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In vitro Osmotic Stress Memory in *Chrisanthemum hybridum*: Structural and Physiological Responses

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Abstract

Structural and physiological responses of chrysanthemum to repeated osmotic stress were studied. Plants were cultured for 2 weeks (for each *stress1* and *stress 2*) on half MS supplemented with mannitol 100 mM (Treatment I) and 200 mM (Treatment II). First stress inhibited growth parameters stronger than second stress in treatment I. In treatment II both stress events strongly inhibited growth parameters of micro-shoots. Proline content exceeded control 6 - 8 times after 1st stress, and 2 - 5 times after the 2nd stress in treatments I and II, respectively. Soluble protein was accumulated in leaves during both stress exposures, and 2 - 2.5 times exceeded control after the 2nd stress. Relative water content in both treatments increased after the 2nd stress exposure. In treatment II chlorophyll *a* and carotenoids contents were 8.78 and 4.62 mg/g comparing to control (4.21 and 2.25 mg/g, respectively) after the 1st stress. But after the 2nd stress there was no difference with control.

Introduction

Osmotic stress in plants leads to disruption of photosynthesis, respiration, translocation of assimilates, absorption of ions, carbohydrate metabolism (Jaleel et al. 2009). Water deficit underlies these disturbances and reduces cells water potential, changes cytosol composition, structure of macromolecules, and eventually inhibits growth and declines crop productivity (Li and Liu 2016).

It is known that plants often exhibit so-called "stress memory" - different reactions in response to similar repetitive stress. Many publications confirm that first stress event leads to changes of plant reactions to subsequent stress events.

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So called priming can contribute to more quickly and effectively plant response to future stresses (Walter et al. 2011, Ramirez et al. 2015, Li and Liu 2016). Four overlapping strategies were revealed in plant to improve its stress tolerance: (1) increased synthesis of protective, damage-repairing, and detoxifying functions; (2) coordinating photosynthesis and growth under repetitive stress; (3) re-adjusting osmotic and ionic equilibrium to maintain homeostasis; and (4) re-adjusting interactions between dehydration and other stress/hormone regulated pathways (Ding et al. 2013).

Despite numerous publications, there are many gaps in research of hardening mechanisms and stress memory, for example, which organs and to what extent are responsible for the presence of stress memory? What mechanisms underlie the transfer of memory signals from the gene to the ecosystem (Munne-Bosch and Alegre 2013)? Moreover, despite the numerous examples of priming and presence of epigenetic memory in many plants such memory does not occur, and the reasons for this remain unclear (Crisp et al. 2016). The most researches in this field were conducted on the genetic level (Wu et al. 2016, Xu et al. 2013, Piwowarczyk et al. 2014, Kinoshita and Seki 2014). Only a few publications show physiological and structural changes in response to repeated osmotic stress (Radik and Pevalek-Kozlina 2010, Fleta-Soriano and Munné-Bosch 2016, Leufen et al. 2016).

In this paper, we studied the physiological and structural changes occurring in chrysanthemum microshoots exposed to repeated osmotic stress. *Chrysanthemum* - one of the most important flower crops in the world, and the drought is the major factor limiting its global production. Recently, transcriptome sequencing in drought conditions was performed on chrysanthemum and candidate genes were identified (Xu et al. 2013, Wu et al. 2016). Even so, physiological reactions on repeated stress in this species were not shown. Importance of the classical approaches based on organ and organism levels, and not just "omics" technologies to study stress memory mechanisms was convincingly shown in recent studies (Walter et al. 2014, Fleta-Soriano and Munné-Bosch 2016). Therefore, the aim of present study was to identify structural and physiological changes in chrysanthemum plants in response to repetitive osmotic stress induced *in vitro*. Analyzing the previous work, we have put forward the hypothesis that stress memory effect in chrysanthemum will be decreasing in physiological and biochemical reactions after 2nd stress comparing to 1st stress.

Materials and Methods

Chrisanthemum hybridum cv. Vesuvius was obtained from *in vitro* collection of our Research Institute. Shoots of this cultivar were multiplied according to previously published protocol (Kolomiets and Malyarovskaya 2013).

Half MS supplemented with 0.5 mg/lNAA, 20 g/l sucrose, 2.5 g/l Phytagel + 100 μM mannitol (Treatment I) or 200 μM mannitol (Treatment II) was used. Control - medium without mannitol. pH was adjusted to 5.9, then 20 ml of the medium were poured into culture vessels of 150 ml and covered with a polypropylene film. Medium was autoclaved for 25 min at 101 Pa and temperature of 110°C. Elongated shoots were cut into 15 mm length and placed into test medium by 4 shoots per vessel. Explants were cultured in growth chamber at 16 hrs photoperiod, light intensity of 3000 lux and a temperature of 16 - 18ºC.

Two series of observations were conducted: after the first two weeks of stress exposure (*stress1*) and after the second two weeks of stress exposure (*stress2*). In between all the plants were recovered for 2 weeks in a medium without mannitol. In our experiments control was used in each stress periods, and the differences with controls were evaluated separately in first and second stress.

Height gain (the difference between the final and initial shoot length), roots number and length were measured.

Fresh leaves were weighed (fresh weight), then dried in oven at 105ºC for half an hour (dry weight). Relative water content (%) was calculated using as follows:

$$\text{RWC} = \frac{(\text{fresh weight} - \text{dry weight} \times 100}{\text{Fresh weight}}$$

Proline content in leaves (mg/g fresh leaf weight) was evaluated by simplified ninhydrin method (Shihaleeva et al. 2014). Briefly: 200 mg of leaf tissue was dipped in hot distilled water and heated on a water bath for 15 minutes at 100ºC. 2 ml of this leaf extract were mixed with 2 ml of glacial acetic acid and 2 ml of acidic ninhydrin reagent then heated in a water bath for 20 minutes. After cooling of the mixture absorbance at 520 nm was measured on spectrophotometer PA-5400vi (Russia) and recalculated using standard formula.

Water soluble protein content was determined by precipitation of filtrate obtained from a suspension of 500 mg grinded leaf tissue mixed with hot distilled water. 4 ml of the precipitant (10 % ferrocyanide solution 2 ml and 15 % zinc sulfate solution 2 ml) was added to 50 ml of the filtrate to precipitate soluble proteins. After precipitation proteins was dried in an oven at 105 °C followed by weighing the dry residue. Results were recalculated per one gram of leaf fresh weight.

Chlorophyll and carotenoids content (mg/g fresh leaf weight) was evaluated using 85 mg of leaf sample. Pigments were extracted using acetone, and filled up volume to 50 ml. The absorbance of extracts was measured at 662 and 644 nm (for chlorophyll *a*, *b*, respectively) and 440.5 nm (for carotenoids) using spectrophotometer PA-5400vi (Russia). The concentration of chlorophyll *a*, *b* and total carotenoids was calculated as published by Shlyk AA (1971).

Fluorescence of chlorophyll of photosynthetic activity and vitality index were analyzed using a portable fluorometer LPT-3C. Coefficient of photosynthetic activity Kf_n - reflects the effectiveness of light utilization during photosynthesis and vitality index F_m / F_T (F_m - maximum of fluorescence and F_T - stationary level of fluorescence) were measured.

Data were analyzed by one-way analysis of variance (ANOVA), and differences between treatments and corresponding controls were considered as statistically significant at $p < 0.05$. The results were calculated as the mean value of at least 9 replicates (or 3 replicates for physiological analyses) ± standard deviation.

Results and Discussion

In vitro induced osmotic stress by addition of 100 and 200 mM mannitol neither led to death of chrysanthemum shoots nor drop of leaf and change of color, so all experimental plants looked healthy. However, differences in growth parameters occurred between the treatments. Two times decrease of height gain was observed in treatment I after the 1st stress. However, after the 2nd stress the difference with control was not significant (Fig. 1A). In treatment II differences between 1st and 2nd stresses were not significant, and in both periods height gain decreased to 0.2 - 0.5 cm comparing to control 1.5 - 2.5 cm.

In treatment I the root length was 4 times less after the 1st stress and 2 times less after the 2nd stress in comparison with the control. In treatment II after the 2nd stress roots did not develop at all. Rooting was strongly inhibited in treatment II. The average root number per explant was 4 - 5 (control) and 0 - 3 (treatments I, II) after the 1st stress (data are not presented). Results showed that our plants responded to repeated stress depending on mannitol concentration. These data were consistent with the findings of other workers who showed that stress reactions depended on the strength and duration of the stress exposure (Fleta-Soriano and Munné-Bosch 2016). Also, Walter et al. (2011) showed that severe drought not only resulted in a decrease in biomass of grasses, but inhibited photosynthesis during repeated stress.

The relative water content decreased significantly after the 1st stress. It was of 89% in treatment I and in 86% treatment II compared to control (93%). But 2nd stress resulted raising the relative water content in chrysanthemum leaves in both treatments (Fig. 1B). This was consistent with the results of other researchers, who reported on stabilization of water metabolism in plants after repeated drought (Fleta-Soriano and Munné-Bosch 2016, Walter et al. 2014). This can be achieved by reducing either transpiration, or the plant size, as well as reducing its photosynthetic activity and changes of pigments content, which was confirmed by our experiments.

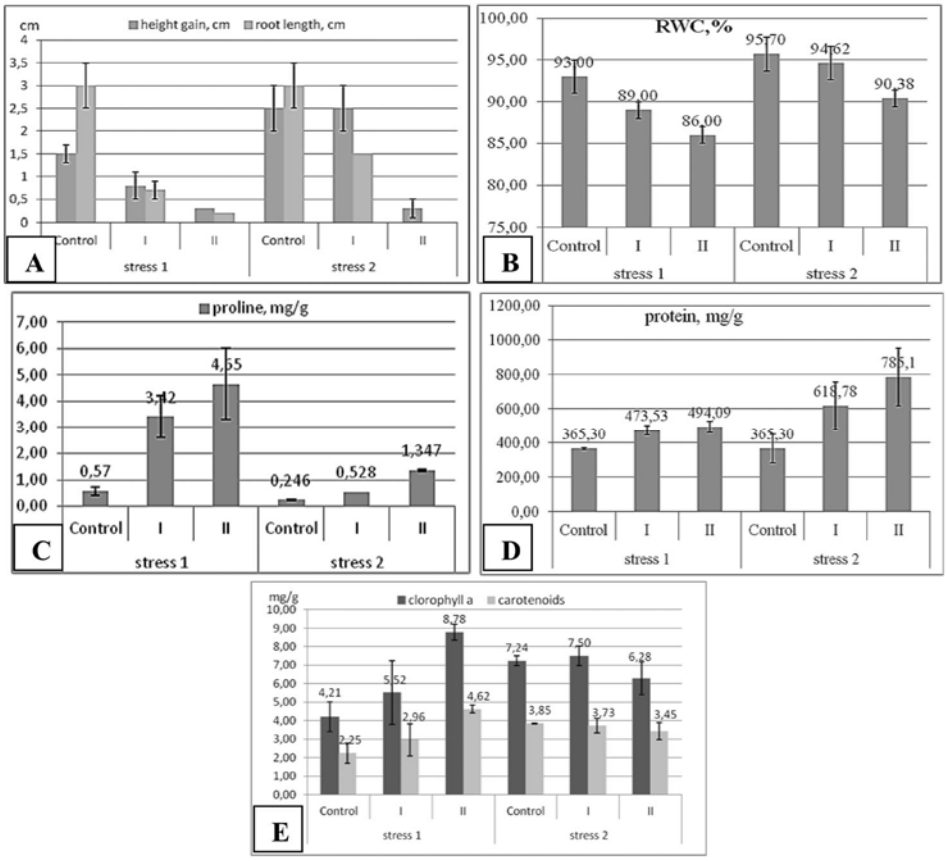

Fig. 1. Effect of 1st and 2nd osmotic stresses on: A. Growth parameters. B. Relative water content. C. Proline content, D. Soluble protein content. E. Chlorophyll *a* and carotenoids content in leaves of *Chrysanthemum hybridum in vitro*.

Proline not only acts as a stress indicator, but also among other osmolytes significantly contributes osmotic regulation (Molinari et al. 2007). After the 1st stress proline content in leaves was 3.42 and 4.65 mg/g in treatments I and II,

respectively that is 6 - 8 times higher than in control (Fig. 1C). However, after the 2nd stress proline content was dramatically reduced in both treatments and became 0.53 and 1.35 mg/g in I and II treatments, respectively compared to control 0.25 mg/g. Nevertheless even after the 2nd stress it was 2 - 5 times higher than control. Our data were consistent with other findings in sugar beet which showed less changes of proline after 2nd stress than after the 1st (Leufen et al. 2016). This was explained by the improvement of osmotic adjustment, because second osmotic stress increased water use efficiency by plant.

In plants soluble proteins are also involved in osmotic adjustment. For example, the total protein content in wheat was a representative indicator of adaptation to drought (Li and Liu 2016). The total soluble protein content was increased during the 1st stress in both treatments I and II and it was 473 - 494 mg/g compared to control 365 mg/g, differences significant (Fig. 1D). Second stress exposure increased protein content till 619-785 mg/g (in I and II treatments) that was 2 - 2.5 times exceeded control. Differences between the treatments I and II were insignificant in both stress exposures. Other studies also indicated that protein content and ratio of structural and soluble proteins were changed during osmotic stress (Suseela et al. 2015).

Evaluation of pigments content was shown as an informative criterion to study plants physiological activity under osmotic stress. In present study, concentration of chlorophyll *b* did not differ significantly between treatments I and II as well as between 1st and 2nd stress exposures and it was of 1.65-2.88 mg/g in fresh leaf weight (data are not presented). Chlorophyll *a* was 8.78 mg/g significantly higher than control (4.21 mg/g) in treatment II after the 1st stress (Fig. 1C). But after the 2nd stress there were no significant differences observed, moreover all the differences between treatments I and II were insignificant after the 2nd stress. A similar trend was observed on carotenoids: in treatment II it was 4.62 mg/g, it is two times higher than after the 1st stress. After the 2nd stress the difference became non significant.

Increased chlorophyll and carotenoids in 1st stress was due to adaptation, but 2nd stress did not decrease photosynthetic activity and accumulation of carotenoids in the leaves, which may indicate the presence of memory at this physiological level. Increase in photosynthetic activity and stabilization of electron transfer during photosynthesis was reported as one of the basic mechanisms of drought tolerance in apple (Zhou et al. 2015). Other researchers also reported that chlorophyll *a* was higher in single-stressed plants in the end of the experiment (stresses lasted 6 days and 6 days of recovery in between) (Fleta-Soriano and Munné-Bosch 2016).

The intensity of the red fluorescence, and its changes under the stress exposure was suggested as a quick indicator of photochemical deviations. Simple fluorescence ratio (SFR), the index obtained from dark red and red fluorescence spectrum strongly correlates with the chlorophyll content (Leufen et al. 2013, Ben Ghozlen et al. 2010). However, we found no differences in the coefficients of photosynthetic activity and plant vitality index in all treatments. The vitality index F_m/F_T of micro shoots was 1.5 - 1.7, and photosynthetic activity coefficient Kf_n was 0.39 - 0.42 in all studied variants (data are not shown). Some authors also showed that in *Silenedioica* photosynthetic parameters (the chlorophyll and photosynthetic activity ratio) did not differ after a single and repeated stress (Fleta-Soriano and Munné-Bosch 2016). Other workers revealed that maximum quantum efficiency (Fv/Fm) and maximum fluorescence (Fm) were reduced in plants that were exposed to recurrent drought. Their findings indicated improved photo protection in double-stressed plants (Walter et al. 2014). We suppose that fluorescent analysis is sensitive to time of its implementation during the stress exposure. In our work, we measured these indicators in the end of stress period. Other colleagues proved high efficiency of this analysis measuring fluorescence level every 2 days during the two-week stress (Leufen et al. 2016). Maybe this is the reason why we have not revealed differences between treatments in chlorophyll fluorescence.

Conclusion

The results of our studies have shown that repeated osmotic stress resulted to changes in the morphological, physiological and biochemical parameters in chrysanthemum micro shoots. However, the average stress and severe stress showed different responses to the 1st and 2nd stress evens. Our data on the relative water, proline, soluble protein and pigments contents in leaves showed its changes after repeated stress, which indicated the presence of stress memory in Chrysanthemum. Proline, pigments and relative water content became closer to control values, but soluble proteins content, in contrast, increased after the 2nd stress. In addition, analysis of growth parameters showed that stress memory also depended on the strength of osmotic pressure: the weak stress provided adaptive responses to repeated stress. On the other hand, strong stress seems to accumulate negative effect during the 1st and 2nd periods. To summarize the results we can conclude that overlapping strategies employed by chrysanthemum to improve its osmotic stress reactions were: changes in synthesis of proline and soluble proteins, coordinating photosynthesis and growth under repetitive stress and stabilization of water regime in microshoots.

References

Ben Ghozlen N, **Cerovic ZG**, **Germain C**, **Toutain S** and **Latouche G** (2010) Non-destructive optical monitoring of grape maturation by proximal sensing. Sensors **10**: 10040-10068.

Ding Y, **Liu N**, **Virlouvet L**, **Riethoven JJ**, **Fromm M** and **Avramova Z** (2013) Four distinct types of dehydration stress memory genes in *Arabidopsis thaliana*. BMC Plant Biology **13**: 229-240.

Crisp PA, **Ganguly D**, **Steven RE**, **Borevitz JO** and **Pogson BJ** (2016) Reconsidering plant memory: Intersections between stress recovery, RNA turnover, and epigenetics. Sci. Adv. **2**: e1501340.

Kinoshita T and **Seki M** (2014) Epigenetic Memory for Stress Response and Adaptation in Plants.Plant Cell Physiol. **55**(11): 1859-1863.

Kolomies TM and **Malyarovskaya VI** (2013) Development of biotechnological techniques to produce *Chrysanthemum* planting material of a high quality.Subtrop. and Ornamental Hortic. **48**: 115-122.

Fleta-Soriano E and **Munné-Bosch S** (2016) Stress Memory and the Inevitable Effects of Drought: A Physiological Perspective. Front. Plant Sci. **7**: 143. doi: 10.3389/fpls. 2016.00143

Jaleel CA, **Manivannan P**, **Wahid A**, **Farooq M**, **Somasundaram R** and **Panneerselvam R** (2009) Drought stress in plants: A review on morphological characteristics and pigments composition. International Journal of Agriculture and Biology **11**: 100-105.

Leufen G, **Noga G** and **Hunsche M** (2016) Drought stress memory in sugar beet: mismatch between biochemical and physiological parameters. J. Plant Growth Regul. DOI 10.1007/s00344-016-9571-8

Leufen G, **Noga G** and **Hunsche M** (2013) Physiological response of sugar beet (*Beta vulgaris*) genotypes to a temporary water deficit, as evaluated with a multiparameter fluorescence sensor. Acta Physiol. Plant. **35**: 1763-1774

Li X and **Liu F** (2016) Drought stress memory and drought stress tolerance in plants: Biochemical and molecular basis. *In:* Drought Stress Tolerance in Plants M.A. Hossain *et al.* (eds.) **1.** DOI 10.1007/978-3-319-28899-4_2.

Li X, **Cai J**, **Liu F**, **Dai T**, **Cao W** and **Jiang D** (2014) Cold priming drives the sub-cellular antioxidant systems to protect photosynthetic electron transport against subsequent low temperature stress in winterwheat. Plant Physiol. Biochem. **82**: 34-43.

Molinari HBC, **Marur CJ**, **Daros E**, **de Campos MKF**, **de Carvalho JFRP** and **Filho JCB** (2007) Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): Osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiol. Plant **130**: 218-229.

Munne-Bosch S and **Alegre L** (2013) Cross-stress tolerance and stress "memory" in plants: An integrated view. Environ. Exp. Bot. **94**: 1-2.

Piwowarczyk B, **Kamińska I** and **Rybiński W** (2014) Influence of PEG generated osmotic stress on shoot regeneration and some biochemical parameters in *Lathyrus* culture. Czech J. Genet. Plant Breed. **50**(2): 77-83.

Radik S, **Pevalek-Kozlina B** (2010) Effects of osmotic stress on antioxidative system of duckweed (*Lemna minor* L.). Periodicum Biologorum **112**(3): 293-299.

Ramirez DA, **Rolando JL**, **Yactayo W**, **Monneveux P**, **Mares V**, **Quiroz R** (2015) Improving potato drought tolerance through the induction of long-term water stress memory. Plant Sci. **238**: 26-32.

Shihalyeyeva GN, **Budnyak AK**, **Shihalyeyev II**, **Ivaschenko OL** (2015) A modified method for determination of proline in plants. The Journal of V.N. Karazin Kharkiv National University. Series: biology **21**(1112): 168-172.

Shlyk AA (1971) Assessment of chlorophyll and carotenoids in extracts of green leaves. Biochemical methods in plant physiology. Moscow: 154-171.

Suseela V, **Tharayil N**, **Xing B**, **Dukes JS** (2015) Warming and drought differentially influence the production and resorption of elemental and metabolic nitrogen pools in *Quercusrubra*. Glob Chang Biol. doi: 10.1111/gcb.13033 .

Xu Y, **Gao S**, **Yang Y**, **Huang M**, **Cheng L**, **Wei Q**, **Fei Z**, **Gao J** and **Hong B** (2013) Transcriptome sequencing and whole genome expression profiling of chrysanthemum under dehydration stress. BMC Genomics **14**: 662 http://www.biomedcentral.com/1471-2164/14/662

Walter J, **Nagy L**, **Hein R**, **Rascher U**, **Beierkuhnlein C**, **Willner E** and **Jentsch A** (2011) Do plants remember drought? Hints towards a drought-memory in grasses. Environ. Exp. Bot. **71**: 34-40.

Wu Y-H, **Wang T**, **Wang K**, **Liang Q-Y**, **Bai Z-Y** and **Liu Q-L**, et al. (2016) Comparative Analysis of the chrysanthemum leaf transcript profiling in response to salt stress. PLoS ONE **11**(7): e0159721.

Zhou S, **Li M**, **Guan Q**, **Liu F**, **Zhang S**, **Chen W**, **Yin L**, **Qin Y** and **Ma F.** (2015) Physiological and proteome analysis suggest critical roles for the photosynthetic system for high water-use efficiency under drought stress in Malus. Plant Sci. **236**: 44-60.