Callus growth and somatic embryogenesis as affected by putrescine and salicylic acid in date palm bream cv.

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ABSTRACT

Different concentrations of putrescine and salicylic acid were examined for their effect on embryonic callus and subsequent embryogenesis in *Phoenix dactylifera* cultivar Bream. Shoot tips were excised from 2-3 years old offshoots, surface sterilized and inoculated onto Murashiege and Skoog, 1962 (MS) medium supplemented with 50 mg/L picloram and 3 mg/L N6-2-isopentyl adenine (2ip). Primary callus was obtained after 24 weeks on the nutrient medium. Calli were then transferred onto fresh MS medium containing 0.0, 0.5, 1.0, 2.0 or 3.0 mM of putrescine or salicylic acid individually. Results were recorded after 12 weeks. A significant increase in embryonic callus fresh and dry weights was recorded reached 2.3 and 0.3 g respectively at 2.0 mM of putrescine and 5.0, 0.27 g at 3.0 mM of salicylic acid. After inoculation of such embryonic callus onto a fresh medium containing the same concentrations of putrescine or salicylic acid, number of mature embryos increased up to 10.3 achieving 1.7 g fresh weight for ten embryos at the concentration of 3.0 mM putrescine. Number of embryos reached 10.0 with a mean fresh weight reached 1.7 g for ten embryos at 0.5 mM of salicylic acid. It is concluded that both putrescine and salicylic acid may play a positive role in increasing callus growth and regulation of somatic embryogenesis in *Phoenix dactylifera* var. Bream tissue cultures.

Key words: *Phoenix dactylifera* L. embryos, *in vitro, r*egeneration, salicylic acid, putrecine

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) (2n=2x=36) is a dioeciously, perennial, monocotyledon fruit trees belong to the family of Arecaceae (Barrow, 1998). Dates are the major fruit crop of arid climate region in Middle East and North Africa. The heterozygosis of date palm makes its progeny strongly heterogeneous (Munier, 1981). Thus the propagation of date palm through offshoots is preferred over the seedlings. Since propagation through offshoots is slow and affected by their low survival rate, tissue culture of female plants has been preferred widely for mass production of true-to-type plants of elite varieties in demand.

Since the first attempts of date palm propagation via tissue culture that proposed by Schroeder (1970) and Reuveni et al. (1972) until now, two methods of propagation were developed, somatic embryogenesis and direct organogenesis. The production of somatic embryos from embryogenic callus was reported by many researchers (Reuveni, 1979; Mater, 1983; Omar, 1988 and Al Musawi, 2001) as well as from axillary branching of shoot tips (Tisserat, 1991 and Hameed, 2001). While Al-Maari, and Al Ghamdi (1997) and Al Khateeb et al. (2002) succeeded in enhancing adventitious bud formation on shoot tips. The first is the most common micropropagation method in commercial plant tissue culture labs.

Recently, Ibrahim (2012) initiated callus after the addition of 50 mg/L Picloram and 3.0 mg/L 2iP, re-cultured at 4 week intervals until transfer to embryogenic callus proliferation medium. The medium composition varies from researcher to another, for example it consisted of MS salts and vitamins without hormone (Omar 1992) or the same supplements described for callus induction medium (Jasim and Saad 2001; Ibrahim, 2012) for eight weeks. Embryogenic calli are transferred to hormone free MS medium (Omar, 1992) or supplemented with 0.1 NAA (Jasim and Saad 2001). The low rate of asexual embryo formation and germination prompted many researchers to enhance the processes.

It was found that supplementing the culture medium with 1 g/L apple seed powder (Saleh et al. 2006), 25 g/L corn seed powder (Jasim et al. 2008), 100 mg/L vitamin E (Al-Meer and Al-Ibresam, 2010), and 20% (v:v) coconut water or casein hydrolysate at 2.0 g/L (Khierallah and Hussein 2013). All these treatments increased somatic embryogenesis and germination percentages for several Iraqi date palm cultivars.

Mature or germinating embryos initiated roots on a medium consisted of MS salts plus 0.1 NAA and 0.01 BA (Omar *et al.,* 1992). Jasim and saad (2001) used half strength MS salts, 0.1 mg/L NAA, 30g/L sucrose, and 3g/L activated charcoal to improve rooting in Barhi cv. This stimulates root induction and shoots elongation which led to full growth plantlets having about 5-cm-long shoots. Maintenance of these plantlets until they reached 8 to 10 cm long increased their survival rate in soil. Jain et al. (2011) indicated that embryogenic cell suspension cultures have shown higher morphogenesis capacity when compared with other in vitro methods.

Putrescine, or tetramethylenediamine, is an organic chemical compound NH2(CH2)4NH2 (1,4-diaminobutane or butanediamine) that is related to cadaverine; both are produced by the breakdown of amino acids in living and dead organisms. It is found that putrescine enhances growth and morphogenesis in plant tissue culture (Goerge *et al.,* 2008). Hegazy and Aboshama (2010) suggested an efficient novel pathway in date palm micropropagation protocol by induction of direct somatic embryogenesis from bud tissues through putrescine incorporation into the culture medium at 150 mg/L plus a cretin combination of plant growth regulators. They also recorded the highest multiplication rate and growth of date palm embryos by adding 100 mg/L putrescine.

Salicylic acid is a monohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid. It is widely used in organic synthesis and its function as a plant hormone. It appears to have a role in systemic acquired resistance to pathogens and is able to induce various pathogen resistance proteins (Goerge *et al.,* 2008).

Therefore the aim of this study is to examine various concentrations of putrescine and salicylic acid on enhancing embryonic callus and subsequent embryogenesis in *Phoenix dactylifera* Bream cv.

MATERIALS AND METHODS

Young offshoots of Bream cultivar (2-3 years old) were chosen and detached from mother palm. Leaves were dissected acropetaly. Shoot tips of 3 cm in length (apical meristem with soft inner leaves), were excised along with immature fiber of 2 cm in diameter. Explants were dipped in antioxidant solution consisted of 150 mg/L citric acid plus 100 mg/L ascorbic acid (Tisserat, 1991). Explants were surface sterilized with 2.0% sodium hypochlorite solution containing eight drops of Tween-20 as emulsifier for 20 minutes under vacuum, and rinsed three times with sterile distilled water. They transferred to Petri dishes where leaf primordia were removed except the two pairs surrounding the apical meristem which then divided longitudinally into four equal segments and cultured in jars aseptically. The medium of initiation stage was composed of Murashige and Skoog (1962) (MS) salts plus the following (in mg/L); thiamine-HC1 1.0; pyridoxine-HCl 1.0; adenine sulfate.2H2O 40; myo-inositol 100; NaH2PO4.2H2O 170; sucrose 30000 activated charcoal 2000 and agar-agar 7000. The pH of the medium was adjusted to 5.7 with 0.1N NaOH or HC1, before the addition of agar. The medium was dispensed into culture jars with aliquots of 25 ml in each, then covered with polypropylene caps and autoclaved under 1.04 kg/cm² at 121 °C for 15 minutes. Callus initiation medium was supplemented with 50 mg/L picloram and 3 mg/L N6-2-isopentyl adenine (2ip). Primary callus was obtained after 24 weeks of growth in full darkness.

Calli were then transferred onto fresh MS medium containing 0.0, 0.5, 1.0, 2.0 or 3.0 mM of putrescine or salicylic acid individually. Cultures were incubated in a growth room under low light intensity of 1000 lux for 16 hours daily at 27±1 °C for four weeks. Results of callus fresh and dry weights, number of germinated embryos and mean fresh weight of ten embryos were recorded after 12 weeks.

Experiments were conducted as factorial using Complete Randomized Design (CRD), with ten replicates. Least significant differences (LSD) were used to compare means at 5% level probability.

RESULTS

Supplementation of the callus initiation medium with putrescine (table 1) exhibited a significant increase in both fresh and dry weights at the concentration 2.0 mM of putrescine while other levels, although increased the weights but not up to a significant level.

Addition of salicylic acid (table 2) as a supplement to the embryonic callus medium at concentrations 2.0 and 3.0 mm led to a significant increase in callus fresh and dry weights reached 5.0, 5.0 and 0.25, 0.27 respectively.

The number of formed embryos increased proportionally after the inclusion of putrescine to the medium till reached to a significant level at the concentrations 2.0 and 3.0 mM recording 9.1 and 10.3 embryos respectively. However, mean fresh weights for these emerged embryos fluctuated with a maximum weight reached 1.70 g after inclusion of 0.5 mM of putrescine. All concentrations of putrescine caused no significant differences in the mean of 10 embryos fresh weights compared with those grown on putrescine free medium.

Table 4 shows that all levels of salicylic acid resulted in a significant increase in number of formed embryos compared with those initiated on a medium lacking salicylic acid. Number of embryos was doubled at the concentration 2.0 mM and continued increasing up to 10.6 embryos at 3.0 mM of salicylic acid. These increments in number of embryos although led to increasing the mean fresh weight of 10 emerging embryos, but the highest was recorded at 1.0 mM of salicylic acid recording 2.40 g.

DISCUSSION

Since propagation via somatic embryogenesis has become a vital mean for propagating many plant species, optimization of the culture medium may require some supplements other than plant growth regulators which are normally added to the nutrient medium. It is clear from the reported data (tables 1 & 2) that putrescine and salicylic acid has increased callus fresh and dry weights when supplemented to the callus maintenance medium separately. Putrescine is a polyamine with low molecular weight. It has been implicated in many cellular processes such as cell division, protein synthesis and DNA replication. The recent work of Ravindra and Nataraja (2013) reported that putrescine enhances callus growth, somatic embryogenesis and plant regeneration in many plant species including *Pinus gerardiana* at a concentration of 2.0 mg/L, however it is a genotype dependent. Hossein et al. (2011) speculated that the capability of a certain plant tissue to onset embryogenesis differs with regard to media composition, genotype, organ ontology and stage of differentiation. The results of their studies unequivocally suggest that, irrespective of the type of explants and media culture, salicylic acid increments beyond than 75 µM negatively affect somatic embryogenesis in carrots.

This study has proved (tables 3&4) that putrescine and salicylic acid have improved embryogenesis represented by almost doubling the number of embryos when both were supplemented individually at concentrations exceeded 2 mM. They may trigger competent cells to form cell aggregates which then developed to embryos. Our results are in agreement with those Lewis et al. (2003) who reported that putrescine improves embryogenesis in cotton plants and disagree with those of Husseini et al (2011) who reported that high concentrations of salicylic acid inhibit somatic embryogenesis in carrot tissue cultures. The doubling in embryo numbers obtained after the addition of putrescine or salicylic acid has accompanied by a decrease in embryo weights as a result of the competition on nutrients available in the culture medium. It is concluded from the current study that inclusion of 2-3 mM of putrescine or salicylic acid to the embryonic date palm culture medium has improved somatic embryogenesis. The finding can be exploited commercially by date palm micro-propagators to almost double their production. Searching for other media supplements with the aim of increasing embryo numbers and weights is a vital aspect and requires intensive investigation.

References

Al-Khateeb, A.A., Abdalla, G.R., Dinar, H.M., Al Khateeb, A.A., and Abugulia, K.A. 2002. Auxin:cytokinine interactions in the *in vitro* Micropropagation of date palm (*Phoenix dactylifera* L.). Egypt. J. Appl. Sci . 17(10):409 415.

Al-Maari, K.W. and Al Ghamdi, A.S. 1997. Micro-propagation of five date palm cultivars through *in vitro* axillary buds proliferation. Du. J. Agric. Sci. 13:55-71.

Al-Meer, U.N. and Al-Ibresam. O.T. 2010. Effect of vitamin E on some callus and embryos characteristics of date palm cv. Barhee propagated *in vitro*. Basrah J. Date palm Res. 9(2):1-12.

Al-Musawi, A.H. 2001. Effect of auxin, cytokinine, and activated charcoal on embryo development and germination of date palm (*Phoenix dactylifera* L.) cultured *in vitro*. Basrah Date Palm Res. J. 1: 8-18.

Barrow, S. 1998. A monograph of phoenix L. (palmae: Coryphoideae). Kew Bull. 53:513-575.

George, E.F., Michael, A. and Klerk, G.D. 2008. Plant Propagation by Tissue Culture. Third Edition. Springer, Netherland.

Hameed, M.K. 2001. Propagation of some date palm (*Phoenix dactylifera* L.) cultivars through tissue culture technique. PhD thesis, Univ. of Basra (in Arabic) Iraq.

Hegazy, A.E. and Aboshama, H.M. 2010. An efficient novel pathway discovered in date palm micropropagation. Acta Hort. 882:167-176.

Hosseini, S.S., Gorgan, I.R., Mashayekhi, K., Alizadeh, M. Ebrahimi, P. and Gorgan, I.R. 2011. Effect of salicylic

acid on somatic embryogenesis and chlorogenic acid levels of carrot (*Daucus carota* cv. Nantes) explants. J. of Ornam. and Hort. Plants, 1(2):105-113.

Ibrahim, N.H. 2012. Some Factors Affecting Embryogenic Callus Initiation of Date Palm (*Phoenix dactylifera* L.) cv. Bream in vitro.Msc. Thesis, College of Agriculture, University of Baghdad. Iraq.

Jasim, A.M., Abass, K.I. and Abdul Samed, A.R. 2008. Effect of some plant substances on contamination and growth somatic embryos of date palm (*Phoenix dactylifera* L.) cv Ashkar cultured *in vitro* Basrah J. Date palm Res. 7(2):1-16.

Jasim, A.M. and Saad, A.A. 2001. Effect of some media component on growth and somatic embryos formation and germination of date palm (*Phoenix dactylifera* L.) cultured *in vitro*. Basrah Date Palm Res. J. 1:1-7.

Khierallah, H.S.M. and Husien, N.H. 2013. The role of coconut water and casein hydrolysate in somatic embryogenesis of date palm and genetic stability detection using RAPD markers. Res Biotechnol. 4(3):20-28.

Lewis, K., Vicker, J. and Chee, P.W. (2003). Putrescine improves somatic embryogenesis and plant regeneration in cotton. ASA-CSSA-SSSA Annua Meeting Abstracts.

Mater, A.A. 1983. Plant regeneration from callus culture of *Phoenix dactylifera* L. Date Palm J. 2:57-73.

Munier, P. 1981. Origine de la culture sur palmier dattier et sa propagation en Afrique. Fruits, 36 (7-8): 437-450.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473 497.

Ravindra, B. and Nataraja, K. (2013). Putrescine influences somatic embryogenesis and plant regeneration in *Pinus gerardiana*. Amer. J. Plant Physiol. 2:107-114.

Reuveni, O., Adato, Y. and Kipnis, H.L.1972. A study of new and rapid methods for the vegetative propagation of date palms. Date Growers Inst. Rep. 49: 17 -24.

Reuveni, O. 1979. Embryogenesis and plantlet growth of date palm (*Phoenix dactylifera* L.) derived from callus tissues. Plant. Physiol. 63. 138.

Omar, M.S. 1988. Callus initiation, asexual embryogenesis and plant regeneration in (*Phoenix dactylifera* L.) Date Palm J. 6:265-275.

Omar, M.S., Hameed, M.K. and Al-Rawi, M.S. (1992) Micro-propagation of Date Palm (*Phoenix dactylifera* L.) In: Bajaj, Y.P. S. (Ed): Biotechnology In Agriculture and Forestry. Vol. 18 High- Tech. and micro-propagation II. Springer, Heidelberg.

Tisserat, B. 1984. Propagation of date palm by shoot tip culture. Hort Sci. 19:230-231.

Tisserat, B. 1991. Clonal propagation of palms. Plant tissue culture manual, C2:1- 14.

Saleh, A.M, Waheed, A.M., Kadhim, M.J. and Husien, A.S. 2006. Effect of some fruits seed powders addition on the growth and development of embyrogenic callus of date palm *Phoenix dactylifera* L. cultivar Berhi. Basrah J. Date palm Res. 5(2):127-136.

Schroeder, C.A. 1970. Tissue culture of date shoots and seedlings. Date Growers Inst. Rep. 47:25-47.

Tables

Table 1: Callus fresh and dry weights initiated on MS medium supplemented with 50 mg/L picloram and 3 g/L 2iP at different concentrations of putrescine for 24 weeks.

Putrescine (mM)	Callus fresh wt (g)	Callus dry wt (g)
0	1.16	0.05
0.5	1.4	0.07
1	1.85	0.08
2	2.3	0.3
3	1.79	0.1
LSD 0.05	0.759	0.094

Table 2: Callus fresh and dry weights initiated on MS medium supplemented with 50 mg/L of picloram and 3 g/L of 2iP at different concentrations of salicylic acid for 24 weeks.

Salicylic acid (mM)	Callus fwt (g)	Callus dwt (g)
0	1.16	0.05
0.5	1.67	0.09
1	1.92	0.07
2	5	0.25
3	5	0.27
LSD 0.05	1.956	0.105

Table 3: Mean number of germinating embryos and mean weight of ten embryos initiated from calli after supplementation with different concentrations of putrescine.

Putrescine (mM)	No. of embryos	Mean wt 10 embryos (g)
0	4.3	1.36
0.5	7.6	1.7
1	7.5	1.52
2	9.1	1.45
3	10.3	0.96
LSD 0.05	3.663	0.529

Table 4: Mean number of germinating embryos and mean fresh weight of ten embryos initiated from calli after supplementation with different concentrations of salicylic acid.

Salicylic acid (mM)	No. of embryos	Mean wt 10 embryos (g)
0	4.3	1.36
0.5	10.1	1.85
1	7.5	2.4
2	8.6	1.33
3	10.6	1.79
LSD 0.05	2.719	0.702