

Characterization of Somaclones of *Medicago sativa* L. for Drought Tolerance

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ABSTRACT

Water stress is a serious environmental problem throughout the world. It is a consequence of both drought and salinity which may be relieved by breeding cultivars that can tolerate low soil water potentials. Alfalfa (*Medicago sativa* L.) is normally grown in arid and semi-arid zones. One approach to improving the ability of the crop tolerate drought is to make use of the variation provided by tissue culture derived from somaclonal variation. Seedlings of a moderately salt tolerant line CUF 101-1S derived from the commercial variety CUF 101 were used as a source of explants to initiate and regenerate tissue cultures. Regenerant plantlets were allowed to grow to maturity, selfed, and set seed. These seeds (R1) were germinated and grown in a nutrient medium containing 0, 200 and 250 g l⁻¹ polyethylene glycol (PEG) 6000 and additional calcium as CaCl₂ (4.0 mol m⁻³) for 14 days to assess their ability to withstand stress in the progeny generation. Tolerance to PEG was assessed using measurements of root and shoot lengths. Proline levels and activity of the antioxidant enzymes, catalase (EC 1.11.1.6) and glutathione reductase (GR) (EC 1.6.4.2) were also determined. Selected somaclones were more tolerant than the parent. A large increase in the level of proline was observed in the somaclones compared with the parent variety in response to PEG stress. The activity of catalase and glutathione reductase increased in the tolerant genotypes but remained unchanged in the parent when they were subjected to PEG stress.

Keywords: Antioxidant, Drought, *Medicago sativa*, Proline, Tissue culture.

INTRODUCTION

Drought is the most important environmental factor that causes osmotic stress and reduces plant growth and crop productivity. Alfalfa (*Medicago sativa* L.) is grown in arid and semi-arid zones and is subjected to water shortages. One approach to improving the ability of the crop to tolerate drought is to make use of the variation provided by somaclonal variation. The responses of plants to water stress induce physiological adaptive processes such as the biosynthesis of intracellular compatible solutes (Delauney & Verma, 1993; Smith *et al.*, 2002), changes in ion transport and metabolic functions (Niu *et al.*, 1993; Rotein *et al.*, 2002) and activation of antioxidant enzymes to

reduce oxidative stress (Foyer *et al.*, 1994). The present study was undertaken to screen for high osmotic tolerance of alfalfa regenerant derived from tissue cultures and to evaluate the regenerants progeny for drought tolerance using morphological and biochemical analysis.

MATERIALS AND METHODS

Plant Material

Seeds of the non-salinity tolerant cultivar CUF 101 were supplied by Cleanseed Pty Ltd. (POBox 31, Bongendore NSW, Australia 2621). A moderately salt tolerant line CUF 101-1S produced at the University of

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Liverpool (Al-Khatib *et al.*, 1993) was used as the source of salinity tolerant seed for tissue culture.

Initiation and Regeneration of Tissue Culture

Seeds were surface sterilized using 0.1% w/v mercuric chloride and 0.1% w/v sodium dodecyl sulfate for 10 minutes, followed by 15% v/v commercial bleach for 20 minutes, then washed in five changes of sterile distilled water. A single seed was placed on the surface of 10 ml aliquots of a germination medium containing either Murashige and Skoog (1962) (MS), or half strength Schenk and Hildebrandt (1972) (SH) media, with 1% w/v sucrose, 0.8% Difco Bacto Agar, but without growth regulators contained in Universal vials. The vials were maintained at 25 °C under fluorescent light with a 12 hour photoperiod. After eight days when seeds had germinated, 1 cm long hypocotyls were placed on the surface of 10 ml BII medium (Sunders and Bingham, 1975) in Universal vials, each hypocotyl in a separate vial. After two weeks, each complete explant with its attached callus was transferred to a regeneration medium, Boi2y (Saunders & Bingham, 1975), and incubated as before. After two to five weeks, buds were visible on the callus. When larger individual shoots were transferred to a modified Schenk and Hildebrandt medium (1972) (GS) containing 1% w/v sucrose, 0.8 % Difco Bacto Agar and without growth regulators. Well rooted plantlets were transferred to Jiffy-7 peat moss containers in a glasshouse mist bed for 10 days, and subsequently transferred into pot containing a soil-sand-peat moss mixture (3:1:1) and grown to maturity. Inflorescences were self pollinated by hand then covered immediately with non moisture-proof glassine bags for 10 days (Sayers & Murphy, 1966) and the bags were then removed to allow seed pods and seed to mature.

Plant Growth under Osmotic Stress

Seeds of uniform size were surface sterilised in 2% v/v sodium hypochlorite for two minutes, rinsed in sterile water and dried at room temperature. For each replicate, 20 seeds were evenly spaced on a raft of alkathene beads in pots containing 300 ml of modified half strength nutrient solution (Hevitt, 1966) plus 4 mol m⁻³ CaCl₂ with PEG treatments. The pots were arranged randomly in a tray that was filled with water and placed in a clear plastic chamber in a growth room at 25-27 °C under fluorescent light with a 12 hour photoperiod. After two weeks, shoot length and root length were measured and leaves collected for estimates of proline and catalase and glutathione reductase enzymes.

Preparation of Extracts for Proline Estimation

Seedling tissue was frozen in liquid nitrogen and stored until required, during which time it was homogenized in a methanol:chloroform: water (MCW 12:5:1 /V) solution using 0.2 gram tissue per 2 cm³ of MCW at room temperature. Proline concentrations were estimated using a modification of the method described by Singh *et al.* (1973), which involved heating the tubes in a boiling water bath for 65 minutes.

Preparation of Extracts for Enzyme Assays

Fresh seedlings (400 mg) were homogenized in 400 µl 0.1 M K₂HPO₄ (pH 7) in braying mortars. The homogenized samples were washed with 2 × 200 µl 0.1 M K₂HPO₄ into Eppendorf tubes and centrifuged for 10 minutes at 10000 rpm in an Eppendorf microfuge. The resulting pellet was resuspended in 800 µl 0.1 M K₂HPO₄ and centrifuged. The combined supernatant was used for the determination of activity of glutathione reductase following Halliwell and

Table 1. Analysis of variance (ANOVA) of root and shoot lengths of *M. sativa* cv. CUF 101 and somaclones (7R1, 3R1, 4R1, 6R21V) grown for 2 weeks in 0 and 200 gr⁻¹ PEG, in half strength Rorison solouction culture plus 4 mol m⁻³ CaCl₂.

Root Length					
SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	138.769	69.385	5.66	0.0037
Genotype (GE)	4	516.695	129.174	10.53	0.0001
PEG	1	6288.942	6288.942	512.84	0.0001
GE*PEG	4	548.0825	137.020	11.17	0.0001
GE*REP	8	840.094	105.012	8.56	0.0001
GE*PEG*REP	7	211.996	30.285	2.47	0.0169
PEG*REP	2	101.82	50.91	4.15	0.0163
Error	505	6192.744	12.263		
Total	533	1439.141			

Shoot Length					
SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	13.97	6.986	3.60	0.0281
Genotype (GE)	4	104.739	26.185	13.48	0.0001
PEG	1	2410.041	2410.041	1240.83	0.0001
GE*PEG	4	190.77	47.693	24.56	0.0001
GE*REP	8	63.42	7.928	4.08	0.0001
GE*PEG*REP	7	26.92	3.846	1.98	0.0560
PEG*REP	2	1.374	0.687	0.35	0.7023
Error	505	980.849	1.9423		
Total	533	3792.091			

Foyer (1978) and catalase following Beers and Sizer (1952).

RESULTS

Growth Measurement

Shoot and root lengths of 14-day-old seedlings were reduced with an increase in PEG concentration (Table 1). Shoots showed a nearly linear decrease in length in the presence of PEG. Root length was reduced in all genotypes in the presence of osmotic stress (Figure 1a) induced by PEG. The reduction was 63.5% of the control in zero PEG for 7R1, 42% of the control for 4R1 and 50% of the control in CUF101 (Figure 1b). Root/Shoot ratios increased with increasing PEG concentrations. In 250 g l⁻¹ PEG, the ratio was 4 compared with 2.1 at zero PEG.

Proline Estimation

There was no significant difference in proline accumulation in the three genotypes in the absence of PEG. At 200g l⁻¹ PEG, proline accumulation increased in all genotypes, but there was a significantly greater increase in 4R1, 7R1 and CUF101 respectively. Proline accumulation in 4R1 and 7R1 at 200 g l⁻¹ PEG increased 47.3 and 22.75 fold compared with 0 g l⁻¹ PEG levels and 2.37 fold in 4R1 and 1.52 fold in 7R1 higher than measured in CUF101.

Enzyme Assay

There was no significant difference in glutathione reductase activity among genotypes in the control and in the 200g l⁻¹ PEG treatment (Figure 3). There was significant difference in catalase activity between the control and those treatments containing PEG (Figure 3b). However, catalase activity in

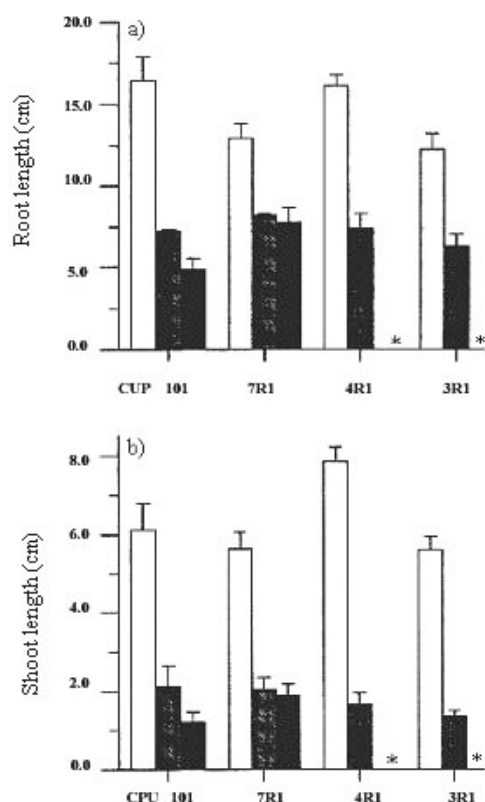


Figure 1. Growth of 14-day-old seedling of *M. sativa* cv. CUF 101 and somaclones (7R1, 4R1, 3R1) in response to osmotic stress (PEG).

a) Mean root length \pm SE.

b) Mean shoot length \pm SE.

Concentrations of PEG were: 0-□, 200-□, 250 g l⁻¹ -■ and no survivors *.

7R1 in the presence of 200 g l⁻¹ PEG increased 2.52 fold above the 0 g l⁻¹ PEG level. At 200 g l⁻¹ PEG, catalase activity was 1.4 fold increased in 7R1 more than that in CUF 101 compared with 0 g l⁻¹ PEG levels.

DISCUSSION

The results from this study show that one of the somaclones (7R1) was significantly more tolerant at 200 g l⁻¹ PEG 6000 levels than CUF 101.

The PEG-induced increase in proline in CUF 101 and in somaclones 7R1 and 4R1

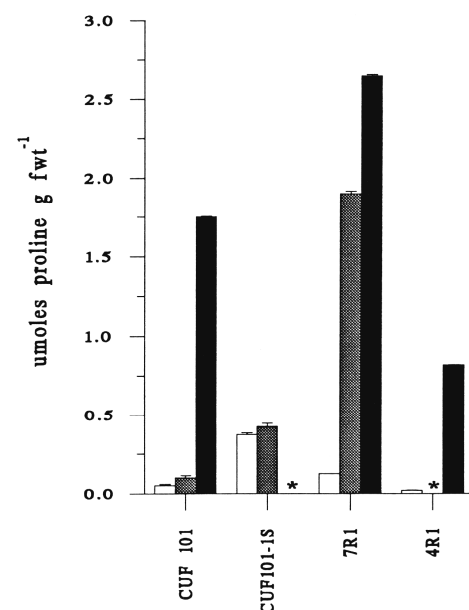


Figure 2. Proline levels in 14-day-old seedlings of *M. sativa* cv. CUF 101, somaclones (7R1, 4R1) and parent CUF101-1S in response to osmotic stress (PEG).

Means of three parallel measurements from each of three independent extracts \pm SE.

Concentrations of PEG were: 0-□, 200-□, 250 g l⁻¹ -■ and not measured *.

indicated a positive correlation between proline accumulation and osmotic stress. Indeed, the accumulation of this amino acid may be part of general response to adverse environmental conditions (Delauney and Verma, 1993; Yordanov and Tsoev, 2000), due to the fact that a proline increase depends on the length of time and concentration of osmotic stress, the type of tissue, the age of the plant and genotype (Bray *et al.*, 1991). Proline accumulation differs between cultivars adapted to certain growth conditions or regions, as well as within species more or less tolerant to drought (Heuer *et al.*, 1994). In alfalfa plants, the most tolerant varieties exhibited the highest potential for proline accumulation in response to osmotic stress (Heuer *et al.*, 1994) suggesting that it was involved in the osmotic adjustment of alfalfa plants during stress. This study does

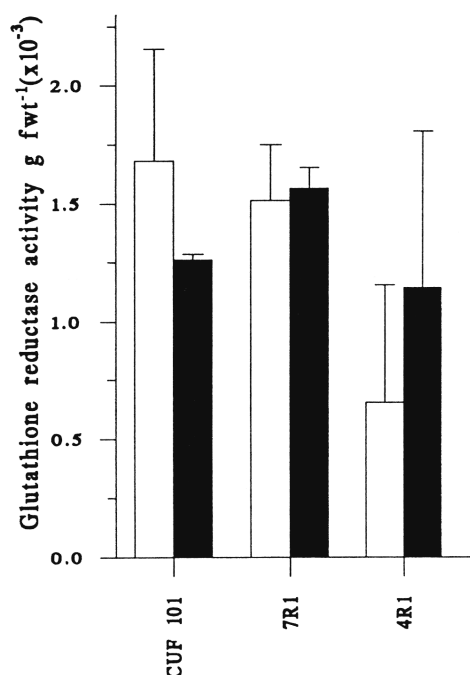


Figure 3. Activity of antioxidant enzyme (Glutathione reductase) in 14-day-old seedlings of *M. sativa* cv. CUF 101 and somaclones (7R1, 4R1) in response to osmotic stress (PEG). Means of three parallel measurements from each of three independent extracts \pm SE. Concentrations of PEG were: 0-□ and 200 g l⁻¹ -■.

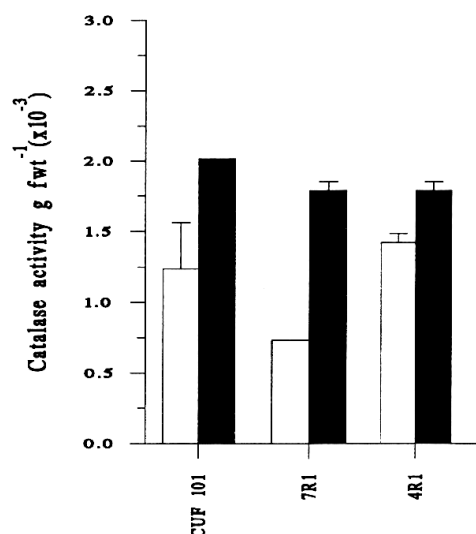


Figure 4. Activity of antioxidant enzyme (Catalase) in 14-day-old seedlings of *M. sativa* cv. CUF 101 and somaclones (7R1, 4R1) in response to osmotic stress (PEG). Means of three parallel measurements from each of three independent extracts \pm SE. Concentrations of PEG were: 0-□ and 200 g l⁻¹ -■.

not show a correlation between increased GR activity and osmotic tolerance in seedlings of alfalfa but does show a positive correlation between increased catalase activity and osmotic tolerance.

REFERENCES

1. Al-Khatib, M., McNeilly, T. and Collins, J. C. 1993. The Potential of Selection and Breeding for Improved Salt Tolerance in Lucerne (*Medicago sativa* L.). *Euphytica*, **65**: 43-51.
2. Beers, F. R. and Sizer, I. W. 1952. A Spectrophotometric Method for Measuring the Breakdown of Hydrogen Peroxide by Catalase. *J. Biol. Chem.*, **195**: 133-140.
3. Bray, L., Chriqui, D., Gloux, K., LeRudulier, D., Meyer, M. and Peduzzi, J. 1991. Betaines and Free Amino Acids in Salt Stressed *in Vitro* Plants and Winter Resting Buds of *Populus Teichcarpa* \times *deltoides*. *Physiologia Plantarum*, **83**: 136-143.
4. Collins, H. A. and Dix, P. J. 1990. Culture Systems and Selection Procedures. In: "*Plant Cell Line Selection, procedures and application*", (Ed.) Dix P. J., VCH, Weinham, Germany. pp. 3-18.
5. Delauney, A. J. and Verma, D. P. S. 1993. Proline Biosynthesis and Osmoregulation in plants. *Plant J.* **4**(2): 215-223.
6. Doyle, J. J., Doyle, J. L. and Bailey Hortorum, L. H. 1990. Isolation of Plant DNA from Tissue. *Focus*, **12**(1): 13-15.
7. Foyer, C. H., Descourvieres P. and Kunert, K. J. 1990. Protection Against Oxygen Radicals: an Important Defence Mechanism Studied in Transgenic Plants. *Plant, Cell Environ.*, **17**: 507-523.
8. Halliwell, B. and Foyer, C. H. 1978. Properties and Physiological Function of a Glu-



- tathione Reductase Purified from Spinach Leaves by Affinity Chromatography. *Planta*. **139**: 9-17.
9. Hawitt, E. J., 1966. Sand and Water Culture Methods used in the Study of Plant Nutrition. *Comm. Agric. Bur. Tech. Comm.*, No. 22.
 10. Heuer, B. 1994. Osmoregulatory Role of Proline in Water- and Salt-stressed Plants. In: "*Handbook of plant and Crop Stress*", (Ed.) Pessarakli, M., pp. 363-383.
 11. Hurkman, W. J., Fornari, C. S. and Tanaka, C. K. 1989. A comparison of the Effect of Salt on Polypeptides and Translatable mRNAs in Roots of a Salt-tolerant and Salt-sensitive Cultivar of Barley. *Plant Physiol.*, **90**: 1444-1456.
 12. Larkin, P. J. and Scowcroft, W. R. 1981. Somaclonal Variation: a Novel Source of Variability from Cell Cultures for Plant Improvement. *Theor. Appl. Genet.*, **60**: 197-214.
 13. Murashige, T. and Skoog, F. 1962. A Revised Medium for Rapid Growth and Bioassay with Tobacco Tissue Cultures. *Plant Physiol.*, **15**: 473-573.
 14. Niu, X., Narasimhan, M. L. Salzman, R. A. and Hasegawa, P. M. 1993. NaCl regulation of Plasma Membrane H⁺-ATPase Gene Expression in a Glycophyte and Halophyte. *Plant Physiol.*, **103**: 713-718.
 15. Rontein, D., Basset, G. and Hanson, A. D. 2002. Metabolic Engineering of Osmoprotectant Accumulation in Plants. *Metab. Eng.*, **4**: 49-56.
 16. Safarnejad, A., Collin, H. A. Bruce K. D. and McNeilly, T. 1996, Characterization of Alfalfa (*Medicago sativa* L.) Following *In Vitro* Selection for Salt Tolerance, *Euphytica*, **92**: 55-61.
 17. Saunders, J. W. and Bingham, E. T. 1975. Growth Regulator Effects on Bud Initiation in Callus Cultures of *Medicago sativa*. *Am. J. Bot.* **62**: 850-855.
 18. Sayers, E. R. and Murphy, R. P. 1966. Seed Set in Alfalfa as Related to Pollen Tube Growth Fertilization Frequency, and Post Fertilization Ovule Abortion. *Crop Sci.*, **6**: 365-368.
 19. Schenk, R. U. and Hildebrandt, A. C. 1972. Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Cultures. *Can. J. Bot.*, **50**: 199-204.
 20. Singh, T. N., Paleg, L. G. and Aspinall, D. 1973. Stress Metabolism. Variations in Response to Water Deficit in the Barley Plant. *Aust. J. Biol. Sci.* **26**: 65-76.
 21. Smith, B. N., Girija, C. and Swamy, P. M. 2002. Interactive Effects of Sodium Chloride and Calcium Chloride on the Accumulation of Proline and Glycinebetaine in Peanut (*Arachis hypogaea* L.). *Environ. Exp. Bot.*, **47**: 1-10.
 22. Yordanov, V. and Tsoev, T. 2000. Plant Responses to Drought, Acclimation and Stress Tolerance. *Photosynthetica*, **38**(1): 171-186.

ویژه گیهای یونجه برای تحمل به خشکی

ع. صفرنژاد

چکیده

کمبود آب یکی از مشکلات جدی در سراسر جهان است که در اثر دو تنش خشکی و شوری ایجاد می گردد. یکی از راههای کاهش این مشکل اصلاح گیاهان بمنظور افزایش تحمل به خشکی می باشد. یونجه (*Medicago sativa* L.) یکی از محصولات عمده مناطق خشک و نیمه خشک است. یکی از روشهای بهبود تحمل به خشکی این محصول استفاده از تغییرات ایجاد شده بوسیله کشت بافت و تولید

سوماکلونهای مقاوم می‌باشد. گیاهچه‌های نیمه مقاوم به شوری *CUF101 - IS* بدست آمده از واریته زراعی *CUF 101* بعنوان منبع ریز قلمه برای کشت بافت استفاده شد. از گیاهان باززایی شده بوسیله خودکشتی و یا گرده‌افشانی در میان گیاهان یک کلون بذر تولید گردید. بذور حاصله در محیط کشت حاوی غلظتهای مختلف پلی اتیلن گلایکول (PEG) ۶۰۰۰ کشت و پس از ۱۴ روز مورد ارزیابی قرار گرفتند. تحمل به تنش ایجاد شده بوسیله پلی اتیلن گلایکول بوسیله اندازه‌گیری طول ریشه و ساقه، میزان پرولین و فعالیت آنزیمهای آنتی اکسیدانت کاتالیز (*Ec 1.11.1.6*) و همچنین گلوتاسیون رداکتیز (*Ec 1.6.4.2*) تعیین گردید. بعضی از سوماکلونها مقاومت بیشتری از والدین در برابر تنش نشان دادند. یک افزایش معنی داری در میزان تجمع پرولین در سوماکلونهای مقاوم در مقایسه با واریته والد در عکس‌العمل به تنش پلی اسمزی مشاهده گردید. فعالیت کاتالیز در ژنوتیپهای مقاوم (*7RI*) در مقایسه با واریته والدین (*CUF 101*) در عکس‌العمل به تنش افزایش یافت در حالیکه فعالیت گلوتاسیون رداکتیز تغییر نمود.