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Original Paper

Comparison of two subspecies of a halophytic multi-use plant *Mertensia maritima in vitro* and *ex vitro*: propagation, salinity tolerance and mineral nutrition

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Abstract

The aim of the present study was to compare morphological and physiological responses to increasing salinity of the two subspecies of *Mertensia maritima* during *in vitro* propagation in two different systems, and subsequent *ex vitro* cultivation. Plants were brought into culture using seeds and further propagated on Murashige and Skoog medium supplemented with thidiazuron and naphthaleneacetic acid either on agar-solidified or liquid medium with a temporary immersion system (Plantform bioreactors). Salinity tolerance were tested both in tissue culture and with *ex vitro* greenhouse-cultivated plants. Low concentration of NaCl increased explant biomass in agar-solidified medium, with no negative consequences even at high concentration. However, the effect of salinity on liquid medium was genotype-specific. Explants in Plantform bioreactors showed several-fold higher biomass in comparison to that on agar-solidified medium, but the multiplication potential was not significantly affected. *Ex vitro* plants were negatively influenced by increasing NaCl concentration in substrate. In contrast to *in vitro* plants, accumulation potential of Na^+ in *ex vitro* plants was low. One of adaptations to salinity at the whole plant level was maintenance of adequate tissue mineral nutrient homeostasis, as mineral nutrient concentrations were not negatively affected by increasing salinity.

Key words: halophyte, ion accumulation, *Mertensia maritima*, mineral nutrition, propagation, salinity, temporary immersion system, tissue culture.
Abbreviations: MS, Murashige and Skoog.

Introduction

The use of tissue culture methods in studies of halophytic plant species is important both from fundamental and practical points of view (Custódio et al. 2022). Development of effective methods of clonal propagation allows to rapidly obtain the required amount of genetically identical plant material for research as well as for purposes of saline agriculture. Cultivation of plant cells and tissues of halophytes in a controlled environment, on the other hand, gives the opportunity to develop various biotechnology applications.

Mertensia maritima (L.) Gray (Boraginaceae) is a perennial species with arctic circumpolar distribution on sand or shingle beaches of coastal shores. The North Atlantic population is known as *Mertensia maritima* subsp. *maritima*, while the North Pacific population is known as *Mertensia maritima* subsp. *asiatica* (Scott 1963). These subspecies are difficult to distinguish morphologically, but it is suggested that plants of subsp. *asiatica* are more robust (Alton, FitzGerald 2009). Natural populations of *M. maritima* are endangered by both climate change and anthropogenic factors (Alton, FitzGerald 2009). Resilience of *M. maritima* plants to highly disturbed conditions of shingle beaches is associated with their morphological adaptations, i.e. ability of annual shoots to form bud-bearing root-producing dimorphic rhizomes, which can persist for several years (Barykina, Alyonkin 2019). As a result of disturbance these rhizome fragments can act as propagules for daughter plant establishment. Mechanical strength of individuals against uprooting by waves and storms is associated with formation of numerous root strands, creating a massive cable-like root structure (Scott 1963).

M. maritima has highly ornamental value, but plants of subsp. *maritima* are rather difficult for cultivation in contrast to the plants of subsp. *asiatica*, which are relatively easy to grow as a garden plant (Alton, FitzGerald 2009). The species has large potential as a source of biologically active metabolites with possible pharmacological importance

(Fedoreyev et al. 2012; Park et al. 2019; Song et al. 2020). Leaves of *M. maritima* have been traditionally used as food (Ager, Ager 1980), and the plant has a great potential for saline agriculture (Farzana et al. 2023).

Propagation of *M. maritima* plants by seed has low efficiency because of a high dormancy rate (Skarpaas, Stabbetrop 2001). Therefore, the use of tissue culture methods in this case seems to be a suitable approach for both scientific and practical needs. Tissue culture allows to assess plant performance at different levels of biological organization: unorganized cellular level (callus cultures), organized tissue level (shoot cultures), and physiologically integrated level (rooted explants). Most importantly, after acclimation to *ex vitro* conditions, genetically homogeneous tissue-culture propagated plants can be used for assessment of the whole-plant level responses. Several studies have investigated the possibility of *in vitro* propagation of *M. maritima* using agar-solidified media (Park et al. 2019; Song et al. 2020), and only one study so far used a temporary immersion liquid culture bioreactor system (Copetta et al. 2021).

As *M. maritima* plants characteristically grow on coastal beaches with a high probability of the impact from seawater, a halophytic nature of the species can be proposed. Indeed, *M. maritima* has been listed in the database of halophytic plants, eHALOPH (Santos et al. 2016). Only recently the effect of salinity on *M. maritima* has been studied in controlled conditions (Farzana et al. 2023). Obligate halophytes require increased salt concentration for optimum growth, but this type of information is not available for *M. maritima*. Most importantly, for the context of plant biotechnology, it is not clear if this requirement also appears in conditions of tissue culture. Several obligate halophyte species indeed require increased medium NaCl concentration for optimum explant growth. These include *Arthrocaulon macrostachyum* (Jurado-Mañogil et al. 2024); *Glaux maritima* (Freipica, Ievinsh 2010), *Salicornia brachiata* (Joshi et al. 2012), and *Sesuvium portulacastrum* (Lokhande et al 2011). However, effect of NaCl on *M. maritima* has not been assessed in tissue culture conditions, and comparison of the two subspecies of *M. maritima* has not been performed in previous studies.

The aim of the present study was to compare morphological and physiological responses to increasing salinity of the two subspecies of *M. maritima* during *in vitro* propagation in two different systems, and subsequent *ex vitro* cultivation.

Materials and methods

Establishment of tissue culture

Seeds of *M. maritima* subsp. *maritima* (*M. m. maritima*) were purchased from AlsaGarden (Strasbourg, France) and those of *M. maritima* subsp. *asiatica* (*M. m. asiatica*) from Jelitto Staudensamen (Schwarmstedt, Germany). Initiation of tissue culture was performed as described previously with some modifications (Purmale et al. 2022b). For surface sterilization, seeds (20 per subspecies) were immersed for 15 min in 15% (v/v) commercial bleach solution Domestos (Unilever, London, Great Britain). After rinsing three times with deionized sterile water (2 min each), the seeds were placed in tubes (55 mL) on agar-solidified (6 g L^{-1}) half-strength Murashige and Skoog (MS) medium (Murashige, Skoog 1962) containing 30 g L^{-1} sucrose, pH 5.8. Explants were placed in a growth cabinet under a 16 h photoperiod provided by fluorescent lamps with photon flux density of 50 µmol m^{-2} s^{-1} of photosynthetically active radiation, 25 °C.

After four weeks, seedling explants were transferred to multiplication medium based on half-strength agar-solidified (6 g L^{-1}) MS medium with g L^{-1} sucrose, pH 5.8. Three combinations of growth substances were compared: (i) 0.5 mg L^{-1} 6-benzylaminopurine; (ii) 1.0 mg L^{-1} thidiazuron and 0.2 mg L^{-1} naphthaleneacetic acid (Copetta et al. 2021); and (iii) 2 mg L^{-1} kinetin and 0.2 mg L^{-1} naphthaleneacetic acid (Song et al. 2020). For cultivation 55 mL tubes were used, one plant in each, with 30 replicates. Cultivation conditions were the same as above. After 4 weeks the experiment was terminated and the number of newly formed shoots per explant was determined.

Effect of NaCl in tissue culture

In the first experiment, effect of increasing concentration of NaCl on growth and multiplication of *M. maritima* explants was assessed using agar-solidified multiplication medium with 1.0 mg L^{-1} thidiazuron and 0.2 mg L^{-1} naphthaleneacetic acid. Different amounts of NaCl were added, with a final concentration of Na in a medium 0, 1.0, 2.5, 4.0; and 5.0 g L^{-1}. Explants were placed in 200 mL plastic jars, five explants per jar, four jars per treatment, for each subspecies of *M. maritima*. Cultivation conditions were the same as above. After four weeks the experiment was terminated and the number of newly formed shoots was determined. Explant biomass was measured before and after drying for 4 days at 60 °C. The dried tissues were used for determination of concentration of soluble ions. Water content was calculated in g per g of dry mass.

In the second experiment, effect of 1 g L^{-1} Na was compared in two cultivation systems: (i) agar-solidified multiplication medium with 1.0 mg L^{-1} thidiazuron and 0.2 mg L^{-1} naphthaleneacetic acid in 200 mL jars and (ii) liquid multiplication medium with 1.0 mg L^{-1} thidiazuron and 0.2 mg L^{-1} naphthaleneacetic acid in a temporary immersion liquid culture bioreactor system (Plantform, Hjärup, Sweden). The bioreactor consisted of a transparent polycarbonate body (180 × 150 × 150 mm) closed by a lid, equipped with a system for explant immersion in liquid medium as well as an aeration system. A batch of 15 shoot explants was placed in each bioreactor, three reactors per treatment for each subspecies. The explants were immersed in the medium two times a day for 10 min and aerated

every 2 h for 10 min. Cultivation conditions were the same as above. Jars and bioreactors were placed in such a way that the explants were equidistant from the light source. After four weeks the experiment was terminated and the number of newly formed shoots was determined. Explant biomass was measured before and after drying for 4 days at 60 °C. The dried tissues were used for determination of concentration of soluble ions. Water content was calculated in g per g of dry mass.

Rooting and ex vitro *acclimatization*

For rooting of explants, they were placed on half-strength MS medium supplemented with 0.5 mg L^{-1} indole-3-butyric acid (Song et al. 2020). Rooted explants were transferred to peat moss substrate (Laflora KKS, Laflora, Kaigi, Latvia) over a layer of expanded clay aggregate and placed in a growth room under 16 h photoperiod provided by fluorescent lamps with photon flux density of 50 µmol m^{-2} s^{-1} of photosynthetically active radiation, 25 °C, for *ex vitro* acclimatization. For the first two weeks increased air humidity conditions were provided. Root formation occurred within four weeks

Ex vitro *cultivation and effect of NaCl*

To mimic natural conditions of a shingle beach, *ex vitro* acclimatized *M. maritima* plants were planted in 5 L plastic containers containing a mixture of 2 L expanded clay pellets (LECA 8-15, Leca International, København S, Denmark), 2 L garden soil (Biolan, Eura, Finland), and 1 L quartz sand (Saulkalne S, Saulkalne, Latvia). After planting, a 2-cm layer of pellets was poured on top of the substrate. Plants were placed in an experimental automated greenhouse (HortiMaX, Maasdijk, the Netherlands) with supplemented light provided by Master SONTPIAGreen Power CG T 400W (Philips, Amsterdam, The Netherlands) and Powerstar HQI-BT 400 W/D PRO (Osram, Munich, Germany) lamps (photon flux density of photosynthetically active radiation, 380 µmol m^{-2} s^{-1} at the plant level) with a 16 h photoperiod. The day/night temperature was 23/16 °C, and the relative air humidity was maintained at 60 to 70%.

The plants were fertilized weekly with Yara Tera Kristalon Red and Yara Tera Calcinit fertilizers (Yara International, Oslo, Norway). A stock solution was prepared for each fertilizer (100 g L^{-1}), and the working solution contained 25 mL of each per 10 L of deionized water, used at a rate of 250 mL per container. Watering was performed with deionized water every other day, adding 250 mL to the bottom plates of individual plants. Plants were gradually treated with NaCl solution in 50 mM increments within two weeks until the amount of salt reached in substrate 50, 100, and 200 mM. Control plants were treated with deionized water. For each subspecies, five plants per treatment were used. The experiment was terminated five weeks after the full treatment.

At termination, plants were individually separated in rosette leaf blades, rosette leaf petioles, stem leaf blades, stem leaf petioles, stems, flowers, and roots. Length of the flowering shoot was measured. The plant material was weighed before and after drying in an oven at 60 °C until a constant mass was reached. The dry plant material was used for the analysis of soluble ions (all parts) and for the measurement of the total concentration of mineral elements (only leaf blades).

Measurements

Chlorophyll *a* fluorescence measurements were performed twice during cultivation of *M. maritima* plants *ex vitro*, two and four weeks after the full treatment with NaCl, as described previously (Jēkabsone et al. 2024). Three leaves per plant were independently measured using a Handy PEA fluorometer (Hansatech Instruments, King's Lynn, UK).

For the analysis of soluble ions, plant material was crushed by hand. A homogenous tissue sample (0.1 g) was placed in a 2 mL Eppendorf tube and destructed using a mixer mill MM 400 (Retsch, Haan, Germany), 300 Hz, 3 min. After extraction with deionized water for 1 min and filtration through a nylon mesh cloth (No. 80), electrical conductivity was measured using a LAQUAtwin B-771 conductivity meter, Na^+ concentration using a LAQUAtwin B-722 compact meter, and K^+ concentration using a LAQUAtwin B-731 compact meter (Horiba, Kyoto, Japan). Three or four tissue samples were independently measured for each treatment and plant part for both subspecies.

Analysis of the total concentration of Na^+, Cl^- and mineral nutrients in leaves of *M. maritima* plants was performed using dried and mineralized tissue samples, as described previously (Andersone-Ozola et al. 2021). The levels of Na, K, Ca, Mg, Fe, Cu, Zn and Mn were estimated using an Agilent 4200 microwave plasma atomic emission spectrometer (Agilent Technologies, Santa Clara, CA, USA), P levels were analyzed by colorimetry with ammonium molybdate in an acid-reduced medium using a Jenway 6300 spectrophotometer (Cole-Palmer Instrument Company, St. Neots, Cambridgeshire, UK), and values of Cl were obtained by $AgNO_3$ titration via the distilled water extraction of plant ash. All analyses were performed in triplicate using representative tissue samples from individual biological replicates.

Data analysis

The results were analyzed using KaleidaGraph software (v. 5.0.6, Synergy Software, Reading, PA, USA). The statistical significance of differences was evaluated by one-way ANOVA using the Tukey post hoc test with honestly significant difference ($p < 0.05$).

Results

Effect of media composition

Initial screening of different types of agar-solidified media using various combinations of growth substances showed

Table 1. Effect of medium composition on multiplication potential of explants of *Mertensia maritima* plants of the two subspecies. Data are means from five replicates ± SE. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$)

Growth substances (concentration)	Average number of new shoots formed	
	Mertensia maritima subsp. *maritima*	*Mertensia maritima* subsp. *asiatica*
6-Benzylaminopurine (0.5 mg L^{-1})	2.90 ± 0.83 a	2.10 ± 0.78 a
Thidiazuron (1.0 mg L^{-1}) + 1-naphthaleneacetic acid (0.2 mg L^{-1})	3.00 ± 0.50 a	2.60 ± 0.46 a
Kinetin (2 mg L^{-1}) + 1-naphthaleneacetic acid (0.2 mg L^{-1})	2.13 ± 1.17 a	0.00 ± 0.00 b

that both benzylaminopurine and thidiazuron plus naphthaleneacetic acid were appropriate for multiplication of shoot explants of both subspecies of *M. maritima* (Table 1). However, a medium with kinetin plus naphthaleneacetic acid was not suitable for *M. m. asiatica*. While there were no statistically significant differences in multiplication potential between the suitable media, the combination of thidiazuron plus naphthaleneacetic acid was selected for further experiments as explants tended to have the highest number of newly formed shoots in this medium for both subspecies.

Effect of NaCl in agar-solidified medium

Shoot explants of *M. m. maritima* tended to have higher biomass in non-saline medium in comparison to that of *M. m. asiatica*, but the difference was not statistically significant (Fig. 1A). Biomass of shoots was not negatively affected by increasing concentration of NaCl in the medium. At 1 g L^{-1} for *M. m. maritima* and both 1 and 2 g L^{-1} for *M. m. asiatica*, explant biomass tended to be higher than that in control conditions, but the effect was not statistically significant due to relatively high variability within and between the replicates. Explants of *M. m. maritima* had higher potential for multiplication, as seen by statistically higher number of shoots formed from each explant (Fig. 1B). For both subspecies, number of shoots was not affected at 1 g L^{-1} Na, but it significantly decreased starting from 2.5 g L^{-1} Na. Shoot water content was similar for explants of the both subspecies, and it significantly decreased at 4 and 5 g L^{-1} Na (Fig. 1C).

Electrical conductivity in shoot tissue extracts of explants was similar for both subspecies and it continuously increased with NaCl concentration in the medium (Fig. 2A). Accumulation capacity for Na^+ was very high, and shoots of M. m. maritima showed significantly higher concentration at 5 g L^{-1} Na than that for *M. m. asiatica* (Fig. 2B). In contrast, concentration of K^+ decreased with increasing medium Na concentration for both subspecies (Fig. 2C).

Effect of NaCl in agar-solidified and liquid medium

Addition of 1 g L^{-1} Na in a form of NaCl to agar-solidified medium resulted in significantly increased dry shoot biomass per explant for both subspecies (Fig. 3A). Cultivation in conditions of the temporary immersion system (Plantform bioreactor) resulted in highly improved tissue growth, but significant differences between the subspecies appeared. Explant biomass was significantly larger for *M. m. maritima*, and it decreased by NaCl treatment, while biomass of *M. m. asiatica* explants was stimulated by the treatment. Also, multiplication potential tended to be lower in Plantform bioreactors and it was further decreased by NaCl for *M. m. maritima*, but the effect was not statistically significant (Fig. 3B). Addition of 1 g

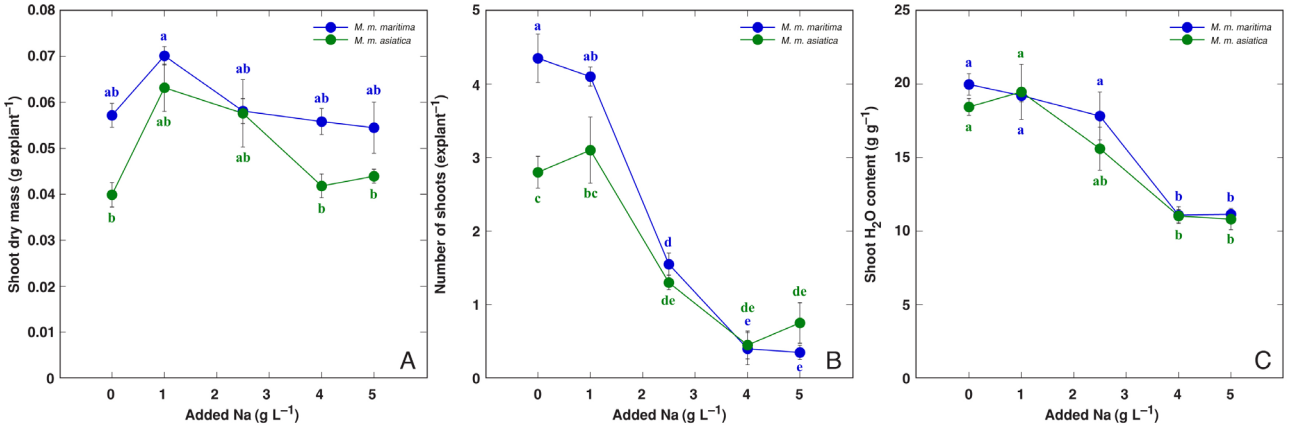

Fig. 1. Effect of increasing NaCl in medium on dry biomass of explants (A), number of shoots (B), and water content in shoots (C) of explants of the two subspecies of *Mertensia maritima* cultivated *in vitro* on agar-solidified medium. Data are means ± SE from four replicates with five explants each. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

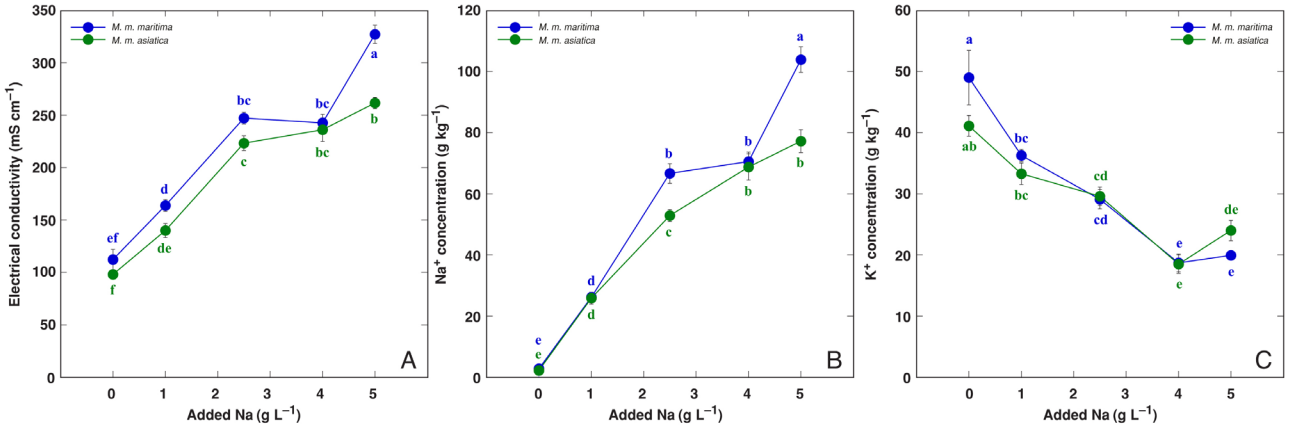

Fig. 2. Effect of increasing NaCl in medium on electrical conductivity of tissue extracts (A), Na^+ concentration (B), and K^+ concentration (C) of explants of the two subspecies of *Mertensia maritima* cultivated *in vitro* on agar-solidified medium. Data are means ± SE from four samples. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

L^{-1} Na to the medium had no significant effect on number of shoots. Tissues of explants cultivated in Plantform bioreactors had significantly higher water content than that in explants on agar-solidified medium (Fig. 3C). NaCl treatment had no significant effect on tissue water content.

Electrical conductivity of tissue extracts of control explants was lower in Plantform bioreactors, but for explants in the presence of NaCl it was higher in the

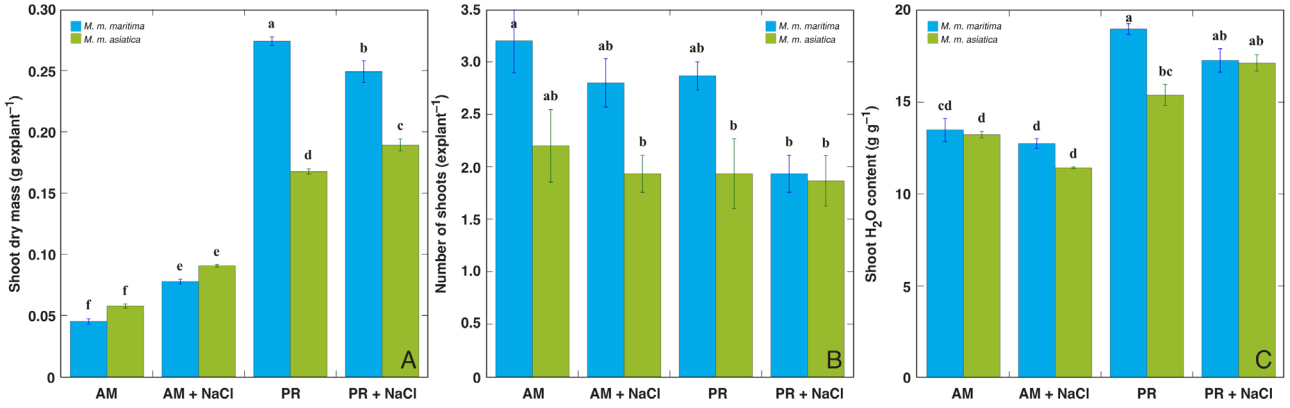

Fig. 3. Effect of cultivation system type (AM, agar-solidified medium; PR, Plantform reactor) and 1 g L^{-1} Na on dry biomass of explants (A), number of shoots (B), and water content in shoots (C) of explants of the two subspecies of *Mertensia maritima*. Data are means ± SE from three replicates. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

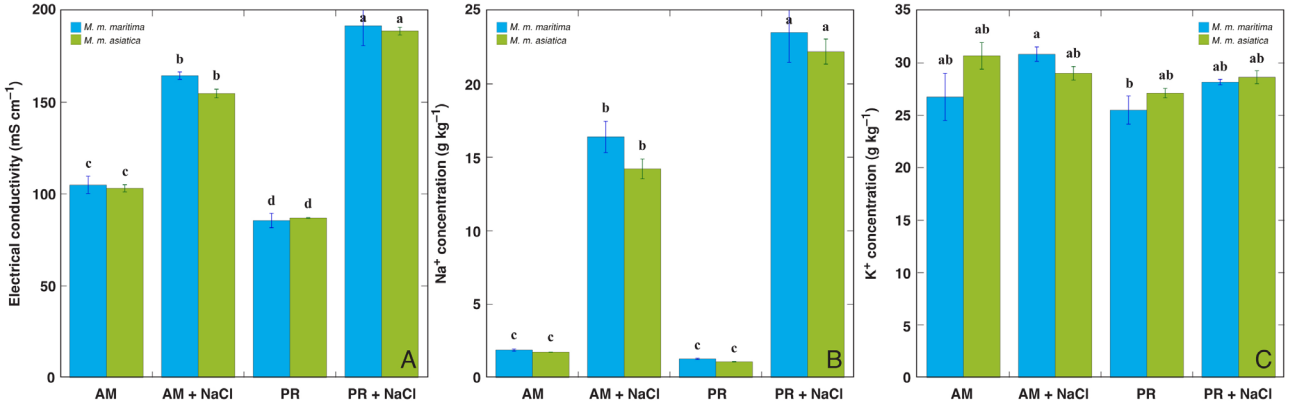

Fig. 4. Effect of cultivation system type (AM, agar-solidified medium; PR, Plantform reactor) and 1 g L^{-1} Na on electrical conductivity of tissue extracts (A), Na^+ concentration (B), and K^+ concentration (C) of explants of the two subspecies of *Mertensia maritima*. Data are means ± SE from three replicates. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

bioreactors (Fig. 4A). A similar effect was evident for Na^+ accumulation capacity in tissues (Fig. 4B). For K^+ concentration, neither cultivation system or added NaCl had significant effect (Fig. 4C).

Effect of NaCl ex vitro

The most striking morphological difference between the two subspecies of *M. maritima* in *ex vitro* conditions was delayed onset of flowering of *M. m. maritima* plants, especially, under salinity treatment (Fig. 5A) in comparison to *M. m. asiatica* plants (Fig. 5B). Number of leaves was identical in control plants of both subspecies, but it was more negatively affected by increasing salinity in the case of *M. m. asiatica* (Fig. 6A). The total stem length was similarly negatively affected by increasing salinity for both subspecies (Fig. 6B), but length of the flowering stem, which was much lower for control plants of M. m. maritima before treatment, was dramatically inhibited by increasing salinity (Fig. 6C). Dry biomass of leaves (both blades and petioles) was significantly higher for *M. m. maritima* plants, but it was relatively more depressed by increasing NaCl concentration than that for *M. m. asiatica* plants (Fig. 6D, E, F). Both dry biomass of roots and stems was relatively similar for both subspecies, and was equally fast inhibited by increasing NaCl concentration (Fig. 6G, H).

Water content in different parts of *ex vitro* plants of *M. maritima* was relatively stable (Table 2). Significant increase over the control level was evident for roots at 100 mM NaCl for both subspecies. Water content tended to increase also in leaf petioles at moderate salinity, but this effect was significant only for *M. m. asiatica* at 50 and 100 mM. Significant increase was seen also for stems of *M. m. asiatica* at 100 and 200 mM NaCl. In contrast, water content significantly decreased in leaf blades of both subspecies at 200 mM NaCl.

Total concentration of mineral elements was determined only in leaves of *ex vitro* cultivated *M. maritima* plants under increasing salinity (Table 3). Capacity for Na^+ accumulation was relatively low in comparison to that in *in vitro* cultivated shoots. Concentration of all mineral elements tended to increase with increasing salinity, but it varied depending on the element. The most pronounced increase was for K^+ and Mn^{2+}.

Electrical conductivity in tissue extracts and concentration of soluble Na^+ and K^+ was measured in different plant parts of *ex vitro* cultivated *M. maritima* plants (Table 4). In general, leaf petioles accumulated more soluble ions than leaf blades, as indicated by electrical conductivity measurements, but the lowest level was in roots. This was mostly due to the preferential accumulation of both Na^+ and K^+ in leaf petioles. No large differences were evident in ion accumulation characteristics between rosette and stem leaves of *M. maritima* plants.

Chlorophyll *a* fluorescence measurements were performed twice during cultivation of *M. maritima* plants, two and four weeks after the full treatment with NaCl (Fig. 7). Salinity led to increase in both photochemical activity of photosystem II, measured as F_v/F_0 (Fig. 7A, B), as well as overall photochemical reactions, measured by the Performance Index (Fig. 7C, D). Photochemical activity

Fig. 5. Morphology of typical plants of *Mertensia maritima* subsp. *maritima* (A) and *Mertensia maritima* subsp. *asiatica* (B) in *ex vitro* conditions under the effect of NaCl. From left to right: control, 50, 100, 200 mM NaCl.

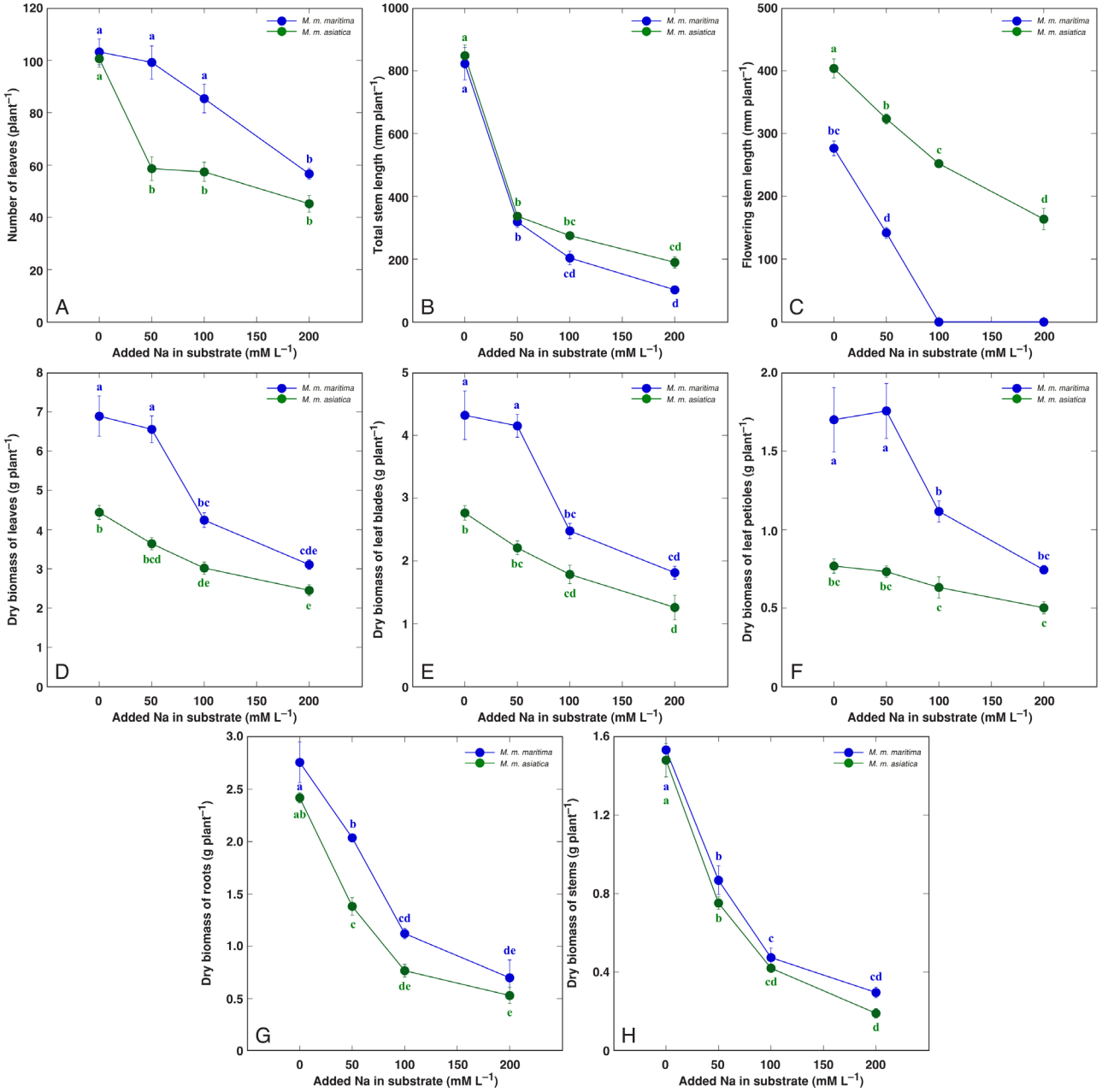

Fig. 6. Effect of salinity on number of leaves (A), total stem length (B), flowering stem length (C), dry biomass of leaves (D), dry biomass of leaf blades (E), dry biomass of leaf petioles (F), dry biomass of rooots (G), dry biomass of stems (H) of *Mertensia maritima* plants of different subspecies in *ex vitro* conditions. Data are means ± SE from five replicates. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

of *M. m. asiatica* plants was more sensitive to high NaCl treatment in comparison to that of *M. m. maritima* plants. Similarly, the Performance Index increased in NaCl-treated *M. m. maritima* plants at all doses, but only at 50 mM or 50 and 100 mM NaCl for *M. m. asiatica*, two and four weeks after the full treatment, respectively.

Discussion

Among the suggested advantages of temporary immersion liquid culture bioreactor systems over agar-solidified ones are higher rate of multiplication and better acclimatization potential of the produced explants *ex vitro* (Etienne, Berthouly 2002; Ruffoni, Savona 2003; De Carlo et al. 2021), but the effects seem to be highly genotype-specific (Mirzabe et al. 2022). In particular, it is desirable to achieve higher growth rates using liquid media (Adelberg et al. 2006). In a previous study comparing agar-solidified and liquid temporary immersion cultivation systems for propagation of *M. maritima*, it was found that shoot cluster biomass on multiplication medium was not affected by the choice of the cultivation system, but number of shoots even

Table 2. Effect of salinity on water content (g g^{-1} DM) in various parts of *Mertensia maritima* plants of the two subspecies in *ex vitro* conditions. Data are means from five replicates ± SE. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$). For sake of comparison, values in red and blue indicate statistically significant increase or decrease, respectively, over control values for the particular plant part and subspecies. DM, dry mass

Plant part	*Mertensia maritima* subsp. *maritima*				*Mertensia maritima* subsp. *asiatica*			
	Control	NaCl 50 mM	NaCl 100 mM	NaCl 200 mM	Control	NaCl 50 mM	NaCl 100 mM	NaCl 200 mM
Roots	3.7 ± 0.1 ef	3.6 ± 0.1 f	4.0 ± 0.2 cd	3.6 ± 0.1 f	4.4 ± 0.1 bc	4.6 ± 0.1 ab	5.0 ± 0.1 a	4.6 ± 0.1 ab
Rosette leaf petioles	10.6 ± 0.4 bc	11.0 ± 0.6 bc	12.1 ± 0.4 ab	9.8 ± 0.4 c	10.0 ± 0.2 c	13.0 ± 0.3 a	12.4 ± 0.4 ab	10.9 ± 0.5 bc
Rosette leaf blades	9.6 ± 0.1 a	9.1 ± 0.3 a	9.1 ± 0.3 a	7.7 ± 0.2 b	9.8 ± 0.2 a	10.2 ± 0.3 a	10.0 ± 0.2 a	8.0 ± 0.2 b
Stems	6.1 ± 0.3 de	5.6 ± 0.2 e	6.1 ± 0.4 de	5.9 ± 0.2 de	7.1 ± 0.4 cd	7.8 ± 0.4 bc	9.0 ± 0.3 ab	10.3 ± 0.2 a
Stem leaf petioles	10.8 ± 0.4 b	11.0 ± 0.2 b	11.7 ± 0.2 b	9.8 ± 0.2 b	11.4 ± 0.8 b	15.0 ± 0.1 a	14.8 ± 0.7 a	10.8 ± 0.5 b
Stem leaf blades	8.2 ± 0.2 ab	7.4 ± 0.2 bc	7.5 ± 0.1 bc	6.6 ± 0.1 cd	7.3 ± 0.3 ab	8.0 ± 0.1 ab	8.6 ± 0.5 a	6.3 ± 0.2 d

decreased for explants in Plantform bioreactors (Copetta et al. 2021). However, when explants produced on agar-solidified medium were further cultivated on a rooting medium, both shoot fresh biomass and number of roots were higher for explants in Plantform bioreactors, leading to higher survival rate during acclimatization. In contrast, in the present study, explants of *M. maritima* cultivated in Plantform bioreactors had several times higher dry biomass and higher water content, but multiplication potential was not affected (Fig. 3). These differences can be related to variation in immersion and aeration frequency, as well as light and temperature conditions (Mirzabe et al. 2022). Thus, the flooding regime was every 3 h for 3 min in the previous study with *M. maritima* (Ceopetta et al. 2021) and only two times a day for 10 min in the present study.

There was an improvement of shoot growth of *M. maritima* by 1 g L^{-1} Na treatment in agar-solidified medium, but the multiplication rate was not affected (Fig. 1). In contrast, in other studies, 2.5 and 5.0 g L^{-1} NaCl in medium doubled shoot multiplication of a halophyte *Limoniastrum monopetalum*, but shoot elongation was not affected (Martini, Papafotiou 2020). Interestingly, the definite positive effect of increased salinity on explant biomass in agar-solidified medium changed in conditions of a Plantform bioreactor: salinity had negative effect on biomass of *M. m. maritima* explants while it was positive for *M. m. asiatica* explants (Fig. 3A). Apparently, the effect of salinity is also affected by growth regulators used for plant multiplication in tissue culture (Joshi et al. 2012).

In natural conditions, *M. maritima* plants were observed to establish from seed, and flowers only in a second vegetation season or even later (Scott 1963). In the present study, plants propagated *in vitro* developed flower-bearing stems early during cultivation in greenhouse conditions, but the degree of flowering in these conditions was a genotype-specific feature (Fig. 5). Most likely, photoperiodic requirements for flowering were not fully met for *M. m. maritima* plants. Other requirements for flowering induction can be important as well, such as prolonged length of the inductive night period or the number of inductive nights (Imaizumi, Kay 2006). Similar differences in flowering ability between different accessions have been shown also for another coastal species, *Plantago maritima* (Ozoliņa et al. 2024).

Combining experimental approaches of tissue culture with whole plant studies allows to search for possible

Table 3. Effect of salinity on mineral element concentration in leaves of *Mertensia maritima* plants of the two subspecies in *ex vitro* conditions. Data are means from three replicates ± SE. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$). For sake of comparison, values in red indicate statistically significant increase over control values for the respective element and subspecies.

Element	*Mertensia maritima* subsp. *maritima*				*Mertensia maritima* subsp. *asiatica*			
	Control	NaCl 50 mM	NaCl 100 mM	NaCl 200 mM	Control	NaCl 50 mM	NaCl 100 mM	NaCl 200 mM
Na (g kg^{-1})	0.50 ± 0.06 d	3.60 ± 0.20 c	3.93 ± 0.05 c	13.19 ± 1.84 a	0.45 ± 0.03 d	3.08 ± 0.19 c	4.79 ± 1.32 c	8.64 ± 0.45 b
K (g kkg^{-1})	37.7 ± 4.1 b	58.1 ± 2.5 a	59.1 ± 2.2 a	57.3 ± 3.0 a	37.3 ± 3.1 b	69.8 ± 5.1 a	70.7 ± 1.6 a	71.6 ± 3.9 a
Cl (g kg^{-1})	2.5 ± 0.8 d	21.5 ± 1.6 c	23.8 ± 0.2 bc	44.5 ± 5.2 a	3.5 ± 0.8 d	35.5 ± 3.8 ab	42.1 ± 3.3 a	45.0 ± 3.6 a
Ca (g kg^{-1})	21.7 ± 4.3 abc	25.4 ± 1.4 ab	22.7 ± 1.1 bc	30.1 ± 3.2 a	14.1 ± 2.3 c	15.5 ± 0.8 bc	19.5 ± 0.3 bc	21.8 ± 1.5 abc
P (g kg^{-1})	1.82 ± 0.32 c	2.46 ± 0.09 bc	2.88 ± 0.05 b	4.63 ± 0.25 a	1.67 ± 0.10 c	3.07 ± 0.13 b	3.10 ± 0.16 b	4.84 ± 0.02 a
Mg (g kg^{-1})	1.31 ± 0.20 bc	1.40 ± 0.14 abc	1.35 ± 0.09 abc	2.25 ± 0.38 a	0.85 ± 0.13 c	1.16 ± 0.10 bc	1.61 ± 0.20 abc	2.00 ± 0.14 ab
Fe (mg kg^{-1})	45.3 ± 3.6 a	58.0 ± 2.5 a	55.4 ± 4.3 a	62.1 ± 13.3 a	43.0 ± 4.1 a	56.3 ± 3.8 a	53.2 ± 3.2 a	49.5 ± 0.2 a
Mn (mg kg^{-1})	17.8 ± 2.8 d	49.8 ± 1.2 c	68.4 ± 9.9 bc	85.9 ± 4.0 ab	18.6 ± 3.2 d	66.8 ± 6.4 bc	91.9 ± 7.7 ab	99.4 ± 0.1 a
Zn (mg kg^{-1})	21.9 ± 2.0 cd	23.3 ± 0.4 c	36.7 ± 1.5 b	48.6 ± 2.2 a	15.4 ± 0.6 d	19.6 ± 1.6 cd	25.4 ± 1.0 c	38.6 ± 0.4 b
Cu (mg kg^{-1})	10.5 ± 0.8 c	11.6 ± 0.3 bc	12.9 ± 0.3 bc	18.8 ± 0.9 a	10.6 ± 0.2 c	11.5 ± 0.4 bc	12.0 ± 0.8 bc	13.9 ± 0.2 b

Table 4. Effect of salinity on electrical conductivity and soluble ion concentration in various parts of *Mertensia maritima* plants of the two subspecies in *ex vitro* conditions. Data are means from three replicates ± SE. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$). For sake of comparison, values in red indicate statistically significant increase over control values for the respective parameter and subspecies. na, not available

Plant part	*Mertensia maritima* subsp. *maritima*				*Mertensia maritima* subsp. *asiatica*			
	Control	NaCl 50 mM	NaCl 100 mM	NaCl 200 mM	Control	NaCl 50 mM	NaCl 100 mM	NaCl 200 mM
				EC (mS m^{-1} kg^{-1})				
Roots	23.1 ± 0.5 d	27.6 ± 0.9 d	48.0 ± 5.6 bcd	106.7 ± 12.0 a	18.2 ± 1.1 d	40.9 ± 8.6 cd	69.8 ± 7.8 bc	76.0 ± 9.0 ab
Dry leaves	154 ± 10 b	197 ± 4 ab	213 ± 16 ab	233 ± 21 a	154 ± 7 b	195 ± 14 ab	227 ± 17 a	256 ± 4 a
Rosette leaf petioles	111 ± 17 e	213 ± 4 d	282 ± 23 abc	316 ± 18 ab	90 ± 13 e	220 ± 8 cd	267 ± 12 bcd	344 ± 9 a
Rosette leaf blades	80 ± 5 d	113 ± 18 bcd	171 ± 22 ab	198 ± 5 a	70 ± 3 d	93 ± 16 cd	112 ± 3 bcd	157 ± 17 abc
Stems	79 ± 1 e	102 ± 3 de	146 ± 10 cd	216 ± 4 b	60 ± 6 e	131 ± 12 cd	178 ± 16 bc	322 ± 17 a
Stem leaf petioles	106 ± 4 e	211 ± 11 d	266 ± 18 bc	344 ± 2 a	83 ± 11 e	232 ± 10 cd	267 ± 1 c	307 ± 4 ab
Stem leaf blades	67 ± 3 d	89 ± 9 bcd	112 ± 15 abcd	117 ± 9 abc	74 ± 2 cd	106 ± 5 abcd	133 ± 12 ab	139 ± 12 a
Flowers	89 ± 2 b	90 ± 3 b	na	na	96 ± 3 ab	101 ± 2 ab	114 ± 2 a	102 ± 11 ab
				Na (g kg^{-1})				
Roots	0.54 ± 0.02 d	1.02 ± 0.07 c	1.26 ± 0.22 bc	4.71 ± 2.98 ab	0.58 ± 0.02 d	2.01 ± 0.38 bc	2.70 ± 0.21 bc	5.51 ± 0.69 a
Dry leaves	1.15 ± 0.04 b	4.21 ± 0.17 ab	4.97 ± 0.19 ab	9.78 ± 3.41 a	1.13 ± 0.09 b	3.76 ± 0.15 ab	6.86 ± 1.30 ab	8.89 ± 1.12 a
Rosette leaf petioles	1.19 ± 0.36 c	7.28 ± 0.56 b	9.94 ± 0.48 b	18.33 ± 3.44 a	1.12 ± 0.22 c	6.67 ± 1.05 b	7.67 ± 0.26 b	12.11 ± 1.73 ab
Rosette leaf blades	0.72 ± 0.12 d	2.16 ± 0.46 bcd	3.73 ± 0.54 b	7.11 ± 0.53 a	0.46 ± 0.02 d	1.39 ± 0.41 cd	1.93 ± 0.19 cd	2.77 ± 0.35 bc
Stems	0.77 ± 0.10 d	2.34 ± 0.20 c	5.02 ± 0.17 bc	8.72 ± 0.56 ab	0.87 ± 0.24 d	4.54 ± 1.81 bc	5.95 ± 1.26 bc	12.00 ± 2.60 a
Stem leaf petioles	0.87 ± 0.14 d	5.38 ± 0.25 bcd	7.17 ± 0.88 bc	13.33 ± 1.39 a	1.37 ± 0.22 d	4.83 ± 0.35 cd	6.28 ± 0.42 bc	9.67 ± 2.18 ab
Stem leaf blades	0.54 ± 0.03 c	1.58 ± 0.28 bc	2.21 ± 0.31 b	3.36 ± 0.41 a	0.54 ± 0.03 c	1.51 ± 0.19 bc	1.95 ± 0.15 b	2.31 ± 0.23 ab
Flowers	0.53 ± 0.03 b	0.65 ± 0.05 b	na	na	0.58 ± 0.08 b	0.68 ± 0.06 b	1.12 ± 0.23 ab	1.62 ± 0.28 a
				K (g kg^{-1})				
Roots	6.2 ± 0.2 cd	7.3 ± 0.3 cd	14.3 ± 2.0 bc	27.9 ± 3.3 a	4.0 ± 0.3 d	9.9 ± 2.8 bcd	17.4 ± 2.2 b	13.3 ± 2.4 bcd
Dry leaves	33.4 ± 1.6 c	40.8 ± 0.4 bc	46.8 ± 2.8 abc	42.9 ± 1.6 bc	43.3 ± 0.6 bc	48.0 ± 4.6 abc	52.1 ± 6.0 ab	60.0 ± 2.9 a
Rosette leaf petioles	31.4 ± 4.5 cd	53.8 ± 0.6 ab	71.1 ± 8.1 a	66.7 ± 5.9 ab	23.0 ± 4.3 d	49.4 ± 1.9 bc	59.6 ± 3.8 ab	71.7 ± 1.0 a
Rosette leaf blades	21.4 ± 1.9 b	28.8 ± 3.8 ab	42.3 ± 3.9 a	40.9 ± 0.3 a	17.8 ± 0.3 b	24.5 ± 2.7 b	29.1 ± 1.2 ab	40.3 ± 5.2 a
Stems	27.5 ± 0.9 ef	38.5 ± 1.0 cde	48.9 ± 3.2 bc	64.4 ± 1.5 b	16.4 ± 2.0 f	31.8 ± 5.3 def	43.9 ± 5.6 cd	82.8 ± 3.4 a
Stem leaf petioles	29.0 ± 1.3 cd	56.7 ± 3.5 b	63.9 ± 11.2 ab	85.6 ± 2.4 a	14.3 ± 1.7 d	46.7 ± 3.0 bc	50.3 ± 4.2 bc	64.4 ± 4.4 ab
Stem leaf blades	19.6 ± 0.8 bc	25.2 ± 3.1 abc	33.3 ± 5.8 ab	29.2 ± 2.6 abc	19.5 ± 0.5 c	28.5 ± 1.0 abc	34.0 ± 3.4 a	37.3 ± 2.5 a
Flowers	28.2 ± 1.7 a	26.0 ± 2.0 a	na	na	27.1 ± 16 a	29.6 ± 0.5 a	33.6 ± 0.3 a	31.3 ± 2.5 a

differences in plant salinity responses at different levels of biological organization. Thus, when unorganized callus cells of *Sesuvium portulacastrum* were cultivated in the presence of 100 mM NaCl, relative growth rate decreased by more than 30% (Lokhande et al. 2010), but when shoot explants of the same plants were cultivated in the presence of 100 mM NaCl, their growth and multiplication potential was significantly stimulated (Lokhande et al. 2011), identically to the response of whole plants (Lokhande et al. 2009). Similar results were found for halophytes *Atriplex uncunlata* and *Suaeda australis* (Smith, Mc Comb 1981). Therefore, it can be proposed that at least part of the characteristics of whole plant salinity tolerance are associated with integrated functioning of the main organ systems of a plant, shoots and roots. However, many salt tolerance mechanisms like ion accumulation in cell vacuoles, maintenance of osmotic balance, and enzymatic antioxidative protection, are associated with the cellular level (Flowers et al. 2015). If the particular plant species relies mostly on adaptive features at the cellular level, relative salinity tolerance is similar at different levels of plant organization. Thus, similar salinity responses between callus cultures and whole plants have been shown for a number of halophyte species, as *Spartina patens* (Li et al. 1995) and *Atriplex halimus* (Bajji et al. 1998). However, another salt tolerant coastal species *Armeria maritima* showed higher salinity tolerance in *ex vitro* conditions when cultivated in substrate, in comparison to that in tissue culture conditions (Purmale et al. 2022a). In particular, shoot and root biomass of *A. maritima* was not significantly affected even at 200 mM NaCl or KCl, but it was inhibited by about 50% in tissue culture conditions at the same salinity level.

However, in the present study with *M. maritima*, NaCl had more positive effect on plant growth in tissue culture conditions in comparison to that in *ex vitro* conditions. Dry biomass accumulation in cultivated explants was stimulated by 1 g Na with no negative effect up to 5 g L^{-1} Na (Fig. 1). No stimulative effect of NaCl appeared during *ex vitro* cultivation, but leaf growth was negatively affected at 100 mM NaCl, while root and stem growth was even more sensitive to salinity (Fig. 6). In a previous study, both leaf and root biomass of *M. maritima* plants cultivated in

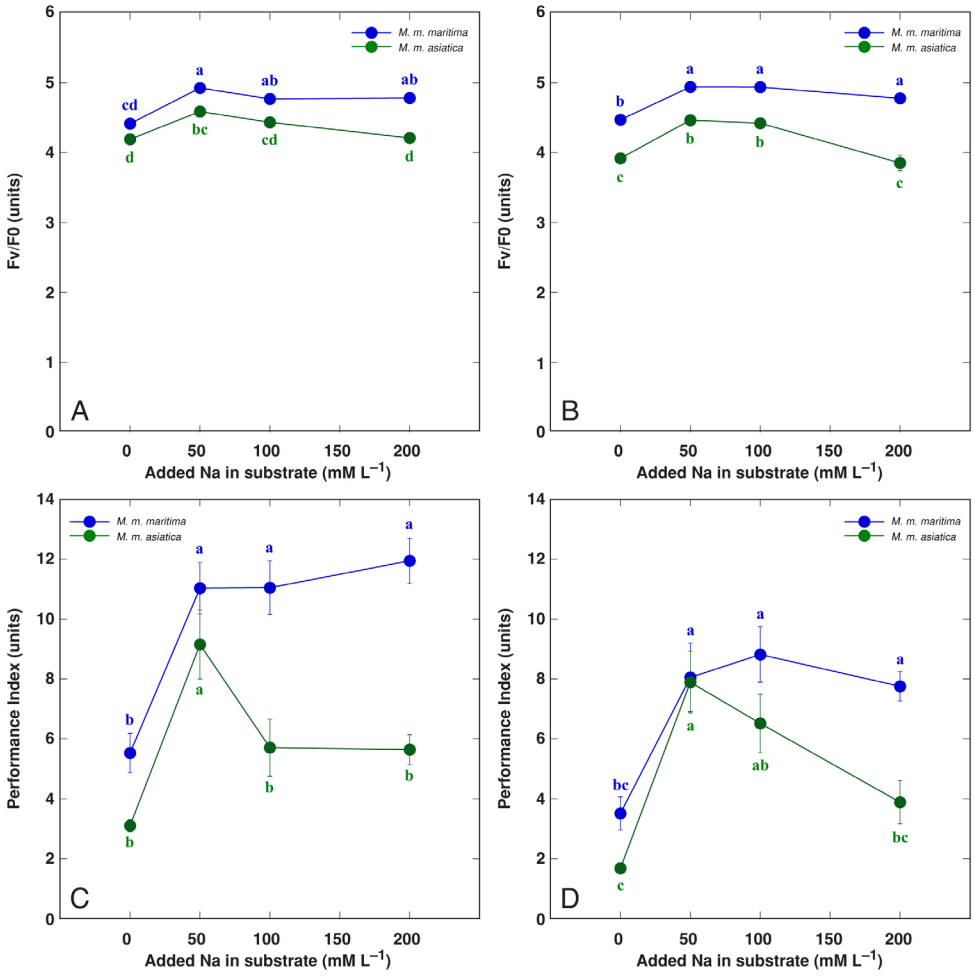

Fig. 7. Effect of salinity on chlorophyll fluorescence parameters F_v/F_0 (A, B) and Performance Index (C, D) measured in leaves of *Mertensia maritima* plants of different subspecies in *ex vitro* conditions two (A, C) and four (C, D) weeks after the full treatment with NaCl. Data are means ± SE from 15 independent measurement for each point. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$). In A and B, SE values were smaller than symbols.

conditions of inert substrate hydroponics was significantly decreased at 100 mM NaCl (Farzana et al. 2023).

In addition, physiological integrity of *M. maritima* plants dramatically affected ion accumulation characteristics. Firstly, accumulation potential for Na^+ was extremely high in shoot explants, reaching 100 g kg^{-1} (Fig. 2B), but it was only 12 to 18 g kg^{-1} in leaf petioles of *ex vitro* plants and even lower in leaf blades (Table 4). Secondly, under the effect of increasing NaCl concentration, K^+ concentration decreased from 40 – 50 to 20 g kg^{-1} in explants (Fig. 2C), but it increased in all parts of *ex vitro* plants under salinity (Table 4). Thus, at the tissue level, *M. maritima* was able to withstand extremely high internal concentration of Na^+ (more than 100 g kg^{-1}), which is comparable to that in salt-hyperaccumulating species (Ievinsh et al. 2021), but this ability was not fulfilled at the whole plant level.

Mineral nutrient deficiency is one of adverse consequences of increased substrate salinity (Grattan, Grieve 1998). Thus, competition of Na^+ and Cl^- with K^+, Ca^{2+} and NO_3^- can cause deficiency of these ions (Hu, Schmidhalter 2005). A recent study showed that macronutrient K, Ca, Mg and micronutrient Cu, Zn, Mn concentrations decreased with increasing salinity when *M. maritima* plants were grown in an inert substrate (Farzana et al. 2023). This contradicts the results of the present study, where concentrations of all nutrients were stable or increasing with increase in NaCl concentration (Table 2). In particular, concentration of K^+ decreased from 45 g kg^{-1} in the control to 29 g kg^{-1} in plants treated with 600 mM NaCl (Farzana et al. 2023), but it increased in the present study from 37 g kg^{-1} in control plants to 55 – 57 and 70 – 72 g kg^{-1} in NaCl-treated plants of *M. m. maritima* and *M. m. asiatica*, respectively (Table 3). Concentration of macronutrients P, Mg and micronutrients Mn, Zn and Cu also increased in leaves of *M. maritima* in saline conditions (Table 3), suggesting that one of adaptations to salinity at the whole plant level in this species is related to maintaining adequate tissue mineral nutrient homeostasis, possibly associated

with selective ion accumulation (Koyro et al. 2011). Similar increase in tissue mineral nutrient concentrations has been documented also for other halophyte species (Romero, Marañón 1996; Jēkabsone et al. 2024). It is highly possible that the observed differences in responses of mineral nutrition to salinity between various studies are related to differences in mineral nutrient availability in substrate.

Among physiological indicators, chlorophyll *a* fluorescence-related parameters are often used in salinity tolerance studies. Usually, at moderate salinity, photochemistry of photosynthesis is activated for relatively salinity-tolerant species (Redondo-Gómez et al. 2006; Zhou et al. 2013; Jēkabsone et al. 2023; Jēkabsone et al. 2024). For *M. maritima* plants of the two subspecies in *ex vitro* conditions, higher values of F_v/F_0 and Performance Index in *M. m. maritima* plants in comparison to *M. m. asiatica* plants (Fig. 7) indicated better adaptability of photochemical reactions of the former subspecies to saline conditions. Maintenance of water homeostasis in tissues is another indicator of salinity tolerance, and water content increased more in leaf petioles and stems of *M. m. asiatica* plants with increasing salinity in comparison to that in *M. m. maritima* plants (Table 2). An ability to maintain adequate tissue water content in conditions of increased substrate salinity is one of characteristic features of salt tolerance in halophyte species (Ievinsh 2023).

Conclusions

The approach used by comparing the effects of salt on two subspecies of *M. maritima* in different tissue culture systems and also *ex vitro*, gave an opportunity to evaluate the dependence of the results on the experimental conditions and genotype. Cultivation of explants of both subspecies of *M. maritima* on multiplication medium was more efficient in conditions of the temporary immersion system, Plantform bioreactor, in comparison to the ordinary agar-solidified medium. While the multiplication rate was not significantly affected, dry biomass of explants increased several times along with increase in tissue water content. However, positive effect of 1 g L^{-1} Na on explant growth, which was clearly pronounced for both subspecies in agar-solidified medium, was not unambiguous in Plantform bioreactor. Certain differences have been observed between the two subspecies, both regarding the response to cultivation conditions and to salt treatment. However, larger differences were observed at the whole plant level in *ex vitro* plants, the most significant being related to flowering. Consequently, both subspecies of *M. maritima* are suitable for the solution of various biotechnological measures, both in terms of the reproduction of living plants for *ex vitro* cultivation, and the extraction of biomass for further practical use. Further studies with *M. maritima* need to include optimization of immersion frequency in liquid medium in combination with reduced sucrose and growth substance concentration on the background of variable salinity, in order to balance production vs. cost efficiency.

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