Journal of Cell and Molecular Research (2010) 2 (2), 86-92

In vitro selection for NaCl tolerance in *Thymus vulgaris* L.

Akram Zia[1]*, Farkhonde Rezanejad[1] and Abbas Safarnejad[2]

[1]*Biology Department, Bahonar University of Kerman, Kerman, Iran*

[2]*Research Center of Agriculture and Natural Resources of Khorassan Razavi, Mashhad, Iran*

Received 15 October 2010 Accepted 18 December 2010

Abstract

Thyme (*Thymus vulgaris* L.) is an aromatic and medicinal plant which is very important for the herbal industry. In this research, *in vitro* selection for NaCl tolerance was investigated in this plant. Hypocotyl and shoot explants (apical meristem and cotyledonary leaves) of sterilized seedlings were cultured in MS medium supplemented with different combinations of 2, 4-dichlorophenoxyacetic acid (2, 4-D), napthaleneacetic acid (NAA), kinetin (KIN) and N_6-benzyladenine (BA) for callus induction. Results showed that the maximum frequency of callus induction was obtained on MS medium containing 1:0.5 ratio of 2, 4-D:Kin (mgl^{-1}), while optimal callus induction with the best quality and regeneration potential was achieved in 1:1 ratio of NAA:BA (mgl^{-1}). The calli grown in 1:1 ratio of NAA:BA (mgl^{-1}) were transferred to NaCl supplemented medium at 50, 75 and 100 mM concentrations. Fresh and dry weights, percentage of necrosis and regeneration of calli were determined after 4 weeks. There were significant differences between fresh and dry weights of calli in different concentrations of NaCl. The highest and the lowest of fresh and dry weights of calli from hypocotyl explants were observed in 0 (control) and 100 mM NaCl concentrations, respectively. But fresh weight of shoot-derived calli induced from shoots reduced only in 100 mM NaCl and the dry weight of them was not significantly different. The necrosis percentage increased by increasing the salt concentration. Callus regeneration just occurred at the concentration of 50 mM of NaCl and all regenerated shoots well rooted on half strength MS medium, with 0.8% (w/v) sucrose and without growth regulators.

***Keywords*:** callus, salinity, *in vitro* selection, *Thymus vulgaris* L.

Introduction

Thyme (*Thymus vulgaris* L.) belongs to the Lamiaceae family, and is an aromatic and medicinal plant, which is very important for the horticultural industry. Thyme is a perennial shrub with woody stems and reaches a height of 30-50 cm. It is a typical plant of the Mediterranean region. Thyme possesses antiseptic, antispasmodic, carminative, diaphoretic, disinfectant, deodorant, diuretic, expectorant, sedative, tonic and antihelmintic properties. Its properties are due to its main components: thymol and carvacrol (Soto-Mendivil et al., 2006). High concentrations of salts account for a large yield decrease of a wide range of crops all over the world (Yildirim et al., 2006). The present numerous mechanisms of NaCl tolerance in halophytes are proposed including ion compartmentalisation, osmolyte production, osmotic adaptation, succulence, selective transport and uptake of ions, enzyme responses, salt excretion and genetic control (Cheesman, 1988). The presence of high concentrations of Nacl and other salts in the soil solution induces a wide range of physiological and biochemical perturbations at the whole plant level (Almansouri et al., 1999**)**. The adverse effects of saline soil include: 1) nutrient constrains caused by reduced uptake of potassium, phosphorous, nitrate and calcium; 2) ion cytotoxicity due to accumulation of sodium, chloride and sulfate; 3) osmotic stress that results from relatively high solute concentration in the soil and 4) oxidative stress caused by the accumulation of reactive oxygen species that damage membrane lipids, proteins and nucleic acids (Katiyar-Agarwal et al., 2005).

Tissue culture techniques have been widely used for the breeding purpose, especially in selection for stress tolerance (Encheva et al., 2004). This technique is a source of genetic variability that gives rise through genetic modifications during the process of *in vitro* culture, a phenomenon called somaclonal variation (Tam and Lang, 2003). Somaclonal variation is manifested as cytological

∗Corresponding author E-mail: *zia136.akram@gmail.com*

abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, and gene activation and silencing (Kaeppler et al., 2000). For example, somaclonal variation which represents new variation commonly occurs in many plant species following regeneration from tissue culture (Kaeppler et al., 2000). Selection can be carried out *in vitro*, by culturing callus pieces, cell suspension, protoplasts, and embryo microspores in the presence of a screening agent (Collin and Dix, 1990). The objective of this study is to examine *in vitro* selection for NaCl tolerance in *Thymus vulgaris*.

Materials and Methods

Seed germination and cultivation of sterile seedlings

In vitro 10-d old seedlings were used as a source of explants. The seeds were surface sterilized with 70% (v/v) ethanol for 90 sec, and then with 1.5% (v/v) sodium hypochlorite (The commercial bleach is mainly hypochlorite) for 20 min. Surface sterilized seeds were rinsed in sterile distilled water three times. The seeds were then air dried in a laminar flow hood and subsequently sown on sterile distilled water solidified by 0.8% (w/v) agar and placed in dark at 28°C for 24 to 48 hours. Germinated seeds were transferred to growth chamber under fluorescent light (40 μM $m^{-2}s^{-1}$) on a 16 hour light per day for another 4 to 8 days and maintained at 25 ± 2°C. After 10 days, hypocotyls and shoot tips having 1 cm length with cotyledonary leaves were placed on the surface of medium in universal vials.

Callus induction and regeneration

Explants of 10 days seedlings were cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and various concentrations of growth regulators including combinations of 2, 4-D, NAA, Kin and BA. The pH of the media was adjusted to 5.7 before solidifying with 0.8% (w/v) agar. The media were contained in universal vials and then were sterilized by autoclaving at 121°C for 15 min with 1.2 kg/cm2 pressure.

In vitro selection for NaCl tolerance

In vitro selection for salt stress tolerance was implemented at the concentrations of 50, 75 and 100 mM NaCl. The callus cultures were allowed to grow in 1:1 ratio NAA: BA (mgl^{-1}) and containing NaCl for 28 days in the culture room at 25±1°C. Fresh and dry weights and the percentage of necrosis of callus were determined to evaluate callus growth. Fresh weight of the calli was recorded after collection from the medium. The calli were dried at 70°C for 48 hours and then the dry weight was recorded.

Rooting and hardening

For root induction, half strength MS medium at half strength, with 0.8% (w/v) sucrose and without growth regulators was used. Root induction was observed within two weeks of culture. Well rooted plantlets were isolated and washed in running tap water for clearing of agar. Then, they were transplanted into hydroponic culture containing sterile MS medium. After one week, plants were transferred to Jiffy peat moss containers for one week more and then potted into a soil-sand-peat moss mixture (3:1:1).

Statistical analysis

All experiments were carried out as a completely randomized design with four replications for callus induction and ten replications for salt experiment. Data were statistically analyzed for significance by analysis of variance with the mean separation by Duncan's multiple range test (DMRT) and means were compared using error rate at the 5% level of significance using SPSS version 14.

Results

Callus induction frequency of hypocotyl and shoot (apical meristem and cotyledonary leaves) explants of *T. vulgaris* in the ratio of 1:0.5 of 2, 4-D:Kin (mgl^{-1}) were 72% and 65%, respectively (figure 1). Callus formation started 18 days after transferring to the medium, but most of calli in this medium were brownish, large and soft. These calli mostly became necrosis after subculturing on the same medium. In 1:1 ratio NAA:BA (mgl^{-1}), callus induction in both explants was started after two weeks. Calli were nodular and cream colored and some of them also green in color and compact in morphology. The callus induction percentages in htpocotyl and shoot explants were 64.3% and 36.6%, respectively (figure 1). Calli grew excellently and became voluminous after subculturing. It was shown that there are differences between the other ratios of growth regulators and explants (figure 1). Hypocotyl explants formed more calli than shoot explants in all the combinations examined with exception of 1:1 ratio of 2, 4-D and Kin.

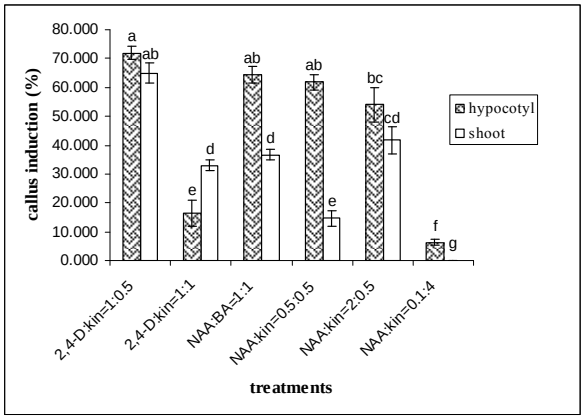

Figure 1. Effects of various combinations of growth regulators on callus formation from different explants.

Fresh weight of calli

The growth and fresh weight of calli induced from hypocotyls decreased with increasing salt concentration in medium (figure 2, 4). The maximum fresh weight with an average of 269 mg was obtained in control (medium without NaCl), following by 50, 75 and 100 mM NaCl with an average weight of 220.4, 173.7 and 145.5 mg, respectively (figure 4). Fresh weight of shoot-derived calli had not any significant difference in the control and 50 and 75 mM NaCl concentrations, but then decreased at 100 mM (figure 3, 4). In shoot-derived calli, the fresh weight was lower than that of hypocotyl-derived calli in control medium, whereas no significant differences were observed between two explants in different concentrations of NaCl (figure 4).

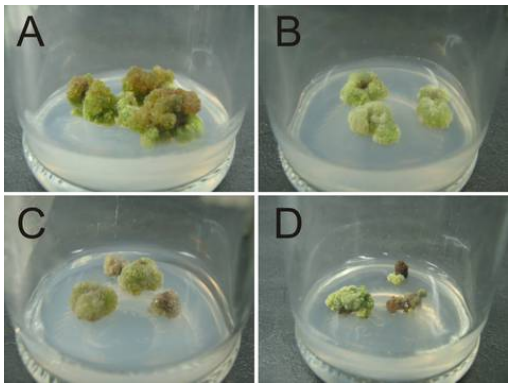

Figure 2. Growth rate of hypocotyl-derived calli in various NaCl concentrations. A, B, C and D show 0, 50, 75 and 100 mM.

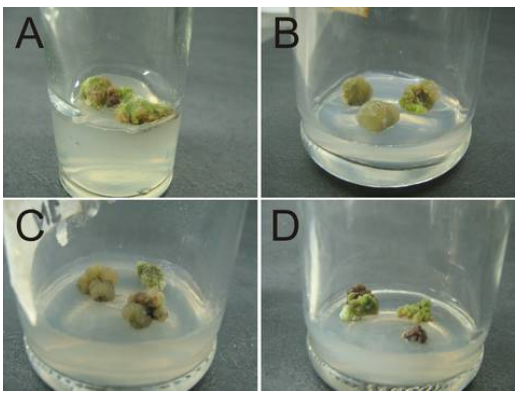

Figure 3. Growth rate of shoot-derived calli in various NaCl concentrations. A, B, C and D show 0, 50, 75 and 100 mM.

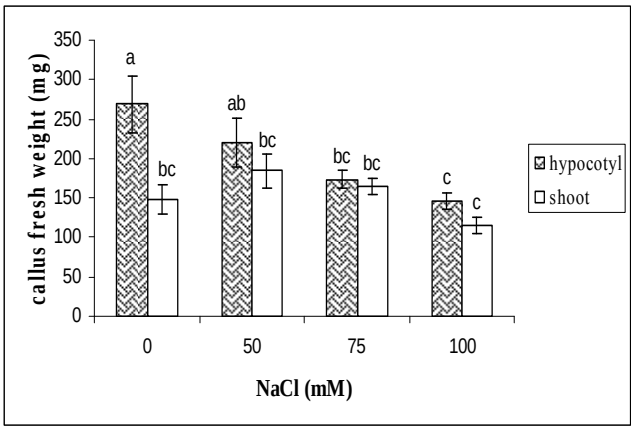

Figure 4. Fresh weight of hypocotyl and shoot-derived calli in various NaCl concentrations.

Dry weight of calli

Salinity affected the dry weight of hypocotyl-derived calli wich was reduced significantly in 50, 75 and 100 mM NaCl concentrations; however, they did not show any significant differences in these concentrations (figure 5). While the dry weight of shoot-derived calli was not significantly affected by the same concentration (figure 5).

Callus necrosis percentage

Two weeks after transferring of the calli to salty medium, they showed signs of necrosis. Its percentage increased significantly by NaCl increasing during growth of calli (figure 6). There were no significant differences between two explants in different NaCl concentrations (figure 6).

Plant regeneration

After transferring the calli to the media, containing various NaCl concentrations, shoot regeneration occurred only on calli derived from shoots at 50 mM NaCl concentration (figure 7A). These regenerated shoots grew thoroughly and produced some branches after transferring to medium containing 0.1:4 ratio of NAA:Kin (mgl^{-1}) (figure 7B). The shoots separated from each other in aseptic conditions and cuttings transferred to vials containing root induction meida. After two weeks, all cuttings were rooted (figure 7C). Finally, *in vitro* plantlets were successfully grown in soil (figure 7D).

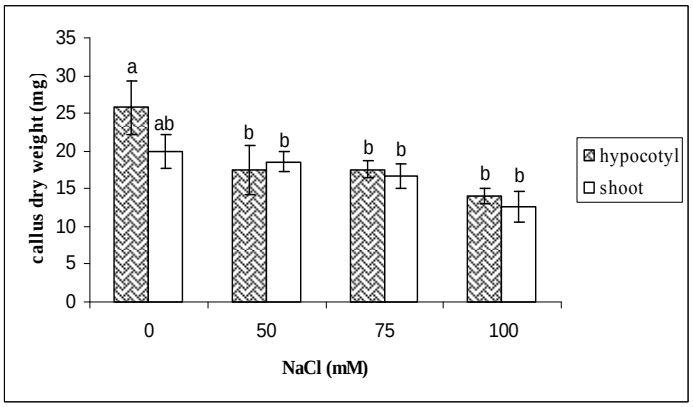

Figure 5. Dry weight of hypocotyl and shoot-derived calli in various NaCl concentrations.

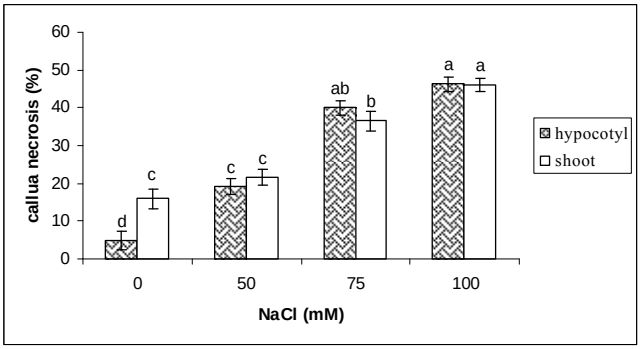

Figure 6. The amount of calli necrosis formed on hypocotyl and shoot explants in various NaCl concentrations.

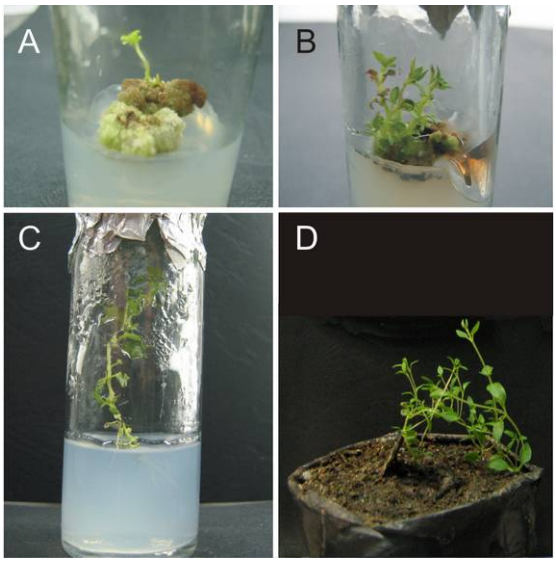

Figure 7. Indirect regeneration of plants in presence of 50 mM NaCl; A) Shoot regeneration from callus B) Propagation of regenerated shoots; C) Rooting of regenerated shoots (cutting); D) Potted plants.

Discussion

A range of plant growth regulators were tested for callus initiation via hypocotyl and shoot segments. The results indicated that all treatments induced callus. However, differences in callus morphology were observed based on plant growth regulators. Calli grown in the media containing 1:0.5 of 2, 4-D:Kin (mgl^{-1}) were fast growing, with brownish and soft, morphology but those grown in 1:1 ratio NAA:BA (mgl^{-1}) were slow growing, with creamy-white and compact. Morphology these findings are in agreement with data by Shirin et al. (2007) who reported similar results about callus morphology in presence of 2, 4-D and NAA for callus induction from internode and leaf explants in potatos. Among all concentrations and combinations, 1:1 ratio NAA:BA (mgl^{-1}) was found to be the most effective hormone concentration for callus induction. Ki-Jong and Bu-Young (2003) showed that in basil, callus was initiated from leaves on an agar-based MS medium supplemented with NAA (mgl^{-1}) and BA (mgl^{-1}). NAA in combination with BA was the best growth regulator for callus induction as reported by several authors (Bicca Dode et al., 2003; Mederos-Molina 2004). The results showed that the explant type has a considerable role for callus induction. Generally, hypocotyls showed a higher potential than shoots. These results correspond to the findings of Zouzou et al. (2008) on cotton who showed that hypocotyl is more callogenic compared to shoot. Explant type and probably its anatomical structure seems to play a significant role in thyme callus initiation. Variation in callus forming ability of different explant types has been reported in many other plants (Ishii et al., 2004; Zouin and El hadrami, 2004). Calligenesis specificity of explant type would be explained by their differential reactivity to media components (Ikram 2005).

The thyme calli showed different response to various concentrations of NaCl so that fresh and dry weights of hypocotyl-derived calli decreased. But fresh weight of shoot-derived calli reduced only in 100 mM NaCl and dry weights of them were not significantly affected. Heckenberger et al.

(1998) pointed out that the relationship between the rate of cell division and the rate of cell elongation may be a direct function of the kinetics of stress development. High salinity causes hyperosmotic stress and ion-disequilibrium that produce secondary effects on growth of calli (Hasegawa et al., 2000; Zhu, 2001). The reduction in callus fresh weight might be a result of reduced water availability in the culture medium due to increased NaCl concentrations. Some researchers found similar response to salt stress on fresh and dry weights (Makhlouf et al., 2002; Abebe et al., 2003). Necrosis percentage increased by salt increment in two explants. Bohnert and Jensen (1996) reported that NaCl decrease cell division and restrict the growth activities. Shoot regeneration occurred just at the concentration of 50 mM NaCl in shoot-derived calli. It could be speculated that the presence of increased concentration of salt inhibits regeneration. Occurrence of regeneration in this treatment may be due to the presence of salt tolerant cells in these calli.

Different studies on genera of Lamiaceae employed various growth regulators for root induction (Dronne et al., 1999; Mederos-Molina, 2004; Misra, 2004; Amutha et al., 2008). It is notable that in this research, in contrast with most studies on this family, the regenerated shoots were rooted on half strength MS medium without growth regulators, with rate of up to 100%.

In conclusion, it is proposed that *Thymus vulgaris* is a sensitive plant to the salinity stress, since the increment of salinity concentration up to 100 mM results in a decrease in the fresh and dry weights and an increase in necrosis of the calli. While in halophyte plants, 150 mM level of the salt seems to be the optimum concentration (Gorham, 1996).

Acknowledgment

Authors gratefully acknowledge the Research Center of Agriculture and Natural Resources, Mashhad, Iran and the Council of Graguate Study of the University of Kerman for their collaboration and support.

References

1- Abebe T., Guenzi A. C., Martin B., Cushman J. C. (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiology 13: 1748-55.

2- Almansouri M., Kinet J. M. and Lutts S. (1999) Compared effects of sudden and progressive impositions of salt stress in three durum wheat (*Triticum durum* Desf.) cultivars. Journal of Plant Physiology 154: 743-752.

3- Amutha R., Jawahar M. and Ravi Paul S. (2008) Plant regeneration and *in vitro* flowering from shoot tip of *Basilicum polystachyon* (L.) Moench -An important medicinal plant. Journal of Agricultural Technology 4: 117-123.

4- Bicca Dode L., Bobrowski V. L., Bolacel Braga E. J., Kömmling Seixas F. and Wulff Schuch M. (2003) *In vitro* propagation of *Ocimum basilicum* L. (Lamiaceae). Biological Sciences 25: 435-437.

5- Bohnert H. J. and Jensen R. G. (1996) Metabolic engineering for increased salt tolerance the next step. Australian Journal of Plant Physiology 59: 661-667.

6- Cheeseman J. M. (1988) Mechanisms of salinity tolerance in plants. Plant Physiology 87: 547-550.

7- Collin H. A., Dix P. J. (1990) Cultures systems and selection procedures. Plant Cell Line Selection, procedure and application, Germany 3-18 pp.

8- Dronne S., Jullien F., Caissard J. C. and Faure O. (1999) A simple and efficient method for *in vitro* shoot regeneration from leaves of lavandin. Plant Cell Reports 18: 429-433.

9- Encheva J., Tsvetkova F. and Ivanov P. (2004) Heritable tissue culture induced genetic variation in sunflower (*Helianthus annuus* L.) as a tool for crop improvement. Helia 27: 163-172.

10- Gorham J. (1996) Mechanisms of salt tolerance in halophytes. In: Halophytes Ecologic Agriculture. (eds: R. C. Allah, C. V. Nalcolm and A. Aamdy). Marcel Dekker. Inc. 30-53.

11- Hasegawa P. M., Bressan R. A., Zhu J. and Bohnert H. J. (2000) Plant cellular and molecular responses to high salinity. Annual Review of Plant Physiology and Plant Molecular Biology 51: 463-499.

12- Heckenberger U., Roggatz U. and Schurr U. (1998) Effect of drought stress on the cytological status in *Ricinus communis*. Journal of Experimental Botany 49: 181-189.

13- Ikram U. H. (2005) Callus proliferation and somatic embryogenesis in cotton (*Gossypium hirsutum* L.). African Journal of Biotechnology 4: 206-209.

14- Ishii Y., Takamura T., Goi M. and Tanaka M. (2004) Callus induction and somatic embryogenesis of phalaenopsis. Plant Cell Reports 17: 446-457.

15- Kaeppler S. M., Kaeppler H. F. and Rhee Y. (2000) Epigenetic aspects of somaclonal variation in plants. Plants Molecular Biology 43:179-188.

16- Katiyar-Agarwal S., Verslues P. and Zhu J. (2005) Mechanisms of salt tolerance in plants. Plant nutrition for food security, human health and environmental protection. Genome Biology 1: 2-3.

17- Ki-Jong L. and Bu-young Y. (2003) Rapid multiplication of basil (*Ocimum basilium*); factors affecting callus formation and plant regeneration. Acta Horticulture 625: 265-269.

18- Makhlouf A., Mabrouk Y., El-Saied M. and Mahdy M. (2002) *In vitro* selection for drought tolerance in Sorghum (*Sorghum bicolor* L.) and regeneration evaluation of selected genotypes. Alexandra Journal of Agricultural Research 47: 77-88.

19- Mederos-Molina S. (2004) *In vitro* callus induction and plants from stem and petiole explants of *Salvia*

canariensis L. Plant Tissue Culture 14: 167-172.
20- Misra M. (2004) Regeneration of patchouli (*Pogostemon cablin* Benth.) plants from leaf and node calli and evaluation after growth in the field. Plant Cell Reports 15:991-994.
21- Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Plant Physiology 15: 473-497.
22- Shirin F., Hossain M., Kabir M. F., Roy M. and Sarker S. R. (2007) Callus induction and plant regeneration from internodal and leaf explants of four potato (*Solanum tuberosum* L.) cultivars. World Journal of Agricultural Sciences 3: 1-6.
23- Soto-Mendivil E. A., Moreno-Rodriguez J. F., Estarron-Espinosa M., Garcia-Fajardo J. A. and Obledo-Vazquez E. N. (2006) Chemical composition and fungicidal activity of the essential oil of *Thymus vulgaris* against *Alternaria citri*. e-Gnosis [online] 4:16.
24- Tam D. M. and Lang N. T. (2003) *In vitro* selection for salt tolerance in rice. Omonrice 11: 68-73.
25- Yildirim E., Taylor A. G. and Spittler T. D. (2006) Ameliorative effects of biological treatments on growth of squash plants under salt stress. Scientia Horticulture 111: 1-6.
26- Zhu J. K. (2001) Plant salt tolerance. Trends in Plant Science 6: 66-71.
27- Zouine J. and El hadrami I. (2004) Somatic embryogenesis in *Phoenix dactylifera* L.: Effect of exogenous supply of sucrose on proteins, sugar, phenolics and peroxidases activities during the embryogenic cell suspension culture. Biotechnology 3: 114-118.
28- Zouzou M., Kouakou T., Kone M., Amani N. G. and Kouadio Y. J. (2008) Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.). Australian Journal of Crop Science 2: 1-9.