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PHYSIOLOGICAL RESPONSE OF ENDANGERED XEROPHYTE *CAMPANULA SCLEROPHYLLA* (KOLAK.) OGAN. TO REPEATED OSMOTIC STRESS *IN VITRO*

LS SAMARINA*, VI MALYAROVSKAYA, NB PLATONOVA AND KV KLEMESHOVA

Federal State Budgetary Scientific Institution «Russian Research Institute of Floriculture and Subtropical Crops», Sochi, Russia, 354002 J. Fabritsiusa st. 2/28

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Abstract

To survive in the arid conditions with little soil and air precipitations, *Campanula sclerophylla* (Kolak.) Ogan. xerophyte has a set of unique strategy for stress response and stress adaptation. Structural and physiological responses of this xerophyte were studied under *in vitro* induced repeated osmotic stress. Plants were cultured for 2 weeks (for each stress 1 and stress 2) on half MS media supplemented with two mannitol concentrations (100 and 200 mM for treatment I and treatment II, respectively). Results showed that first stress inhibited shoot length, but after the second stress it did not differ from the control in treatment II. On the other hand, root number and root length were strongly inhibited in both stress events in treatment II. Relative water content in leaves in these experiments decreased significantly in treatment II compared to control, and repeated stress showed the same results. However, first stress increased pigment content compared to control, but stress 2 resulted in decreasing it till the level of control. Proline content increased significantly after the stress 2 periods, but there were nonsignificant changes after the stress 1. It is evident that stress 2 needed more compensation of high osmotic pressure comparing to the stress 1 in case of *C. sclerophylla*. Thus results suggest that in *C. sclerophylla* the structural strategy of adaptation to repeated stress is a redistribution of growth processes, but not a complete inhibition of plant growth. On the other hand, pigment content did not decrease during both stresses compared to control indicating stable physiological state during repeated stress.

Introduction

The negative effect of drought, salinity and extreme temperatures is primarily due to a decrease in the osmotic potential, so they all induce similar metabolic changes in plant cells (Jewel *et al*. 2010). As result of numerous studies of plant reactions to osmotic stress, the basic mechanisms underlying their adaptation were revealed. These mechanisms were identified at the genetic, biochemical, cellular, organ and organism levels in many agricultural crops (Li and Liu 2016). Increased synthesis of osmolytes (including proline), changes in the content of soluble proteins, sugars, pigments, and other physiological mechanisms are included in response to osmotic stress (Munné-Bosch and Alegre 2013, Piwowarczyk *et al*. 2014, Li *et al.* 2015, Fleta-Soriano, and Munné-Bosch 2016). In addition, it has been shown that the response of plants to drought and salinity is strongly affected by the genotype (Gupta and Huang 2014, Crisp *et al*. 2016). Furthermore, many plant species showed stress memory mechanisms, usually expressed by less intensive physiological reactions to repeated stress (Li and Liu 2016). The study on the physiological mechanisms of xerophytes adaptation to osmotic stress will provide deeper understanding of plant drought resistance. In recently published researches devoted to the identification of specific drought resistance mechanisms in some xerophytes, a transcriptome and

*Author for correspondence: <samarinalidia@gmail.com>.

proteome analysis was performed in several species from arid habitats (Pang *et al.* 2015, Liu *et al.* 2015). However, there are a few researches on the physiological and structural level published for xerophytes, and the presence of stress memory mechanisms in arid species has not been studied yet.

Campanula sclerophylla (Kolak.) Ogan. (Fam.: Campanulaceae) is a relict endangered species, endemic of the Western Caucasus, with less than 100 plants growing in the canyon of the Mzymta River, in the cracks of limestone rocks. This plant is a typical xerophyte, sicioliophyte, calciphil, lithophyte (Kolakovskii 1995). Adaptabilities of *C. sclerophylla* to drought include a strong root system to provide absorption of any available water, rough leaves with trichomes and to constrain transpiration. For surviving on the rocks with little soil and precipitation, *C. sclerophylla* must have a set of unique strategies for stress response and stress adaptation: perceiving rapidly a stress stimulus, switching on the signal transduction pathways and resulting in physiological changes in the plant cell (Chen and Jiang 2010). The aim of present study was to identify the reactions of this species to *in vitro* induced single and repeated osmotic stress.

Materials and Methods

Campanula sclerophylla microplants were collected from 2-year-old *in vitro* collection from Russian Research Institute of Floriculture and Subtropical Crops. Explants were previously obtained from meristems and propagated on half MS (Murashige and Skoog 1962) medium according to published protocol (Kolomiets *et al*. 2016).

As it was reported earlier that 100 - 200 mM of mannitol in culture medium induces osmotic stress similar to that which occurs during a drought *in vivo* (Watanabe *et al*. 2001). So these concentrations were used in the present experiments. Test medium composition: half MS supplemented with 0.5 mg/l NAA, 20 g/l sucrose, 2.5 g/l phytagel + 100 μM mannitol (treatment I), or 200 μM mannitol (treatment II). 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. The medium was autoclaved for 25 min at 101 Pa and temperature of 110°C. Elongated shoots were cut into (15 mm long small pieces and 4 shoots were placed into test medium per vessel. Explants were cultured in a growth chamber at 16 hrs photoperiod, the light intensity of 3000 lux and a temperature of + 16 - 18^{o}C.

Two series of observations were conducted: after the first two weeks of stress exposure (Stress 1) and after the second two weeks of stress exposure (stress 2). In between all the plants were recovered for 2 weeks in mannitol free medium. Height gain (the difference between the final and initial shoot length), roots number and root length were measured.

The relative water content (RWC) of fresh leaves was calculated by weighing after removal of any moisture attached and expressed as fresh weight. The sample was then dried in an oven at 105^{o}C for one hour and weighed further. This value was considered as dry weight. Relative water content (%) was calculated as follows:

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

Proline in leaves (μg/g fresh leaf weight) was evaluated by simplified ninhydrin method (Shihaleeva *et al*. 2014). The sample contained 100 mg of leaf tissue which was dipped in 5 ml hot distilled water and heated on a water bath for 15 min at 100^{o}C. After that, 2 ml of leaf extract was mixed with 2 ml of glacial acetic acid and 2 ml of acidic ninhydrin. The mixture was heated in a water bath for 20 min. After cooling, the absorbance of the mixture was measured

spectrophotometrically against (PA-5400vi , Russia) at 520 nm and recalculated using the standard formula.

Chlorophyll and carotenoids content of the leaf sample was measured by taking 85 mg of leaf sample and the result has been expressed as mg/g fresh leaf weight. Pigments were extracted using acetone to a final volume of 50 ml. The absorbance of the extracts was measured spectrophotometrically at 662 and 644 nm for chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*), respectively and at 440.5 nm for carotenoids. The concentration of chlorophyll *a*, *b* and total carotenoids were calculated after Shlyk (1971).

The 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) ± Sd (Dospekhov 1985).

Results and Discussion

An addition of 200 mM of mannitol (treatment II) decreased height gain significantly in stress 1 period, but in the stress 2, there was no difference with control (Fig. 1A). In treatment I, there was no difference with control on height gain in both stress periods. The number of roots per explant significantly decreased in treatment II and after the stress 2 root inhibition effect was intensified whilst the average number of roots per explant became 0.5 that is significantly lower than in control (3 roots per explant) and in stress 1 shoots (1 root per explant). In treatment I root number did not differ significantly with control in both stress events. On the other hand, root length significantly decreased in both the treatments I and II compared to control.

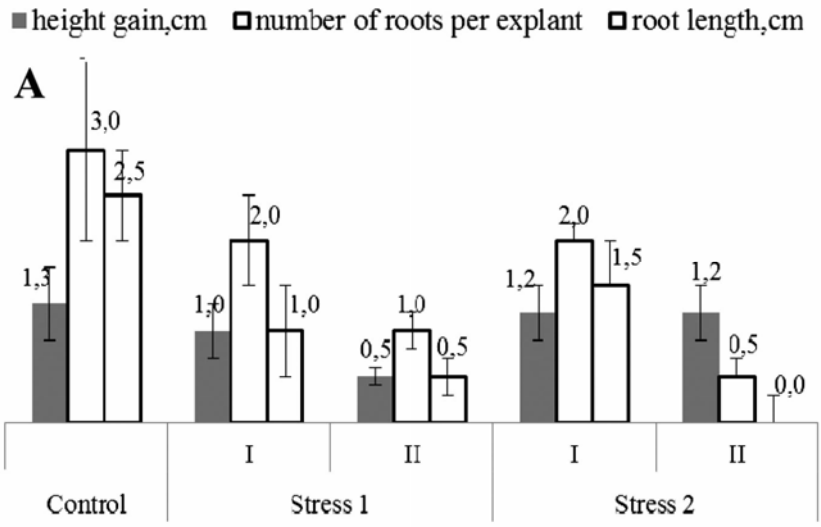

Fig. 1A. Growth parameters of *Campanula sclerophylla* under stresses 1 and 2 on culture media with 100 mM mannitol (I) and 200 mM mannitol (II).

RWC in leaves decreased significantly in treatment II, it was 80 - 85% comparing with the control 93% (Fig. 1B). In this experiment, the difference with control was significant, but there was no difference between stress 1 and stress 2 in RWC. Treatment I was not affected significantly on RWC.

Data on proline content in leaves showed that stress 1 event was not lead to increase its content, there was no difference between two treatments as well as with the control (Fig. 1C). On the other hand, stress 2 event resulted a significant increase of proline content. Its maximum level was observed in treatment II and it was 924.5 µg/g and in treatment I it was 683 µg/g fresh weight compared to the control 397.8 µg/g fresh weight.

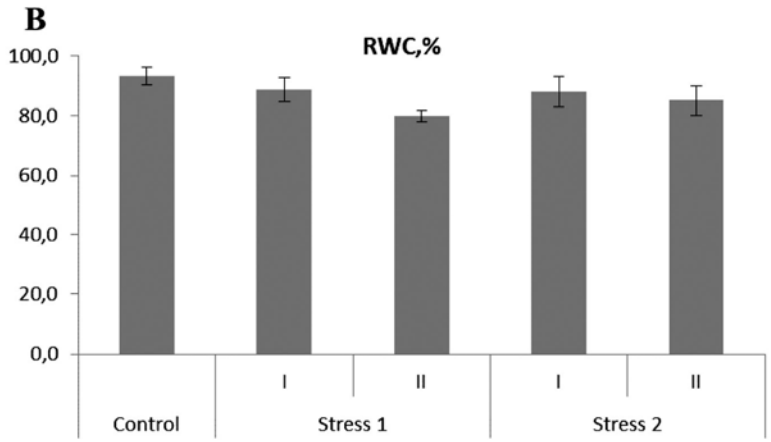

Fig. 1B. Relative water content of *Campanula sclerophylla* under stress 1 and stress 2 on culture media with 100 mM mannitol (I) and 200 mM mannitol (II).

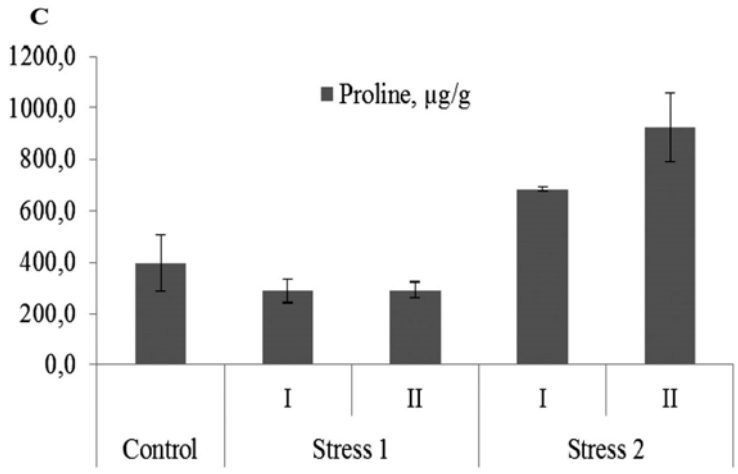

Fig. 1C. Proline content of *Campanula sclerophylla* under stress 1 and stress 2 on culture media with 100 mM mannitol (I) and 200 mM mannitol (II).

Chl *a*, *b* and carotenoid contents of leaves showed an increasing trend in the stress 1 event. There was a significant difference in pigment content between stress 1 and stress 2 events. After the stress 1, chl *a* was 7.1 and 9.9 mg/g in treatments I and II, respectively compared to control 5.4 mg/g. However, after the stress 2, the difference with control became insignificant. Chl *b* increased in the same manner in both the treatments, but after the stress 2, difference with control

became insignificant. In addition, carotenoid content increased to 3.9 - 5.5 mg/g compared to control 2.9 mg/g and this trend was observed in both the treatments. In stress 2 carotenoid also behaved in the same manner as those observed by the chlorophylls. Total chlorophyll to carotenoid ratio and chlorophyll a/b ratio were not affected significantly by any of the treatments.

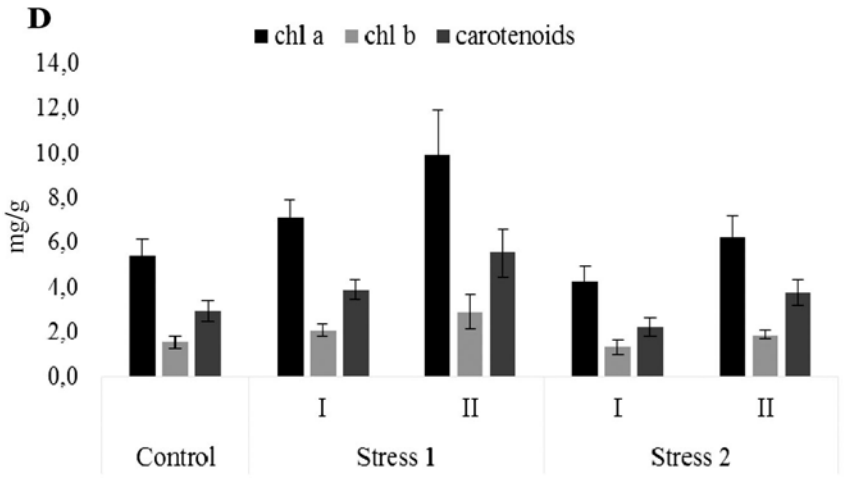

Fig. 1D. Pigment content of *Campanula sclerophylla* under stress 1 and stress 2 on culture media with 100 mM mannitol (I) and 200 mM mannitol (II).

C. sclerophylla is an ancient, evergreen, broad leaved, perennial and endemic plant of semi-arid region of Western Caucasus, Russia. This species is able to survive on rocks without rich soil surface (Kolakovskii 1995). The present research showed that mannitol-induced osmotic stress led to inhibition of the development of the root system, and after stress 2 this negative effect was intensified. The relative water content in the leaves after the first and second stress was not different and was lower than in the control. However, the proline content in the leaves increased sharply after the second stress, compared to the first. On the other hand, photosynthetic pigments and carotenoid levels in leaves increased after the stress 1, but in the stress 2, the differences with the control became insignificant.

In treatment II first stress inhibited shoot length, but after the stress 2, it did not differ from the control. On the other hand, root number and root length were strongly inhibited in both the stresses in treatment II. So it can be assumed that, in *C. sclerophylla*, the strategy of adaptation to repeated stress is a redistribution of growth processes, but not a complete inhibition of plant growth.

In the present experiment, RWC in the leaves decreased significantly in treatment II compared to control but there was no difference between stress 1 and 2. Other results showed stabilization of water metabolism after repeated stress that can be achieved by reducing either transpiration, or the size of the plant, as well as reducing its photosynthetic activity and changes of pigment content (Walter *et al*. 2011).

Results of the present study show that stress 1 led to increase of pigment content in leaves, but stress 2 resulted insignificant difference between treatments and control. The change in pigments composition might be due to the reduction of pigments receptors after stress 2, which helps plants to reduce the content of reactive oxygen species in the chloroplasts in subsequent stress events.

These results correspond with other researchers who reported that chlorophyll content was higher in single-stressed plants than in double-stressed plants (Fleta-Soriano and Munné-Bosch 2016). The increased rate of photorespiration in plant that observed during the onset of drought stress can be seen as an acclimation process to avoid an over-excitation of PS-II under more severe drought conditions (Li and Liu 2016). In cotton, photosynthetic electron transport is promoted during the onset of drought stress due to a higher efficiency of the open PS-II reaction centers (Massacci *et al*. 2008). The proteomic study with a drought-tolerant apple (*Malus domestica* Borkh) cultivar suggested that the main regulatory mechanisms under moderate drought stress included stabilizing photosynthetic electron transfer, and keeping reactive oxygen species at normal level by regulating the photosynthetic electron transfer chain (Zhou *et al*. 2015).

In present study on *C. sclerophylla*, proline content increased significantly after the stress 2 period only, but there were no significant changes after the stress 1. This osmolyte not only acts as a stress indicator, but also among other osmolytes significantly contributes osmotic regulation (Molinari *at al*. 2007). Other studies showed that after the stress 2 period, proline content increased less then after the stress 1. Authors explained that there was improvement of osmotic adjustment and second osmotic stress increased of water use efficiency by plant (Fleta-Soriano and Munné-Bosch 2016). In the present experiments contradictory results and sharp increase of proline after stress 2 may be that stress 2 needed more compensation of high osmotic pressure comparing to the first stress event in *C. sclerophylla*.

In the conclusion, the present research is the first of its kind on the structural and physiological mechanisms related to osmotic stress response and stress memory of endangered endemic xerophyte *Campanula sclerophylla*. The results demonstrated that this species have specific drought-resistance pathways on structural and physiological levels. Second stress reactions was not typical as in many other previously studied species, in which first stress event leaded stronger structural and physiological effect than second stress event. Results obtained during this study indicated that the xerophytes is interesting for further deep investigation of drought resistance and stress memory mechanisms.

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