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EXOGENOUS SODIUM CHLORIDE EXPLORES THE MECHANISMS FOR HYPEROSMOTIC STRESS ACCLIMATION IN LIVERWORT *MARCHANTIA POLYMORPHA*

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Abstract

In contrast to angiosperms, the mechanisms of hyperosmotic stress responses in liverworts, the evolutionarily important land plants remain more or less elusive. The present investigation was set with liverwort, *Marchantia polymorpha* to find out the approaches essential for hyperosmotic stress acclimation in distant land plants. The gemmae of *M. polymorpha* cultured in ½ B5 medium were subjected to control (0 mM NaCl) and osmotic stressed conditions led by 50 mM and 100 mM NaCl to find their morphological, biological and physiological responses. Compared to non-stress control, the thallus body of *M. polymorpha* showed strong growth inhibition while acclimating to hyperosmotic stress led by 100 mM NaCl. The significant reduction of chlorophyll content and tissue damage were recorded by hyperosmotic stress led by 100 mM NaCl compared to control. The negligible tissue damage and more or less similar chlorophyll content were recorded in control and by 50 mM NaCl. Further, the gemmalings showed enhanced accumulation of osmolytes proline and soluble sugar by 100 mM NaCl compared to the control which was consistent with the increased accumulation of soluble sugar by hyperosmotic stress led by 0.2 M sucrose. Hyperosmotic stress led by 100 mM NaCl showed a higher rate of electrolyte leakage in the gemmalings which was consistent with the higher amount of lipid peroxidation; malondialdehyde and hydrogen peroxide in the gemmalings treated with 100 mM NaCl compared to the control. However, the activity of enzymatic antioxidants such as superoxide dismutase; catalase; ascorbate peroxidase; dehydroascorbate reductase and glutathione *S*-transferase were greatly induced by 100 mM NaCl as compared to control. Therefore, the findings suggest that the basal land plants liverworts followed morpho-physiological alterations during acclimation to hyperosmotic stress which were crucial for the terrestrialization of land plants.

Keywords: Liverworts, gemmalings, antioxidant defense and oxidative stress.

Introduction

During the evolution, adaptation and thereafter radiation process, the land plants followed numerous changes at their cellular and molecular levels and investigation of which has been a major research topic for several years (Seki *et al*., 2003; Saruhashi *et al*., 2015; Ghosh *et al*., 2021; Jahan *et al*., 2022). Due to the wide nature of the environment, plants are

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being frequently exposed to different abiotic stresses. Among those, the most important one is salinity stress which causes a considerable yield loss of crop plants across the globe (Seleiman *et al*., 2022). The excess occurrence of sodium in soil leads to hyperosmotic stress resulting in imbalance condition in cellular osmotic adjustment and ionic homeostasis which ultimately cause cellular desiccation. The plants having the capability to survive in cellular desiccation are known as desiccation tolerant species. Although such tolerance is common among plant's propagules, such as spores, pollen grains, and seeds, the vast majority of vegetative and reproductive tissues do not survive under desiccation (Proctor *et al*., 2007). As compared with angiosperms, the higher degree of desiccation tolerance is present in early diverging land plants though the mechanism is yet to be clarified completely. It has been postulated that among today's land plants, the plants with bryophytes type organization are the first land plants (Kenrick and Crane, 1997; Wellman *et al*., 2003). The bryophytes comprised of mosses, liverworts and hornworts possess very simple morphology with alteration of generation and haploid dominating like cycle which makes them ideal for biochemical and molecular studies in response to abiotic stress. This type of unique desiccation tolerance attribute of bryophytes is very similar to the ancestral traits of land plants which has been lost from the vascular plants during evolution (Oliver *et al*., 2000; 2005). Among the bryophytes, moss *Physcomitrium patens (Physcomitrella patens)* and liverworts *Marchantia polymorpah* have been widely used as the models to answer the evolutionary questions (Decker *et al*., 2006; Rensing *et al*., 2008; Bowman *et al*., 2016; Ghosh *et al*., 2016; Jahan *et al*., 2019). Therefore, analysis of hyperosmotic stress acclimation process of this basal representatives at biochemical and molecular levels should reveal the novel mechanisms of stress tolerance. As the ancestors of land plants; bryophytes had to face a lot of challenges for cellular desiccation during their evolution and thereafter adaptation, analysis of hyperosmotic stress acclimation ability with this sort of plants might explore some mechanisms crucial for today's land plants.

Although, likewise angiosperms, the basal representative of land plants follows adequate changes in their morphology while acclimating to osmotic stress and cellular desiccation (Takezawa *et al*., 2015; Ghosh *et al*., 2021; Jahan *et al*., 2022), the mode of changes while facing to salinity stress is still to examined in bryophytes. Along with growth, the information regarding the maintenance of photosynthetic pigments during abiotic stress adaptation of bryophytes is very poor. Additionally, plants develop the protective mechanisms at their cellular levels by accumulating different osmolytes like proline and soluble sugar for enhancing tolerance to abiotic stress including drought and salinity (Liang *et al*., 2013). Though the desiccation tolerance of moss *P. patens* and liverworts *M. polymorpha* mediated by phytohormone abscisic acid (ABA) was confirmed by several investigations (Minami *et al*., 2003; Takezawa *et al*., 2015; Akter *et al*., 2014), the direct role of the cellular osmolytes is yet to be clarified during hyperosmotic stress acclimation of basal land plants. Additionally, the higher plants accumulate different cellular enzymatic antioxidants such as superoxide

dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), etc. as the scavengers of excess Reactive Oxygen Species (ROS), overproduction of which result in cellular damage and death (Raja *et al*., 2017; Laxa *et al*., 2019), but the clarification of the role of those components in basal land plants is not considered yet. Even though, our most recent investigation on *M. polymorpha* demonstrated that cellular protectants such as proline and enzymatic antioxidants including SOD, CAT, APX, DHAR and GST are greatly induced under physiological drought (Ghosh *et al*., 2021), the information regarding those are still to be examined under hyperosmotic stress led by NaCl.

Considering the above facts, we set our present efforts and submitted the gemmae (asexual reproductive unit of liverwort *M. polymorpha*) to sodium chloride (NaCl)-induced hyperosmotic stress for showing the alteration in morpho-physiological attributes such as growth, occurrence of photosynthetic pigments, accumulation of proline and soluble sugars, occurrence of ROS and the activity of enzymatic antioxidants. Thus, by this investigation, we explored that hyperosmotic stress acclimation in liverworts was regulated by limiting morphology, and by accumulating higher level of proline, soluble sugar and enzymatic antioxidants and the characters of those are conserved in evolutionarily important land plant liverworts.

Materials and Methods

Plant materials' culture and their growth

The gemmae; asexual reproductive unit of liverwort *M. polymorpham* (accession Takaragaike-1; TAK-1) were cultured in Gamborg ½ B5 medium containing 2% sucrose by maintaining standard conditions such as temperature range; 23°C-24°C with a light cycle of 16 h of light/8 h of darkness and a light intensity of 55 μmol s^{-1} m^{-2} which was followed by Ghosh *et al*. (2021).

Treatments combination for hyperosmotic stresses

For the analysis of growth, after taking initial weight, 15-d old cut thallus body was directly cultured in ½ B5 solid medium (control) or osmotic stresses provided with ½ B5 solid medium supplemented with 50 mM and 100 mM NaCl for another 15-d by following previous study (Tanaka *et al*. 2018). We also standardized the doses of NaCl by using different concentrations and found 150 mM showed very lethal effects to the gemmalings by only 2-d (data not shown). The growth performances of the thallus body were measured by making comparison of both final and initial weights of fresh tissue after 15-d of culture. The weight gained by 15-d was recorded in both control and treated thallus body. For measuring the degree of tissue damaged, we supposed the thallus body to the Evan's blue staining as followed by Takezawa *et al*. (2015). For photosynthetic pigments and proline accumulation assay, liquid culture was followed. After 3-d normal culture in ½ B5 liquid medium, the gemmalings were subjected to either ½ B5 liquid medium (control) or 1/2B5 liquid medium supplemented with 50 and 100 mM NaCl for another 2-d. For showing electrolyte leakage, ROS accumulation and antioxidant assay, after 3-d normal culture, the gemmalings were subjected to either ½ B5 liquid medium (control) or 1/2B5 liquid medium supplemented with 100 mM NaCl for 2-d. For soluble sugar assay, the gemmalings were subjected to either ½ B5 liquid

medium (control) or 1/2B5 liquid medium supplemented with 100 mM NaCl and 0.2 M sucrose. The experiment was followed by maintaining at least three independent replications.

Determination of chlorophyll content and electrolyte leakage

Chlorophyll content in the treated and non-treated gemmalings was estimated from the thallus body of *M. polymorpha* using the method described by Porra (2002). Electrolyte leakage from the damaged tissue was measured by an electrical conductivity meter as described by Takezawa *et al*. (2015).

Determination of proline and soluble sugar content

Proline extraction from treated and non-treated gemmalings was done by following the method of Bates *et al.* (1973). A standard curve was prepared using different concentration of proline as standard and proline concentration was calculated as µmole g^{-1} FW. The soluble sugar content in the gemmalings was quantified by the anthrone-sulfuric acid assay using glucose as a standard (Yemm and Willis, 1954).

Phylogenetic analysis for sugar biosynthesis gene

Phylogenetic analysis of sucrose biosynthesis gene (*sugar phosphatase 1*) was made using protein sequences of representative bryophyte models; *P. patens* and *M. polymorpha* and angiosperms' models; *Arabidopsis thaliana* and *Oryza sativa*. The blast search was made using the website of Phytozome v13 and amino acid sequences were aligned by ClustalW program. The tree was made using MEGA 6 Program by following neighbor-joining method (Saitou and Nei, 1987).

Determination of MDA and H_2O_2 content

The amount of lipid peroxidation product; malondialdehyde (MDA) in both treated and non-treated gemmalings were determined using Trichloroacetic acid (TCA) and thioberbutyric acid (TBA) by following the method used by Ghosh *et al*. (2021). The MDA content was measured as nmol g^{-1} FW. The H_2O_2 was measured as µmol g^{-1} FW using the method followed by Ghosh *et al*. (2021).

Analysis of enzymatic antioxidant activities

For analyzing the activities of various antioxidant enzymes, gemmalings (0.1 g) were crushed in 300 µl of extraction buffer comprised with 50 mM ice-cold KP buffer (pH 7.0), potassium chloride (100 mM), ascorbate (AsA, 1 mM), β-mercaptoethanol (5 mM) and glycerol (10%; v/v). The homogenates were centrifuged at 11,500 × g for 12 min, and the supernatant was collected for estimating enzyme activities. Protein concentration was determined using Bradford reagent assay using Bovine Serum Albumin (BSA) as standard. The activity of The SOD activity was estimated using xanthin-xanthin oxidase system as suggested by Beyer and Fridovich (1987). SOD activity was calculated as unit; U mg^{-1} protein (inhibition of NBT reduction by 50% per minute). The activity of Catalase (CAT) was determined by using the procedure of Aebi (1984). The activity of CAT was determined as µmol $min^{-1}mg^{-1}$ protein. The activity of APX was determined according to the protocol used by Nakano and Asada (1981). The activity of APX was determined as µmol $min^{-1}mg^{-1}$ protein. GST activity was measured following the procedure of Hossain *et al*. (2010). The activity of

DHAR was determined according to the protocol used by Nakano and Asada (1981). The activity of DHAR was determined as nmol $min^{-1}mg^{-1}$ protein. Finally, the activity of GST was determined as nmol $min^{-1}mg^{-1}$ protein.

Microscopy and image analysis

The damaged tissue of the gemmalings stained with Evans blue dye was marked with the help of advanced microscopy by following the method used by Takezawa *et al*. (2015).

Statistical analysis

Completely Randomized Design (CRD) was followed for setting experiments. Least significant difference (LSD) at $P < 0.05$ and student t-test was used for comparison of treatments. The numerical values used in the figures represented as means ± standard errors (SEs). The experiment was conducted by following at least three independent replications. The data were analyzed by Statistix 10 program.

Results and Discussion

The findings regarding morphological, physiological and biochemical attributes of the gemmalings exposed to hyperosmotic stress led by different concentrations of NaCl are presented, discussed and explained as different sub-heads.

Effect of hyperosmotic stress on the growth of *M. polymorpha*

While acclimating to drought and osmotic stresses, land plants follow numerous changes in their morphology especially in the reduction or alteration of vegetative growth to mitigate excess water loss (Ansari *et al*., 2019). To see the morphological alteration of *M. polymorpha*, the gemmae were cultured in ½ B 5 solid medium for 15 days of culture. Then the thallus body was cut and after taking initial weight, that was supposed to subculture in control (1/2 B5 solid medium), and hyperosmotic conditions provided with 1/2 B5 solid medium supplemented with 50 mM and 100 mM NaCl. After 15 days of subculture, the final weight of the thallus body was recorded. The weight gain was calculated from final and initial weight. When cut thallus body of *M. polymorpha* were supposed to hyperosmotic stressed condition, it showed suppression of phenotypic growth significantly (Fig.1A). The highest rate of thallus weight gain was observed at control condition (0.325g), and the lowest (0.041g) was found by 100 mM NaCl (Fig.1B). Since the quality and quantity of plant growth depend on many cellular events like cell division, differentiation, development and involves genetic, physiological, ecological and morphological events, the changing of growth pattern should be the important strategy of plants for the acclimation to the existing condition. As compared to angiosperms, very few reports were made in the growth inhibition of basal land plants during abiotic stress. As for example, very strong growth inhibition was reported in basal land plant moss *P. patens* and liverwort *M. polymorpha* in response to phytohormone ABA (Akter *et al*., 2014; Takezawa *et al*., 2015; Arif *et al*., 2019). Another reports on *M. polymorpha* suggested the interruption of growth appearance in response to salinity and drought stress (Tanaka *et al*., 2018; Godinez-Vidal *et al*., 2020). Our most recent investigation supported strong growth inhibition of *M. polymorpha* thallus under physiological drought induced by polyethelene glycol

(PEG), sucrose and mannitol (Ghosh *et al*., 2021). In our observation, the gemmaling growth was drastically reduced in responses to hyperosmotic stress induced by 100 mM NaCl (Fig. 1) which was consisted to the previous findings. Therefore, along with the findings of previous efforts, the present observation suggest that growth inhibition caused by drought and osmotic stresses are very common to all sort of land plants and the attributes were gained in early and distant land plants liverworts.

Effect of hyperosmotic stress on the chlorophyll content and the tissue damage of *M. polymorpha*

As proper maintenance of photosynthetic efficiency is a big challenge for land plants during acclimation to abiotic stresses, synthesis of photosynthetic pigments specially chlorophyll should be good indicator for stress tolerance. Hence, the gemmalings of *M. polymorpha* after 3 days of normal culture were submitted to control (1/2 B5 liquid medium) and 1/2 B5 liquid medium supplemented with hyperosmotic stressed

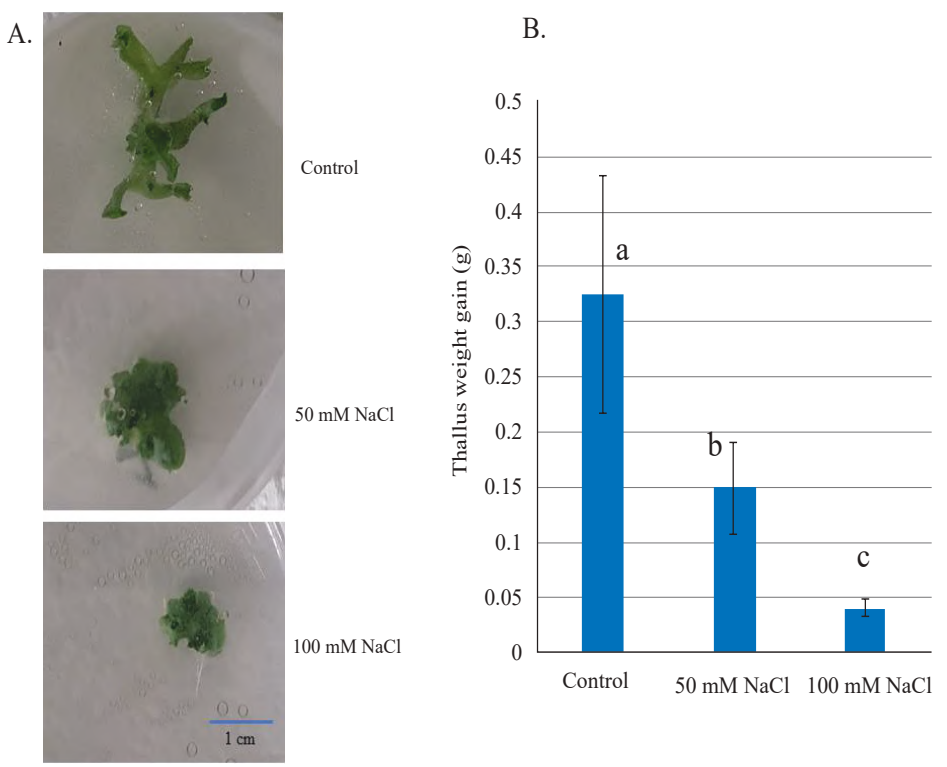

Fig. 1. Effect of NaCl-induced hyperosmotic stress on the size (A) and weight (B) of the thallus body of *Marchantia polymorpha*. Suppression of thallus size was observed when 15-d old cut thallus body was exposed to osmotic stressed condition led by 50 and 100 mM NaCl for another 15-d (A). As compared to control (1/2 B5 solid medium), thallus weight gain was significantly reduced under osmotic stressed conditions provided with ½ B5 medium supplemented with 50 and 100 mM NaCl (B). Error bar indicates standard error (n=3). Different alphabetic letters above the bars show significant differences among the treatments ($P<0.05$).

condition led by 50 and 100 mM NaCl. The chlorophyll content; Chl *a* and Chl *b* and Chl (*a*+*b*) were determined from control and treated gemmalings after 2 days of culture. The hyperosmotic stress showed profound effects on the chlorophyll content of the gemmalings of *M. polymorpha* (Fig. 2). The lowest Chl *a* content; 1.72 mg g^{-1} FW was recorded in 100 mM NaCl treatment while the highest value 2.49 mg g^{-1} FW was observed in control treatment (Fig. 2A). Likewise, Chl *a*, the effect of hyperosmotic stress on chl *b* content was also significant. The Lowest amount of Chl *b* content; 1.11 mg g^{-1} FW was recorded in 100 mM NaCl treatment while the highest value; 1.39 mg g^{-1} FW was observed in control treatment (Fig. 2A). Therefore, the highest amount of chl (*a*+*b*) (3.88 mg g^{-1} FW) was found in control condition followed by 50 mM NaCl (3.81 mg g^{-1} FW) and 100 mM NaCl (2.90 mg g^{-1} FW) (Fig. 2A). The chl *a* (2.44 mg g^{-1} FW), Chl *b* (1.37 mg g^{-1} FW) and Chl (*a*+*b*) (3.81 mg g^{-1} FW) content under 50 mM NaCl were more or similar to control condition; 2.49, 1.39 and 3.89 mg g^{-1} FW respectively which indicates little osmotic pressure created by 50 mM NaCl (Fig. 2A). To see the tissue damaged, the treated and non-treated gemmalings were stained with Evan's blue dye and damaged tissue was monitored by recoding blue-colored death tissue. The Evan's blue test indicated higher degree of tissue damage by 100 mM NaCl rather than 50 mM NaCl which showed little tissue damage as compared to control (Fig. 2B). The finding was consistent to the lower and higher reduction of photosynthetic pigments in the gemmalings by 50 mM and 100 mM NaCl respectively (Fig. 2A). A lot of research efforts in angiosperms suggested that drought or water limiting environment led to the drastic reduction of photosynthetic pigments specially chlorophylls (Thaloot *et al*., 2006; Liu *et al*. 2018; Fallah, 2020). Along with that, salt stress reduced chlorophyll contents in the plant that might be the malfunctioning of the pigment molecules, and protein-pigments complexes, and structural instability of light harvesting complexes (Geissler *et al*., 2009). However, increased salinity also leads to the higher generation of ROS in chloroplast which further damages chloroplast membrane and thereby causes leakage of chlorophyll from thylakoids (Sun *et al*., 2010). As contrast to angiosperms, basal land plants mosses and liverworts also compromised to photosynthetic pigments while facing physiological drought and osmotic stresses (Pizarro *et al*., 2019; Ghosh *et al*., 2021). The possible reasons for decreasing chlorophyll content might be the damaging of photosynthetic pigments as well as thylakoid membranes during drought stress. Along with those findings, significant reduction of chlorophyll content under hyperosmotic stress in evolutionary important land plant *M. polymorpha* (Fig. 2A) reflects that chlorophyll pigments were very indispensable during the first acclimation of land plants to dry terrestrial habitats.

Effect of NaCl-induced hyperosmotic stress on the proline content of *M. polymorpha*

Enhanced accumulation of compatible solutes or osmolytes are very common in angiosperms during acclimation to drought stress. Therefore, we submitted gemmalings to hyperosmotic stress to find out the possible accumulation of one of the essential osmolyte; proline. We found a remarkable increase in proline content by100 mM NaCl where more

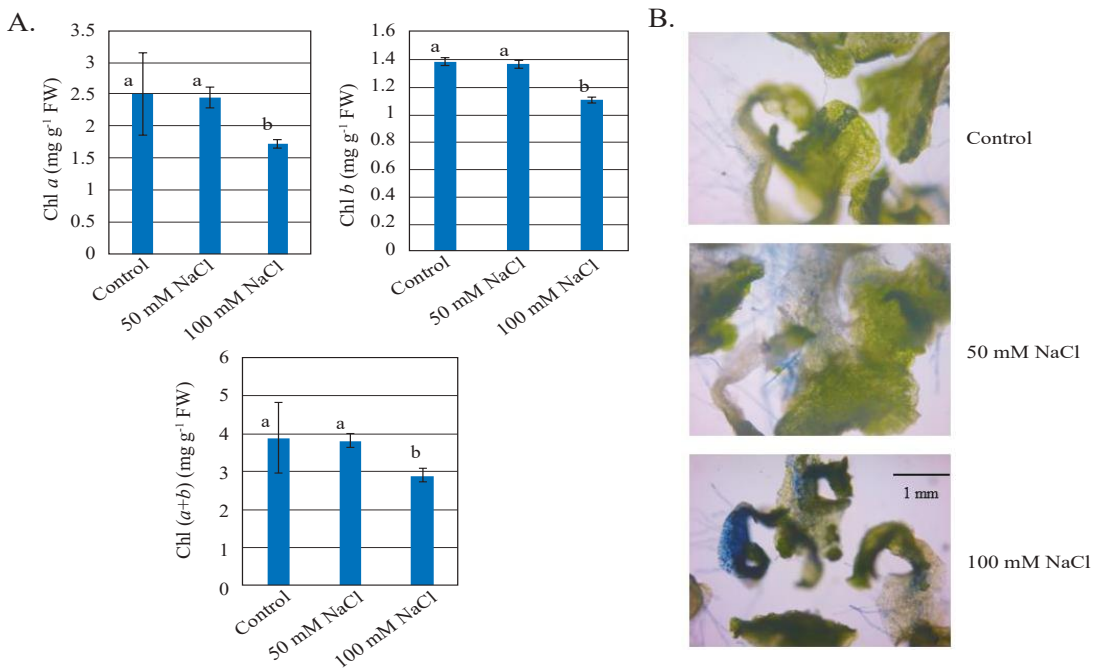

Fig. 2. Effect of NaCl-induced hyperosmotic stress on the chlorophyll content (A) and tissue damage (B) of the gemmalings of *Marchantia polymorpha*. As compared to control, Chl *a*, Chl *b* and total Chl (*a*+*b*) content (mg g^{-1} FW) were significantly reduced when 3-d culture gemmalings were treated with 100 mM NaCl for another 2-d (A). Error bar indicates standard error (n=3). Different alphabetic letters above the bars show significant differences among the treatments ($P<0.05$). Treated and non-treated gemmalings were stained with Evan's blue dye for measuring tissue damage (B). Scale bar indicates 1 mm.

or less similar pattern of proline accumulation in control (0.11 µmol g^{-1} FW) and 50 mM NaCl (1.13 µmol g^{-1} FW mM NaCl) indicates negligeable osmotic pressure by 50 mM NaCl (Fig. 3). Maximum proline accumulation**;** 0.33 µmol g^{-1} FW was observed in 100 mM NaCl treatment. In contrast, minimum proline accumulation; 0.11 µmol g^{-1} FW was found in control condition. Enhanced accumulation of proline was previously reported in response to drought stress in *Petunia hybrid*a (Yamada, 2005), pea (Alexieva *et al*., 2001) and rice (Saddique *et al*., 2020). Very recent investigation on *M. polymorpha* resulted the higher accumulation of proline under physiological drought induced by PEG, Sucrose and Mannitol (Ghosh *et al*., 2021). The accumulation of proline is therefore, a physiological strategy used by many plants to overcome abiotic stresses including drought and osmotic stresses. Increased accumulation of proline and soluble sugar under salt stress contributed to osmotic adjustment by reducing cell water potential, maintaining cellular turgidity and protecting metabolic processes (Munns and Tester, 2008; Azeem *et al*., 2023). In our observation, proline accumulation was found to be enhanced under hyperosmotic stress induced by 100 mM NaCl (Fig. 3) indicating that the machinery for the accumulation of cellular osmolytes was developed during the earlier stages of land plants' terrestrialization

and the characters are conserved throughout the land plants. However, the occurrence of proline biosynthesis gene *Δ[1]-pyrroline-5-carboxylate synthase 1* (*P5CS1*) in *M. polymorpha* by phylogenetic analysis made further confirmation of the protective role of osmolyte proline during land plant evolution (Ghosh *et al*., 2021).

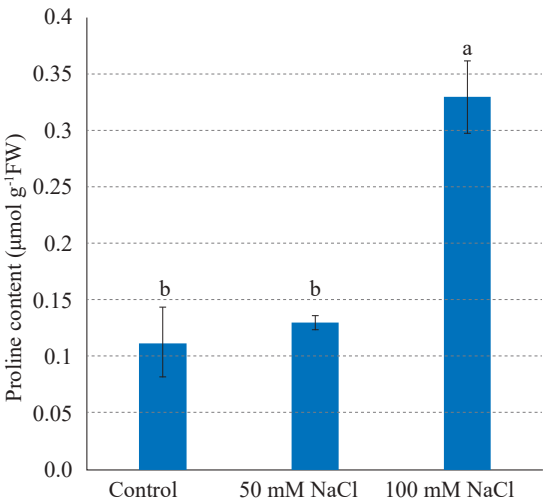

Fig. 3. Effect of NaCl-induced hyperosmotic stress on the proline content of the gemmalings of *Marchantia polymorpha*. As compared to control, proline content was significantly increased when 3-d culture gemmalings were treated with100 mM NaCl for another 2-d. No significant difference was found when treated with 50 mM NaCl. Error bar indicates standard error (n=3). Different alphabetic letters above the bars show significant differences among the treatments ($P<0.05$).

Effect of NaCl-induced hyperosmotic stress on the sugar content of *M. polymorpha*

As plants follow accumulation of soluble sugar during acclimating to different abiotic stresses (Takezawa *et al*., 2015), we measured sugar content in treated and non-treated gemmalings. The present investigation showed higher accumulation of soluble sugar by both 100 mM NaCl (27.66 mg g^{-1}FW) and 0.2 M sucrose (32. 14 mg g^{-1}FW) as compared to control (17.64 mg g^{-1} FW) (Fig. 4A). Enhanced accumulation of soluble sugar by salt stress was reported to maintain osmotic adjustment by means of lower cell water potential in plant (Azeem *et al*., 2023). Higher accumulation of soluble sugar was also reported in moss *P. pates* under a variety of abiotic stresses including cold, ABA and osmotic stress (Bhyan *et al*., 2012; Takezawa *et al*., 2015). In the present investigation, significant increase of soluble sugar under osmotic stress led by 100 mM NaCl and 0.2 M sucrose suggesting that evolutionarily important land plants liverworts engaged sugar against hyperosmotic stress acclimation process (Fig. 4A). Phylogenetic analysis of present investigation showed that the presence of sugar biosynthesis gene in all sort of land plants including basal land plants moss, *P. patens* and liverwort *M. polymorpha* (Fig. 4B) which was consistent to the higher accumulation of soluble sugar in *M. polymorpha* under hyperosmotic stress led by 100 mM and 0.2 M sucrose in this study (Fig. 4A).

Effect of NaCl-induced hyperosmotic stress on the electrolyte leakage, lipid peroxidation and H_2O_2

Since cell membrane stability is greatly injured during drought stress, the measurement of electrolyte leakage suggests the rate of injury caused at cellular level. To measure the level of injury, the gemmalings were submitted to hyperosmotic stress induced by 100 mM NaCl and electrolyte leakage was measured by EC meter. As compared to control, a significant increase in electrolyte leakage was recorded

while acclimation to hyperosmotic stress (Fig. 5A). The higher electrolyte leakage (92.42%) was recorded in gemmae of *M. polymorpha* by 100 mM NaCl as compared to control (32.63%) (Fig. 5A). Since electrolyte leakage is related to ROS and membrane lipid peroxidation, we determined H_2O_2 and MDA in the gemmalings treated or non-treated with hyperosmotic stress led by 100 mM NaCl. Our observation resulted the significant induction of H_2O_2 and MDA under hyperosmotic stress. The higher rate of H_2O_2 (12.9 µmol min^{-1} mg^{-1} FW) and MDA (94.5 nmol min^{-1} mg^{-1} FW) were recorded in the gemmalings treated with 100 mM NaCl (Figs. 5B and 5C). The values of H_2O_2 (6.3 µmol min^{-1} mg^{-1} FW) and MDA (33.5 nmol min^{-1} mg^{-1}) were recorded in control condition (Figs. 5B and 5C). Abiotic stress including drought and osmotic stress causes disruption of membrane integrity due to peroxidation of membrane lipids by the enhanced activity of ROS resulting in the increased membrane permeability and decreasing the stability (Guo *et al*., 2018). Likewise, angiosperms, the membrane instability with higher degree of electrolyte leakage was reported in basal representative *P. patens* and *M. polymorpha*

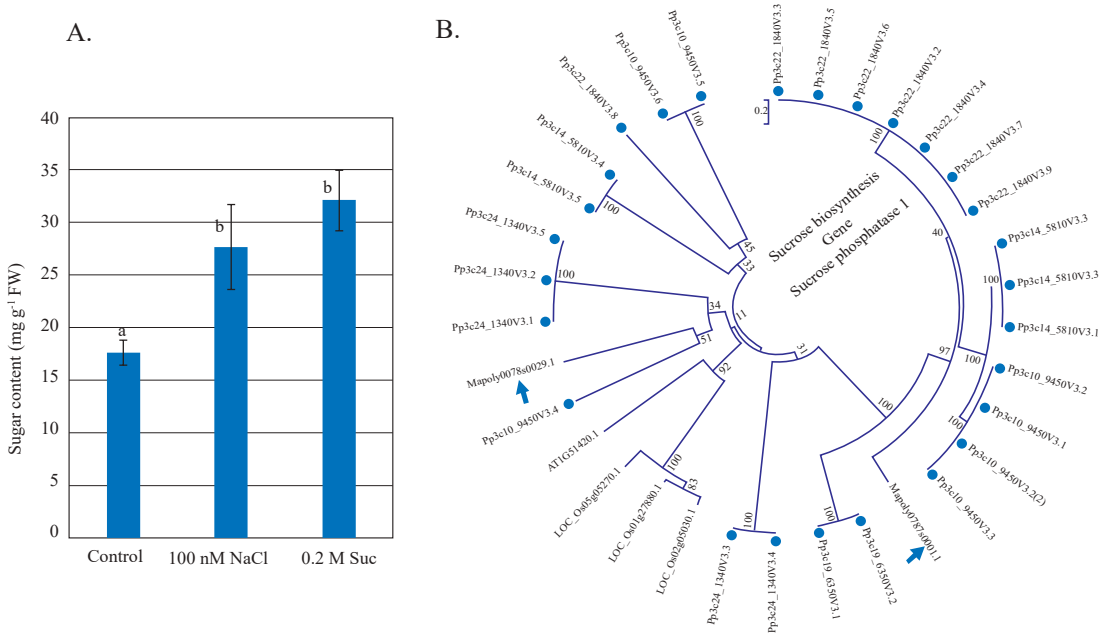

Fig. 4. Effect of hyperosmotic stress on the soluble sugar content of the gemmalings of *Marchantia polymorpha*. As compared to control, sugar content was significantly increased when 3-d culture gemmalings were treated with100 mM NaCl and 0.2 M Sucrose (Suc) for another 2-d (A). Error bar indicates standard error (n=3). Different alphabetic letters above the bars show significant differences among the treatments ($P<0.05$). Phylogenetic analysis of sucrose biosynthesis gene *sucrose phosphatase 1* using the representative plant's models; angiosperms *Arabidopsis thaliana* and *Oryza sativa*, and moss *Physcomitrella* patens, and liverwort *Marchantia polymorpha* (B). Protein sequences were collected by blast search using Phytozome v13 databases and alignment of the sequences was made by ClustalW program. The tree was built using neighbor Joining method. Bar indicates the number of substitutions per site. Black circle indicates the presence of conserved member in moss *P. patens* and arrow indicates the representative member in liverwort *M. polymorpha.*

during acclimation to osmotic stresses induced by PEG, sucrose and mannitol (Takezawa *et al*., 2015; Ghosh *et al*., 2021). In our observation, hyperosmotic stress induced by 100 mM NaCl greatly enhanced the amount of electrolyte leakage in the gemmalings of *M. polymorpha* as compared to control (Fig. 5A). However, the enhanced accumulation of reactive oxygen species ROS; H_2O_2 and lipid peroxidation product MDA in the present study was very consistent to the previous findings. In the current study, enhanced accumulation of H_2O_2 and MDA in the gemmalings of *M. polymorpha* by higher concentration of NaCl (Figs. 5B and 5C) suggesting the implication of ROS in osmotic stress acclimation process in evolutionarily important land plants liverworts.

Hyperosmotic stress-induced antioxidant activity in the gemmalings of *M. polymorpha*

Since accumulation of ROS was induced by 100 mM NaCl in gemmalings, we analyze the activity of enzymatic antioxidants which are very essential for the recovery of stress induced cell death. In the present study, the higher value of SOD was recorded as 26.69 U mg^{-1} protein by 100 mM NaCl where the lower value; 17.91 U mg^{-1} protein was recorded under control condition (Fig. 6A). The CAT activity was significantly increased in the gemmalings under hyperosmotic stress (16.6) as compared to control (6.22) (Fig 6B.). The APX activity was higher; 0.30 µmol min^{-1} mg^{-1} protein in 100 mM NaCl and lower; 0.078 µmol min^{-1} mg^{-1} in control condition (Fig. 6C). We determined the activity of DHAR in gemmalings exposed to control and hyperosmotic stress induced by 100 mM NaCl. Likewise other antioxidants, the activity of DHAR was also found to be increased during acclimation to physiological drought (Fig. 6D). The activity of DHAR were recorded as 92.72 and 75.70 nmol min^{-1} mg^{-1} under 100 mM NaCl and control conditions respectively (Fig. 6E). The GST activity was also significantly increased in the gemmalings exposed to hyperosmotic stress induced by 100 mM NaCl (Fig. 6E). The higher activity; 0.80 nmol min^{-1} mg^{-1} protein was recorded under 100 mM NaCl where the lower activity; 0.67 nmol min^{-1} mg^{-1} was found under control condition (Fig. 6E). During abiotic stress, enhanced accumulation of ROS in angiosperms has pivotal roles in increasing lipid peroxidation; MDA and damaging membrane integrity and interfering cellular homeostasis. Therefore, the alteration of antioxidant metabolisms is fundamental metabolic processes that influences the drought and osmotic stress tolerance of plants (Laxa *et al*., 2019). In enzymatic systems, SOD was reported as first line defense to scavenges $O_2^{\bullet-}$ to H_2O_2 for protecting cellular damage (Bowler *et al*., 1992). POD, CAT, APX and DHAR were responsible for decomposing H_2O_2 to H_2O at different cellular locations during oxidative stress (Mittler, 2002). APX converts H_2O_2 into water by using ascorbate in choloroplast whereas CAT and GPX detoxify it in the cytoplasm. Salt stress in plant caused a substantial increase of enzymatic antioxidants such as SOD, CAT, APX and GPX to counteract the effects of excess ROS burst all over the cell (Azeem *et al*., 2023). Therefore, in angiosperms, stimulation of antioxidant capacity has been considered as the integral part of the defense mechanisms to combat ROS toxicity under abiotic stresses including drought and osmotic and salinity

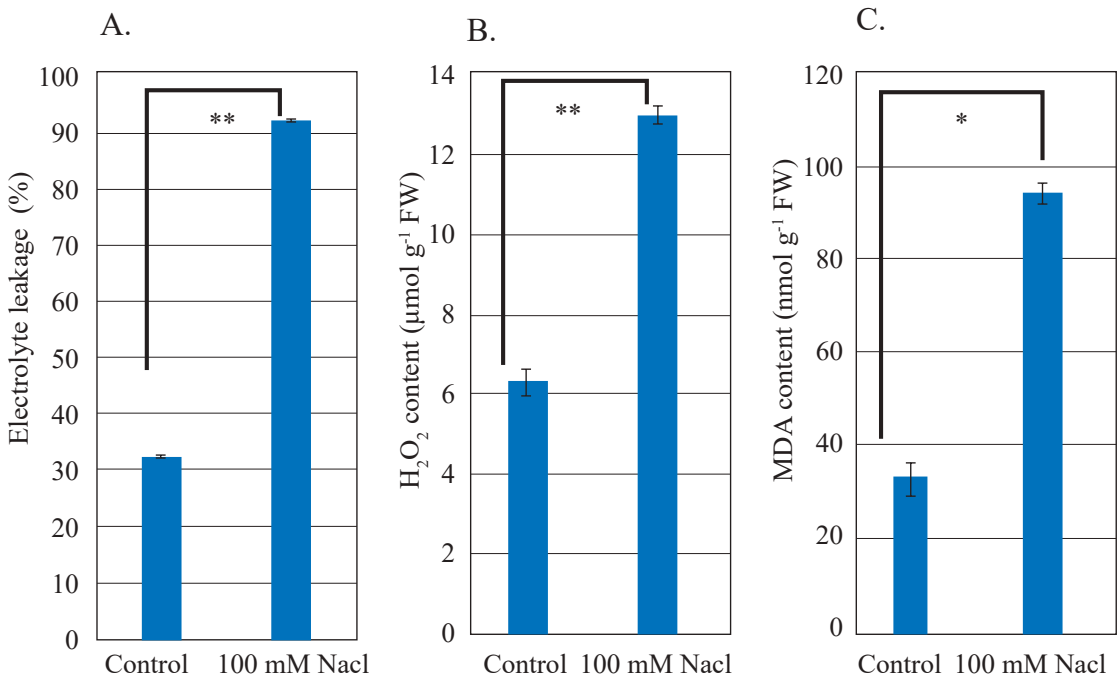

Fig. 5. Effect of NaCl-induced hyperosmotic stress on the electrolyte leakage (A), hydrogen peroxide (H_2O_2) (B) and lipid peroxidation product malondialdehyde (MDA) (C) content in the gemmalings of *Marchantia polymorpha*. As compared to control, significantly higher amount of electrolyte leakage (A), H_2O_2 (B) and malondialdehyde; MDA (C) content were recorded when gemmalings were treated with hyperosmotic stress led by 100 mM NaCl. Asterisk indicates significant differences among the treatments (*$P<0.05$, **$P<0.01$).

stresses. In contrast to angiosperms, very little reports about the activity of enzymatic antioxidant were made in basal land plants. As for example, the activity of SOD was found to be increased in moss *Sanionia uncinata* in responses to abiotic stresses (Pizarro *et al*. 2019). Our most recent investigations suggested the enhanced activity of SOD under physiological drought (Ghosh *et al*., 2021). Along with that, enhancement of SOD activity by NaCl-induced hyperosmotic stress in this investigation (Fig. 6A) claimed the early adaptive role of this enzymatic antioxidant in hyperosmotic stress acclimation of land plants. Notably, plant triggers CAT activity in order to eliminate the detrimental H_2O_2 which is produced during metabolic processes and cause damage to the cell functioning (Gasper *et al*., 2002). CAT activity was also found to be enhanced in the primitive plant *Selaginella tamariscina* and *M. polymorpha* under abiotic stresses (Wang *et al*., 2010; Ghosh *et al*., 2021). In the present observations, the enhanced accumulation of CAT activity in *M. Polymopha* under hyperosmotic stress indicates that the mechanism was developed during the earlier stages of land plant's terrestrialization (Fig. 6B). Several investigations also reported the increased activity of APX, an important member of ascorbate-reduced glutathione (ASA-GSH) cycle enzyme in angiosperms under abiotic

stresses (Wang *et al*., 2017). Paciolla and Tommasi (2003) reported the activity of APX to the removal of H_2O_2 in moss *Brachythecium velutinum* and liverwort, *M. polymorpha*. With the findings of elevated activity of APX in *M. polymorpha* in response of physiological drought (Ghosh *et al*., 2021), our observations of the enhanced activity of APX upon getting NaCl-induced hyperosmotic stress suggest the activity of APX are conserved throughout the land plants including liverworts (Fig. 6C). We further analyzed the activity of DHAR

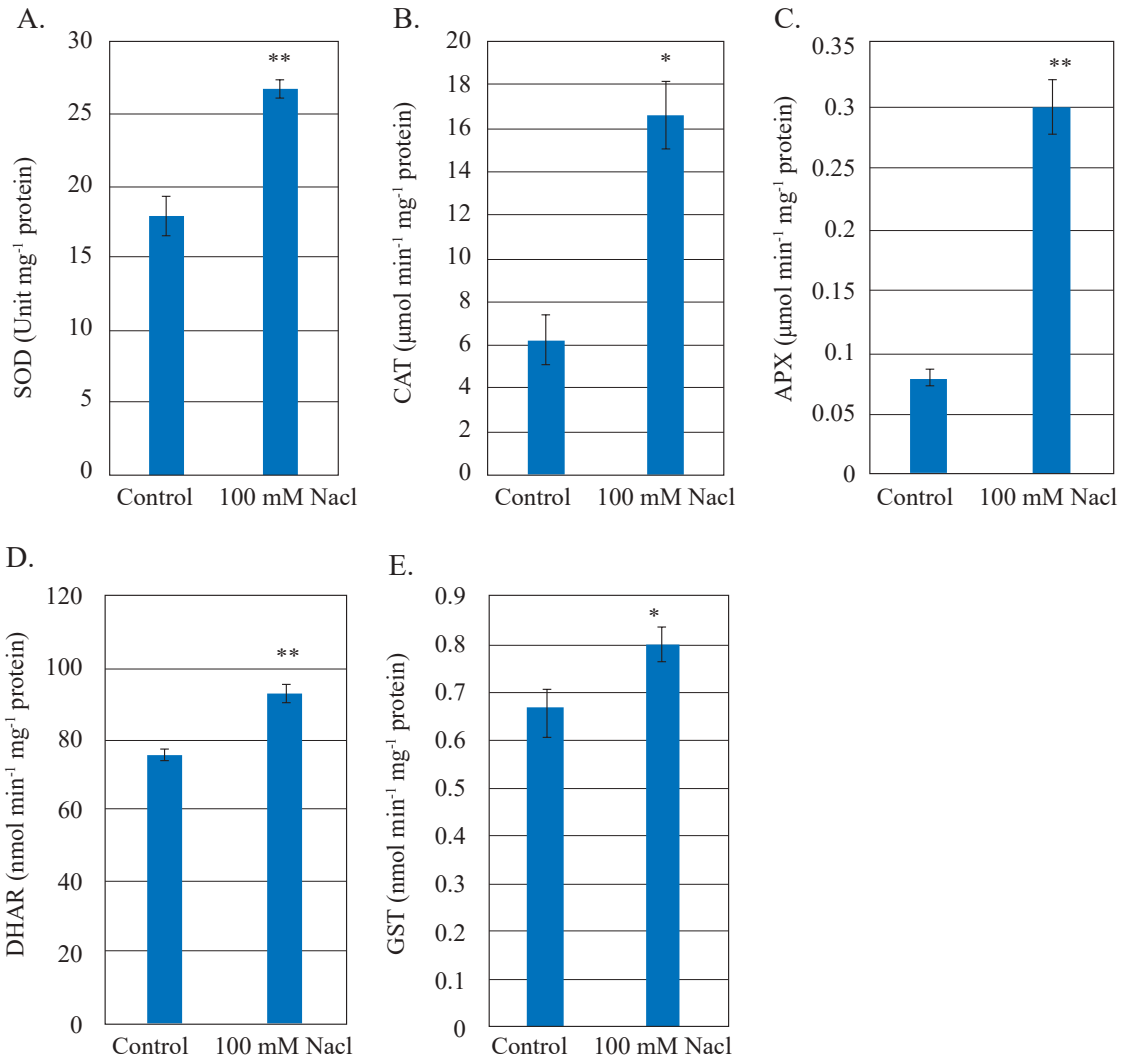

Fig. 6. Effect of NaCl-induced hyperosmotic stress on the activity of enzymatic antioxidants in the gemmalings of *Marchantia polymorpha*. When 3-d old gemmalings were treated with hyperosmotic stress led by 100 mM NaCl for another 2-d, the superoxide dismutase; SOD activity (A), catalase; CAT activity (B), ascorbate peroxidase; APX activity (C), dehydroascorbate reductase; DHAR activity (D) and glutathione S-transferase; GST activity (E) were significantly increased as compared to non-stress control. Asterisk indicates significant differences among the treatments (*$P<0.05$, **$P<0.01$).

which is another key component of AsA-GSH cycle enzyme regulating the level of AsA and its redox state under oxidative stress. Increased activity of DHAR was reported in plant during acclimation to various osmotic stresses induced by PEG, sucrose and mannitol in evolutionarily important land plant *M. polymorpha* (Ghosh *et al*., 2021). In the present investigation, increased activity of DHAR in *M. polymorpha* under NaCl-induced hyperosmotic stress suggests the primitive role of this enzyme for stress acclimation (Fig. 6D). Besides these, the activity of GST was reported to be greatly induced by various abiotic stresses in plants (Dixon *et al*., 2010). Ghosh *et al*. (2021) reported enhanced activity of GST in liverwort *M. polymorpha* under physiological drought and the results of which supported our present findings regarding enhanced level of GST activity in *M. Polymopha* under hyperosmotic stress (Fig. 6E) suggesting the early adaptive roles of this superfamily enzymes in regulating glutathione metabolism.

Conclusion

Based on the morpho-physiological and biochemical strategies of liverwort *M. polymorpha* for acclimation to hyperosmotic stress, it can be summarized that the gemmae; asexual reproductive unit of *M. polymorpha* showed the reduction of thallus growth under hyperosmotic stress induced by 100 mM NaCl. The induction of H_2O_2, MDA, and electrolyte leakage by 100 mM NaCl suggest that *M. polymorpha* was supposed to be affected by oxidative stress during acclimation to hyperosmotic stress. The enhanced level of proline and soluble sugar accumulation in *M. polymorpha* under hyperosmotic stress led by 100 mM NaCl and possible occurrence of sugar biosynthesis gene in this basal representative indicate the evolutionary role of those osmolytes in hyperosmotic stress tolerance. Additionally, the significant induction of the NaCl-induced enzymatic antioxidants such as SOD, CAT, APX, DHAR and GST activities in *M. polymorpha* indicates the cellular mechanisms for the detoxification of ROS during hyperosmotic stress acclimation were developed during the terrestrialization land plants. However, further comprehensive studies for the accumulation of stress related proteins and transcriptomic analysis of stress tolerance genes are needed to fine-tune the understanding of the hyperosmotic stress tolerance mechanisms in basal land plants.

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Author's declaration

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