BULG. J. PLANT PHYSIOL., 1996, 22(3–4), 30–39

IN VITRO SELECTION FOR OSMOTIC TOLERANCE IN ALFALFA (*MEDICAGO SATIVA* L.)

***Rumiana Dragiiska*[†1], *Dimitar Djilianov*[1]*, *Plamen Denchev*[1], *Atanas Atanassov*[2]**

[1] *Institute of Genetic Engineering; 2232 Kostinbrod-2, Bulgaria*
[2] *The DeMontford University, Norman Borlaug Centre for Plant Sciences, Institute of Genetic Engineering, 2232 Kostinbrod-2, Bulgaria*

Received November 19, 1996

Summary. A system for *in vitro* selection during somatic embryogenesis in alfalfa was developed. PEG was used as a selective agent for osmotolerance. The procedure involved screening of seeds and seedlings till cotyledonary stage on 10% PEG with further growth of the plants on PEG-free medium. The initiation of somatic embryogenesis proceeded till the end of globular stage on PEG. Further development and growth was possible only if the osmoticum was omitted. Four regenerants were obtained which were cloned and potted. The preliminary studies revealed their high osmotolerance compared with the explant-source genotype. Additional studies are in progress on the breeding behaviour at controlled and field conditions.

Key words: alfalfa, *in vitro* selection, PEG-tolerence

Abbreviations: PEG – polyethylene glycol 6000; ABA – abscisic acid; 2,4-D – 2,4-dichlorophenoxyacetic acid; 2,4,5-T – trichlorophenoxyacetic acid; Dicamba – 3,6-dichloro-*o*-anisic acid

Introduction

The most prevalent environmental stresses have in common the reduction of water availability for the plants, thus impairing its numerous biological roles (Bonnert et al., 1995). The classical breeding for abiotic stress tolerance is quite difficult, time-, space- and labour-consumming because of the reduced genetic background, the in-

[†] Deceased 19 May 1996; Dedicated to her memory.

* Corresponding author: *E-mail*: atanas@icgeb.trieste.it or dmuije@bgcict.acad.bg

compatibility between the wild resistant species with the crop varieties etc. The gene transfer approach gaining recently considerable results (Tarczynski et al., 1993; Pilon-Smits et al., 1995; Ishitani et al., 1995; Yoshiba et al., 1995; Kavi Kishor et al., 1995) is still leaving the main question unanswered – how introduction of stress tolerance genes will affect crop productivity.

It appears that *in vitro* selection for stress tolerance will continue to have its significant place in the strategy of establishing plant systems with optimal stress reaction and productivity. Polyethylene-glycols (PEG) of high molecular weights have been long used to simulate drought stress in plants as non-penetrating osmotic agent lowering the water potential in a way similar to soil drying (Bressan et al., 1981; Larher et al., 1993). After the first selection of osmotic tolerant tobacco cell lines (Heyser and Nabors, 1979) there were several reports on habituated but not genetically modified suspension cultures (Bressan et al., 1981; 1982; Handa et al., 1982; 1983). On the other hand, promising somaclonal variants were obtained after *in vitro* selection for PEG tolerance in *Sorghum* (Smith et al., 1985) and rice (Kavi Kishor and Reddy, 1985; Adkins et al., 1995).

Alfalfa is the world's main forage legume and, at the same time, it is a model culture for numerous genetic engineering studies. While the *in vitro* selection for salt tolerance achieved considerable results (Smith and McComb, 1983; Stavarek and Rains, 1984; Winicov, 1991), studies at a whole plant level revealed that the nature of correlation between high productivity and salt tolerance is currently not well established and understood (Winicov, 1994). No strictly confirmed reports on PEG-tolerant alfalfa cell lines and regenerants are available.

The aim of the present study was to obtain tolerant to PEG alfalfa genotypes applying *in vitro* selection along regeneration procedure.

Materials and Methods

Plant material

Several highly productive and relatively drought tolerant genotypes (*Medicago sativa* L.) resulting from an extensive breeding programme at the Institute of Forage Crops, Pleven, Bulgaria were used (Radeva et al., 1987).

Regeneration and selection procedure

The genotypes were tested simultaneously for osmotic tolerance and regeneration ability. In preliminary experiments (data not presented) 10% of PEG 6000 was chosen as a selective concentration for further studies. The osmoticum was added to the media before autoclaving. The selection pressure was tested at all stages of culture (seeds, seedlings, explants, calli, somatic embryos, regenerants). The culture took place on filter bridges in Petri dishes or tubes where necessary.

The procedures for somatic embryogenesis, developed in our Institute (Atanassov and Vlahova, 1985; Denchev et al., 1990; Denchev et al., 1991) were applied with some modifications. System of media, based on B5 (Gambourg et al., 1968) and MS (Murashige and Skoog, 1962) was used. Particular attention was paid to the induction stage where several auxins and concentrations were tested. Five replications with 30 seeds per genotype were used and compared to plants grown on osmoticum-free B5 medium. All plant materials were cultured in growth chambers at 26 °C, 70% humidity, a 16/8 h light/dark photoperiod and 2500–3000 lx light intensity. After developing a good root system the *in vitro* cloned regenerants were planted in autoclaved soil. Adaptation took place in a greenhouse.

Preliminary tests to confirm the osmotolerance of the regenerants

After cloning, the obtained regenerants were used as explant sources in a recurrent selection scheme with PEG 6000 (10%). Cuttings from the potted under greenhouse conditions plants (40 in 4 randomized replications) of the regenerants and the explant-source genotype were rooted under non-sterile conditions. Later, their roots were placed in special vessels filled with buffer solution supplemented by 15% PEG 6000 for 2 weeks (McCoy, 1987). Control cuttings were placed in vessels with PEG-free solution. The following parameters were measured: fresh weight, stem length, number and length of internodes per cutting.

The trials were repeated three times.

Results

Screening of the donor materials for PEG tolerance

The tested genotypes varied in their reaction to PEG at germination but the differences decreased with the further plantlet development (Fig.1). The growth was almost fully blocked at cotyledonary stage. Thus, a procedure of culture establishment was accepted where germination and cotyledon development occurred under osmotic pressure, but further seedlings growth proceeded on basal medium.

About 20 seedlings per genotype survived after the PEG screening. When regeneration procedure was applied, only those of the Breeding line 2 (T-I) responded embryogenically (data not presented). Thus, all further experiments were performed with the seedlings of this line.

Protocol for regeneration and *in vitro* selection for PEG tolerance

Cut leaves and petioles detached from 30-day-old plantlets were used as explants. Callus formation and embryo induction occured simultaneously at the end of the first

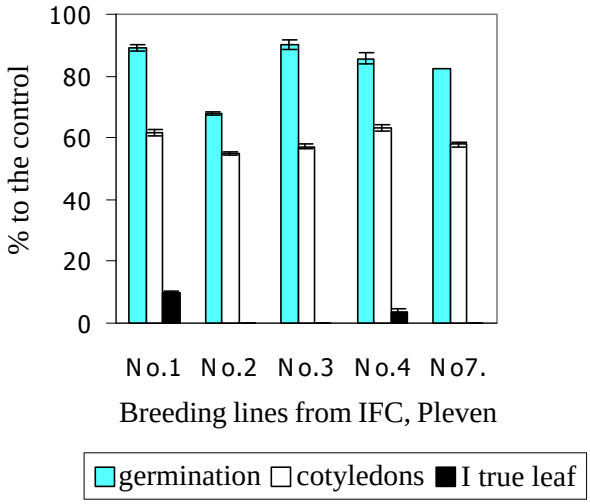

Fig. 1. Inhibition of germination and growth under 10% PEG stress in various breeding lines of alfalfa

week of culture. Significant differences were found between culture growth on various auxin types and doses (Fig. 2). Most abundant calli with simultaneous embryo proliferation was achieved on 10 µM 2,4-D. No differentiation was observed on 2,4,5-T and Dicamba containing media. With the increase of the auxin concentrations the cultures' fresh weight decreased (Fig. 2).

The growth of the cultures was significantly inhibited by the continuous presence of PEG. When the torpedo-shaped embryos were transferred to maturation

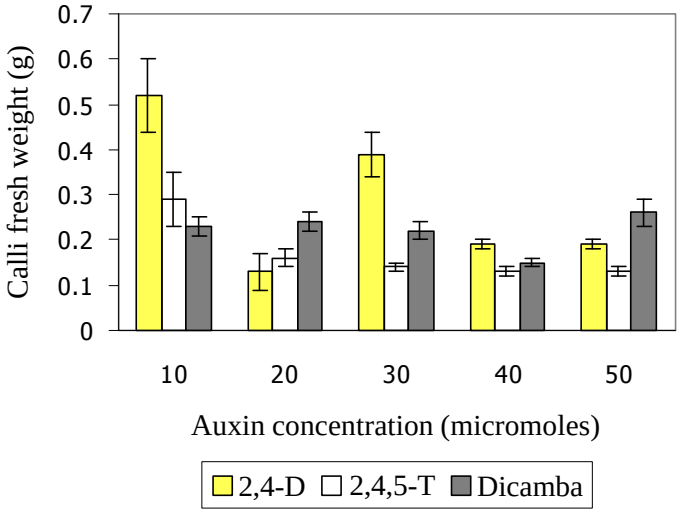

Fig. 2. Effect of various auxins and their concentration on culture fresh weight

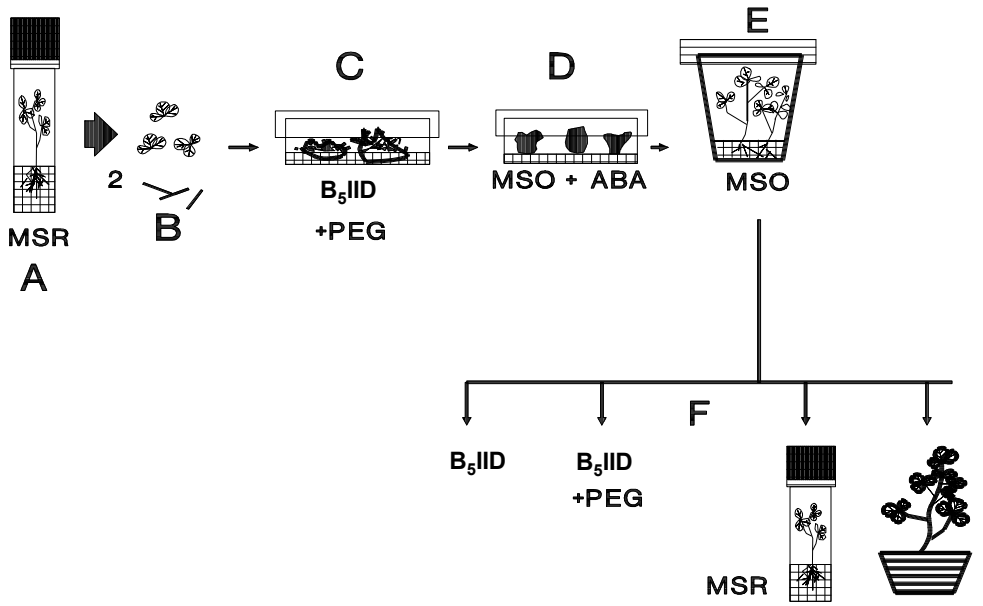

Fig. 3. Protocol for *in vitro* selection for PEG tolerance during somatic embryogenesis: A – seedlings after screening at germination; B – initial explants; C – simultaneous induction of callus and globular embryoids (4–5 weeks); D – maturation (4 weeks); E – conversion (4 weeks); F – recurrent selection and clonal propagation (4–5 weeks)

medium their further development was fully blocked. Thus, the selection pressure was omitted this step further and a protocol for selection along indirect somatic embryogenesis was established (Fig. 3). About 20 regenerants were obtained which developed quite slowly. Most of them were unable to root. Finally 4 regenerants were successfully cloned and potted.

In vitro and *in vivo* preliminary tests of the osmotolerance

Recurrent selection. Since the selective agent was omitted at the late stages of regeneration, a recurrent selection was applied to confirm the putative osmotolerance of the regenerants obtained. The results obtained with cultures initiated from the regenerants showed their advantage to the cultures where T-I seedlings were used as donors of explants (Fig. 4). More than 3 times higher fresh weight of calli and up to 6 times greater number of embryos and plantlets on PEG were observed. This is a clear evidence that if the regenerants were used as explant sources they exceed the initial form in osmotic tolerance.

Cuttings. A strong inhibition of growth occurred in the PEG-treated cuttings of all genotypes. This was particularly true for the fresh weight and stem length where

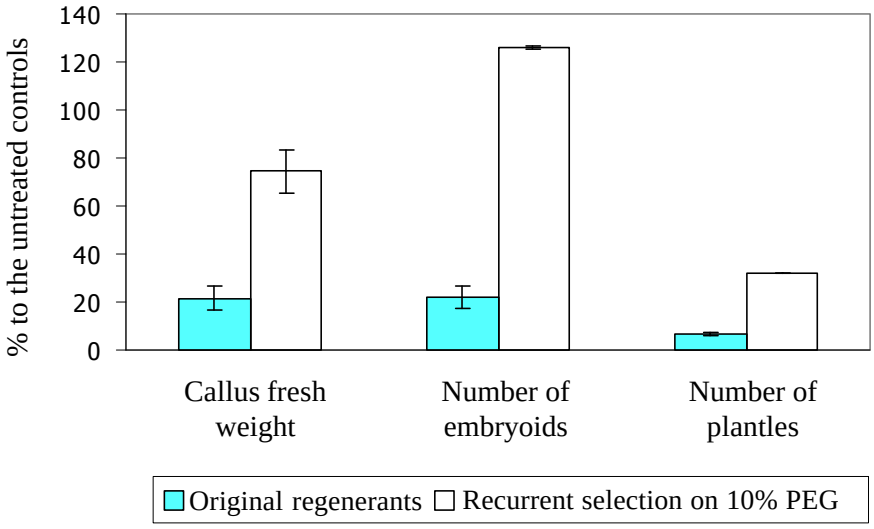

Fig. 4. Growth of calli from the selected regenerants compared with cultures initiated from the explant-source genotype

the osmotic reduced the cuttings' performance 3–10 times compared to the untreated controls (Fig.5). Decrease was also observed for number and length of internodes

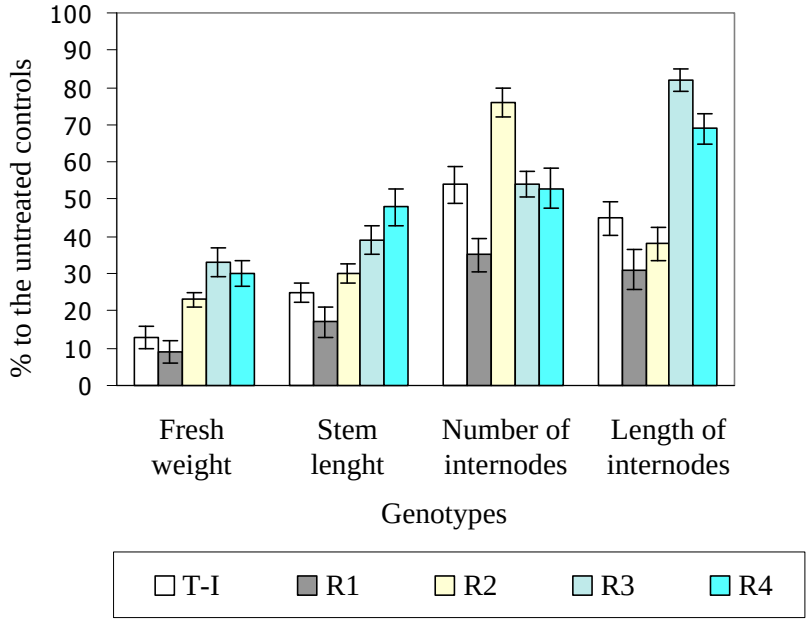

Fig. 5. Growth of cuttings from the explant-source line and the regenerants when their roots are placed in 15% PEG-supplemented nutrient media for 2 weeks

(1.5–2 times). R3 and R4 cuttings exceeded significantly T-I in fresh weight, stem and internode length while the number of their internodes was similar. The cuttings of R1 and R2 also performed similar or better than T-I (Fig. 5).

Discussion

Germination and seedling development under laboratory conditions have been accepted as suitable growth stages for testing the salt tolerance in alfalfa (Smith and McComb, 1983; Carlson et al., 1983). PEG-induced drought was reported as unreliable marker for the reaction at whole plant level in wheat (Laszlo, 1990). On the other hand, good possibilities to use such preliminary tests for successful further breeding have been pointed out for other crops (Carlson et al., 1983; Bouslana and Scapaugh, 1984; Zahid and Hughes, 1995). Our recent studies with alfalfa genotypes, different from the tested here (Petkova et al., 1995) revealed a positive correlation between germination on PEG-supplemented media and the whole plant behaviour under drought stress in the field.

As a rule, exogenous auxins are used to trigger somatic embryogenesis. Similar to other systems (Amirato, 1983; Merkle et al., 1990; Denchev et al., 1991; Komamine et al., 1992) in our case 2,4-D appeared to be most suitable to induce globulae formation. Transfer of developed embryoids to a cascade of media containing PEG and ABA is a widely accepted procedure in alfalfa somatic embryogenesis (Denchev et al., 1991). Thus, their development is arrested at the end of torpedo stage, preventing the precocious germination (Kiyosue et al., 1993), which occurs probably due to the relatively low endogenous ABA at that developmental stage (Ivanova et al., 1994).

It is widely accepted (Hsiao, 1973) that under decreased water potential the plant cell turgor and growth decline. The data for long-term habituated suspensions on PEG which loose their regeneration competence (Bressan et al., 1981; 1982; Handa et al., 1982; 1983) support this statement. It could be speculated that the presence of increased concentrations of PEG during the growth of the initial material and somatic embryo initiation inhibits further development. Inhibition of regenerants growth compared to the initial lines was reported with *in vitro* selection for salt tolerance in alfalfa (McCoy, 1987). In our case, with the exception of R1, the regenerants performed similar or better than the initial line T-I.

Our results confirmed the possibility for successful *in vitro* selection for osmotic tolerance. PEG inhibited in greater extend the differentiation but there are species-specific variations in the regeneration response (Smith et al., 1985; Kavi Kishor and Reddy, 1985; Adkins et al., 1995).

The results obtained so far showed that the osmotic tolerance of our regenerants was higher than that of the explant-source line T-I. In progress are the studies on the breeding behaviour of our selected regenerants and their drought response at controlled and field conditions.

References

Adkins, S. A., R. Kunanuvatchaidach, I. D. Godwin, 1995. Somaclonal variation in rice-drought tolerance and other agronomic characters. Austral. J. Bot., 43, 201–209.

Amirato, P. V., 1983. The regulation of somatic embryo development in plant cell cultures: suspension cultures and hormone requirenments. Bio/Technology, 1, 68–74.

Atanassov, A., M. Vlahova, 1985. Somatic embryogenesis in callus and cell suspension cultures of three species of *Medicago*. In: Tissue Culture in Forestry and Agriculture, Eds: R. Henke, K. W. Hughes, M. S. Constantin and A. Hollaender, Plenum Press, New York, 18–19.

Bonnert, H. J., D. E.Nelson, R. G. Jensen, 1995. Adaptations to environmental stresses. Plant Cell, 7, 1099–1111.

Bouslana, M., W. T. Scapaug, 1984. Stress tolerance in soybeans: I. Evaluation of three screening techniques for heat and drought tolerance. Crop Sci., 24, 933–937.

Bressan, R. A., A. K. Handa, S. Handa, P. M. Hasegawa, 1982. Growth and water relations parameters of cultured tomato cells after adjustment to low external water potentials. Plant Physiol., 70, 1303–1309.

Bressan, R. A., P. M. Hasegawa, A. K. Handa, 1981. Resistance of cultured higher plant cells to polyethylene glycol-induced water stress. Plant Sci. Lett., 21, 23–30.

Carlson, J. R. Jr., R. L. Ditterline, J. M. Martin, D. C. Sands, R. E. Lund, 1983. Alfalfa seed germination in antibiotic agar containing NaCl. Crop Sci.,23, 882–885.

Denchev, P., M. Velcheva, A. Atanassov, 1991. A new approach to direct somatic embryogenesis in *Medicago*. Plant Cell Rep.,10, 338–341.

Denchev, P., M. Velcheva, R. Dragiiska, A. Kuklin, A. Atanassov, 1990. Somatic embryogenesis in *Medicago*. Biotechn. & Biotechn. Eq., 4, 66–70.

Gambourg, O., L. Miller, K. Ojima, 1968. Nutrient requirements of suspension culture of soybean root callus. Exp. Cell. Res., 50, 151–158.

Handa, A. K., R. A. Bressan, S. Handa, P. M. Hasegawa, 1982. Characteristics of cultured tomato cells after prolonged exposure to medium containing polyethylene glycol. Plant Physiol., 69, 514–521.

Handa, A. K., R. A. Bressan, S. Handa, P. M. Hasegawa, 1983. Clonal variation for tolerance to polyethylene glycol-induced water stress in cultured tomato cells. Plant Physiol., 72, 645–653.

Heyser, J. W., M. W. Nabors, 1979. Growth, water content and solute accumulation of two tobacco cell lines cultured on sodium chloride, dextran and polyethylene glycol. Plant Physiol., 68, 1454–1459.

Hsiao, T., 1973. Plant responses to water stress. Ann. Rev. Plant Physiol., 24, 519–570.

Ishitani, M., T. Nakamura, S. Y. Han, T. Takabe, 1995. Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. Plant Mol. Biol., 27, 307–315.

Ivanova, A., M. Velcheva, P. Denchev, H. Van Onckelen, A. Atanassov, 1994. Endogenous hormone levels during direct somatic embryogenesis in *Medicago falkata*. Physiol. Plant., 92, 85–89.

Kavi Kishor, P. B. K., G. M. Reddy, 1985. Resistance of rice callus tissues to sodium chloride and polyethylene glycol. Curr. Sci., 54, 1129–1131.

Kavi Kishor, P. B., Z. Hong, C.-H. Miao, C.-A. A. Hu, D. P. S. Verma, 1995. Overexpression of Δ^1-pyroline-5-carboxylate synthetase increase proline production and confers osmotolerance in transgenic plants. Plant Physiol., 108, 1387–1394.

Kiyosue, T., S. Satoh, H. Kamada, H. Harada, 1993. Somatic embryogenesis in higher plants. J. Plant Res. (Spec. Iss.), 3, 75–82.

Komamine, A., R. Kawahar, M. Matsumoto, S. Sunabori, T. Toja, A. Fujiwara, M. Tsukahara, J. Smith, M. Ito, H. Fukuda, K. Nomura, K. Fujimura, 1992. Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry and molecular biology. In Vitro Cell. Dev. Biol., 28, 11–14.

Larher, F., L. Leport, M. Petrivalsky, M. Chappart, 1993. Effectors for the osmoinduced proline response in higher plants. Plant Physiol. Biochem., 31(6), 911–922.

Laszlo, C., 1990. Testing of winter wheat (*Triticum aestivum* L.) varieties for drought resistance by simple laboratory methods. Novenytermeles, 39(30), 227–233.

McCoy, T. J., 1987. Characterisation of alfalfa (*Medicago sativa* L.) plants, regenerated from selected NaCl-tolerant cell lines. Plant Cell Rep., 6, 417–422.

Merkle, S., W. Parrott, E. Williams, 1990. Applications of somatic embryogenesis and embryo cloning. In: Plant Cell Cultures – Applications and Limitations. Ed. S. S. Bhojwani, Elsevier, Amsterdam, pp. 67–101.

Petkova, D., D. Nedjialkov, D. Djilianov, 1995. Early screening for drought tolerance in cultivated alfalfa. Bulg. J. Agric. Sci., 1(4), 429–432.

Pilon-Smits, E. A. H., M. J. M. Ebscamp, M. J. Paul, M. J. W. Jeuken, P. J. Weisbeek, S. C. M. Smeekens, 1995. Improved performance of transgenic fructan-accumulating tobacco under drought stress. Plant Physiol., 107, 125–130.

Radeva, V., Z. Georgiev, A. Topchieva, 1987. Studies on the reaction of some promising alfalfa cultivars and breeding lines to drought. Bulg. Plant Sci., 6, 28–31.

Smith, R. H., S. Bhaskaran, F. R. Miller, 1985. Screening for drought tolerance in Sorghum using cell culture. In Vitro Cell. Dev. Biol., 21, 541–545.

Smith, M. K., J. A. McComb, 1983. Selection for NaCl tolerance in cell cultures of *Medicago sativa* and recovery of plants from NaCl-tolerant cell lines. Plant Cell Rep., 2, 126–128.

Stavarek, S. J., D. Rain, 1984. The development of tolerance to mineral stress. HortSci., 19(3), 377–382.

Tarczynski, M. C., R. G.Jensen, H. J. Bonnert, 1993. Stress protection in transgenic tobacco producing a putative osmoprotectant, manitol. Science, 259, 508–510.

Winicov, I., 1991. Characterization of salt tolerant alfalfa (*Medicago sativa* L.) plants regenerated from salt tolerant cell lines. Plant Cell Rep., 10, 561–564.

Winicov, I., 1994. Gene expression in relation to salt tolerance. In: Stress-induced Gene Expression in Plants, Ed. A. S. Basra, Harwood Academic Publ., pp. 61–86.

Yoshiba, Y., T. Kiyosue, T. Katagiri, H. Ueda, T. Mizoguchi, K. Yamaguchi-Shinosaki, K. Wada, Y. Harada, K. Shinozaki, 1995. Correlation between the induction of a gene for Δ^1-pyroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant J., 7(5), 751–760.

Zahid, A., H. Hughes, 1995. Water loss and polyethyleneglycol-mediated acclimatisation of in vitro-grown seedlings of 5 cultivars of date palm plantlets. Plant Cell Rep., 14, 385–390.