*, *23*(5), 272-276.
24. Sánchez, F. J., De Andres, E. F., Tenorio, J. L., & Ayerbe, L. (2004). Growth of epicotyls, turgor maintenance and osmotic adjustment in pea plants (Pisum sativum L.) subjected to water stress. *Field Crops Research*, *86*(1), 81-90.