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PREVENTION OF HYPERHYDRICITY PHENOMENON AND IMPROVING SOMATIC EMBRYOGENESIS IN DATE PALM (*Phoenix dactylifera* L.)

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ABSTRACT

The present work was carried out at the Central Laboratory of Date Palm Research and Development, Agriculture Research Center, Giza, Egypt. throughout the period from 2010 to 2013. Hyperhydricity is a serious problem during *in vitro* propagation of date palm which directly affects the production at the commercial level. Thus, this work aimed to reduce the hyperhydricity and improve somatic embryos of date palm cv. Gundila. throughout two experiments. The first experiment was conducted to investigate the effect of different substances of different concentrations 0.2 g/l Silver nitrate ($AgNo_3$), 0.05g/l Calcium nitrate ($CaNo_3$), 0.05g/l Calcium Chloride ($CaCl_2$) and 0.5 g/l Calcium Pantothenate (CaP). The second experiment was conducted in order to investigate the effect of different amino acids to improve somatic embryos. Adding 0.2g/l $AgNo_3$ to culture medium decreased hyperhydricity percentage to 22.22% compared with control medium (55.55%). It achieved the highest significant value of differentiation percentage (77.77%). Moreover, the superior significant results of differentiation percentage was achieved when 100mg/l Glutamine + 100mg/l Alanine was added to the regeneration medium (100%).

Key Words: *amino acid, date Palm, embryonic callus, hyperhydricity,somatic embryo.*

1. INTRODUCTION

Hyperhydricity is a serious problem during *in vitro* propagation of date palm which directly affects the production at commercial level. Thus, *in vitro* cultured plantlets do not survive when transferred to soil due to yellowing, swelling, glassiness and leaf curling of plantlets (Wetzstein and Sommer, 1982; Donelly and Vladaver, 1984). These morphological changes have been related to the low photosynthetic capacity of the leaves (Kevers *et al*., 1984; Paek *et al*., 1991). Accumulation of gases such as ethylene and CO_2 have also been found to be responsible for hyperhydricity (De Proft *et al*., 1985). Various approaches to overcome hyperhydricity are included containers with good gaseous exchange. Hyperhydricity is not restricted to shoots, some yellowish and hyperhydric callus types may be considered as vitreous (Crevecoeur *et al*., 1987). The high degree of friability therefore is related to the breakability of vitrified organs (Gaspar, 1991). The uptake of calcium is affected by low transpiration rate and calcium levels in the developing plant tissues become deficient. Perhaps a plant physiologist could explain the physiological link between calcium and vitrification (Petersen, 2004). In recent years, basic studies on ethylene regulation opened new vistas for applied research in the area of micro-propagation, somatic embryogenesis, *in vitro* growth promotion, and sex expression. Silver nitrate has proved to be a very potent inhibitor of ethylene action and is widely used in plant tissue culture. Few properties of silver nitrate such as easy availability, solubility in water, specificity and stability make it very useful for various applications in exploiting plant growth regulation and morphogenesis *in vivo* and *in vitro*. Silver ion mediated responses seem to be involved in polyamines, ethylene- and calcium- mediated pathways, and play a crucial role in regulating physiological process including morphogenesis (Kumar, *et al*., 2009) . In date palm (*Phoenix dactylifera* L.), callus proliferation that normally occurs prior to de-differentiation upon callus transfer to hormone-free regeneration medium, as well as subsequent somatic embryogenesis, were shown to be stimulated by silver nitrate added to the regeneration medium in cv. Barhee (Al-Khayri and Al-Bahrany,2001).

Stuart and Strickland (1984) mentioned that, the effect of adding amino acids to cultures of alfalfa (*Medicago sativa* L.) undergoing *in vitro* somatic embryogenesis has been investigated. By supplementing Schenk-Hildebrandt (SH) medium with 50–300 mM L-proline, somatic embryogenesis was stimulated by 3-fold when compared with SH medium without proline. Several other proline analogs were also effective. Alanine, glutamine, arginine, lysine, serine, asparagine and ornithine also stimulated the numbers of somatic embryos formed in alfalfa cultures. The structural quality of embryos was also enhanced by glutamine, arginine or alanine in the regeneration medium. Both embryo size and conversion of embryos to plantlets were increased by these amino acids. It is suggested that adding proline with other amino acids which stimulate embryo conversion may be one method for achieving high frequency and high quality somatic embryogenesis. The objective of the present work was to study the effect of 0.2g/l Silver nitrate ($AgNo_{3)}$, 0.05g/l Calcium nitrate ($CaNo_{3)}$, 0.05g/l Calcium Chloride ($Cacl_{2)}$, 0.5g/l Calcium Pantothenate (CaP) on normal somatic embryo production and hyperhydricity from vitrified callus and improve somatic embryo conversion by amino acids of date palm cv. Gundila.

2. MATERIALS AND METHODS

The experimental work was performed at the laboratory of date palm researches and development during the period from 2010 to 2012. Using date palm cv. Gundila. Shoot apecies were sliced longitudinally into 4 pieces and then cultured on Murashige and Skoog (MS) basal nutrient medium (1962) supplemented with 170 mg $NaH_2Po_4.2H_2O$; 200 mg glutamine; 40 mg adenine sulfate; 0.4 mg Thiamine HCL, 3g activated charcool (AC), 30g sucrose; 6 g agar and 100mg 2,4-D+3mg 2ip /liter as described by Mater (1986) to obtain embryonic callus.

The pH of all culture media was adjusted to 5.8±0.1 prior to the addition of agar, and then 35 ml of the medium dispensed into small jars (150 ml), the jars were autoclaved at 121°C and 1.2 kg/cm² for 20 min.

During germination cycle of embryonic callus vitrified embryonic callus appeared and used as explant materials. 0.5 g approximately of vitrified embryonic callus (0.5 g in weight and 1-2 mm in diameter) were used in the two experiments. Each treatment included 3 replicates each replicate of 3 small jars (150 ml) and each jar contained one explant. Culture jars were incubated at temperature-controlled room at 24± °C under 16 h daily exposure to low light intensity of 1000 lux illumination.

The first experiment was conducted to investigate the effect of some substances with different concentrations as 0.2g/l Silver nitrate ($AgNo_{3)}$, 0.05g/l Calcium nitrate ($CaNo_{3)}$, 0.05g/l Calcium Chloride ($Cacl_{2)}$, 0.5g/l Calcium Pantothenate (CaP) and the control treatment on callus growth and hyperhydricity percentage. Data were calculated after 8 weeks during two subcultures on fresh weight, dry weight, growth value (Ziv, 1992), relative water, hyperhydricity percentage and calcium content by the method described by Chapman and Pratt (1961).

The second experiment was conducted to investigate the effect of different amino acids on improveing somatic embryo conversion and form good plantlets. The concentrations of the amino acids were 200 mg/l of Glutamine, L-Alanine, L-Lysine Hcl, L-Leucine, L-Systeine, Tyrosine and 100mg/l Glutamine + (100mg/l of L-Alanine, L-Lysine Hcl, L-Leucine, L-Systeine, and Tyrosine). Data were calculated after 8 weeks during two subcultures on differentiation percentage, fresh weight, No. of somatic embryos and protein content using the method described by Bradford (1976).

2.1.Statistical analysis

The randomized factorial design was used and data were subjected to analysis of variance. Separation of means among treatments was determined using L.S.D test at 5% according to Snedecor and Cochran (1972).

3. RESULTS AND DISCUSSION

3.1.The effect of some substances with different concentrations as 0.2g/l Silver nitrate ($AgNo_{3)}$, 0.05g/l Calcium nitrate ($CaNo_{3)}$, 0.05g/l Calcium Chloride ($Cacl_{2)}$, 0.5g/l Calcium Pantothenate (CaP) and the control on callus growth and hyperhydricity percentage.

3.1.1. Fresh weight(g): Data in Table (1) showed that, the vitrified callus of date palm cv. Gundila when cultured on MS basal nutrient medium supplemented with 0.05g/l $Cacl_2$ increased significantly fresh weight to 3.53g compared with 1.52 g of the vitrified callus when cultured on

0.2g/l $AgNo_3$ whereas, data recorded 2.33 g of fresh weight on the control.

3.1.2.Dry weight(g): Dry weight of vitrified callus recorded the highest values on 0.05g/l $CaNo_3$ or 0.5g/l CaP treatment without significant differences in between (0.73 and 0.70 g, respectively) while, the control and 0.2g/l $AgNo_3$ treatment gave the lowest values of dry weight without significant differences (0.28 and 0.23 g, respectively).

3.1.3. Growth values: Concerning the growth values, 0.05g/l $CaNo_3$ treatment recorded the highest result of growth value (6.06) while, 0.5g/l CaP and the control recorded the lowest values of growth value without significant differences between (3.18 and 3.02, respectively).

3.1.4. Hyperhydricity percentage: It was clearly noticed from Table (1) that adding 0.05g/l $CaNo_3$ to the culture medium gave the superior significant value of hyprhydricity percentage (77.77%), while adding 0.2g/l $AgNo_3$ to the culture medium decreased hyperhydricity percentage to 22.22% compared with the control medium (55.55%) (Table 1).

(0.3mg/g).

The results of this study proved that the addition of silver nitrate to the differentiation medium improved the differentiation rate and somatic embryo numbers.

From our previous experiment vitrified calli were improved by adding 0.2g/l $AgNo_3$ to the culture medium, as it reduced fresh and dry weight as well as the percentage of hyperhidricity. Also, it increased differentiation percentage and somatic embryos average. The increase in fresh weight by 0.05g/l $CaNo_3$ treatment was due to the increase of cell water. Somatic embryogenesis was induced by transferring the callus to hormone-free MS medium containing corresponding $AgNO_3$ concentrations. Callus growth of date palm cvs. Barhee, Naboot Saif, Ruzaiz, and Hillali was significantly promoted in response to 37.5 μM $AgNO_3$ but optimum growth occurred at 50 μM in the former three cultivars, whereas cv. Hillali grew best at 62.5 μM. (Al-Khayri and Al-Bahrany,2004). Also the results agree with Mayor, *et al*. (2003) who reported the response of three sunflower inbred lines examined on regeneration

Table (1): Effect of silver nitrate, calcium nitrate, calcium chloride and calcium pantothenate on growth and development of vitrified callus of date palm cv. Gundila after 2 months from culturing on modified germination medium

Concentration g/l	Fresh weight(g)	Dry weight(g)	Growth value	Hyperhydricity %	Differentiation %
Control	2.33c	0.28bc	3.02c	55.55c	33.33d
0.2$AgNo_3$	1.52d	0.23c	1.36d	22.22d	77.77a
0.05$CaNo_3$	2.77b	0.73a	4.54b	77.77a	44.44c
0.05$Cacl_2$	3.53a	0.38b	6.06a	66.66b	55.55b
0.5CaP	2.86b	0.70a	3.18c	44.44	55.55b

Growth value = (Final fresh weight – initial fresh weight) by Ziv,1992
Initial fresh weight

3.1.5. Differentiation percentage: There were high significant differences among all treatments used in this experiment. 0.2g/l $AgNo_3$ treatment achieved the -highest significant value (77.77%). Whereas, the control gave the lowest value 33.33% of differentiation percentage.

3.1.6. Calcium content (mg/g): Data in Figure (1) indicated that, the best calcium content **(**mg/g): values were obtained from 0.2g/l ANo_3 or 0.5g/l CaP treatment without significant differences (0.6 mg/g) followed by the control and 0.05g/l $Cacl_2$ which recorded the same value of calcium content (0.4mg/g) while 0.05g/l $CaNo_3$ treatment recorded the lowest value of calcium content

media containing various concentrations of kinetin, silver nitrate, and casein hydrolysate, calcium nitrate and cobalt nitrate. The addition of silver nitrate showed to be useful in improving the quality of sunflower micropropagated plants by reducing this undesirable phenomenon.

3.2.The effect of different amino acids on improving somatic embryo conversion and form good plantlets.

The effect of adding amino acids to cultures of date palm cv. Gundila undergoing *in vitro* somatic embryogenesis was investigated (Table 2).

3.2.1. Fresh weight (g): Data revealed that adding 100 mg/l of glutamine recorded the best results of

fresh weight whereas adding 200mg/l Tyrosine to regeneration medium recorded the least value of fresh weight. Overall there were no clear and significant differences among the different treatments for fresh weight. These results agree with Zein El Din (2010) who noted that, increasing polyamines level from 0 to 300 mg/l had no significant effect on embryonic callus fresh weight.

3.2.2. Number of somatic embryos: Adding 200mg/l Glutamine, 200mg/l Alanine or 100mg/l Glutamine + 100mg/l Alanine to the regeneration medium significantly stimulated conversion of somatic embryos compared with other treatments (4.08, 4.60 and 4.60, respectively). Whereas data showed that, 200 mg/l Lysine, 200 mg/l Leucine or 200 mg/l Tyrosine treatment recorded the lowest value of somatic embryo number without significant differences among them (0.6, 0.8 and 1.00 respectively). Stuart and Strickland (1984) stated that, alanine, glutamine, arginine, lysine, serine, asparagine and ornithine stimulated the numbers of somatic embryos formed in alfalfa cultures. The structural quality of the embryos was also enhanced by glutamine, arginine or alanine in the regeneration medium. Both embryo size and conversion of embryos to plantlets were increased by these amino acids. El-Shiaty *et al*. (2004) declared that, growth of date palm callus tissue was significantly stimulated by the addition of amino acids and vitamins specifically glutamine and biotin. This stimulation may be attributed to the role of organic nitrogen as a growth-limiting factor in date palm cultures. There were gradual increase in the percentage of vitrified embryonic callus differentiation to normal somatic embryos by increasing glutamine levels from 0.0 to 400 mg/l. Glutamine at the lowest level (50mg/l) increased significantly the number of vitrified somatic embryos (Zayed *et al*.,2012).

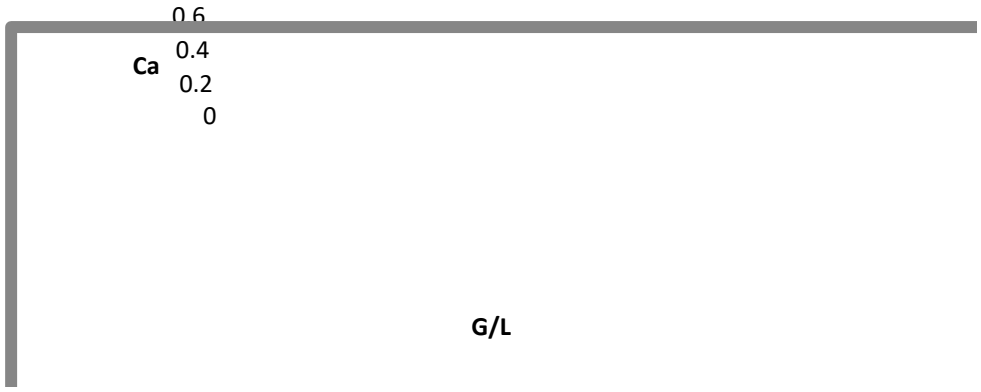

Fig. (1): Calcium content (mg/g) of vitrified callus of date palm cv. Gundila in the control, silver nitrate, calcium nitrate, calcium chloride and calcium Pantothenate.

Table (2): Effect of amino acids on the growth and development of somatic embryos of date palm cv. Gundila after 8 weeks from culturing.

Amino acid (mg/l)	Fresh weight(g)	Differentiation (%)	No. of S.Embryos
200 Glutamine	0.69ab	40c	4.08a
200 Alanine	0.73ab	60b	4.60a
200 Lysine	0.72ab	20d	0.60e
200 Leucine	0.77a	40c	0.80e
200 Systeine	0.70ab	60b	2.80c
200 Tyrosine	0.61b	20d	1.00e
100 Glu +100 Ala	0.65ab	100a	4.60a
100 Glu +100 Lys	0.81a	60b	2.00d
100 Glu + 100 Leu	0.73a	40c	1.20de
100 Glu + 100 Syst	0.64ab	60b	3.40b
100 Glu + 100 Tyr	0.63b	20d	1.40de
Mean			

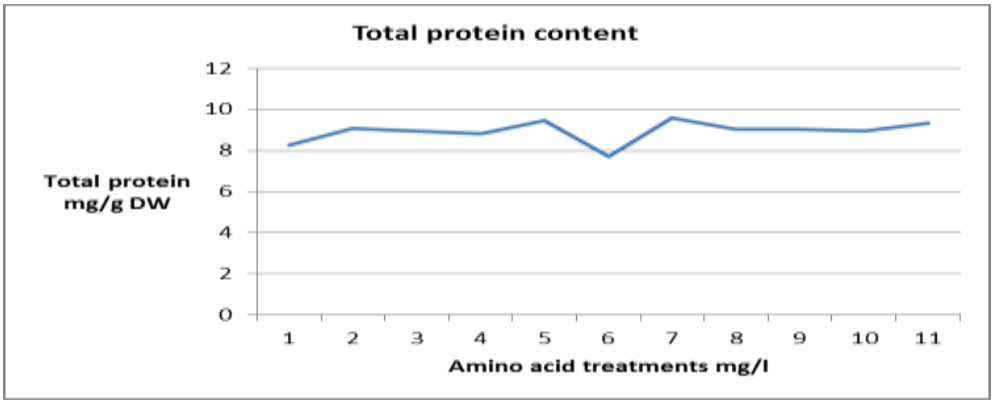

Fig. (2): Total protein (mg/g DW) of vitrified callus of date palm cv. Gundila at different concentrations of amino acids.

3.2.3. Total protein content mg/g DW): Data in Figure (2) show the effect of different amino acids on total protein content of date palm cv. Gundila.

There were increases of total protein content when 200mg/l Systeine, 100mg/l Glutamine + 100mg/l Alanine or 100mg/l Glutamine + 100mg/l Tyrosine were added to the regeneration medium without significant differences among them. However, adding 200mg/l Tyrosine to the culture medium gave the least value of total protein content. The SDS-PAGE protein profiles showed a significant difference between treated and untreated SE. A protein band of 22 kDa, identified as glutelin in a previous work, was accumulated after treatment with 20 µM ABA or 3 mM arginine. These findings may contribute to further understanding of the mechanisms involved in the accumulation of specific storage proteins in several plants (Sghaier *et al*. 2009).

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إعاقة الظاهرة الزجاجية لنباتات نخيل البلح وتحسين إنتاج الأجنة الجسدية بواسطة إستخدام الأحماض الأمينية

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المعمل المركزى للأبحاث وتطوير نخيل البلح – مركز البحوث الزراعية – مصر

ملخص

أجريت هذه الدراسة فى المعمل المركزى للأبحاث وتطوير نخيل البلح خلال الفترة من 2010 – 2013م. تمثل الظاهرة الزجاجية مشكلة خطيرة خلال الزراعة المعملية لنباتات نخيل البلح وتؤثر على الانتاج التجارى. يسعى هذا العمل لتخفيض التزجج وتحسين إنتاج الأجنة الجسدية فى نباتات نخيل البلح صنف الجنديلة من خلال تجربتين. التجربة الأولى: أجريت لبحث تأثير بعض المواد بمختلف تركيزاتها 0.2جم/لتر نترات فضة، 0.05 جم/لتر نترات كالسيوم، 0.05 جم/لتر كلوريد كالسيوم، 0.5 جم/لتر كالسيوم بانتوسيانات. التجربة الثانية: أجريت لبحث تأثير الأحماض الأمينية المختلفة على تحسين الأجنة الجسدية. تبين أن اضافة جم/ليتر نترات فضة إلى بيئة الزراعة خفضت النسبة المئوية للت زجج إلى 22.22 % مقابل 55.55% فى البيئة الكنترول وكذلك تحققت القيمة الأعلى معنوية للنسبة المئوية للتكشف وهى 77.77%. على صعيد أخر تحققت النتائج الأعلى معنويا للنسبة المئوية لتكشف الأجنة الجسدية تحققت عندما اضيف 100ملليجم/لتر جلوتامين+100ملليجم/لتر الأنين إلى بيئة التكشف.