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Effect of desiccation and Cold hardening on germination of Somatic embryos in Date Palm (*Phoenix dactylifera* L.) Berhi cultivar *in vitro*

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ABSTRACT

Embryogenic suspension cultures of Date palm (Phoenix dactylifera L.) allow mass propagation of somatic embryos; Partial desiccation (0, 1, 2, 3 and 4 hours) and low temperature (0 °C for 2 hr, 0 °C for 4 hr, 4 °C for 24 hr and 4 °C for 48 hr) treatments were applied to improve germination of somatic embryos in vitro of date palm cultivar Berhi with or without AC. The highest germination percentage was achieved when embryos were desiccated for three hours as well as treatment of low temperature in 4 °C for 24 hr. Also, the results proved that found activated charcoal (AC) in liquid media produced the highest somatic embryos number and weight and improving percentage of germination. Further, Partial desiccation and low temperature increased embryos proline content. The improvement of the germination of somatic embryos via low temperature and especially via partial desiccation embryos somatic is successful can be used for the purpose of commercial propagation especially for Berhi cultivar.

Key words: Cell suspension, somatic embryos, germination, partial desiccation, low temperature.

INTRODUCTION

Micropropagation of date palm has been achieved from several genotypes through organogenesis and somatic embryogenesis using various meristematic explants including zygotic embryos, shoot tips and lateral buds [1]. However, the date palm remains a recalcitrant species to *in vitro* techniques because of the influence of genotype factors that affect the responsiveness of explants in culture and the frequency of maturation and germination of embryos, thus hindering the establishment of simple, reliable, and reproducible protocols [2, 3]. In fact, date palm tissue cultures grow very slowly: the initiation phase may require more than 24 months, especially when low amounts of plant growth regulators are used in order to avoid somaclonal variation [4]. Somatic embryogenesis is considered the most efficient regeneration process for date palm micropropagation [5]. It is reported to be a quick and efficient method for large scale propagation of date palm and could also be highly useful for breeding programs [6]. Liquid media were eventually utilized in date palm tissue culture. Several researchers successfully obtained *in vitro* plant regeneration using suspension cultures established from date palm embryogenic callus [7, 8]. Moreover, adding low concentration of auxin and activated charcoal (AC) enhanced the number of induced somatic embryos in the liquid cultures of date palm [9]. Meanwhile, [8] enhanced plant regeneration of date palm *in vitro* cultures through partial desiccation of somatic embryos. Also, [10] found that cold hardening useful in germination of somatic embryos. The present work aims to develop an effective system for desiccation and Low temperature of tissue cultures of date palm by investigate the role of dehydration and AC medium on germination of somatic embryos tissue cultures to Date Palm Berhi cultivar.

MATERIALS AND METHODS

2.1 Plant material and explants preparation

Shoot tips of date palm Berhi cultivar were separated from healthy offshoots (3-4 years old) of 5-7 kg in weight; Outer leaves were acropetally removed, exposing the hearts of the offshoots (15-20 cm length, 6-8 cm width). To prevent browning, the hearts were immersed in a chilled antioxidant solution consisting of 100 mg l^{-1} ascorbic acid

and 150 mg l^{-1} citric acid until the time of culture. The outer leaves of the offshoot hearts were removed under aseptic conditions exposing the shoot tip region (3-4 cm length, 1-1.5 cm width) with 3-4 primary leaves. The shoot tips were disinfected by immersion in 0.3% $HgCl_2$ with 3 drops of Tween 20 for 5 min under agitation and then washed three times in sterile distilled water before dividing them to small squares (0.5-1 cm^2) which formed our initial explants.

2.2 Callus induction

The explants were cultured on a medium consisting of MS salts (Murashige and Skoog, 1962) supplemented with (per liter) 170 mg NaH_2PO_4, 100 mg myo-inositol, 200 mg glutamine, 2.5 mg thiamine-HCl, 0.2 mg biotin, 0.2 mg pyridoxine-HCl, 30 g sucrose and 7 g agar. The medium was enriched with 3 mg l-1 N-(3-methyl-2-butenyl)-1Hpurin-6-amine (2iP) and 1.5 g l^{-1} activated charcoal (AC) during the first 8 months of culture, then the explants were transferred to a medium supplemented with 10 mg l^{-1} 2,4- Dichlorophenoxyacetic acid (2,4-D), 3 mg l^{-1} 2iP and 1.5 g l^{-1} AC for additional 2-3 months [9]. The pH was adjusted to 5.7 and distributed into 100-ml Magenta vessels containing 35 ml of the medium/vessel, caped with Magenta B-cap and autoclaved at 121°C for 15 min. Culture incubation conditions consisted of complete darkness and 24 ± 2°C. Resultant callus from the explants served as a source of callus for the following experiment.

2.3 Cell suspension establishment

Portions of embryogenic friable callus (200 mg) were chopped and cultured in 50 ml liquid MS media dispensed in 150 ml flasks. The suspension cultures were incubated on a rotary shaker set at 100 rotations per minute (rpm) using electric shaker (Certomat® R), (Plate 1, B).

2.4 Culture medium

Two medium compositions were chosen according to [5] ½ MS with 1 mg l^{-1} 2,4-D without AC and ½ MS with 1 mg l^{-1} 2,4-D 300 mg l^{-1} AC. The pH was adjusted to 5.7 before the addition of 30 g l^{-1} sucrose. The media were autoclaved at 121°C for 15 min. The cultures were incubated under 16-hr photoperiods of cool-white florescent light (35 µmol m^{-2} S^{-1}) at 24 ± 2°C.

2.5 Somatic embryo induction

At the end of the 16th week the calli were transferred to a somatic embryo induction medium with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC for 18 weeks. The calli were subculture every 6 weeks and the number of formed somatic embryos was determined after 18 weeks on this medium. Maturate Somatic embryo (01mm and more).

2.6 Desiccation of embryo cultures

Formed embryos were transferred to Petri dishes with double layer of filter paper (Whatman no. 40). Clumps of non-germinated somatic embryos (each contained 3 to 5 embryos and weighed of 0.702 to 0.883 g) were held in the air current of Laminar flow cabinet for the periods of desiccation (1, 2, 3 and 4hr). After each period, each sample was weighed to calculate reduction in fresh weight (water content).Then the embryos cultured in the maturation medium to 6 weeks.

2.7 Cold hardening Procedures

The tubes (contained date palm cultures in the maturation medium) were kept at (0 °C for 2 hr, 0 °C for 4 hr, 4 °C for 24 hr and 4 °C for 48 hr).

2.8 Germination of somatic embryos and conversion into plantlets

Advanced cotyledonary somatic embryos were picked from the maturation medium after 6 weeks of culture and transferred to germination medium without any post-maturation treatment. The germination medium was MS medium supplemented with 1 mg l^{-1} NAA and 0.1 g l^{-1} AC. The cultures were kept under the same conditions as for maturation. Somatic embryos were considered germinated as soon as radical emergence occurred with plantlet conversion based on shoot greening and elongation [8].

2.9 Determination of proline concentration according to [11].

2.10 Experimental design and statistical analysis

The experiment was set up as a factorial in Completely Randomized Design (CRD) comprising two main factors: levels desiccation and/or low temperature and AC as 2 levels. Each treatment consisted of 10 replications. The data present an average of the repetitions. Data were subjected to analysis of variance (ANOVA) and the means were separated using RLSD test by using the program SPSS 16 for Windows.

RESULTS

3.1 Effect of AC on embryo numbers

Data showed in Fig. 1 that increasing the number of embryos cultures on liquid medium with AC compared with liquid medium without AC. Also, the cell suspension proliferated callus prior to somatic embryo formation was observed after 32 day of culturing. Somatic embryo formation was obvious in the liquid culture medium, although a small percentage of the cultures commenced embryo formation prior to that, within the first 21 day after introducing to 1 mg l^{-1} 2, 4-D with 300 mg l^{-1} AC medium.

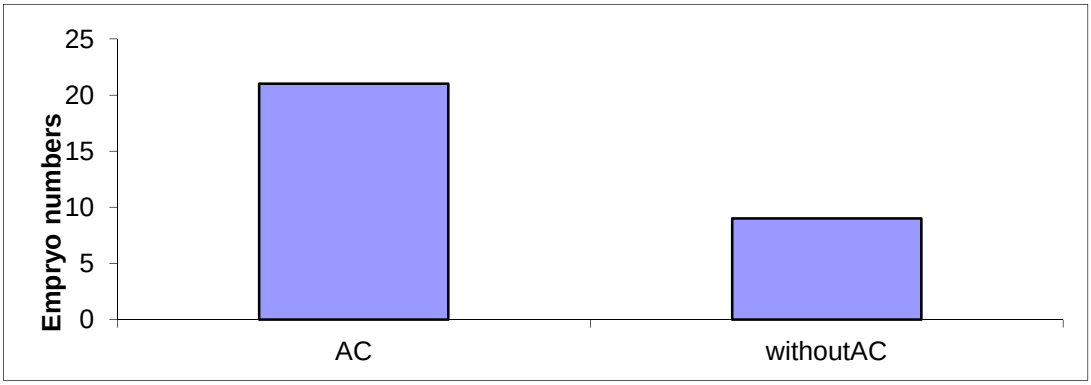


Figure 1: Effect of AC on embryo numbers of date palm Berhi cultivar after 8 weeks culture

3.2 Effect of desiccation on fresh weight of embryo cultures

Data in Table 1 indicated that increasing the time of desiccation to embryo cultures decreased embryos fresh weight, due to the reduction of water content just after treatment. The highest reduction was appeared with four hours period. Liquid medium without AC gave more reduction of fresh weight of embryos than with AC at four hours period. Also, fresh weights of embryos with AC more increase than without AC. In all periods, the embryos of both treatments of AC decreased in size and became fragile as a result of reduction in water content compared with no desiccated embryos.

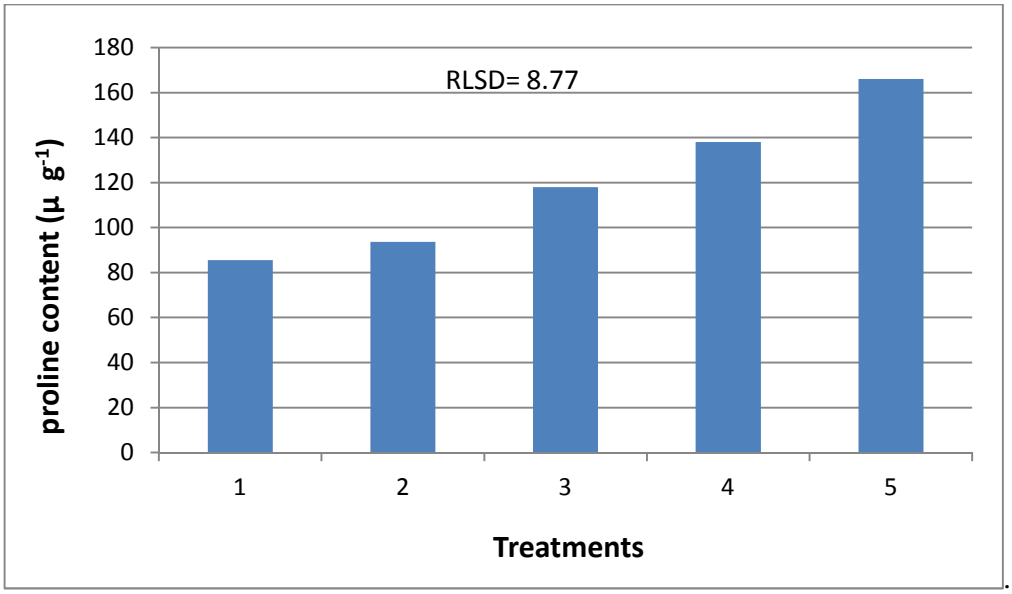
Table 1: Effect of AC and desiccation period on embryo weight (g) of date palm Berhi cultivar after 8 weeks culture

Desiccation period (hr) (B)	Treatments of AC (A)		Mean (B)
	AC	Without AC	
0	0.85	0.68	0.77
1	0.78	0.64	0.71
2	0.75	0.61	0.68
3	0.72	0.59	0.65
4	0.69	0.56	0.62
Mean (A)	0.76	0.62	
RLSD	A=***	B= 6.26	AxB=NS

0: Control without treated

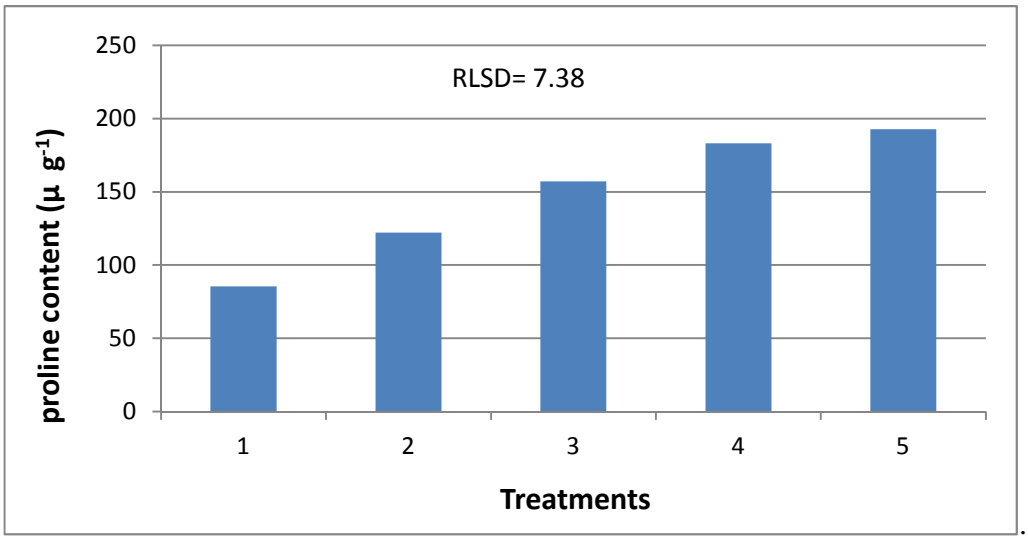
3.3 Effect of AC and desiccation period and cold hardening on embryos proline content

Data in Fig. 2 showed that increasing the time of desiccation to embryo cultures increased embryos proline content, due to the reduction of water content just after treatment. The highest value was appeared with four hours period (192.6 µ g^{-1}). Also, data in Fig. 3 indicated that increasing the time of cold hardening to embryo cultures increased embryos proline content. The highest value was appeared with four treatment period (166.1 µ g^{-1}). However, the results a clear that the treatments of desiccation caused increased proline content more than cold hardening.



1= 0 (control), 2=1hr, 3=2 hr, 4=3hr, 5=4hr

Figure 2: Effect of desiccation period on embryos proline content in medium with AC of date palm Berhi cultivar after 8 weeks culture



1= 0 (control),2= 0 °C for 2 hr ,3= 0 °C for 4 hr ,4= 4 °C for 24 hr, 5= 4 °C for 48 hr

Figure 3: Effect of cold hardening on embryos proline content in medium with AC of date palm Berhi cultivar after 8 weeks culture

3.4 Effect of AC and desiccation period on embryos germination

Partial desiccation of mature embryos corresponding to a decrease in water content followed by their culture on germination medium resulted in a higher germination percentage from 14.50 to 35.50 %. Liquid medium with AC were affected positively by desiccation more than without AC. Increasing desiccation period to three hours increased germination percentage to Liquid medium with AC. Whereas, these percentages reduced when desiccated period extended to four hours (Table 2).

Table 2: Effect of AC and desiccation period on embryo germination % of date palm Berhi cultivar after 8 weeks culture

Desiccation period (hr) (B)	Treatments of AC (A)		Mean (B)
	AC	Without AC	
0	19.00	10.00	14.50
1	31.33	22.00	26.66
2	35.33	28.00	31.66
3	41.00	30.00	35.50
4	32.00	26.00	29.00
Mean (A)	31.73	23.20	
RLSD	A= ***	B= 2.121	AxB=NS

0: Control without treated

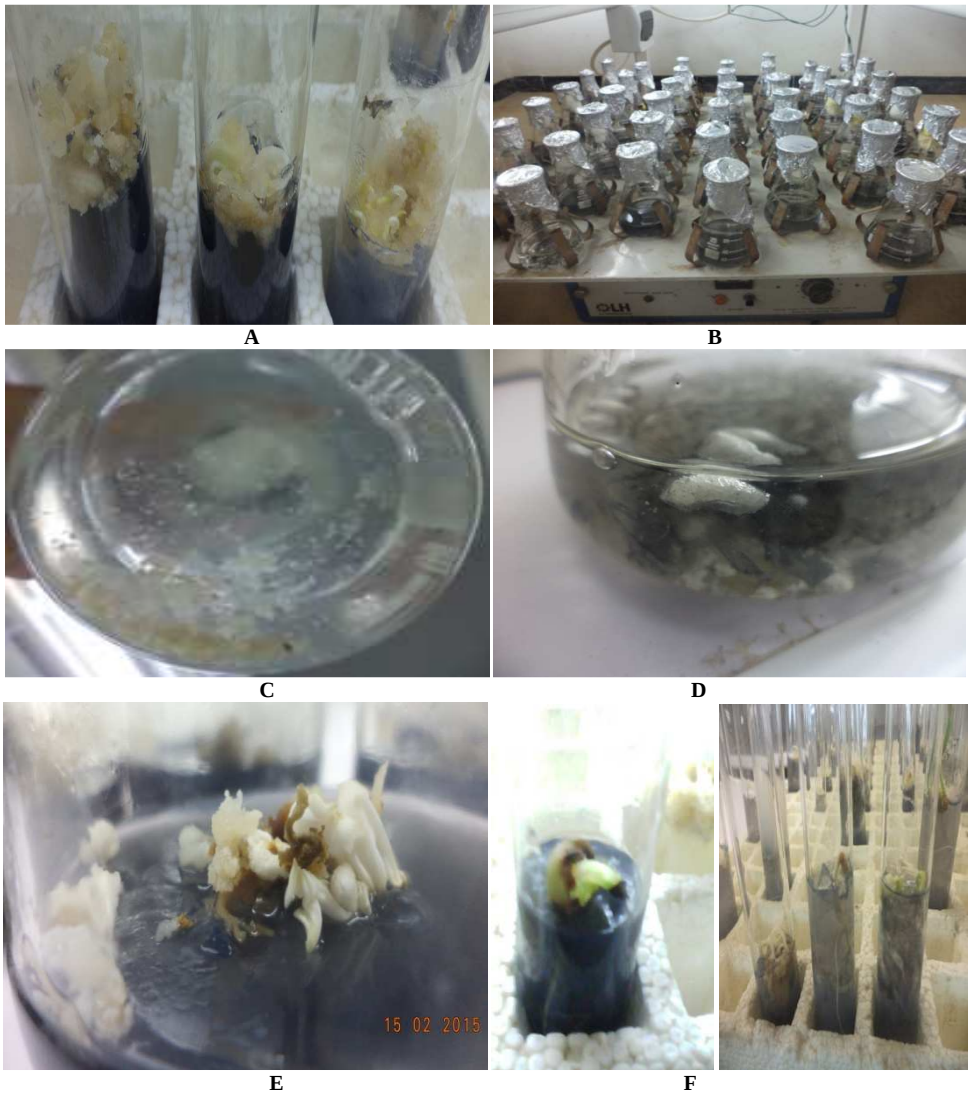
3.5 Effect of AC and cold hardening on embryo germination

Data in Table 3 indicated that the treatment of 4° C 24 hr increased the embryos germination percentage, whereas the treatment of 4 °C for 48 hr decreased embryo germination percentage. Also, liquid medium with AC were affected positively by cold hardening treatments in increase embryos germination percentage more than without AC. The best treatment of low temperature was 4 °C 24hr on liquid medium with AC which increased embryos germination from 10.00 to 37.00 %.

Table 3: Effect of AC and cold hardening on embryo germination% of date palm Berhi cultivar after 8 weeks culture

Cold hardening (B)	Treatments of AC (A)		Mean (B)
	AC	Without AC	
0	19.00	10.00	14.50
0 °C for 2 hr	21.00	16.00	18.50
0 °C for 4 hr	24.00	20.00	22.00
4 °C for 24 hr	37.00	26.00	31.50
4 °C for 48 hr	21.00	17.00	19.00
Mean (A)	24.40	17.80	
RLSD	A=***	B=1.643	AxB= 2.323

0: Control without treated



A **B** **C** **D** **E** **F**

G **H**

Plate 1: Illustration of the different somatic embryogenesis phases in Berhi cultivar, from embryogenic friable callus to in vitro plantlets. (A) embryogenic friable callus (B) suspensions system (a rotary shaker) (C) somatic embryoes derived from embryogenic friable callus after 18 weeks (D) size of somatic embryos in liquid medium with AC (E) Development of embryos on solid medium (maturation medium) (F) Structured somatic embryos after maturation on solid media (G) Germination of somatic embryos (H) Development of somatic embryos into plantlets

DISCUSSION

Liquid culture system is a promising technique for rapid mass propagation of date palm. Somatic embryo production in liquid media is about ten times greater than that on solid media. Furthermore, the suspension cultures are technically easier and more economical than the bioreactors [9]. It's important step to improved formation of somatic embryo. Also, using of AC in Liquid culture system Participated in this improvement and enhanced the number of induced somatic embryos in the liquid cultures of Berhi date palm. However, Partial desiccation of mature embryos followed by their culture on germination medium resulted in a higher germination rate, whereas these percentages reduced when desiccated period extended to four hours. Results in discussion are in line with the findings of [5]. It was reported that partial desiccation of the mature embryos of cultivar Deglet Nour, corresponding to a decrease in water content from 90 to 75% significantly improved germination rate from 25% to 80%. The effects of partial drying may cause a breakdown of endogenous ABA or decreased sensitivity of embryos to ABA which could release embryos from development constraints and allow germination to proceed [12].These results confirm those from [8] on germination of date palm. Also, increase proline content under desiccation and cold hardening treatments may be improved the germination of somatic embryos by increasing enzyme activity as peroxidase enzyme which play role in cell division or it was able to change the expression of genes which are essential for the germination. In turn recent proteomic, genomic and metabolic studies have revealed that the function of proline is not as straight forward as initially believed. Research studies on plants, especially those on proline synthesis and catabolic genes, have demonstrated that the proline produced under stressful conditions can act as a compatible solute in osmotic adjustment, a free radical scavenger, a metal chelator, an activator of detoxification pathways, a cell redox balancer, a cytosolic pH buffer, a source of energy, nitrogen and carbon, a stabilizer for subcellular structures and membranes including photosystem II (PS II), or act as a signaling molecule [13]. Moreover, the effect of cold hardening on proline content of explants was found to increase proline contents in date palm tissues Khenizi cultivar [10]. According to [14] the specific accumulation of this amino acid improves resistance to both severe osmotic stress and cold hardening. Cold acclimation was found to improve post-cryopreservation recovery in several plant species such as apple [15] and date palm [10].

CONCLUSION

The determination of biochemical and physical changes associated with germination of somatic embryos is a very interesting approach to optimize tissue culture date palm protocol. Also, the present study suggests that the improvement of the germination of somatic embryos via low temperature and especially via partial desiccation embryos somatic is feasible, reproducible and offers a tremendous potential for an inexpensive method for large scale propagation of superior date palm cultivars.

REFERENCES

[1] Al-Khayri J M, *Biotechnol.* **2010**, 9: 477-484.
[2] Zaid A, Arias-Jimenez E J, *FAO Plant production andprotection paper* 156 Rev.1. F.A.O., Rome, **2002**.pp153.
[3] Jain M S, Al Khayri J M, DVF J, 1st ed. Springer,Netherlands, **2011**.pp 54
[4] Cohen Y, In: Jain, S.M., Al-Khayri, J.M., Johnson, D.V. (ed.): *Date Palm Biotechnology*, pp. 221-235. Springer, Dordrecht, **2011**.

[5] Fki L, Masmoudi R, Drira N, Rival A, *Plant. Cell. Rep*., **2003**, 21, 517–524.
[6] El-Hadrami I, El-Bellaj M, El-Idrissi A, J'Aiti F, El-Jaafari S, Daaf F, *Cah. Agric.* ,**1998**, 7: 463-468.
[7] Sharma D R, Kumari R, Chowdhury J B, *Euphytica*, **1980**, 29, 169–174.
[8] Othmani A, Bayoudh C, Drira N, Marrakchi M, Trifi M, *Plant Cell Tiss. Org*. **2009**, 97, 71–79.
[9] Ibraheem Y, I Pinker and, M Böhme, *Emir. J. Food Agric*. **2013**, 25 (11): 883-898
[10] Fki L, N Bouaziz, O Chkir, R Benjemaa-Masmoudi, A Rival, R Swennen, N Drira, B Panis, *Biologia Plantarum*, **2013**, 57 (2): 375-379.
[11] Irigoyen JJ, Emerich DW, Sanchez- Diaz M, *Physiol Plant*, **1992**, 84: 55- 60.
[12] Kermode AR, Dumbroff EB, Bewley JD, *J. Exp. Bot.*, **1989**, 40:303-313.
[13] Anwar M A, Hossain M d, Anamul H, David JB, Masayuki F, in: P. Ahmad (Ed): Oxidative Damage to Plants, *Elsevier Inc*, **2014**, :477-522.
[14] Pociecha E, P1azek A, Janowiak F, Zwierzykowski Z , Physiol. mol. *Plant Pathol.* **2009**, 73: 126-132.
[15] Kushnarenko S V, Romadanova N V, Reed B M, *CryoLetters*, **2009**, 30: 47-54.