Influence of Temperature and Light Intensity on Proliferation and Formation of Somatic Embryos of *cv*. Samany Date Palm *in vitro*

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Received 21 May 2015 - Accepted 6 October 2015

ABASTRACT
The aim of this study was to detect the effect of physical factors (temperature and light intensity) on proliferation and formation of somatic embryogenesis stage from callus of Samany *cv*. of date palm. Two temperature degrees (20±2 or 27±2 ºC) and four levels of light intensities (0, 1000, 2000, or 3000 Lux) were used on calluses formed from production stage that was cultured on callus induction medium (DP_1). The calluses were transferred to modified hormones free nutrient medium (DP_2) for 3 months. The embryonic callus was transferred on the modified medium (DP_3) containing (in mg l^{-1}): 2.5 NAA (1-Naphthaleneacetic acid) + 3 BAP (6-Benzylaminopurine) + 2 Kin (Kinetin), for another 7 months to produce the largest possible number of somatic embryos during that period. The results showed that there are significant differences between the eight treatments of physical factors under study as the highest value for embryonic callus formation and highest means for the resultant somatic embryos numbers were for the explants cultured on total darkness and under low temperature of 20±2ºC. Meanwhile, the lowest values came from explants cultured on high temperature of 27±2ºC under high light intensity of 3000 Lux all over the study.

Key Words: Embryogenic callus, Light intensity, Physical factors, Proliferation, Somatic embryogenesis, Temperature.

INTRODUCTION
Embryogenic calluses are randomly arranged undifferentiated parenchymal cells resulted from cell divisions of explant cells cultured on nutrient medium, and it is usually formed at the cut region. Generally, callus passes through several growth and development stages as indicated by both Razdan (2003) and Fehér (2005). These stages are loose fragile callus, off white soft coherent callus, yellowish white granular callus, ending with formation of somatic embryos. Somatic embryos pass through several growth stages as it change into cell clump, induced cells, the globular embryo, heart embryo, and finally torpedo embryo that is transformed into plantlets (Gaj, 2004; Umehara *et al*., 2007).

Somatic embryogenesis in tissue culture laboratories is considered a clear evident for the dormant ability of cell to change its courses and divide till forming a complete organism (plant). The differences between somatic embryos and zygotic ones should be recognized as somatic embryos are formed and developed from somatic cells while sexual embryos are formed as a result of pollination and fertilization processes between male and female gametes (Zimmerman, 1993; Ramos *et al*., 2012).

Physical factors (temperature and light intensity) greatly affect all organisms in general and all internal biological processes in particular. It is known, among researchers of tissue culture, that optimum temperature for *in vitro* growth for most plants especially date palm is 25 ºC as it achieved good results in many studies (Belal *et al*., 2008; Shehata, 2008; Tongtape and Te-chato, 2010; Al-Khayri, 2011). In addition, temperature lower than 15 ºC resulted in growth retardance (Engelmann, 1997; El-Ashry *et al*., 2013). The objective of this study is to detect the effect of these physical factors on somatic

embryogenesis stage of date palm. In this stage, undifferentiated parenchymal cells (callus) turn into differentiated embryonic cells that produce plantlets ready for later acclimatization stage. This stage greatly determines the numbers of the *in vitro* resultant plantlets. Thus, this work aims to determine the best protocol for *in vitro* commercial date palm propagation through embryogenesis in addition to lowering the production costs in concurrence with increasing the resultant number of plantlets during a lower period than the traditional methods.

Subsequently, the study was designed to study temperature degrees and light intensities that are most likely available in laboratories. Two degrees of temperature (20±2 and 27±2 ºC) were used in addition to four different levels of light intensities (0, 1000, 2000 and 3000 Lux) to detect the optimal levels of temperature and light intensity needed for transforming callus into embryonic one and furthermore to somatic embryos.

MATERIALS AND METHODS

This study was carried out, at the Tissue Culture Lab. of Plant Production Department, College of Environmental Agricultural Sciences, El-Arish, North Sinai, Suez Canal University, Egypt during the period from 2006 to 2008.

Callus material:

Callus is obtained from culturing explants (shoot tip with soft inner leaves) cut from cuttings of Female Samany *cv*. offshoots of 5 year old, 5-7 kg weight and about 50-70 cm height. After 6 months of maintaining explant culture of Samany *cv*. on the callus induction modified medium (DP_1), shown in Table 1, according to Zimmerman (1993). The resulted calluses are white, friable, nodular and embryogenic.

Table 1: Nutrient medium composition for *in vitro* somatic embryogenesisstage of date palm.

Modified media	Composition medium (mg l^{-1})			
	Salts Strength	Organic Constituent's	Growth regulators	Complex addenda
Callus induction (DP_1)	Full MS*	100 Myo-Inositol + 80 Adenine Sulfate + 170 $NaH_2PO_4.2H_2O$ + 2 Thiamine-HCl + 2 Glutamine + 2 Ca-Pantothenate + 2 Biotin	10 NAA + 10 IAA + 30 2.4-D	30000 Suc. + 2000 Gelrite + 3000 AC
Embryogenic callus (DP_2)			Hormones free	
Embryogenesis (DP_3)			2.5 NAA + 3 BA + 2 Kin	

* Notes: MS medium (Murashige and Skoog, 1962)

Nutrient medium:

Calluses were taken re-cultured on the embryogenic callus modified medium (DP_2) shown in (Table 1). This medium is growth regulators free and was used for three subcultures to obtain embryonic callus (pro-embryo). After three months, all embryonic callus formed were moved on embryogenesis-modified medium (DP_3) that contain the same constituents in addition to the following hormones (in mg l^{-1}): 2.5 NAA (1-Naphthaleneacetic acid) + 3 BAP (6-Benzylaminopurine) + 2 Kin (Kinetin), for another seven months. The pH level was adjusted at 5.7±0.1 before adding gerlite and autoclaving the medium at 1.2 $Kg.cm^{-2}$ equivalent to 121 ºC for 15 min. The nutrient media were dispensed into small jars 25 ml. for seven subcultures to obtain somatic embryos (Table 1).

Tested physical factors:

The tested calluses treatments were cultured under different culture conditions using two degrees of temperature (low: 20±2 ºC and high: 27±2 ºC), either in the dark or light with light intensities of 0, 1000, 2000 and 3000 Lux, respectively. The photoperiod of

16/8 hrs. of light/dark cycles daily was used for this study, shown in (Table 2). Cool white fluorescence lamp of 36 watt was fixed at a distance of 45 cm above jars was also used.

Table 2: Eight different treatments of physical conditions: two degrees of temperature and four levels of different light intensity.

Temperature (°C)	Light intensity		Lamp number
	Lux	µmol m^{-2} s^{-1}	
	Total darkness	0.00	Nothing
20±2 (18 - 22)	1000	16.50 -20.00	One
	2000	33.00 – 40.00	Two
	3000	49.50 – 60.00	Three
	Total darkness	0.00	Nothing
27±2 (25 - 29)	1000	16.50 -20.00	One
	2000	33.00 – 40.00	Two
	3000	49.50 – 60.00	Three

* Notes: µmol photons m^{-2} s^{-1} = Lux X ~ 0.0165 (Hershey, 1991).

Statistical analysis:

Because of the unequal number of replications among treatments, the study adapted Pottino (1981) method in recording and analysis of views and primary data of somatic embryogenesis in order to determine the optimum physical factors needed for transformation of callus into somatic embryos. The required data were taken by rating samples according to the following scale: 0= Not Detected negative (ND), 1= Weak (1-20%), 2= Below average (21-40%), 3= Average (41-60%), 4= Good (61-80%), and 5= Very good (81-100%). Embryogenic callus formation data were recorded for Samany *cv*. at the end of the fourth subculture (3, 6, 9 and 12 weeks from initial culture, respectively).

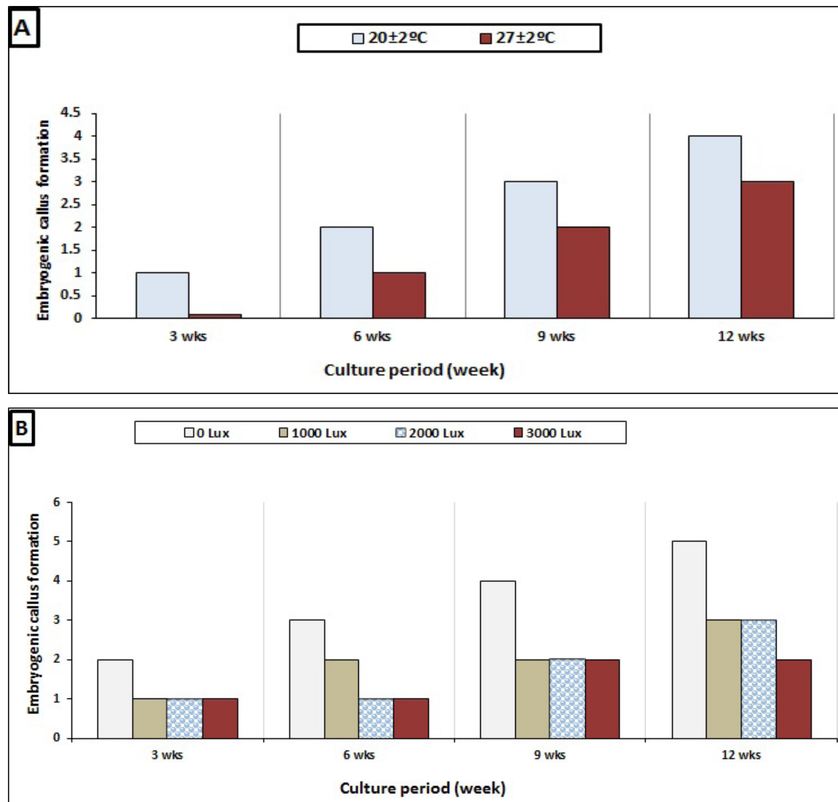
Data of number of embryos during seven months were statistically analyzed according to the technique of analysis of variance (ANOVA) for the completely randomized design according to (Sndecor and Cochran, 1990). The treatment means were compared using least significant difference (LSD) at 5 % level of probability. As the number of replications were five for the eight treatments and the data were recorded every two months, i.e. after (1, 3, 5 and 7 mon., respectively). Mean separations according to Duncan (1955) was performed using Mstat computer program v.4.

RESULTS AND DISCUSSION

Effect of physical factors on proliferation of embryogenic callus:

Concerning the specific effect of temperature, data in Figure (1A) reveals that the best rate of embryogenic callus formation (4.00) was recorded by 20±2 ºC. While, the worst rate (3.00) was noticed with 27±2 ºC during 12 wks. (Belal *et al*., 2008) obtained similar results.

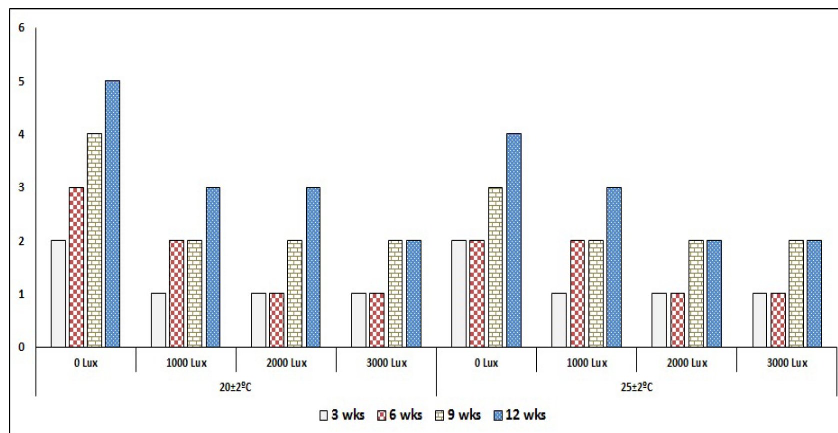
As for the specific effect of light intensity, data from the Figure (1B) showed that the best rate of embryogenic callus formation (5.00) was achieved by total darkness (0 Lux). On the other hand, the worst rate (2.00) was recorded by light intensity of 3000 Lux during somatic embryogenesis stage. Similar results were obtained by (Eke *et al.*, 2005; Al-Khayri, 2011; El-Ashry *et al*., 2013; Boufis *et al*., 2014), and disagreed with those of (Bhargava *et al*., 2003; Zaid *et al*., 2004) who reported opposite results.



* Rating of individual parameters: 0= ND, 1= Weak, 2= Below average, 3= Average, 4= Good, and 5= Very good.

Figure (1): Specific effect of: A, temperature and B, light intensity on proliferation embryogenic callus during three months of Samany *cv. in vitro*.

On the other hand, the combination between temperatures with light intensities in Figure (2) showed that the best rates of embryogenic callus (5.00 and 4.00, respectively) were at 20±2 ºC followed by 27±2 ºC with darkness. While, the worst rates (2.00) were at 27±2 and 20±2 ºC, respectively with high light intensity of 3000 Lux during somatic embryogenesis stage. (Veramendi and Navarro, 1996; Shehata, 2008; Tongtape and Te-chato, 2010) obtained similar results.



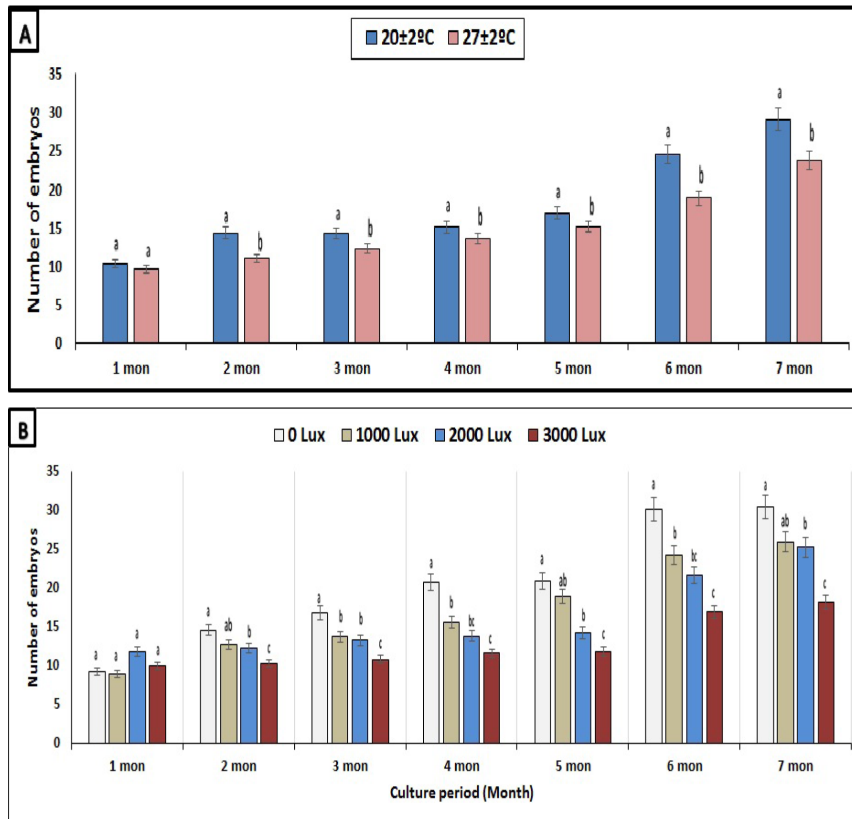
* Rating of individual parameters: 0= ND, 1= Weak, 2= Below average, 3= Average, 4= Good, and 5= Very good.

Figure (2): The interaction effect of temperature and light intensity on proliferation embryogenic callus during 12 weeks of Samany *cv. in vitro*.

Effect of physical factors on formed embryos number:

The results in Figure (3A) show the specific effect of two different temperatures on embryos number during seven months of culture of Samany *cv*. with five replications. The obtained results revealed that, the highest mean of embryos number (29.15a) occurred with 20±2 ºC. While, the lowest mean (23.75b) was at 27±2ºC during the same stage. (Belal *et al*., 2008; Shehata, 2008) obtained similar results.

Data present in Figure (3B) indicated that the highest mean of embryos number (30.40a) occurred with total darkness (0 Lux) that was significantly (P0.05>) surpassed during cultures period. However, the lowest value of number of embryos (18.20b) was noticed with 3000 Lux during period of culture.

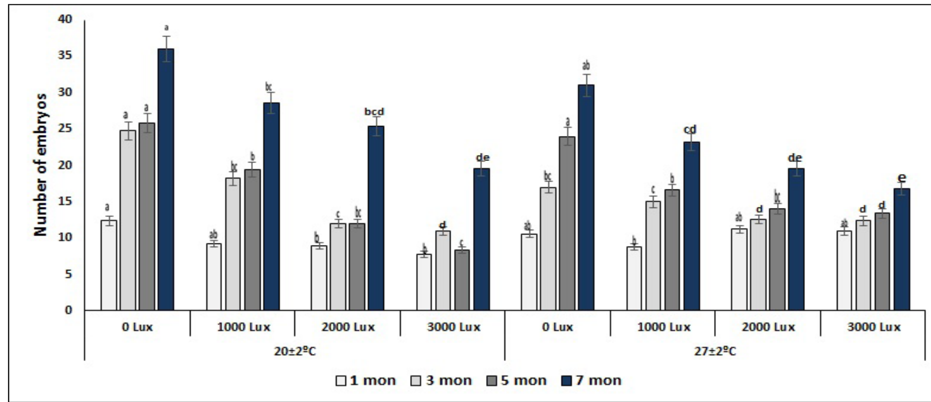


* P-values less than 0.05 are significant.

Figure (3): Specific effect of A) temperature and B) light intensity on formed embryos number during seven months of Samany *cv. in vitro*.

Concerning the interaction effect of temperature and light intensity on No. of embryos, the results in Figure (4 and 5) show the interaction effect of eight different treatments combinations on the number of embryos during seven months of cultures of Samany *cv*. The data reflected that the highest means (36.00a) was achieved at 20±2 ºC in darkness followed by 27±2 ºC under total darkness (0 Lux). Whereas, the lowest mean of number of embryos (16.80b) was noticed at 27±2 ºC with light intensity 3000 Lux during the same stage.

The results of this study showed that, the stage of somatic embryogenesis from undifferentiated somatic cells of callus as well as the callus formation stage does not need lighting contrary to other growth stages that requires lighting. As cells division occurs to form either sexual or asexual zygote at suitable temperature in the absence of light (Ramos *et al*., 2012). This study confirmed that the highest possible number of somatic embryos was achieved under total darkness especially those cultured at low temperature (20±2ºC).



* P-values less than 0.05 are significant.

Figure (4): The interaction effect of temperature and light intensity on formed embryos number during seven months of Samany *cv. in vitro*.

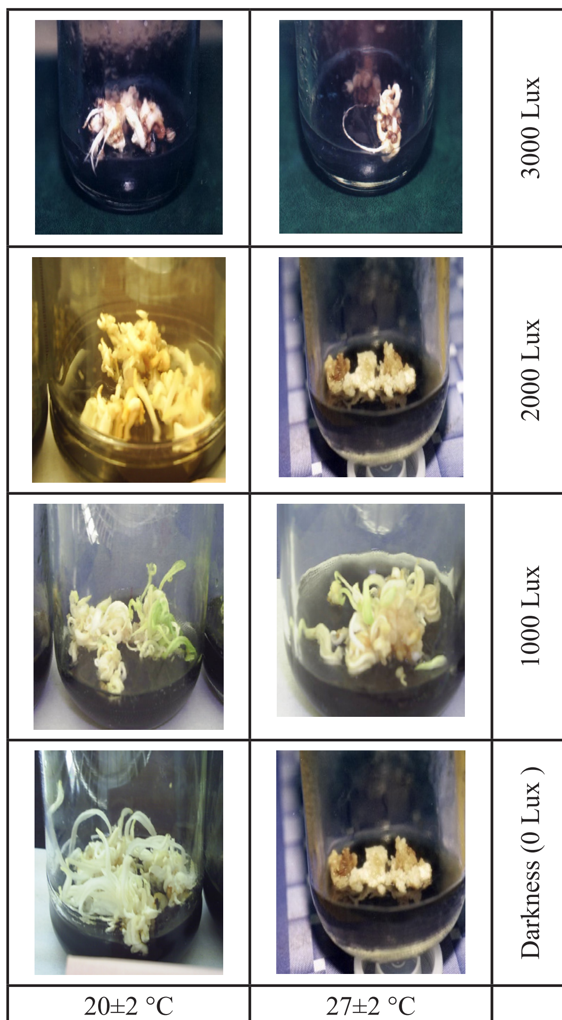


Figure (5): Effect of temperature and light intensity on proliferation and formed somatic embryogenesis during seven months of Samany *cv. in vitro*.

These results agreed with both Veramendi and Navarro (1996) and Belal *et al.* (2008).

Culturing under high temperature (27±2ºC) gave lower numbers with significant differences between the tested temperatures. This indicates the clear effect of the high temperature on all biological processes inside cells, especially cell fission and fusion resulting in lowering the transformation rate of the undifferentiated parenchymal cells of callus into embryonic callus, subsequently into somatic embryos as reported by Engelmann (1997), Razdan (2003), and Fehér (2005). Husaini *et al.* (2011) and Shehata *et al.* (2014) attributed this to the loss of effectivity for some hormones added to the nutrient medium due to hormonal damage in presence of light, in addition to secretion of peroxidase and oxidase oxidative enzymes that are harmful to the callus. This resulted in browning phenomenon that greatly disable callus bioavailability and ability to transform into somatic embryos in addition to retardation of growth extension and transformation process below normal rates.

Light intensity played an important and effective role as there were significant differences between callus cultured under total darkness and those cultured under other levels of light intensity (1000, 2000, and 3000 Lux). The highest transformation rate of cells into somatic embryos was achieved by callus cultured under total darkness followed by that cultured at 1000 then 2000 then 3000 Lux, respectively. Higher light

intensity caused higher catabolism and lower anabolism rates that greatly affect all biological processes and subsequently affect somatic embryogenesis rate (Hothersall and Ahmed, 2013). In addition, it also caused explant deterioration because of the higher probability of browning occurrence which extremely reduce callus bioavailability and activity (Shehata *et al*., 2014).
Finally, it could be concluded that incubation can be carried out in total darkness during the first three stages of growth and development with no need to use lighting. This period is approximately 16 months from the start of culture processes. This should reduce production costs as a result of cultivation of plant parts at room temperature and in darkness during the initial stages of development and differentiation. This is a very important aspect especially for the commercial laboratories.

ACKNOWLEDGEMENT

The authors would like to thank Plant Production Dept., College of Environmental Agricultural Science, Suez Canal University, Arab Republic of Egypt, for supporting this research.

REFERENCES

Al-Khayri, J. M. 2011. Basal salt requirements differ according to culture stage and cultivar in date palm somatic embryogenesis. Am. J. Biochemistry and Biotechnology. 7: 32-42.

Belal, A.H., El-Deeb, M.D. and Shehata, W.F. 2008. Effect of light intensity and temperature on plantlet growth and development of Samany *cv*. *in vitro*. The Third international Conference on Date palm, El-Arish, Egypt, 25-27 April. 265-277.

Bhargava, S.C., Saxena, S.N. and Sharma, R. 2003. *In vitro* multiplication of *phoenix dactylifera* (L). J. Plant Biochemistry and Biotechnology. 12: 43-47.

Boufis, N., Khelifi-Slaoui, M., Djillali, Z., Zaoui, D., Morsli, A., Bernards, M.A., Makhzum, A. and Khelifi, L. 2014. Effects of growth regulators and types of culture media on somatic embryogenesis in date palm (*Phoenix dactylifera* L. *cv*. Degla Beida). Scientia Horticulturae 172: 135-142.

Duncan, B.D. 1955. Multiple ranges and multiple F test. Biometrics. 11: 1-42.

Eke, C.R., Akomeah, P. and Asemota, O. 2005. Somatic embryogenesis in date palm (*Phoenix dactylifera* L.) from apical meristem tissues from 'zebia' and 'loko' landraces. African Journal of Biotechnology. 4 (3): 244-246.

El-Ashry, A.A., Shaltout, A.D., El-Bahr, M.K., Abd El-Hamid, M., Matter, A. and Bekheet, S.A. 2013. *In vitro* preservation of embryogenic cultures of two Egyptian dry date palm cultivars at darkness and low temperature conditions. Journal of Applied Sciences Research. 9 (3): 1985-1992.

Engelmann, F. 1997. *In vitro* conservation methods. *In:* Ford-Lloyd, B. V., Newburry, J. H., and Callow, J. A. (Eds.). Biotechnology and Plant Genetic Resources: Conservation and Use. 119-162. CABI, Wellingford.

Fehér, A. 2005. Why somatic plant cells start to form embryos? *In:* Mujib A., Samaj J. (Eds.). Plant Cell Monographs (volume 2). Somatic Embryogenesis. 85-101. Springer-Verlag, Berlin Heidelberg.

Gaj, M.D. 2004. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regulation. 43: 27-47.

Hershey, D.R. 1991. Plant light measurement & calculations. The American Biology Teacher 53: 351-53.

Hothersall, J. and Ahmed, A. 2013. Metabolic fate of the increased yeast amino acid uptake subsequent to catabolite derepression. J. Amino Acids: e461901. doi:10.1155/2013/461901.

Husaini, A. M., Mercado, J.A., Silva, J.A. and Schaart, J.G. 2011. Review of factors affecting organogenesis, somatic embryogenesis and agrobacterium tumefactions mediated transformation of strawberry. Genes, Genomes and Genomics 5(1): 1-11.

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

Pottino, B.G. 1981. Methods in Plant Tissue Culture. Dept. of Hort., Agric. College, Maryland Univ., College Park, Maryland, USA. 8-29.

Ramos, L.S., Torres, A.A., Carbonell, L.S., Salín, C.O. and Serna, E.C. 2012. Somatic embryogenesis in recalcitrant plants. *In*: Sato, K. I. (Ed.), Embryogenesis. 597-618. InTech, China.

Razdan, M.K. 2003. Introduction to Plant Tissue culture (2nd.ed.). Oxford Publishers Enfield, NH.

Shehata, W.F. 2008. Effect of light intensity and temperature on date palm micro-propagation. PhD. thesis, Suez Canal University, Arab Republic of Egypt.

Shehata, W.F., Al-Turki, S., Aldaj, M. and Ghazzawy, H. 2014. Effect of ammonium nitrate on antioxidants production of date palm (*Phoenix dactylifera* l.) *in vitro*. Biotechnology j. 13(3): 116-125.

Sndecor, G.W. and Cochran, W.G. 1990. Statistical methods. 7th ed. Lowa State Univ. Press, Ames-Iowa, USA. 507.

Tongtape, K. and Te-chato, S. 2010. Physical and Chemical Factors Affecting Growth and Development of Embryogenic Callus of Oil Palm. Proceedings of the 7th IMT-GT UNINET and the 3rd International PSU-UNS Conferences on Bioscience. Prince of Songkla University, HatYai, Songkhla, Thailand. 7-8 October 2010. 87-91.

Umehara, M., Ikeda, M. and Kamada, H. 2007. Endogenous factors that regulate plant embryogenesis: Recent advances. Japanese Journal of Plant Science. 1(1)**:** 1-6.

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

Zaid, Z.E., Gomaa, A.H. and Ibrahim, I.A. 2004. *In vitro* growth and development of different somatic embryo shapes of some date palm genotype. The Second International Conference on Date Palm El-Arish, Egypt. 6-8 Oct. 109-118.

Zimmerman, J.L. 1993. Somatic embryogenesis: A model for early development in higher plants. The Plant Cell 5. 1411-1423.

تأثير درجة الحرارة وشدة الإضاءة على تكشف وتكون الأجنة الجسدية من نخيل التمر صنف سماني معملياً

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استلام 21 مايو 2015م - قبول 6 أكتوبر 2015م

الملخص

تم إجراء هذه الدراسة بهدف معرفة مدى تأثير الظروف الفيزيائية [درجة الحرارة وشدة الإضاءة] على مرحلة إنبات الأجنة الجسدية من كالس نخيل تمر صنف سماني. فقد تم استخدام درجتي حرارة (20±2 أو 27±2°م) مع أربع مستويات من شدة الإضاءة (0، 1000، 2000 أو 3000 لكس)، حيث تم أخذ الكالس الناتج خلال المرحلة الأولى على بيئة (DP_1) قيد الدراسة ونقله على البيئة الغذائية المعدلة (DP_2) الخالية من الهرمونات لمدة 3 أشهر. وبعد ذلك ينقل الكالس الجنيني على البيئة المعدلة (DP_3) التي تتميز باحتوائها على (2.5 NAA + 3 BA + 2 Kin ملجم/ لتر) لمدة 7 أشهر أخرى متتالية لإنتاج أكبر قدر ممكن من الأجنة الجسدية خلال تلك الفترة. وقد أوضحت البيانات أن هناك فروقاً معنوية بين المعاملات الثمانية من الظروف الفيزيائية قيد الدراسة، حيث أثبتت الدراسة أن أعلى قيمة لتكوين الكالس الجنيني وكذلك أعلى المتوسطات لأعداد الأجنة الجسدية الناتجة كانت للأجزاء النباتية المنزرعة في إظلام تام وتحت درجة الحرارة المنخفضة 20±2°م، بينما كانت أقل القيم والمتوسطات لها كانت مع الأجزاء النباتية المنزرعة تحت درجة الحرارة العالية 27±2°م وشدة إضاءة عالية أيضاً 3000 لكس خلال فترة التجربة بشكل ملحوظ.

الكلمات المفتاحية: الأجنة الجسدية، التكشف، درجة الحرارة، شدة الإضاءة، الظروف الفيزيائية، الكالس الجنيني.