Influence of drought stress on growth, protein expression and osmolyte accumulation in sunflower *Helianthus annuus* L. c.v. Peredovik

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ABSTRACT

Drought stress causes considerable yield losses in agriculture and receives high attention in current sunflower breeding programs. For this study, an *in vitro* system based on MS media (Murashige and Skoog, 1962) supplemented with polyethylene glycol 6000 (Korell, 1997) was established to examine sunflower (*Helianthus annuus* L.) seedlings under artificial drought stress conditions. The objective is to identify parameters applicable as markers for drought stress, which would allow breeders to screen their material efficiently for drought tolerance. For this purpose, different morphological and physiological parameters were analysed by comparing control plants with plants grown under drought stress (-0,6 MPa). The evaluation of growth reveals a significant growth deficit of drought stressed plants compared to control plants concerning hypocotyl length, development of cotyledons and primary leaves, whereas for root fresh weight in relation to shoot fresh weight no significant difference could be detected. Qualitative and quantitative changes in osmolyte accumulation were examined by HPLC and gas chromatography. Osmolyte analyses revealed an accumulation of glucose (25-30fold), inositol (20-30fold), proline (10-20fold), fructose (3-6fold) and sucrose (4-5fold) in extracts from leaves of drought-stressed plants. Changes in protein expression of drought-stressed versus control plants were detected in colloidal Coomassie-stained 2D-PAGE gels. In future studies, differentially expressed proteins will be identified by peptide mass fingerprinting and used to develop molecular markers for breeding programs.

Key words: drought stress – PEG – osmolyte – 2D-PAGE.

INTRODUCTION

Drought is a potential major constraint to crop production all over the world, and causes considerable yield losses in agriculture. Global warming, deforestation, and urbanisation will all increase the severity and frequency of drought in the future, leading to a possible decrease in global food production. Therefore, there is a great need for the development of stable food crops that produce high and stable yields also when drought occurs. Water stress in its broadest sense encompasses both drought and salt stress. Water stress results in stomatal closure and reduced transpiration rates, a decrease in water potential of plant tissue, decrease in photosynthesis and growth inhibition. The accumulation of abscisic acid (ABA) serves as a signal, initiating acclimation reactions such as accumulation of compatible compounds like proline, mannitol, and sorbitol, or the formation of scavenging compounds like ascorbate, gluthathione, α-tocopherol etc. The acclimation process employs processes of differential gene expression leading to new proteins and mRNAs (Yordanov et al., 2003). Obviously, water stress acclimation is a multi-gene acclimation process, in which many different physiological processes and many drought stress-inducible genes are involved. Functionally, these gene products can be distinguished into: osmolyte synthases (Chen and Murata, 2002), protection factors for macromolecules (chaperons, LEA/dehydrin-type genes), proteases, membrane proteins (aquaporins, transporters), detoxification enzymes (GST, SOD), and genes of regulatory proteins like transcription factors, protein kinases, protein phosphatases (Wang et al. 2003; Zhu, 2002). Although the alterations in all of these processes related with drought stress have been widely investigated in many model species and a few crop plant species, reports on sunflower are limited. Physiological and molecular responses have been described by Kane and Rieseberg (2007), Kavas et al. (2006), Bailly et al. (2004), Liu and Baird (2003; 2004), Cellier et al. (1998; 2000) and Poormohammad et al. (2007). The aim of our *in vitro* studies of drought tolerance of sunflower seedlings was to analyse physiological key processes after applying drought stress conditions by supplementation of MS media with polyethylene glycol (PEG) 6000. This evaluation should result in an appropriate test system for breeders to select for drought tolerant breeding material while saving time, space and costs.

MATERIALS AND METHODS

Sunflower c.v. Peredovik seedlings were grown in a liquid MS medium (Murashige and Skoog, 1962) using 0.75 l Weck glasses. The plant material was incubated in climate chamber at 21°C, 16/8 hours light/dark cycle at 150 µM photons m^{-2} s^{-1}. After three days of cultivation half of the seedlings were transferred to drought stress medium, achieved by supplementation of MS medium with PEG 6000 to an osmotic potential of -0.6 MPa (MS6), while the control plants were transferred onto fresh MS0 medium without PEG addition. Seven days later plant growth was characterized by measurement of the hypocotyl length, characterizing the development of cotelydons and primary leaves (using a relative scale from 0 to 6 representing area of leaves up to >4 cm^2) and determination of fresh weight of root and shoots. Primary leaves were frozen in liquid nitrogen and stored at -20°C till extraction of proteins and osmolytes.

Total homogenates of leaves were obtained by grinding plant material in Eppendorf tubes on an ice bath with HEPES buffer (10 mM, pH 7.6) containing protease inhibitors (10 mM PMSF, protease inhibitor cocktail, Sigma P9599 according product information). The soluble protein fraction was obtained after centrifugation of the crude extract at 38,000 g and 4°C. After determination of the protein concentration (Bradford, 1976), these protein extracts (400 µg each) were used for 2D-PAGE according to Fulda et al. (2000, 2006) followed by differential analysis of colloidal Coomassie-stained 2D gels from drought-stressed plants versus control plants using Delta2D software (Decodon).

Qualitative and quantitative determination of osmolytes was done by HPLC and gas chromatography (GC). In the case of HPLC analyses, soluble protein extracts containing 400 µg of protein were taken as raw material. The high molecular contaminants were precipitated with 80% ethanol (containing an internal standard: 100 µg sorbitol) overnight at 4°C followed by centrifugation (28,000 g). Supernatants were dried in a vacuum centrifuge. Dry residues were washed once with 80% ethanol and dissolved in A. bidest. HPLC analyses were performed according to Schoor et al. (1995) using the combination of reversed-phase and ion-moderated partition chromatography. To verify the results of HPLC analyses, additional samples were analyzed by gas chromatography using a Focus GC (Thermo Scientific) equipped with a TR-5MS column (30 m x 0.25 mm x 0.25 µm) and an AS 3000 autosampler. For GC-analyses, a separate ethanolic extraction of ground leaves was performed with 80% ethanol at 68°C for two hours at first, followed by a second extraction at 68°C overnight. The combined extracts were purified by centrifugation at 38,000g, 4°C and dried in a vacuum centrifuge. Trimethylsilyl-derivates of sugars were obtained by incubation with 65 µl pyridine/methoxylamine (20 mg/ml, 90 min at 30°C) and 35 µl N, O-Bis(trimethylsilyl)-trifluoroacetamide (Sigma, 60 min at 60°C). A set of the following standards was chosen for qualitative analysis of the osmolytes: trehalose, maltose, glucose, sucrose, inositol, fructose, glycerol, mannitol, sorbitol, glycine betaine, proline. As an internal standard for quantification sorbitol was used (see above).

RESULTS AND DISCUSSION

In five independent experiments we observed a clear growth inhibition of plants cultivated in drought stress MS6 medium in comparison to MS0 plants (Fig. 1). In each experiment, about 200 plants were evaluated for growth. The rating reveals a significant growth deficit of drought-stressed plants compared to control plants concerning hypocotyl length (Fig. 2a) as well as the development of cotyledons (Fig. 2b) and primary leaves (Fig. 2c).

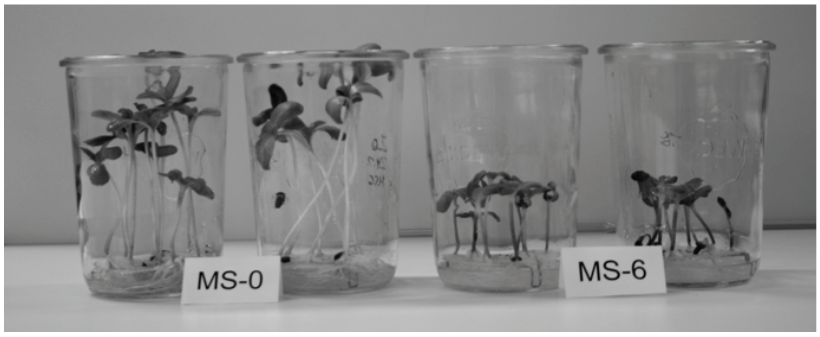

Fig. 1. Growth of 10-days-old *Helianthus annuus* L. c.v. Peredovik plants cultivated in control medium MS0 and drought stress medium MS6.

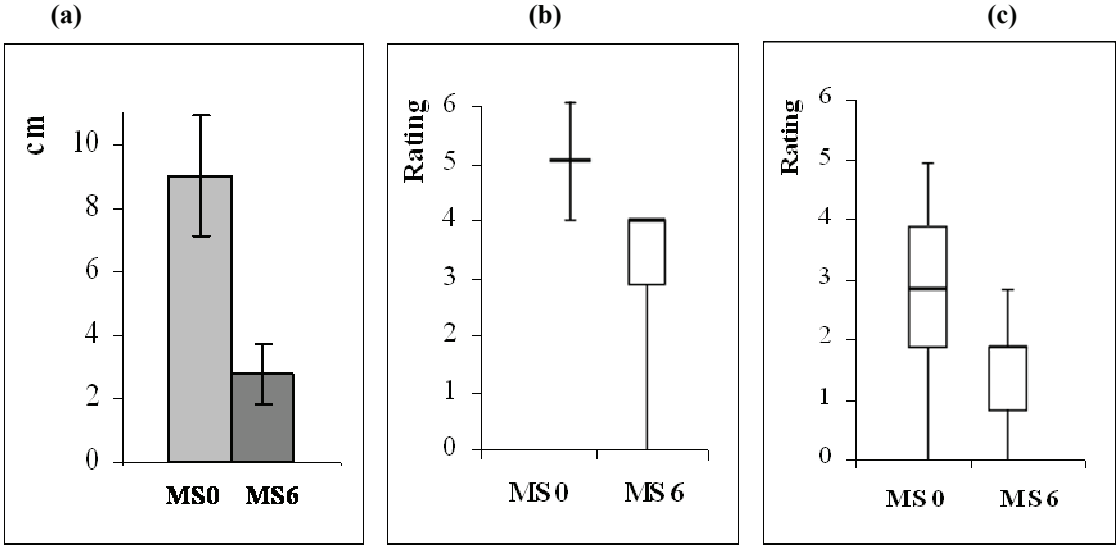

Fig. 2. Development of **(a)** hypocotyl length (cm), **(b**) cotyledons and **(c)** primary leaves of *Helianthus annuus* L. c.v. Peredovik grown in MS media with (MS6) and without (MS0) drought stress. Means and standard deviation (a) or medians (b, c) are given (n for MS0=87, n for MS6=98).

These observations agree with data published by Yordanov et al. (2003). Obviously, drought stress affects growth at the whole plant level leading to a decrease in photosynthesis and associated carbon and nitrogen metabolism. The growth inhibition could be attributed to shrinkage of cells and to the fact that the turgor pressure against cell walls relaxes. Because turgor reduction is the earliest significant biophysical effect of water stress, turgor-dependent activities such as leaf expansion and root elongation are the most sensitive to water deficits. Cell expansion is a turgor-driven process and is extremely sensitive to water deficit so that a decrease in turgor causes a decrease in the growth rate (Taiz and Zeiger, 2007).

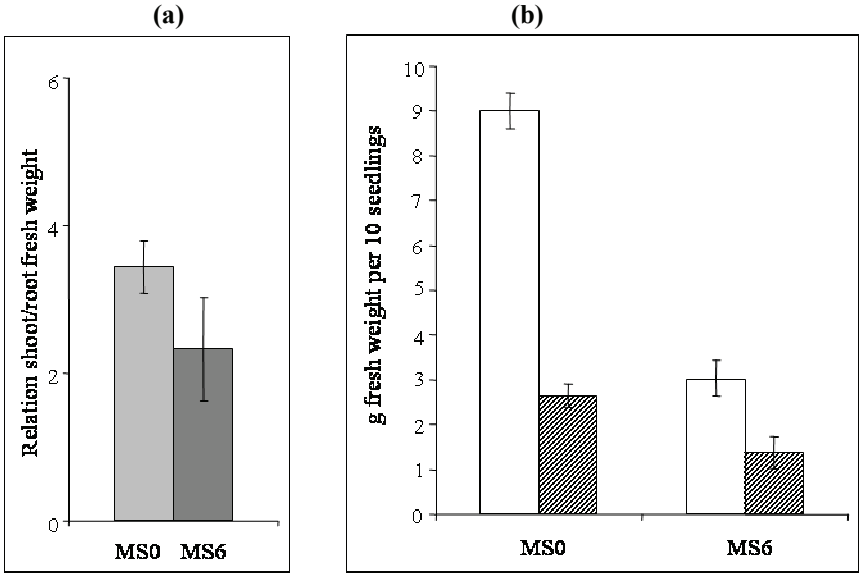

Fig. 3. Comparison of shoot and root development of 10-days-old seedlings of *Helianthus annuus* L c.v. Peredovik grown in MS media with (MS6) and without (MS0) drought stress. Means and standard deviations are given (n for MS0=9, n for MS6=10). **(a)** Fresh weight ratios shoot/root, **(b)** Fresh weight per 10 shoots (white columns) and fresh weight per 10 root systems (grey columns).

For root fresh weight in relation to shoot fresh weight, no significant difference could be detected (Fig. 3b). Karrenberg et al. (2006) investigated responses to salinity in the homoploid species *Helianthus paradoxus* and its progenitors *H. annuus* and *H. petiolaris*. They reported that growth reduction in the

progenitors *H. annuus* and *H. petiolaris* affected roots more than shoots as indicated by a decrease in root mass fraction. In the homoploid hybrid species *H. paradoxus* root biomass allocation did not change in response to salt stress so the relationship between root to shoot growth seems to differ from species to species. On the other hand, development of an optimal root/shoot ratio in relation to water availability is very important for the crop yield. Under natural conditions, plants are able to improve uptake of water by developing an extensive root system, which enables plants to grow into deep soil region with sufficient or improved water supply. Therefore, changes in relative root and shoot growth, leading to an increased root/shoot ratio were often observed with drought stressed plants (Verslues et al., 2006; Sharp et al., 2004). Additional tissue water storage capacity and thickness of the cuticula and water permeability are also of potential importance. Of these, changes in root growth to maximize water uptake are of the greatest importance for crop plants. In our PEG 6000-based hydroponics we observed, for shoot as well as root development, reduced growth in stressed plants (fresh weight, Fig. 3a) that leads to an unaffected root/shoot ratio. This may be due to the fact that PEG 6000-mediated drought stress conditions represent a severe stress.

Accumulation of osmolytes represents one of the central acclimation reactions in drought-stressed plants. Osmolyte analyses, which were done by HPLC and GC, obviously indicate an average accumulation of substances such as glucose (25-30fold), inositol (20-30fold), proline (10-20fold), fructose (3-6fold) and sucrose (4-5fold) in drought-stressed sunflower plants (Fig. 4.).

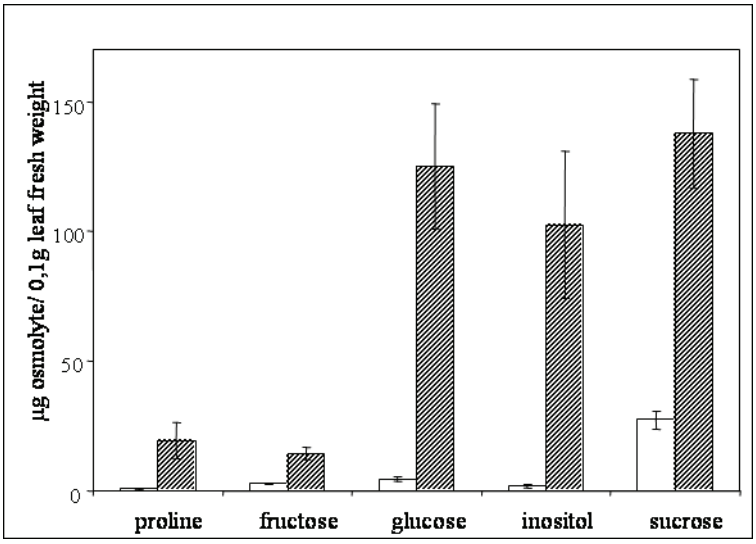

Fig. 4. Osmolyte accumulation in leaf extracts of *Helianthus annuus* L c. v. Peredovik determined by GC. Plants were grown on MS0 medium (white columns) or on MS6 medium (grey columns), respectively. Means of four analyses and their standard deviations are shown.

As expected, decreased water availability requires the accumulation of solutes by cells to decrease cell water potential, which enables plants to absorb water. This osmotic adjustment is a net increase in solute concentration per cell that is independent of the volume changes that result from loss of water. This can be accounted for by an increase in the concentration of a variety of common solutes, including sugars, amino acids, organic acids, polyols and inorganic ions (Taiz and Zeiger, 2007). In the case of our PEG-mediated drought stress system, the hexose glucose, the polyol inositol and sucrose seem to represent the main contributors to osmotic adjustment in primary leaves of *H. annuus* c.v. Peredovik followed by proline and fructose. Sharp et al. (2004), who investigated osmotic adjustment in roots of maize, found that in the maize primary root tip, hexoses are the dominant osmolytes in the basal region of the growth zone, while, in the apical zone proline concentration increases dramatically in water-stressed roots. One of the most frequently found solutes in water-stressed plants is the amino acid proline. Additionally, proline may act as a regulatory or signalling molecule to activate multiple responses that are part of the acclimation process (Maggio et al., 2002). Also, proline is a reliable indicator of the environmental stress imposed on plants (Claussen, 2005). Chechin et al. (2006) found in greenhouse-grown *H. annuus* c.v. Catissol-01 plants a 7-fold increase in proline content in young stressed leaves in comparison to non-stressed plants, but in the case of mature stressed leaves the proline content was increased four fold. In our study, proline was not the dominating compatible compound. These differences may be related to the

use of different sunflower varieties or culture conditions. Myo-inositol and its derivates are commonly studied with respect to cell signalling and membrane biogenesis, but they also participate in response to salinity in plants. Non-methylated inositols are found in all plants but are especially common in legumes. Pinitol and ononitol (methylated derivates) have been reported as a salt-induced response in *M. crystallinum* (Thomas and Bohnert, 1993). The already high concentration of cyclitols in unstressed soybean (*Glycine max*) is further increased in drought-stressed plants, underlying the important role of unmethylated and methylated inositols as osmoprotectants (Schneider et al., 2007; Gagneul et al., 2007).

Adaptation to drought stress requires alterations in the cell machinery that result directly from modifying gene expression. Functional gene expression profiles can be best achieved by proteome analysis. Furthermore, proteins undergo significant levels of post-translational modification of their primary sequences and are readily subjected to targeted proteolysis. Thus, a quantitative analysis of gene expression at the protein level is essential for dissecting responses to drought stress. We used the most common tool for revealing the expression of intact proteins, the two-dimensional gel electrophoresis (2D-PAGE). After staining proteins with colloidal Coomassie stain nearly 250 protein spots could be visualized in sunflower (Fig. 5). Protein pattern of control and drought-stressed sunflower plants were compared. So far, two regions marked on the gels were identified, where remarkable changes in protein expression became obvious. By using same sample replicates (4-5 times), several protein spots could be found, which showed an accumulation in drought-stressed plant material. However, a lot more spots seem to be present in smaller amounts in the drought-stressed plants compared to the control plants. In future experiments, the proteins of these stress-induced protein spots as well as decreased spots will be identified by excising gel plugs from the gel for MALDI-TOF analysis.

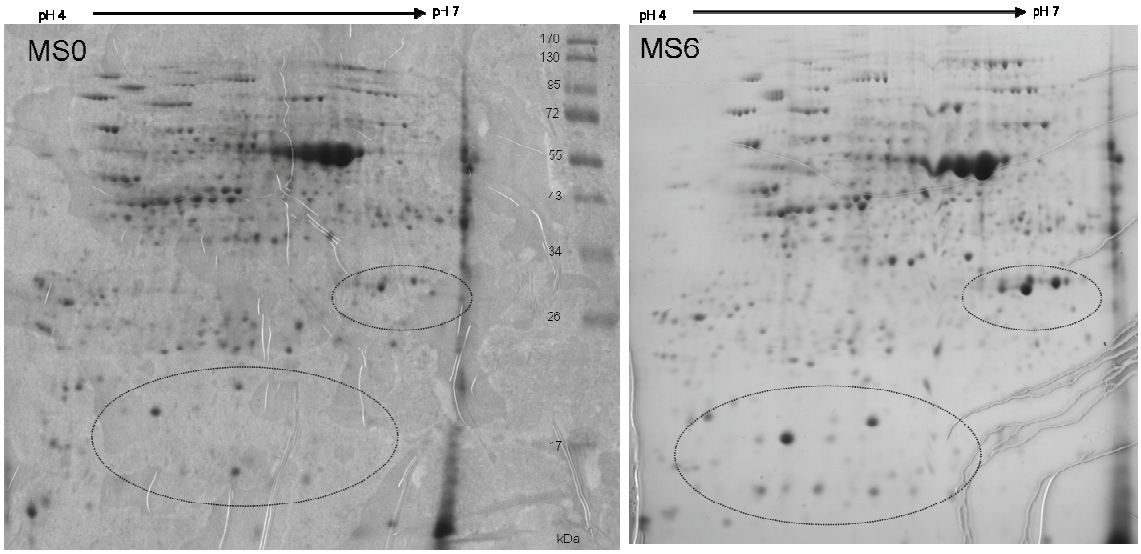

Fig. 5. 2D-PAGE gels of control and drought-stressed protein extracts from *Helianthus annuus* L. c.v. Peredovik. Soluble proteins were separated on 18 cm IPG pH 4-7 strip according to their isoelectric point in the first dimension and then on a SDS-PAG according to their molecular weight in the second dimension. The 2D-PAGE gels were stained with colloidal Coomassie blue. Protein samples were diluted to a load of 400 µg in rehydration buffer.

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