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In vitro Propagation of the Endangered Date Palm (*Phoenix dactylifera* L.) Ghazal cv. 1-Effect of Folic Acid and Biotin on Callus Formation and Differentiation

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Abstract: The present investigation is the first work on *in vitro* propagation of an elite endangered date palm (*Phoenix dactylifera* L.) Ghazal cultivar, through somatic embryogenesis. This cultivar is grown in Siwa Oasis, which is suffering an excessive rise in the subsoil water levels. Consequently, the fertile soils are subjected to deterioration and sanitization. This study was conducted to determine the effect of various concentrations of folic acid (vitamin B9) and biotin (vitamin H), on callus initiation, embryogenic callus formation and embryos number of date palm Ghazal cv. Shoot tip explants were cultured on modified Murashige and Skoog (MS) medium supplemented with 100 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D), 3 mg/l isopentenyl adenine (2iP) and vitamins (B9 and H) at 0.0, 0.1, 0.5 and 1 mg/l for each or in combinations. The best medium for callus initiation and formation was modified MS medium supplemented with vitamin B9 at 0.1 mg/l and 0.5 mg/l vitamin H. The highest percentage of embryogenic callus formation was obtained on modified MS medium, supplemented with 10 mg/l 2, 4-D, 3 mg/l 2iP, 0.5 mg/l vitamin B9 and 1 mg/l vitamin H. This treatment also recorded increase in the embryogenic callus fresh weight. Moreover, the maximum number of embryos was formed at modified MS medium supplemented with 0.1 mg/l NAA, 0.5 mg/l vitamin B9 and 1 mg/l vitamin H, compared control treatment. Organic substances seem to be a limiting factor in *in vitro* date palm culture and their addition as vitamins (B9 and H) have a beneficial effect.

Key words: Date palm · *Phoenix dactylifera* · Ghazal cv. · Somatic embryogenesis · Folic acid · Biotin

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the important fruit species grown in arid regions of the Middle East and North Africa. It is tolerant to adverse environmental conditions [1, 2]. In addition, date palm trees distributed at Nile Valley, Oases and desert districts in Egypt. Siwa Oasis is located within extremely arid zone, which characterized with temperature extreme, high salinity and its excellent cultivars of date palm. These cultivars are well adapted to the local environmental conditions. Date palm Ghazal cv. has a great economic importance as a dry and endangered superior cultivar. The traditional method of date palm propagation is by offshoots. However, insufficient number of offshoots resulting from the mother tree through the vegetative propagation hinders the expansion of date palm cultivated area. Thus, tissue culture technique for vegetative propagation of plants is an alternative rapid method to obtain high numbers of date palm superior cultivars. Several attempts have been made for the *in vitro* propagation of date palm cultivars based on somatic embryogenesis or organogenesis. Somatic embryogenesis has been achieved by Taha *et al.* [3], Al-Khateeb [4], Diab [5], Othmani *et al.* [6], Al- Khayri [7] and Ageel and Elmeer [8]. Vitamins are necessary compounds synthesized and utilized in plants. In tissue culture media, vitamin addition is not always common; since the amount needed by plants is relatively unknown, varies and uncertain. Plant cell in tissue culture have requirements for vitamins, especially thiamine, nicotinic acid, pyridoxine, pantothenate, folic acid and biotin [9]. While, thiamine, nicotinic acid, pyridoxine and myo-inositol found in Murashige and Skoog [10] medium at 0.1 mg/l, 0.5 mg/l, 0.5 mg/l and 100 mg/l, respectively, are the most commonly used. The exogenous vitamins

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such as thiamine and biotin are essential for both callus initiation and induction [11, 12]. Also, Abrahamian and Kantharajah [13] pointed that vitamins, in combination with other media constituents, have been shown to have direct and indirect effects on callus growth, somatic growth, rooting and embryonic development. On the other hand, studies on vitamins requirement of date palm *in vitro* cultures are scarce [14]. Moreover, folic acid is poorly applied in culture media whereas biotin is less common and is usually added at 0.01- 1.0 mg/l [15, 16].

The aim of this study was to investigate the effect of folic acid and biotin vitamins, on callus initiation, embryogenic callus formation and an effort to enhance growth of embryogenic callus of date palm Ghazal cultivar.

MATERIALS AND METHODS

This study was carried out in the plant Tissue Culture Laboratory, Genetic Resources Department, Desert Research Center, Cairo, Egypt during the period from 2011-2014.

Plant Material: Date palm (*Phoenix dactylifera* L.) Ghazal cv. offshoots, a well-known superior cultivar grown at Siwa Oasis region collected from healthy, disease-free mother plants, 3-4 years old, ranging in weight from 5-7 kg and about 50-70 cm in length were used as source of explant materials.

Explants Preparation and Sterilization: The selected off shoots were cleaned and the outer large leaves and fibers were carefully and gradually removed by a sharp knife until the appearance of the shoot tip zone. The apical shoot with a few of primordial leaves was used as explant material. Explants were washed by running tap water for one hour and then immediately placed in a chilled antioxidant solution consisting of 150 mg/l ascorbic acid and 200 mg/l citric acid for 30 minutes to prevent explants browning. Explants were surface sterilized in 70% ethanol for one minute followed by immersion in 0.5 g/l mercuric chloride ($HgCl_2$) for 5 minutes and thoroughly washed with sterilized distilled water. After that, the sheathing leaf base was removed and the explants were re-sterilized by commercial Clorox (5.25% sodium hypochlorite, NaOCl) with two drops of tween-20 per 100 ml solution, firstly by 40% Clorox for 15 minutes and thoroughly washed with sterilized distilled water for two times and secondly by 50% Clorox for 20 minutes. Then, they were rinsed with sterilized water for four times. Outer soft leaves were carefully removed to obtain shoot tip composed of apical meristem and 4-6 primordial leaves. The shoot tips were sectioned longitudinally into four to eight sections and cultured individually on a culture medium.

The Basic Nutrient Medium: The basic salts and vitamins of MS [10] medium were used and modified by the addition of adenine sulfate 40 mg/l, sodium dihydrogen orthophosphate (NaH_2PO_4)170 mg/l, glutamine 200 mg/l, thiamine-HCL 0.5 mg/l, nicotinic acid 1.0 mg/l, sucrose 30 g/l and gelrite 2 g/l. The medium was adjusted to pH 5.7 ± 0.1 with 1N HCl or KOH, dispensed into small jars (200 ml) filled with 35 ml per jar and autoclaved under 1.05 kg/cm^{2}pressure at 121°C for 20 min.

Culture Conditions: All cultures were incubated at constant temperature 27±2°C under complete darkness and recultured onto the same fresh medium every six weeks.

Callus Initiation: To study the effect of folic acid and biotin on the callus initiation. Sterilized shoot tip explants were cultured on modified MS medium supplemented with 100 mg/l 2,4- dichlorophenoxy acetic acid (2,4-D), 3 mg/l 2-isopentenyl adenine (2iP), 3 g/l activated charcoal (AC) and different combinations of folic acid and biotin at 0.0, 0.1, 0.5 and 1.0 mg/l for each. Each treatment consisted of twelve replicates; each replicate was represented by one explant. After six weeks, survival percentage, swelling and callus initiation were recorded.

Callus Formation: Friable callus which resulted from culturing explants on modified MS medium supplemented with 0.1 mg/l folic acid and 0.5 mg/l biotin were divided into pieces approximately 0.5 - 1cm. The pieces were cultured individually in small jars (200 ml) containing 35 ml of the different media used, in the previous experiment under the same conditions for six weeks. Each treatment contains three replicates and each one contained three jars. Callus formation percentages were recorded.

Embryogenic Callus Formation: Calli produced from the previous step were transferred to the modified MS medium supplemented with 10 mg/l 2, 4-D, 3 mg/l 2iP, 2g/l activated charcoal and different combinations of folic acid and biotin at 0.0, 0.1, 0.5 and 1.0 mg/l for each. Each treatment consisted of twelve jars. Cultures were transferred to fresh medium every six weeks. The percentage of embryogenic callus formation was recorded after three months.

Embryogenic Callus Growth Rate: To evaluate callus growth rate in response to different combinations of folic acid and biotin at (0.0, 0.1, 0.5 and 1.0 mg/l) for each were tested. In all treatments, 0.3 g of white friable callus was cultured in each small jar. Each treatment contains three replicates and each one contained two jars. To determine callus growth rate, the calli were weighted after four and eight weeks.

Somatic Embryogenesis Growth: Embryogenic callus and embryogenesis in response to folic acid and biotin. To test the effect of vitamins on embryogenic callus proliferation and embryos number, callus from the maintenance cultures were transferred to modified MS medium hormone-free supplemented with different combination of folic acid and biotin at 0.0, 0.1, 0.5 and 1.0mg/l for each. Each treatment contained two jars.

Experimental Design and Statistical Analysis: A complete randomized design was employed in all of the experiments. Variance analysis of data was carried out using ANOVA program for statistical analysis according to Sendecor and Cochran [17]. Duncan's multiple range test was employed for means comparisons [18].

RESULTS

Callus Initiation

Effect of Folic Acid and Biotin on Callus Initiation: The effect of different concentrations of the selected vitamins on survival, swelling and callus initiation of date palm (*Phoenix dactylifera* L.) Ghazal cultivar with resulted from cultured explants for 6 weeks were presented in Table 1. It was found that, survival percentage ranged from 91.67 to 100%, whereas the different treatments had 100% survival except vitamins free medium, which resulted in 91.67%. Moreover, swelling percentage was responded differently to the used folic and biotin treatments. In this concern, swelling percentage ranged from 41.67 to 100%. The highest significant swelling percentage 100% was recorded by using folic acid at the concentrations of 0.1, 0.5 and 1.0 mg/l combined with biotin at 0.5 mg/l (Fig. 1A). While, the lowest significant swelling percentage 41.67% was recorded for free medium (control). Folic acid at 0.1, 0.5 and 1.0 mg/l without or with biotin at 0.1 mg/l had depressing effect on swelling percentage (50%). Other treatments had positive effect and good results on swelling percentage. Concerning of effect of folic acid and biotin on callus initiation, data indicated that, callus initiation percentage ranged between 16.67 to 83.33%. It was found that the highest significant callus initiation (83.33%) was recorded by using MS medium supplemented with 0.1 mg/l folic acid and 0.5 mg/l biotin followed by (75 %) for 0.5 mg/l biotin. While, the lowest significant callus initiation (16.67%) was recorded

Table 1: Effect of different concentrations of folic acid and biotin on survival, swelling and callus initiation percentage of date palm (*Phoenix dactylifera* L.) Ghazal cultivar

Vitamins concentration (mg/l)				
Folic acid	Biotin	Survival %	Swelling %	Callus initiation %
0.0	0.0	91.67b	41.67f	16.67i
0.1	0.0	100a	50e	16.67i
0.5	0.0	100a	50e	25 h
1.0	0.0	100a	50e	25 h
0.0	0.1	100a	50e	33.33 g
0.1	0.1	100a	50e	33.33 g
0.5	0.1	100a	50e	41.67 f
1.0	0.1	100a	75d	66.67 c
0.0	0.5	100a	91.67b	75b
0.1	0.5	100a	100a	83.33a
0.5	0.5	100a	100a	66.67c
1.0	0.5	100a	100a	58.33d
0.0	1.0	100a	83.33c	58.33d
0.1	1.0	100a	91.67b	50e
0.5	1.0	100a	91.67b	50e
1.0	1.0	100a	91.67b	41.67f

Means within columns followed by the same letters are not significantly ($P < 0.05$) different

Table 2: Effect of different concentrations of folic acid and biotin on callus formation percentage of date palm (*Phoenix dactylifera* L.) Ghazal cultivar

Vitamins concentration (mg/l)		
Folic acid	Biotin	Callus formation %
0.0	0.0	22.5 f
0.1	0.0	22.5 f
0.5	0.0	22.5 f
1.0	0.0	22.5 f
0.0	0.1	33.3 e
0.1	0.1	33.3 e
0.5	0.1	44.4 d
1.0	0.1	55.6 c
0.0	0.5	66.7 b
0.1	0.5	88.8 a
0.5	0.5	66.7 b
1.0	0.5	66.7 b
0.0	1.0	55.6 c
0.1	1.0	44.4 d
0.5	1.0	33.3 e
1.0	1.0	33.3 e

Means within columns followed by the same letters are not significantly ($P < .05$) different

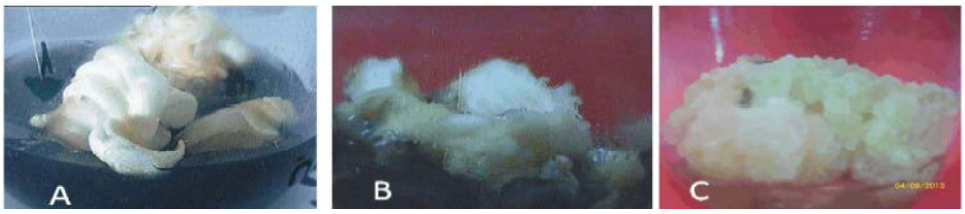

Fig. 1: Effect of modified MS medium containing 100 mg/l 2,4-D + 3mg/l 2iP + 0.1 mg/l folic acid + 0.5mg/l biotin on (A) swelling formation from shoot tip explants of date palm Ghazal cv. (B) Callus initiation (C) callus formation

Fig. 2: Effect of modified MS medium containing 10 mg/l 2,4-D + 3mg/l 2iP + 0.5 mg/l folic acid + 1.0mg/l biotin on (A) embryogenic callus formation (B) embryogenic callus growth after 4 weeks (C) embryogenic callus growth after 8 weeks

with control treatment and 0.1 mg/l folic acid. From the obtained results, it can be concluded that modified MS medium supplemented with 0.1 mg/l folic acid and 0.5 mg/l biotin was the best medium for callus initiation (Fig. 1B).

Callus Formation

Effect of Folic Acid and Biotin on Callus Formation: The results of the effect of different concentrations of folic acid and biotin on callus formation percentage were presented in Table 2 and Fig. 1C. Data clearly indicate that, all treatments used gave callus formation. The percentage of callus formation ranged between 25 and 87.5 %. Using modified MS medium supplemented with 0.1 mg/l folic acid and 0.5 mg/l biotin gave the highest percentage of callus formation (87.5%). This value was significantly higher than any other value resulted from other treatments used.

Embryogenic Callus Formation: To test the effect of vitamins (folic acid and biotin) on embryogenic callus formation, friable calli were transferred to modified MS basal nutrient medium supplemented with 10 mg/l 2, 4-D, 3 mg/l 2iP and different concentrations of the two selected vitamins. Results present in Table 3 and Fig. 2A showed that the highest significant percentage of embryogenic callus formation were obtained with modified MS medium containing 0.5 mg/l folic acid and 1.0 mg/l biotin, which recorded 87.5 %. This percentage was followed by 0.1 mg/l folic acid with 1.0 mg/l biotin and 1.0 mg/l folic acid with 1.0 mg/l biotin (79.17 and 75%), respectively with significant difference in between. While, the lowest significant percentage of embryogenic callus was recorded with control treatment and modified MS medium containing 0.1 mg/l folic acid which gave the same value (12.5%), for each. The percentage of embryogenic calli was increased gradually with an increase in folic acid and biotin concentrations, while the concentration 1.0 mg/l for both folic acid and biotin caused a decrease in embryogenic callus. From the above mentioned results, it can be concluded that, modified MS medium supplemented with 0.5 mg/l folic acid and 1.0 mg/l biotin was the best medium for embryogenic callus formation.

Table 3: Effect of different concentrations of folic acid and biotin on the percentage of embryogenic callus formation of date palm (*Phoenix dactylifera* L.) Ghazal cultivar

Vitamins concentration (mg/l)		
Folic acid	Biotin	Embryogenic callus %
0.0	0.0	12.50 j
0.1	0.0	12.50 j
0.5	0.0	20.83 i
1.0	0.0	33.30 h
0.0	0.1	33.30 h
0.1	0.1	37.50 h
0.5	0.1	41.67 g
1.0	0.1	50.00 f
0.0	0.5	54.17ef
0.1	0.5	54.17ef
0.5	0.5	54.17ef
1.0	0.5	58.33 d
0.0	1.0	62.5 d
0.1	1.0	79.17b
0.5	1.0	87.5 a
1.0	1.0	75.00 c

Means within columns followed by the same letters are not significantly ($P< 0.05$) different

Fig. 3: Effect of modified MS medium containing 10 mg/l 2,4-D + 3mg/l2iP + 0.5 mg/l folic acid + 1.0 mg/l biotin on growth and development of date palm Ghazal cv. somatic embryos during germination stage (A) development of somatic embryos (B) enhanced number of embryoc

Table 4: Effect of optimized concentrations and control of folic acid and biotin on the embryogenic callus growth of date palm (*Phoenix dactylifera* L.) Ghazal cultivar after four and eight weeks

Vitamins concentration (mg/l)		Embryogenic callus fresh weight (g/ jar)	
Folic acid	Biotin	After 4 weeks	After 8 weeks
0.0	0.0	0.39 f	0.47 h
0.1	0.0	0.41 f	0.45h
0.5	0.0	0.43 ef	0.47 h
1.0	0.0	0.43 ef	0.48 h
0.0	0.1	0.47 e	0.50 h
0.1	0.1	0.52 de	0.59 g
0.5	0.1	0.52 de	0.61 g
1.0	0.1	0.53 d	0.67 f
0.0	0.5	0.55 d	0.71 f
0.1	0.5	0.56 d	0.88 e
0.5	0.5	0.56 d	0.90 e
1.0	0.5	0.57d	0.97d
0.0	1.0	0.57 d	0.99 d
0.1	1.0	0.71 b	1.80 b
0.5	1.0	0.88 a	2.94 a
1.0	1.0	0.63 c	1.52 c

Means within columns followed by the same letters are not significantly ($P < 0.05$) different

Table 5: Effect of folic acid and biotin on somatic embryos number formed from embryogenic calli of date palm (*Phoenix dactylifera* L.) Ghazal cultivar

Vitamins Treatment (mg/l)		
Folic acid	Biotin	Number of embryos/ culture
0.0	0.0	14 f
0.1	0.0	20 e
0.5	0.0	20 e
1.0	0.0	21 e
0.0	0.1	23 e
0.1	0.1	24 e
0.5	0.1	29 d
1.0	0.1	30 d
0.0	0.5	31 d
0.1	0.5	31 d
0.5	0.5	33 d
1.0	0.5	39 c
0.0	1.0	41 c
0.1	1.0	47 b
0.5	1.0	68 a
1.0	1.0	52 b

Means within columns followed by the same letters are not significantly ($P < 0.05$) different

Embryogenic Callus Growth Rate: Studies on the effect of folic acid on callus growth rate of date palm are rare. Data presented in Table 4 showed that the effect of folic acid and biotin at optimized concentrations on embryogenic calli fresh weights. Highly effective in stimulating high embryogenic callus production was 0.5 mg/l folic acid and 1.0 mg/l biotin. In the present study, date palm callus growth rate was significantly influenced by the concentrations of folic acid and biotin after 4 weeks (Fig. 2 B). Calli fresh weights were significantly influenced as the concentration of folic acid increased from 0.1 to 0.5 mg/l. Extending the culture duration 8 weeks resulted in more callus production (Fig. 2C). After 8 weeks culture duration Ghazal cv. callus grew better on modified MS medium containing 0.5 mg/l folic acid and 1mg/l biotin as compared to the other tested treatments. This difference was undetectable after only 4 weeks of treatment. These responses were more accurately depict the effect of folic acid and biotin concentrations as differences become larger and more pronounced in comparison to observations made after 4 weeks of treatments.

Somatic Embryogenesis: The influence of vitamins on somatic embryos number from embryogenic callus was presented in Table 5 and Fig. 3 A and B showed that the highest number of embryos (68) was formed with 0.5 mg/l folic acid and 1mg/l biotin. Data about the effect of both folic acid and biotin on number of formed somatic embryos showed that all treatments improved number of embryos compared with control treatment.

DISCUSSION

The current study compared two vitamins, folic acid and biotin at different Concentrations, to test their effect on callus initiation, formation and embryogenic callus formation Ghazal cv. Concerning the effect of vitamins on callus and embryogenic callus formation, Gamborg and Shyluk [9], Al-Khayri [11] and Kumra and Chopra [19] reported that the exogenous vitamins such as thiamine and biotin are essential for both callus initiation and induction. Presence of essential organic acid, folic acid and biotin, in Olive medium plays an important role in directing and transporting cytokinins [20]. Scott *et al*. [21] also reported that foliates, a class of pterdine compounds are essential for normal growth and differentiation. They further suggested that folic acid coenzymes are involved in one carbon transfer reactions such as those necessary for the biosynthesis of methionine, serine, deoxythymidylic acid and purines essential for the cell differentiation. Studies on vitamin and amino acid requirements of date palm *in vitro* cultures are scarce [14]. To contribute and overcoming the recalcitrance of date palm, the present study was conducted to define the optimum requirements of two vitamins, thiamine and biotin, in an effort to enhance growth of embryogenic callus and subsequent formation of somatic embryos. The addition of some vitamins, sources of vitamin B, such as biotin, folic acid and yeast extract, play an important role in plant growth and development metabolism. Al-Khayri [11] reported that biotin and thiamine incorporated in culture medium enhanced date palm height. Biotin, important in; fat, protein and carbohydrate metabolism, promoted growth of leaves and stems in *Paphiopedilum*, while it enhanced root growth in *Cymbidium* [22, 23]. In this respect, Gamborg and Shyluk [9] indicated that plant cell have requirements for vitamins; especially thiamine-HCl, nicotinic acid, pyridoxine, pantothenate, foliate and biotin. Thiamine is an important co-factor in carbohydrate metabolism and biotin is important carboxylation reaction. Both thiamine and biotin are essential for both callus initiation and induction [9, 11, 19]. Presence of essential organic acid, folic acid and biotin in culture medium plays an important role in directing and transporting cytokinins. Alban *et al.* [20] also reported that foliates, a class of pteridine compounds is essential for normal growth and differentiation. They further suggested that folic acid coenzymes are involved in one carbon transfer reactions such as those necessary for the biosynthesis of methionine, serine, deoxythymidylic acid and purines essential for the cell differentiation. Moreover, folic acid is essential for cells to replicate their DNA during cell division. It is also required for RNA synthesis, or the reading of the DNA code. On the other hand, biotin is a vital vitamin in all organisms though numerous plants and mammals are incapable of synthesis it. This vitamin is a cofactor for five key enzymes (carboxylases, enzymes considered essential for metabolism of proteins, fats and carbohydrates. Carboxylases, enzymes includes; acetyl Co enzyme A (Acyl Co-A) which assists in synthesizing of fatty acid, propionyl Co-A carboxylase which is essential for amino acid metabolism [24]. Accordingly, the present study has shown that folic acid and biotin significantly affected somatic embryogenesis of date palm.

In conclusion, this study has shown that vitamins content in the culture medium are an important factor in date palm tissue culture. In addition to determining the requirements of folic acid and biotin also, this study has highlighted the importance for both vitamins as a medium additive. Callus initiation and formation, was the best achieved on a modified MS medium supplemented with 0.1 mg/l folic acid and 0.5 mg/l biotin. Whereas, embryogenic callus formation, callus growth and embryos formation were recorded the best results with 0.5 mg/l folic acid and 1.0 mg/l biotin.

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