Contribuții Botanice, *XLII*, **2007**
Grădina Botanică “Alexandru Borza”
Cluj-Napoca

PHYSIOLOGICAL REACTIONS OF THE SUCCULENT CAM PLANT *BRYOPHYLLUM DAIGREMONTIANUM* TO INCREASED SALINITY

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Abstract: Increasing salinity represents a considerable environmental stress for plants, mainly by causing water deficit and by ion toxicity due to Na^+ accumulation. Salt tolerance that develops during acclimation to salt stress relies mainly on the synthesis of osmoprotectants, on sequestration of Na^+ in the vacuoles and in the apoplast, as well as on the enhancement of antioxidative protection. Because drought resistance may interfere with salt stress tolerance, a succulent non-halophyte (*Bryophyllum daigremontianum* Ham. &Perr.) was selected to study salt stress reactions on the background of drought resistance. The plants originating from the auxiliary buds formed on the edges of a leaf of the mother plant were grown in an environmental test chamber, under controlled light, temperature, humidity and photoperiod conditions, and exposed for 4 weeks to 150 mM and 300 mM NaCl in ¼ strength Hoagland nutrient solution, used to water the sand of the pots. Changes in the water content of leaves, in stomatal conductance and stoma density of newly developed leaves, in the photosynthetic pigment content, in the chlorophyll a/b ratio and in the parameters of induced chlorophyll fluorescence offer an insight into the molecular events associated with physiological reactions to salt stress in a succulent non-halophyte with intense clonal reproduction, able to populate saline habitats. The incomplete resistance of this succulent non-halophyte to dehydration caused by increased salt concentration is compensated by a reduced stomatal density of the leaves and by a decrease in transpiration rate during acclimation to salt stress. The decreased potential photosynthetic quantum use efficiency of the leaves is partly compensated by an elevated light-harvesting capacity ensured by an increased amount of chlorophyll molecules associated with the photosystems. Resistance to desiccation is combined with tolerance of chemical toxicity induced by long-term treatment with high salt concentration, in order to reestablish a suitable water balance and photosynthetic efficiency.

Keywords: Induced chlorophyll fluorescence, photosynthetic pigments, physiological acclimation, potential quantum yield efficiency, salt stress, stomatal conductance, succulent non-halophyte.

Introduction

Salinity is a major environmental factor limiting plant growth and productivity. Nearly 20% of the world’s cultivated area and nearly half of the world’s irrigated lands are affected by high salinity [4, 9]. There are human influences, other than irrigation, that lead to adverse effects of secondary salinization, such as: overgrazing, deforestation in semihumid and semiarid areas, contamination with chemicals and accumulation of airborne or waterborne salts. The earliest response of a vascular plant to salt stress at the whole-organism level is a reduction in the rate of leaf surface expansion. Under salinity stress, the dry weight to fresh weight ratio of many glycophytes will increase as a result of osmotic adjustment [1, 17, 24].

Mechanisms of salt tolerance include: a) selective accumulation or exclusion of Na and Cl ions; b) control of ion uptake by roots and transport into leaves; c) compartmentalization of sodium and chloride ions; d) synthesis of compatible solutes; e) changes in photosynthesis and gas exchange; f) alteration in membrane structure; g) induction of antioxidative enzymes and other protective proteins; h) induction of plant growth regulators [2, 19]. The mechanism of intra-plant allocation is characteristic for many halophytes, which, due to the limited transpiration, can keep excess of salt within their roots and lower parts of the shoot, thus preventing salt accumulation in the photosynthetically active young leaves. Partial stomatal closure under high salinity is induced by the presence of sodium ions in the apoplast surrounding the guard cells, causing a reduction in rates of transpiration and increase of water use efficiency [3, 12, 20].

The loss of water and the invading ions activate a concerted acclimation process that leads to salt-tolerant cells with a new steady state of growth. This acclimation process includes three basal processes: 1. restoration of turgor, 2. regulation of the uptake and export of ions through the cell membranes, and 3. induction of the accumulation of osmoprotecting compatible solutes and stress proteins. These molecules ensure the protection and renaturation of damaged functional and structural proteins, nucleic acids and membrane lipids [10, 18]. Besides these main processes, several secondary responses are needed to ensure a successful salt tolerance, e. g. the scavenging of liberated free radicals, increase in energy-supplying reactions and, finally, the adjustment of the whole metabolism to the new situation. All these efforts are realized with an increased energy consumption and require an adequate photon flux density and photochemical conversion to cope with the stressful conditions [5, 7, 13].

Plants are often more salt tolerant during germination, become more sensitive during the emergence and young seedling stage, and exhibit enhanced tolerance through the reproductive stage with the exception of flower formation. Salt-affected plants appear darker green and are stunted, with shorter and fewer internodes. Succulence, defined as a high water content per unit leaf surface area, is a typical morphological response to salinity in dicotyledonous species but is rarely observed in the monocots [11, 12, 16]. To achieve salt tolerance, the task is either to prevent or alleviate the damage, or to re-establish homeostatic conditions in the new stressful environment. Identification of molecular markers linked to salinity tolerance traits has provided plant breeders a new tool for selecting varieties with improved tolerance [15]. Salt stress and dehydration stress show a high degree of similarity with respect to physiological, molecular and genetic effects. Sub-lethal salt stress condition is ultimately an osmotic effect, similar to some extent with drought, cold as well as heat stresses [14, 21, 22].

The aim of the present study is to characterize the capacity of resistance or tolerance to salt stress of a succulent non-halophyte regarding water balance and photosynthetic light use capacity, and to identify overlapping adaptive characteristics in the physiological reactions to drought stress and excessive salinity.

Material and Methods

2.5 months old clones of *Bryophyllum daigremontianum* Ham. & Perr., originating from the auxiliary buds formed on the edges of the same leaf of a mother plant, grown in vegetation vessels with inert sand, were watered at regular periods with identical amounts of ¼ strength Hoagland’s nutrient solution, without sodium chloride (control) or supplemented with 150 mM and 300 mM NaCl (p.a.). The salt treatment lasted for 4 weeks in a Versatile Environmental Test Chamber MLR–351H (Sanyo), where during the 12 hours of daytime temperature was adjusted to 25 °C, relative air humidity was 50% and the photosynthetically active photon flux density was 170 µM photons $m^{-2}s^{-1}$, while during the 12 hours of darkness the temperature was 18 °C and the relative humidity was 70%. The diurnal photoperiodism was ment to ensure normal developmental conditions for this obligate CAM species [11].

The water content of leaves developed on the 4th node from the base of the stem was determined as the difference between the fresh weight and the dry weight measured after 48 hours of desiccation at 80 °C in an electric dryer, and expressed as percent of the fresh weight [16]. The rate of transpiration (conductance for water vapor of the lower surface of leaves) was determined both during daytime and in darkness with an AP4 diffusion porometer (Delta-T Devices, UK), using the third leaf from the apical bud. Stomatal density of the leaves developed during the salt treatment was evaluated by peeling the lower epidermis of the leaf blades and counting the stomata of 10 different regions of the native slides under the optical field of known area of a microscope (magnification 100X).

The photosynthetic pigment content of the young mature leaves was determined spectrophotometrically (with a Jasco V–530 UV-VIS Spectrophotometer, Japan). Leaf discs with a known weight (between 0.1 and 0.3 g) were immersed in 10 ml dimethylformamide and kept in darkness at low temperature for 48 hours, in order to achieve a complete extraction of chlorophylls and carotenoids from intact tissue samples. The absorbance of the centrifuged extracts was measured at 663.8 nm, 646.8 nm and 480.0 nm [5, 6]. The potential quantum yield efficiency of photosystem II was evaluated by determining the ratio between the variable chlorophyll fluorescence (Fv, representing the difference between the ground fluorescence and the maximal fluorescence) and the maximal chlorophyll fluorescence (Fm) induced in leaves that were dark-adapted for 15 minutes before the induction of chlorophyll fluorescence with a photosynthetic efficiency analyser (MK2 PEA Hansatech, UK) [8].

All experiments were performed in 5 replicates. Data presented are the means ± standard deviation. Significant differences between controls and salt-treated samples were determined by the LSD test performed after the one-way ANOVA, and P values < 0.05 were considered significant [23]. Statistical analyses were performed with the SPSS 8.0 program for Windows.

Results and Discussion

Bryophyllum daigremontianum is a xerophyte with succulent leaves, which exhibits an obligate, but plastic Crassulacean Acid Metabolism, opening its stomata during the night and accumulating large amounts of vacuolar malic acid to deposit the fixed carbon dioxide until daytime. Under conditions of sufficient water supply, this plant is able to open its stomata also during daytime, when the carbon dioxide is directly incorporated into carbohydrates in the Calvin cycle. Whenever water stress installs, transpiration is reduced by opening of the stomata only during the night. Drought stress can be induced not only by water deficit in the environment, but also by low temperatures and by high salt concentration. This is the reason why drought stress tolerance has many common features with chilling stress- and salt stress-tolerance. Succulent plants are typically resistant to water stress, because they are able to avoid desiccation and to maintain a high internal water content. Drought resistance may ensure in the same time resistance to high salinity, but salt stress has not only an osmotic, but also a chemical component, leading to toxicity by inhibition of metabolic processes. Under the condition of a long-term exposure to salt stress represented by 150 mM and 300 mM NaCl in the nutrient solution used to water the sand in which the plants were grown, the water content of mature leaves decreased moderately, but in a statistically significant degree, and this loss of water content was proportional with salt concentration (Fig. 1). This reflects that succulence of this plant does not confer a complete resistance to salt stress, as it does in case of water deficit of the environment.

The development of a physiological tolerance may compensate for the incomplete resistance of the water regime against high salinity stress. This tolerance is achieved on a long-term scale by reducing the stomatal density on the surface of the newly developing leaves, and on a short-term scale by regulation of the intensity of stomatal transpiration. In the lower epidermis of the young leaves formed during the period of salt stress, the density of stomata decreases significantly both in the presence of 150 mM and 300 mM NaCl (Fig. 2). This suggests that the plant is able to adjust its morphogenetic characteristics related to water balance, in order to ensure a more suited structural basis for a reduced water loss by transpiration under the circumstances when the high salt concentration of the soil water exerts a dehydrating effect on the organism. From more than 100 per mm^2, the number of the anisocytic stomata decreased in the presence of salt stress to below 80 per mm^2 of lower leaf surface.

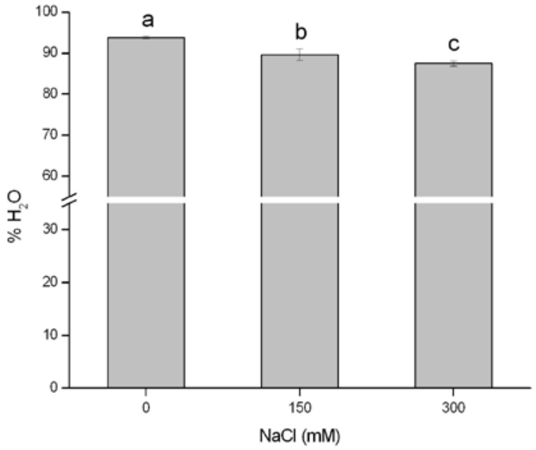

Fig. 1: Water content of the leaves of *Bryophyllum daigremontianum* exposed for 4 weeks to salt stress represented by 150 mM and 300 mM NaCl (0 – control, watered with ¼ Hoagland nutrient solution without sodium chloride). Vertical bars represent standard deviation of average of 5 replicates. Columns marked with different letters are significantly different from each other (P=0.05) according to the post-hoc LSD test

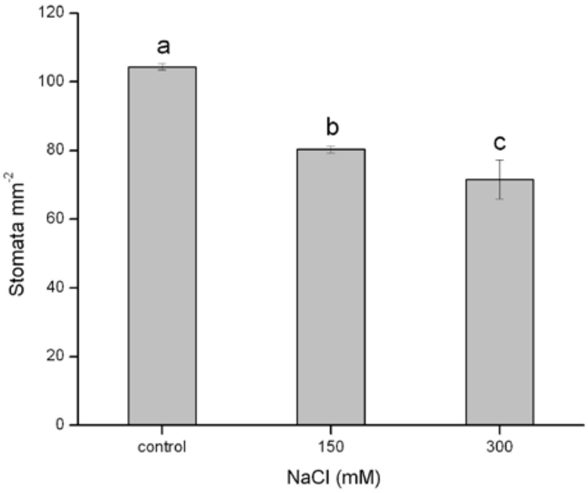

Fig. 2: Density of stomata in the lower epidermis of young leaves developed during the 4 weeks period of exposure to salt stress of *Bryophyllum daigremontianum*. Vertical bars represent standard deviation of average of 5 replicates. Columns marked with different letters are significantly different from each other (P=0.05) according to the LSD test

Being a plastic CAM plant, *Bryophyllum daigremontianum* is able to open its stomata and to take up carbon dioxide both during the period of illumination and during the night. When it is well watered and no salt stress is present, the intensity of transpiration is higher during illumination than in darkness, reflecting that stomata are widely open during daytime. If there is no water shortage, this enables a more efficient fixation of carbon dioxide in the presence of light energy. When salt stress induced water deficit in the plant, the transpiration rate is pronouncedly reduced in the presence of light, but this reduction is not significant in darkness (Fig. 3). This suggests that salt stress enforces the onset of the typical CAM behavior in order to reduce water deficit even if the carbon assimilation becomes less efficient. Under the influence of both salt concentrations applied in the experiments, the transpiration of leaves drops from around 12 mM water $m^{-2}s^{-1}$ to 2 mM water $m^{-2}s^{-1}$. This is an important component of the physiological acclimation of this succulent non-halophyte to high salinity, and the two different salt concentrations induce the same degree of reduction in transpiration rate. On a long-term scale, the diurnal adjustment of stomatal opening, in accordance with the degree of water deficit, and the developmental changes occurring in the stomatal density of leaves, are able to ensure salt tolerance with respect to the maintenance of a sufficient water content, in order to avoid dehydration.

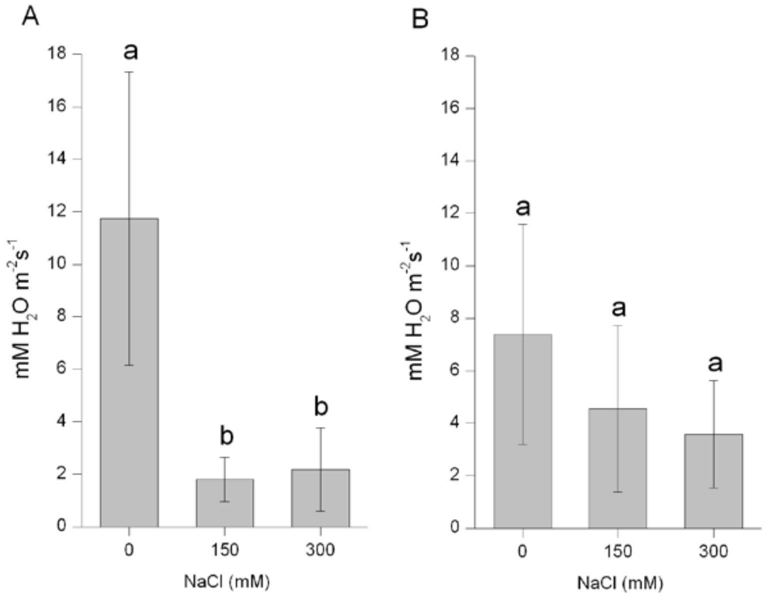

Fig. 3: Transpiration rate of leaves of *Bryophyllum daigremontianum* during daytime (A) and in darkness (B), under the influence of different concentrations of NaCl in the nutrient solution. Vertical bars represent standard deviation of average of 5 replicates. Columns marked with different letters are significantly different from each other (P=0.05) according to the post-hoc LSD test

Survival and growth under salt stress requires not only a well regulated water status, but also an adequat photosynthetic biomass production and energy supply. In this context, the photosynthetic pigment content of the leaves and the efficiency of the conversion of the absorbed light energy into useful chemical energy were investigated in the plants exposed to two degrees of salt stress. Although in different other experiments a significant decrease of chlorophyll content was reported in a variety of plant species exposed to salt stress [3, 5, 16], our findings show that in *Bryophyllum daigremontianum* plants exposed for a longer period to salt stress exerted by 150 mM and 300 mM NaCl, the amount of chlorophylls and carotenoid pigments increased significantly (Fig. 4).

The increment of chlorophyll-*a* and chlorophyll-*b* content was approximately identical in the presence of 150 mM NaCl, while in the plants treated with 300 mM NaCl the chlorophyll-*b* content increased more than the chlorophyll-*a* content of the leaves. This is the reason why the chlorophyll *a*/*b* ratio decreased significantly only in the presence of 300 mM NaCl (Fig. 5). Because the peripheral part of the light-harvesting pigment-protein complexes in the thylakoid membranes has more chlorophyll-*b* molecules than the internal antenna (especially in photosystem II), the unchanged chlorophyll *a*/*b* ratio suggests that not the size, but the number of antennae (associated with the photosystems) is increased in the presence of 150 mM NaCl, while in the plants exposed to 300 mM NaCl the existing antennae become more extended, and the larger peripheral region possesses more chlorophyll-*b* molecules related to the chlorophyll-*a* molecules, resulting in a decreased chlorophyll *a*/*b* ratio.

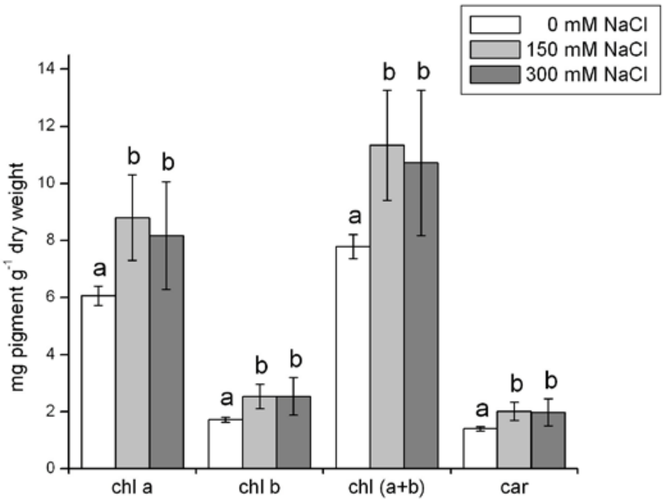

Fig. 4: Photosynthetic pigment content of leaves of *Bryophyllum daigremontianum* exposed to different concentrations of NaCl (car – carotenoids; chl – chlorophyll). Vertical bars represent standard deviation of average of 5 replicates. Columns marked with different letters are significantly different from each other (P=0.05) according to the LSD test

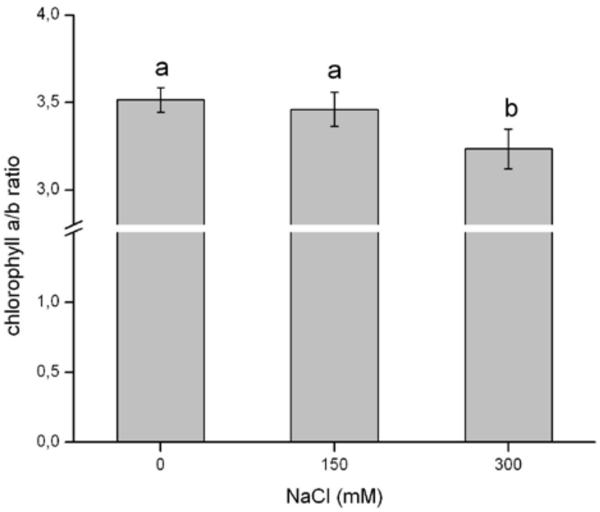

Fig. 5: The chlorophyll *a/b* ratio in leaves of *Bryophyllum daigremontianum* plants exposed to salt stress. Vertical bars represent standard deviation of average of 5 replicates. Columns marked with different letters are significantly different from each other (P=0.05) according to the post-hoc LSD test

The reason why the plants increased their photosynthetic pigment content as a response to salt stress becomes more obvious when the changes in the photochemical efficiency of the conversion of the absorbed light energy is examined. This can be achieved with the method of induced chlorophyll fluorescence, which is largely used as a non-destructive and sensitive tool to examine the influence of different environmental stress factors on the photosynthetic performance of plants [8]. From among the many parameters determined with this method, a very useful one is the potential quantum yield efficiency of photosynthesis (in photosystem II), reflected by the value of the Fv/Fm ratio. Even if only a smaller part of the sodium chloride reaches the leaves of this plant from the nutrient solution used to water the sand around the roots (data not shown), increased salinity causes a slight, but statistically significant decrease in the efficiency of conversion of light energy into chemical energy that can be used in carbon assimilation (Fig. 6). This decrease in the Fv/Fm ratio below 0.8 is proportional with the concentration of sodium chloride used to induce salt stress. In this context, the increment of the amount of light-absorbing photosynthetic pigments may be a compensatory reaction to the reduction in light use efficiency, as part of the salt tolerance reaction regarding the regulation of the functional state of the photosynthetic apparatus.

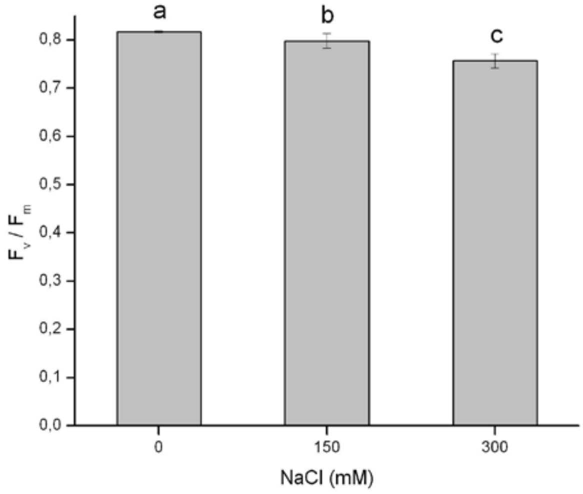

Fig. 6: Potential quantum yield efficiency of photosynthesis reflected by the ratio between the variable and the maximal chlorophyll fluorescence (Fv/Fm) induced in dark-adapted leaves of *Bryophyllum daigremontianum* plants watered with Hoagland's nutrient solution containing different concentrations of sodium chloride. Vertical bars represent standard deviation of average of 5 replicates. Columns marked with different letters are significantly different from each other (P=0.05) according to the LSD test

Reduction of the transpiration rate and of the stomatal density of the leaves compensates for an incomplete resistance to dehydration caused by high salt concentration, while the increased photosynthetic pigment content partly compensates for the functional damages caused by salt stress to the photochemical reactions of photosynthesis. These processes that develop during the acclimation of *Bryophyllum daigremontianum* to increased salinity of the environment are able to ensure a certain degree of tolerance needed for a better survival and development in habitats affected by salt stress. A better understanding of salt stress tolerance of plants that are drought resistant may reveal overlapping protective mechanisms under different environmental stress conditions, while extending the studies directed to identify physiological and biochemical markers of salt tolerance or salt resistance will enable researchers to use plants for an efficient phytoremediation of habitats affected by increased salinity.

Conclusions

Succulence of the leaves of *Bryophyllum daigremontianum* does not confer resistance to salt stress as it does in case of drought stress. The water content of leaves decreases moderately, but proportionally with the increment of salt concentration to 150 mM and 300 mM in the nutrient solution used to water the sand of the vegetation vessels. The incomplete resistance to dehydration caused by high salinity is compensated by a reduced rate of transpiration, and as a result of long-term acclimation, by a decreased density of stomata in the lower epidermis of young leaves developed during the period of salt stress. This reflects that physiological tolerance compensates for insufficient adaptive resistance of the water regime under the influence of increased salinity.

Only a small part of the external salt content reaches the photosynthetic tissue of leaves, but if increased amounts of sodium chloride enter the chloroplasts, a decrease in the quantum yield efficiency of photosystem II occurs. The lower efficiency of the photochemical reactions is reflected by a smaller value of the Fv/Fm ratio resulting from the induced chlorophyll fluorescence measured in dark-adapted leaf samples. The impairment of light energy conversion is partly compensated by an increased amount of photosynthetic pigments responsible for the harvesting of photon energy.

Salt tolerance reflected in the water regime and in the photosynthetic capacity of this plant species, as well as its growth intensity and its capacity to reproduce vegetatively with auxiliary buds, makes it a potential candidate for introduction in plant communities suited for phytoremediation of arid and semiarid habitats affected by increased salinity. In this context of halotolerance, further investigations will be performed in order to establish the antioxidative protective capacity of this plant, destinated to cope with generation of increased amounts of reactive oxygen species as a consequence of high salinity stress.

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REACŢII FIZIOLOGICE ALE PLANTEI SUCULENTE DE TIP CAM *BRYOPHYLLUM DAIGREMONTIANUM* LA EXCESUL DE SALINITATE

(Rezumat)

Datorită aridizării climei şi irigaţiilor excesive, terenuri tot mai extinse devin afectate de sărăturare, iar majoritatea plantelor terestre sunt sensibile la concentraţia salină ridicată a soluţiei apoase a solului. Ca urmare, studiul efectelor fiziologice ale salinităţii excesive în vederea identificării speciilor şi varietăţilor de plante halotolerante prezintă o importanţă practică în remedierea habitatelor afectate de stresul salin. Scopul prezentului studiu este identificarea unor parametri fiziologici prin care se poate determina gradul de halotoleranţă a plantelor, în cazul de faţă la o specie rezistentă la uscăciune (xerofită suculentă), dar nehalofilă. În acest fel se poate studia interrelaţia dintre adaptarea la stresul hidric şi acomodarea funcţională la stresul de salinitate de diferite intensităţi. Având în vedere că salinitatea crescută are efect deshidratant şi contracararea acestuia necesită un surplus energetic provenit din fotosinteză, s-au studiat modificările induse de stresul salin la nivelul regimului hidric şi al proceselor fotochimice ale fotosintezei. Clone de *Bryophyllum daigremontianum* Ham. & Perr., obţinute din mugurii adventivi de pe marginile unei frunze ale unei singure plante, au fost crescute în cameră de vegetaţie cu iluminare, temperatură şi umiditate reglabile, ţinând cont de necesităţile specifice pe timp de zi şi de noapte ale speciilor xerofile suculente cu asimilaţia carbonului de tip CAM. Plantele crescute în vase de vegetaţie cu nisip inert au fost udate regulat cu soluţie nutritivă Hoagland fără adaos de sare (martor), respectiv cu 150 mM şi 300 mM clorură de sodiu chimic pură, o perioadă de 4 săptămâni. Pentru detectarea efectelor stresului de salinitate asupra regimului de apă al plantelor, s-a urmărit prin determinări porometrice, dinamica conductanţei stomatice pentru vaporii de apă, în combinaţie cu determinarea conţinutului hidric al frunzelor şi cu măsurarea densităţii stomatice la nivelul epidermei inferioare a frunzelor tinere dezvoltate în cursul tratamentului salin. În paralel s-a determinat randamentul cuantic al utilizării energiei fotonice în fotosinteză, utilizându-se metoda fluorescenţei clorofiliene induse, metodă foarte sensibilă pentru investigarea perturbărilor funcţionale legate de utilizarea energiei luminii. În aceleaşi frunze în care s-a urmărit intensitatea transpiraţiei şi fluorescenţa clorofiliană, s-a determinat spectrofotometric şi cantitatea principalilor pigmenţi fotosintetici, utilizându-se extracte cu dimetilformamidă. S-a constatat că suculenţa, care conferă rezistenţă totală faţă de stresul hidric, asigură doar o rezistenţă parţială la acţiunea de deshidratare a excesului de sare, însă aceasta este compensată de dezvoltarea toleranţei la salinitate prin reducerea densităţii stomatice pe frunzele formate şi prin scăderea intensităţii transpiraţiei foliare. Acea parte a excesului de sare care pătrunde în cloroplastele celulelor asimilatoare ale frunzelor, provoacă o scădere moderată a randamentului cuantic potenţial al proceselor fotosintetice, scădere care este parţial compensată de creşterea cantităţii pigmenţilor clorofilieni responsabili de captarea energiei fotonice. Raportul dintre clorofilele *a* şi *b* arată că această creştere are loc în primul rând prin mărirea numărului de sisteme fotochimice şi nu prin extinderea sistemului de pigmenţi antenari din jurul sistemelor fotochimice existente. Prin combinarea investigaţiilor efectuate şi prin completarea lor cu parametri fiziologici referitori la osmoprotecţie, la sistemul de protecţie antioxidativă şi la capacitatea de bioacumulare a ionilor de sodiu, devine posibilă o caracterizare complexă a comportamentului adaptiv al plantelor în condiţiile stresului de salinitate, indicatorii fiziologici-biochimici ai halotoleranţei fiind utili în identificarea plantelor capabile de dezvoltare şi propagare în habitatele afectate de salinitate crescută.

Received: 31.05.2007; Accepted: 15.12.2007