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Effects of salt on selected bryophyte species tested under controlled conditions

Marija Ćosić*, Milorad M. VUJIČIĆ, Marko S. SABOVLJEVIĆ and Aneta D. SABOVLJEVIĆ

University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden, Takovska 43, 11000 Belgrade, Serbia

* correspondence: marijac@bio.bg.ac.rs

ABSTRACT:

Bryophytes inhabit all ecosystems on the Earth except seas. Thus, it is commonly accepted to ask are there real halophytes among bryophytes? However, some species do inhabit salty grassland or even brackish waters. Not much research has been done on the physiological reaction of moss species to salt stress. In order to study these responses, we selected three moss species, two of which are considered to be halophytes—*Entosthodon hungaricus* (Funariaceae), *Hennediella heimii* (Pottiaceae) and the non-halophytic model moss *Physcomitrella patens* (Funariaceae) and tested salt effects on them in controlled conditions. The idea was to show if there is tolerance to salt in the selected moss species and to document it if there is a difference in salt tolerance among them. Established *in vitro* moss cultures of gametophores were used to test various developmental parameters for the selected moss species. Morpho-developmental parameters (secondary protonema diameter and the index of multiplication) and biochemical parameters (pigment content and antioxidative capacity) were analysed in relation to salt concentration and time of exposure. All of the tested moss species tolerated salt stress to some extent and during some time of exposure to it. Recovery after salt stress depended both on the concentration of salt and duration of the stress. The three tested moss species did not show similar patterns of response to salt stress.

Keywords:

salt stress, mosses, *Entosthodon hungaricus*, *Hennediella heimii*, *Physcomitrella patens*

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INTRODUCTION

Plants, being sessile organisms, are continuously exposed to variable environmental conditions. Currently, one of the major abiotic factors that reduce agricultural productivity worldwide is salt stress (MUNNS & TESTER 2008). Increased salt content in the substrate has an adverse effect on plant growth and development. During the long course of evolution, plants developed the ability to cope with changes in their habitats. They evolved various mechanisms of tolerance. Plants can respond to stress through anatomical and morphological adaptations, but the most interesting discovered responses are at the biochemical and molecular levels (OLIVER *et al.* 2005; PARIDA & DAS 2005). Salts have various osmotic and ionic effects on vascular plant growth, development and function, but very few data can be found on how salt affects atracheophytic plants (i.e., bryophytes).

Bryophytes are a specific group of higher plants comprised of hornworts, liverworts and mosses. Having no conductive tissue, they belong to the category of non-tracheophytes. Since they were among the first land plants that faced a harsh terrestrial environment, bryophytes had to evolve life strategies that enabled them to survive. Their survival strategies are somewhat different from those of vascular plants. This is mainly due to the absence of quick cell communication through vascular tissue, and it is assumed that bryophytes respond to stresses at the cellular level (OLIVER *et al.* 2000). On the other hand, this feature makes them an ideal model for studying biochemical and physiological responses to stresses (WANG *et al.* 2012). However, compared to vascular plants rather little is known about the tolerance and resistance of bryophytes to salt stress. The available data are related to the moss model system *Physcomitrella patens* (Hedw.) Mitt. (MINAMI *et*

al. 2003; FRANK *et al.* 2005). As the genome of *P. patens* has been sequenced (RENSING *et al.* 2008), many genomic and proteomic analyses are carried out using this species (WANG *et al.* 2008, 2012; KHRAIWESH *et al.* 2015). There are few data on the tolerance of other bryophyte species to salt stress (ĆOSIĆ *et al.* 2019). Many biochemical parameters in plants can demonstrate the stage of stress within the plant organisms. These include the content of photosynthetic pigments, which is also very important in studies of salt stress in vascular plants, and that of phenolic compounds, which indicates the antioxidative capacity of plants. A significant difference in the synthesis of phenolic compounds is evident between salt-stressed and non-stressed plants (LIM *et al.* 2012). It has been shown that plants grown on media with increased salinity synthesise phenolics in higher concentration than those grown on non-saline media (NAVARRO *et al.* 2006). The rate of photosynthesis and the process itself are especially sensitive to salt stress, as salt ions rupture thylakoid membranes, inactivate enzymes, break down chlorophyll and disrupt the electron-transport chain (CHAVES *et al.* 2009). Chlorophyll retention under salt stress can be used as a biochemical parameter to describe the salt tolerance of plant species (PARIDA & DAS 2005).

Although bryophytes are generally considered to be non-halophytes, some species can tolerate high salt content in the substrate (SABOVLJEVIĆ & SABOVLJEVIĆ 2007; ĆOSIĆ *et al.* 2019). Bryophytes can be divided into three groups based on their ability to grow on saline substrates: obligatory halophytes, which grow exclusively on saline substrates; facultative halophytes, which can survive on saline substrates, but grow and develop better on substrates without salt or with low salt concentration; and indifferent species, which can grow on saline substrates as successfully as on substrates without salt (SABOVLJEVIĆ & SABOVLJEVIĆ 2007).

The aim of the present research was to gain insight into salt survival strategies used by the facultative halophyte species *Entosthodon hungaricus* (Boros) Loeske (Funariaceae) and *Hennediella heimii* (Hedw.) Hampe (Pottiaceae) compared to the model species *P. patens*. *Physcomitrella patens* is an ephemeral moss that develops in late summer from overwintered spores and grows on banks of ponds, lakes and rivers that have been exposed by the lowering of water levels (COVE 2005). *Entosthodon hungaricus* and *H. heimii* are both rare and regionally endangered species in different parts of Europe. In ecological terms, they are often taken as indicators of saline lands. These species have been shown to be poor competitors, and they are therefore rarely found growing outside of harsh salt environments, where they are assumed to have survival advantages over non-halophytic plants (i.e., bryophytes) (SABOVLJEVIĆ *et al.* 2018). In order to fill out existing ideas about the responses of these organisms to salt stress, more studies of bryophyte life strategies are needed. There are rather few experimental approaches to the study of salt stress in bryophytes (ĆOSIĆ

et al. 2019). The effects of different NaCl concentrations on morphogenesis and on eco-physiological and biochemical changes in these three species were here studied in fully controlled laboratory conditions.

MATERIALS AND METHODS

Plant material and experimental design. An axenic *in vitro* culture was established and full development of gametophores was achieved. Subsequently, *Entosthodon hungaricus*, *Hennediella heimii* and *Physcomitrella patens* cultures were grown on BCD medium with sucrose added (15.0 g L⁻¹) (SABOVLJEVIĆ *et al.* 2009; SABOVLJEVIĆ *et al.* 2012) prior to experimental tests. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 25 min. The effects of NaCl (0, 5, 10, 50, 100, 300 and 500 mM) on the multiplication index, chlorophyll and carotenoid content and total phenol concentrations of these species were studied. Cultures were grown at 25 ± 2°C under long-day conditions (a 16-h photoperiod), at 47 μmol m⁻² s⁻¹ PFD (provided by cool-white fluorescent tubes). Plants were grown on media with NaCl for 3 days and then transferred to a salt-free BCD medium for up to 3 weeks or alternatively for 3 weeks a on medium containing salts in order to compare short- and long-term effects of salt exposure. Plant material was kept at -70°C after experimentation until the biochemical analyses were performed.

For every treatment, shoot explants were cultured in 90 mm Petri dishes, each containing 10 explants. The multiplication index represents the number of newly grown shoots originating from newly induced buds on secondary protonema derived from one starting shoot.

Analysis of photosynthetic pigments. Chlorophylls were extracted from frozen plant material by applying 96% ethanol and absorbance of the ethanol extract was measured with an Agilent 8453 UV/visible spectrophotometer at three wave lengths: 470 nm, 648 nm and 664 nm. The amount of pigments (chlorophylls and carotenoids) was obtained using the following formulas (LICHTENTHALER 1987):

$C(a+b) = 5.24A_{664} + 22.24A_{648}$, for total chlorophyll content

$Ca = 13.36A_{664} - 5.19A_{648}$, for chlorophyll a content

$Cb = 27.43A_{648} - 8.12A_{664}$, for chlorophyll b content

$C(x+c) = 1000A_{470} - 2.13 Ca - 97.64 Cb / 209$, for total carotenoid content.

Antioxidative capacity. The antioxidative capacity of extracts was measured by the DPPH test using a UV/visible spectrophotometer at 520 nm (BRAND-WILLIAMS *et al.* 1995). Antioxidant activity of the tested materials was expressed as the percentage of free radical scavenging.

Statistical analysis. For every treatment, there were four replications, each representing 10 gametophyte shoots of the same height (7 mm) per moss species. Each experiment

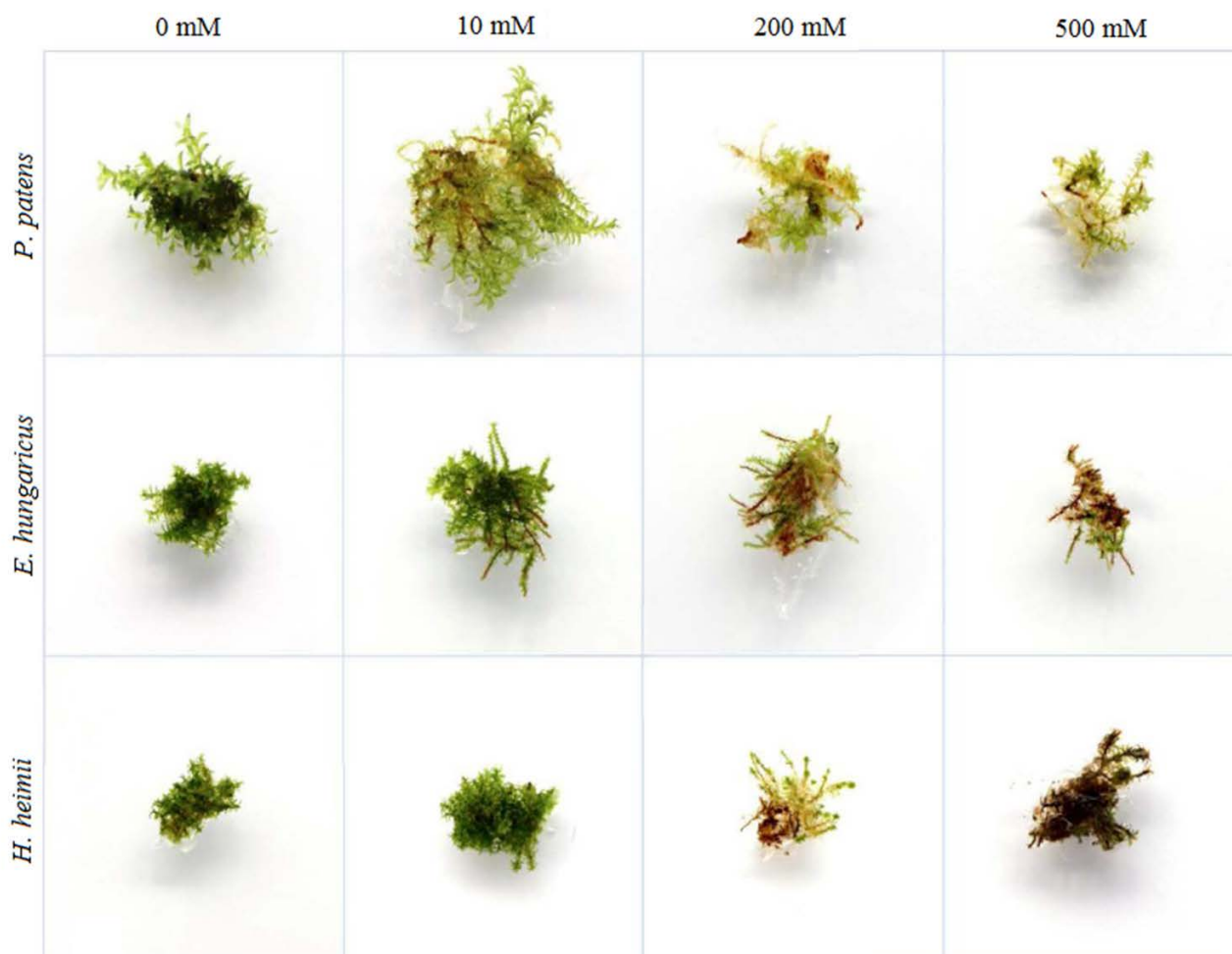


Figure 1. Appearance of tested mosses after three weeks of exposure to media enriched with different concentration of NaCl.

was repeated three times. Data were analysed using Statistica software, version 7 (STSC Inc., Rockville, Maryland, USA), followed by analysis of variance (ANOVA). Separation of mean values was done using Fisher's least significant difference (LSD) test. The term significant was used to indicate differences for which $P \leq 0.05$.

RESULTS AND DISCUSSION

Parameters of morphogenesis. Generally, survival of the tested mosses decreases with increase in the concentration of sodium chloride added to media (Fig. 1). Morphogenetic parameters could not be noted and thus were not measured after short-term salt stress because the time span was too short for us to discern any measurable developmental changes in the treated mosses. In the case of long-term experimental exposure, all of the tested species had a similar pattern of survival in the presence of added salt, i.e., over 90% of the moss plantlets survived up to 100 mM NaCl, while moss survival only slightly decreased at a salt concentration of 200 mM (Table 1). Interestingly, the non-halophytic moss *P. patens* showed better survival (62.7%)

Table 1. Survival (%) of plants after three weeks of exposure to NaCl.

Concentration of NaCl (mM)	<i>P. patens</i>	<i>E. hungaricus</i>	<i>H. heimii</i>
0	100±0.00	100±0.00	100±0.00
5	100±0.00	100±0.00	100±0.00
10	100±0.00	100±0.00	100±0.00
50	100±0.00	100±0.00	93.4±0.62
100	98.0±0.50	98.4±0.40	93.4±1.00
200	73.1±0.06	96.3±0.56	75.8±1.29
300	81.0±0.20	60.81±0.23	33.64±0.33
500	62.7±0.17	11.77±1.06	10.94±1.02

compared to the two halophytic mosses under conditions of the highest salt concentration in the experiments (500 mM) over the course of three weeks. This was more or less expected since it confirms previous results of FRANK *et al.* (2005) that showed *P. patens* to be salt-tolerant species.

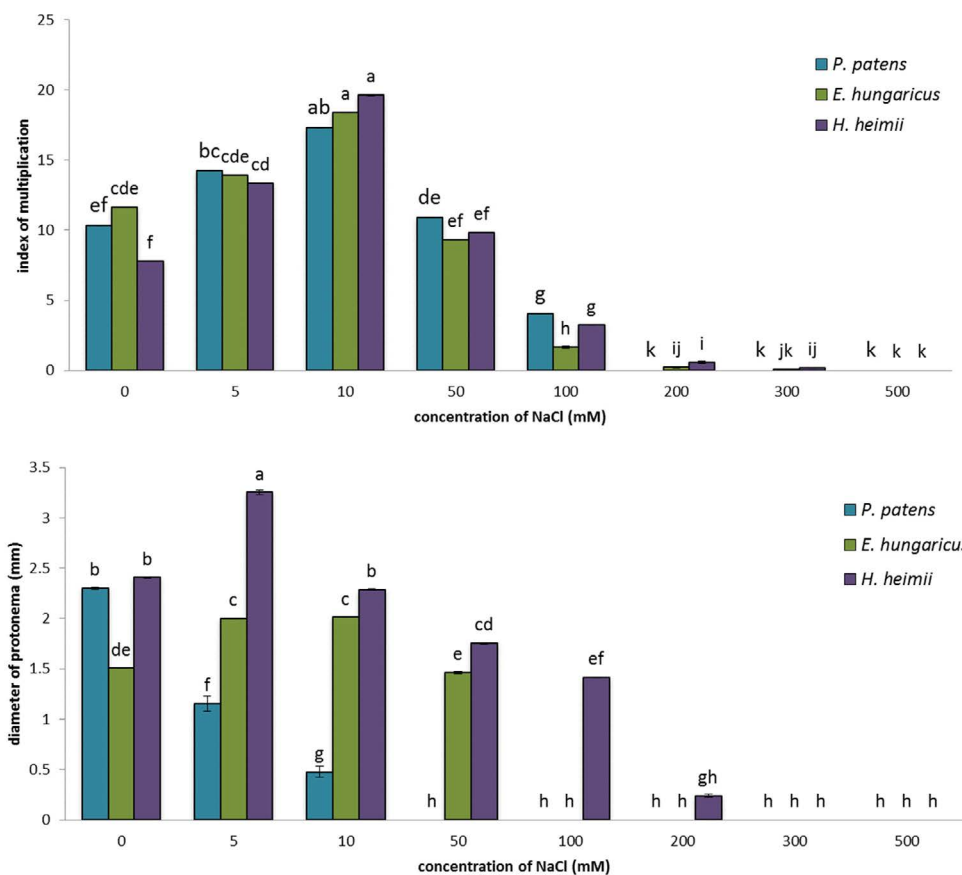


Figure 2. A) Index of multiplication measured after three weeks of exposure to NaCl. B) Diameter of protonema measured after three weeks of exposure to NaCl. The letters above bars indicate statistical similarities vs. dissimilarities among species and treatments.

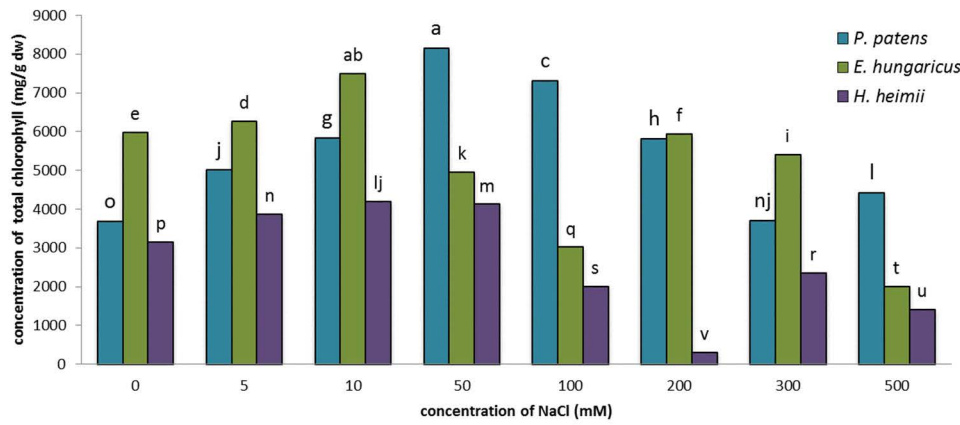
It is probably a consequence of the similar physiological response of *P. patens* to osmotic, salt and drought stresses in accordance with its life strategy. At low NaCl concentrations (5 and 10 mM), a statistically significant increase is recorded in all three of the tested species, as is reflected by the number of newly developed gametophores (shoots) compared to control plants grown on a salt-free medium. Thus, it can be assumed that a relatively low concentration of applied NaCl has a stimulating effect on formation of new buds in the tested species (Fig. 2A). New bud formation, i.e., the index of multiplication, decreased significantly in all three species at concentrations higher than 100 mM.

Development of secondary protonema, i.e., the recorded protonemal diameter, was greatest in *H. heimii* at all tested salt concentrations. However, at NaCl concentrations of 300 mM and 500 mM, secondary protonema development was not recorded in any of the species. Compared to the model moss *P. patens*, it can be seen that the facultative halophytes *E. hungaricus* and *H. heimii* frequently and abundantly formed secondary protonema in the presence of salt concentrations higher than 10 mM (Fig. 2B). The non-halophytic *P. patens* did not form any secondary protonema at salt concentrations higher than 10 mM, suggesting a different survival strategy of this species.

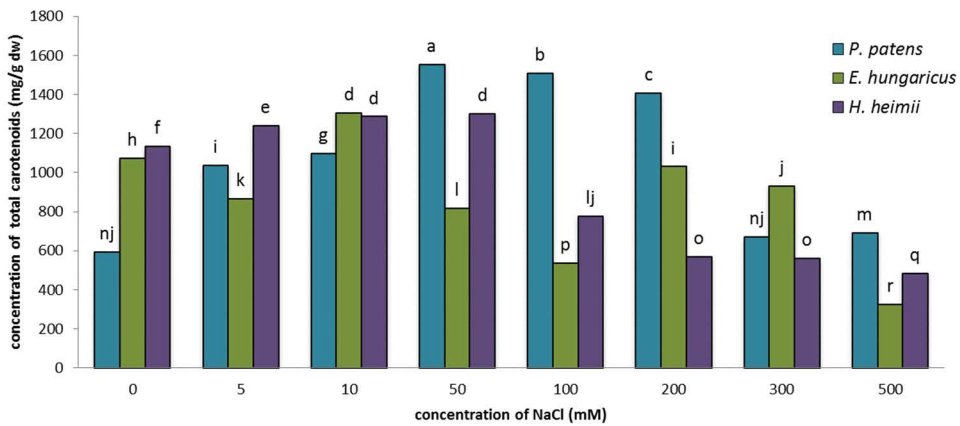
Biochemical parameters could be measured in both short- and long-term experiments.

Total chlorophyll content. Chlorophyll content was affected by an increase of NaCl concentration in all three of the tested species. No general pattern could be seen (Fig. 3A, B). In the case of short-term exposure to salt, none of the three species showed significant suffering, indicating harmlessness of the effect of brief salt stress (if there was any) in all of the tested species. In the case of long-term exposure to salt, decrease of total chlorophyll content can be seen as a general pattern in all of the tested species, especially at an NaCl concentration higher than 200 mM.

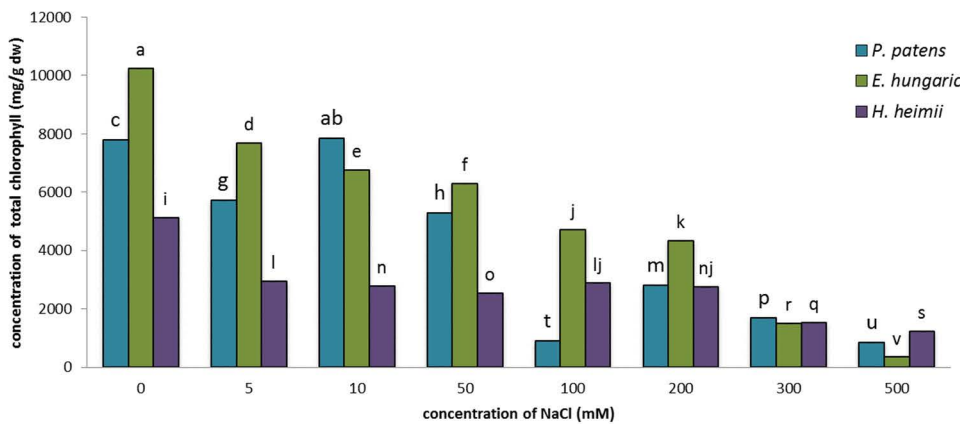
Total carotenoid content. As with total chlorophyll content, short-term stress caused no significant damage to the tested mosses as reflected by carotenoid content, and no common pattern could be observed. In the case of long-term stress, the highest concentration of total carotenoids in *E. hungaricus* was recorded in the control group, and the content of carotenoids decreased with an increase of salt concentration. In *H. heimii* an increase of total carotenoids was detected at NaCl concentrations of 50 mM, 100 mM and 200 mM in comparison with the control plants and in contrast to the pattern seen in *E. hungaricus* and *P. patens*. Even when the highest NaCl concentration was applied, *H. heimii* had the highest carotenoid concentrations compared to the other two species (Fig. 3C, D).



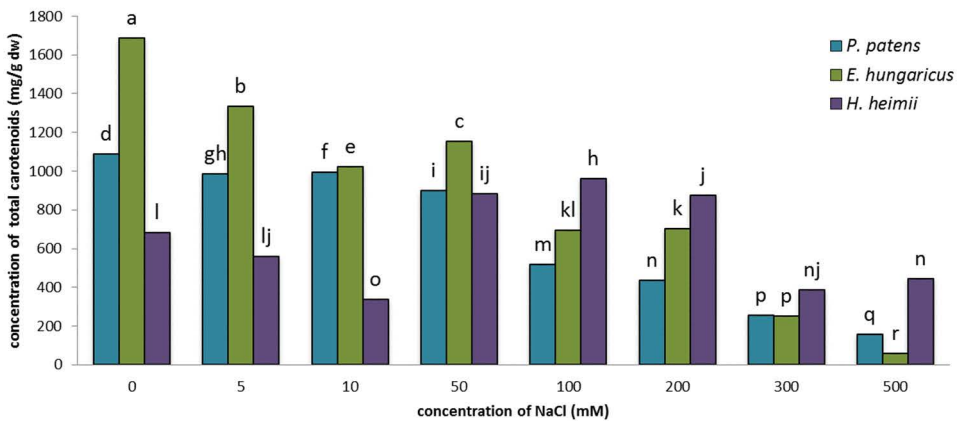
A **Figure 3.** A) Concentration of total chlorophylls measured after three days of exposure to NaCl. B) Concentration of total carotenoids measured after three days of exposure to NaCl. C) Concentration of total chlorophylls measured after three weeks of exposure to NaCl. D) Concentration of total carotenoids measured after three weeks of exposure to NaCl. The letters above bars indicate statistical similarities vs. dissimilarities among species and treatments.



B



C



D

Table 2. DPPH values (%) measured after three days of exposure to NaCl.

Concentration of NaCl (mM)	<i>P. patens</i>	<i>E. hungaricus</i>	<i>H. heimii</i>
0	72.9±0.02	39.0±0.00	30.0±0.00
5	60.5±0.00	47.6±0.00	32.6±0.00
10	52.0±0.00	47.4±0.00	33.2±0.00
50	45.6±0.00	53.7±0.00	35.6±0.00
100	54.9±0.01	60.3±0.02	42.6±0.00
200	22.3±0.03	31.6±0.00	22.3±0.00
300	9.2±0.04	21.3±0.00	23.7±0.00
500	1.6±0.01	7.5±0.00	14.2±0.00

Table 3. DPPH values (%) measured after 21 days of exposure to NaCl.

Concentration of NaCl (mM)	<i>P. patens</i>	<i>E. hungaricus</i>	<i>H. heimii</i>
0	32.9±0.00	20.3±0.00	17.3±0.00
5	37.3±0.00	22.6±0.00	18.9±0.00
10	39.3±0.00	19.6±0.00	22.3±0.00
50	40.9±0.00	24.5±0.00	26.9±0.00
100	44.3±0.00	26.6±0.00	26.5±0.00
200	41.9±0.00	31.6±0.00	35.3±0.01
300	45.6±0.00	33.3±0.00	39.6±0.00
500	52.6±0.00	30.6±0.00	42.9±0.00

Antioxidative capacity. In order to test the antioxidative capacity of plants exposed to stress conditions and seek optimal conditions for each of the three tested species (both halophytic and non-halophytic mosses), the DPPH assay was used to quantify their ability to cope with the salt stress.

In the case of short-term exposure, both halophytic species increased their ability to survive as reflected by their antioxidative capacity, which increased up to the medium salt concentration, while this ability in the non-halophytic species *P. patens* declined with increase of salt concentration, slightly at first and then abruptly (Table 2).

In the case of long-term exposure, surprisingly, antioxidative capacity remained at the same levels as in the control plants in all three species or even increased with salt concentration increase (Table 3).

As expected, salt applied to moss species was harmful. There is rather little evidence about how bryophytes cope with salt stress, tolerate it or even resist it (Ćosić *et al.* 2019). From the available literature, it is known that

halophytic vascular plants can grow and multiply at an NaCl concentration of 300 mM (TESTER & DAVENPORT 2003). Studies on the tolerance of *P. patens* to salt stress indicate that this moss can tolerate salts at a concentration of 350 mM (FRANK *et al.* 2005). However, if the plants are gradually exposed to salts, they can function normally at an NaCl concentration of 600 mM (BENITO & RODRIGUEZ-NAVARRO 2003).

Increased salt concentrations affect many processes in plants, including growth and development. During stress in mosses, there is a decrease in the number of newly formed buds, i.e., reduced development of cauloids, phylloids and lateral branches. A reduced index of multiplication at higher salt concentrations was expected. Plants under stress tend to save energy in order to survive. Energy is invested not in the formation of new branches, but probably in the synthesis of protective compounds.

The index of multiplication was highest in mosses treated with 10 mM NaCl, in which it was higher than in the control group. Given that two of the three investigated mosses grow on saline substrates, these results indicate that such low salt concentrations actually stimulate the formation of new buds. These lower salt concentrations present mild stress for the plant and there is no need for changes in the rate of metabolism. On the other hand, relatively higher salt concentrations prevent the formation of new buds, which was also shown by BOGDANOVIĆ *et al.* (2011).

In general, one would expect that development of protonema is characteristic at low salt concentrations, while high salt concentrations inhibit it. However, protonemal growth in individual plants of *H. heimii* was high at concentrations of up to 200 mM, suggesting a survival strategy based on production of the energy-cheapest vegetative reproductive parts, even developing protonema cells and brachycytes (enabling mosses to survive harsh environmental conditions). This was also the quickest way to move away from the salty environment and try to settle in some less salty place. The species in question has strong development during the rainy and cold season in salty grasslands. The rain washes away high salt concentrations and the water present dilutes the salt for a short period of time. Thus, the strategy is to use resources available for a short period of time as much and as quickly as possible, spending less energy in the process. On the other hand, the non-halophytic moss *P. patens* had no measurable protonema already at an NaCl concentration of 50 mM, while *E. hungaricus* did not form any protonema at 100 mM NaCl, suggesting alternative survival strategies under salt stress in nature. Although the mosses did not form new buds and protonema at NaCl concentrations of 300 mM and 500 mM, there was no absolute growth-inhibitory effect of these concentrations since certain moss plants did survive such conditions. It is assumed that if the treated moss plantlets were further grown on a salt-free medium, they would regenerate since they retained

their viability. Facultative halophytic bryophytes have near-optimal conditions for growth, development and reproduction in a salt-free substrate or one containing low salt concentrations (SABOVLJEVIĆ & SABOVLJEVIĆ 2007).

Reduction of growth and development, as well as physiological changes, depend on many factors, such as salt concentration, the period of time during which plants are exposed to stress and the genotype (PARIDA & DAS 2005; MUNNS & TESTER 2008), an assertion supported by the results obtained in the present study.

Because salt stress affects the photosynthetic apparatus, chlorophyll content can be used as a parameter which reflects tolerance of stress (PARIDA & DAS 2005). Reduction of chlorophyll content during salt stress is a characteristic of stress-sensitive species (DELFINO *et al.* 1999). In stress-tolerant species, there are no drastic changes of chlorophyll content, but an increase can occur in some cases (MIŠIĆ *et al.* 2009).

FRANK *et al.* (2005) showed that there is no complete degradation of chlorophyll in the moss *P. patens* grown at an NaCl concentration of 500 mM. Similar results were obtained for the bryophytes *Atrichum undulatum* and *Bryum argenteum* grown on substrates with different salt concentrations (BOGDANOVIĆ *et al.* 2011). This was also shown for another three moss species in the present study.

In optimal conditions plants have a ratio of chlorophyll a to chlorophyll b of about 3:1, but lower values are present in bryophytes. Deviation from this ratio indicates the presence of stress factors. Decrease of the chlorophyll ratio can be caused by chlorophyll degradation, primarily degradation of chlorophyll a because it is more sensitive to salt stress than chlorophyll b (SINGH & DUNBEY 1995), or by decreased chlorophyll synthesis and changes in thylakoid membranes (KHALEGHI *et al.* 2012). Carotenoids are plant protective pigments whose synthesis occurs during stress. In stress-tolerant species, carotenoid concentrations are higher in situations of more intense stress. Carotenoid synthesis requires more energy than chlorophyll synthesis, so there is no significant change of carotenoid content at lower salt concentrations (AMIRJANI 2011). The increase of carotenoid content during osmotic stress is important for the xanthophyll cycle and its protective role (BORGHESI *et al.* 2011; LIM *et al.* 2012). Bryophytes synthesise a large number of secondary metabolites during their response to stress (ASAKAWA 2007). We can assume that defensive mechanisms based on non-enzymatic components are not equally used in the three species tested here, but further studies are needed to confirm this.

CONCLUSION

Our study of salt stress in three moss species clearly showed that there is some tolerance to salt stress in both the halophytic and the non-halophytic moss species tested. However, they exhibit somewhat different patterns of response to equal and controlled salt stress. They can survive weak-

er and short-term stress rather easily, but express different strategies of survival when longer stress is applied. These findings were documented by measurements of morphogenetical and biochemical parameters, as well as antioxidative capacity. Salt tolerance is known to be present in the non-halophytic moss *P. patens* and unquestionably in the bryo-halophytes *E. hungaricus* and *H. heimii*. However, the last two seem to have different responses, i.e., survival strategies, in such harsh environmental conditions, where they thrive in nature.

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Efekat soli na odabrane vrste briofita u kontrolisanim uslovima

Marija Ćosić, Milorad M. VUJČIĆ, Marko S. SABOVLJEVIĆ i Aneta D. SABOVLJEVIĆ

Briofite naseljavaju sve ekosisteme na planeti osim morskih. Stoga je uobičajeno pitati se da li zaista postoje prave halofite među briofitama. Međutim, neke vrste mogu kolonizovati zaslanjena zemljišta, ali i brakične vode. Zbog svoje male veličine i ne tako velikog ekonomskog značaja, proučavanje fizioloških reakcija mahovina na stres dugo je bilo zapostavljeno. U cilju proučavanja odgovora mahovina na stres izazvan solima, odabrane su tri vrste mahovina, dve halofitne vrste *Entosthodon hungaricus* (Funariaceae), *Hennediella heimii* (Pottiaceae) i nehalofitna model vrsta *Physcomitrella patens* (Funariaceae). Cilj ovog istraživanja je ispitivanje tolerancije na stres izazvan solima kod odabranih vrsta, kao i ukazivanje na međusobne razlike u fiziološkim odgovorima na stres. Analizirani su parametri morfogeneze (prečnik sekundarne protoneme i indeks multiplikacije), kao i biohemijski parametri (sadržaj fotosintetičkih pigmenata) i antioksidativni kapacitet mahovina. Pokazano je da sve tri ispitivane vrste tolerišu stres u određenom opsegu koncentracija natrijum-hlorida. Oporavak mahovina nakon stresa izazvanog solima zavisi od primenjene koncentracije soli i dužine trajanja stresa. Tri ispitivane vrste različito reaguju na stres izazvan solima.

Ključne reči: stres solima, mahovine, *Entosthodon hungaricus*, *Hennediella heimii*, *Physcomitrella patens*