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Research Article

Proline priming: An efficient strategy to mitigate salinity impact at early developmental stages of the oilseed halophyte *Cakile maritima*

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Abstract

Seed germination is a vital process, yet extremely sensitive to salinity. This is particularly true for coastal halophytes like the annual oilseed species *Cakile maritima*, which faces the simultaneous impact of wind, salt-spray and seawater inundations in its natural biotopes. At the early developmental stages, this may jeopardize seed germination, plant establishment capacity and hence its development and persistence. Osmopriming is a pre-sowing approach aiming to improve seedling emergence and establishment in adverse environments. Here, we investigate the effect of proline (at 0, 1, 5, and 20 mM) pre-treatment on salt tolerance of *C. maritima* at the juvenile stage under salinity (0, 100, and 200 mM NaCl). Proline seed priming enhanced the germination rate (28% to 92%) and promoted seedling establishment of *C. maritima* by stimulating α-amylase activity even at the highest salinity (+55 %). Besides, after transfer of non-germinated seeds on distilled water, salt impact was fully reversible. At the seedling stage, chlorophyll fluorescence parameters showed that this osmoticum increased the maximal quantum yield of PSII photochemistry (Fv/Fm) and the quantum yield of photochemical energy conversion [Y(II)]. In contrast, the quantum yield of nonregulated nonphotochemical energy dissipation [Y(NO)] and the quantum yield of regulated nonphotochemical energy dissipation [Y(NPQ)], which might be correlated to the mitigation of the salt deleterious effects on PSII. Proline and carbohydrate concentrations also increased following priming. Overall, our data provide strong arguments for using proline at low doses (1 and 5 mM) as a successful priming agent to alleviate salinity-induced adverse effect on plants.

Keywords: α-amylase; *Cakile maritima*; germination; proline; photosynthesis; salinity; seed priming

Abbreviations: α-Amylase: Alpha-Amylase; COP: Cotyledons Osmotic Potential; Fv/Fm: Maximum Photochemical Efficiency of PSII; GP: Germination Percentage; HP: Hydro-Primed; MDA: Malondialdehyde; P5C: Pyrroline-5-carboxylate; P5CS: Pyrroline-5-carboxylate Synthase; Pro: Proline; PSII: Photosystem II; PS: Primed Seeds; RCWC: Relative Cotyledon Water Content; ROS: Reactive Oxygen Species; SDW: Seedlings Dry Weight; SG: Speed of Germination; SL: Seedling Length; SLVI: Seedling Length Vigour Index; SWVI: Seedling Weight Vigour Index; UPS: Un-Primed Seeds; Y(II): Efficient Quantum Yield

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of PSII; Y(NO): Nonregulated Nonphotochemical Quenching; Y(NPQ): Regulated Nonphotochemical Quenching.

Introduction

Sea rocket (*Cakile maritima*, Brassicaceae) is an annual succulent halophyte, naturally thriving on sandy beaches along the North African coasts. Although it is frequent in Tunisia, this species is currently threatened by mass tourism and the impact of high tides and salt spray. It is recognized for its ecological benefits, such as sand dune stabilization, and its economic potential (Zarrouk *et al.,* 2003). Owing to a deep and dense root system, *C. maritima* is one of the key species to the restoration and anchoring of damaged sandy littoral dunes (Debez *et al.,* 2017). To protect and restore these natural barriers, which provide unique vegetation and wildlife habitats, recent developments in tourism are integrating the ecological dimension.

Germination and seedling stages are the crucial primary phases of plant establishment and the most sensitive stages to abiotic stress, especially for annual plants (Debez *et al.,* 2012). Depending on the species and the accession, moderate and high salinities can inhibit plant establishment by inducing secondary dormancy (Yang and Guo, 2018) due to osmotic stress or specific ion toxicity (Boukraa *et al.,* 2022). For *C. maritima*, previous studies on various Tunisian accessions revealed that their maximal germination was reached under salt-free conditions (Debez *et al.,* 2018; Ghars *et al.,* 2009). Salinity impacted seed germination of this species *via* both osmotic and toxic effects. Using the proteomic approach, Debez *et al.* (2018) showed that 200 mM NaCl salinity impacted and delayed the germination process in *C. maritima* by inhibiting the seed reserve mobilization.

The decline in biomass production observed in plants subjected to salinity stress is frequently associated with a decrease in their photosynthetic capacity (Kwon et *al*., 2019). Numerous reports revealed a linear regression between salinity and growth, and maximum photochemical efficiency of photosystem II (Fv/Fm) (Hnilickova et *al.*, 2021). To successfully cope with salt stress conditions, plants synthesize and accumulate organic compatible solutes like sugars and amino acids. Osmoprotectants not only contribute to osmotic adjustment but also, they are involved in stabilizing proteins and membranes. Exogenous application of compatible osmolytes (small, highly soluble, uncharged, and nontoxic organic molecules) proved to be effective in reducing salt impact on plants due to its higher penetration ability and faster mobilization capacity (Johnson and Puthur, 2021). Priming using these agents acts directly through osmoregulation or indirectly *via* osmoprotection and antioxidant properties (Sghaier-Hammami *et al.,* 2020). Chemical seed priming is suggested as a feasible and affordable approach in order to alleviate seed dormancy and to improve seed germination, seedling growth. This positive effect was concomitant with an improvement of the maximum quantum yield of photosystem II in various crops and non-crop species under abiotic stresses (Yang *et al.* 2018). Messedi *et al.* (2016) reported that foliar-applied proline (20 mM) improved the quantum yield of photochemical energy conversion in PSII [Y(II)] and electron transfer rate (ETR). This was associated with lower nonphotochemical quenching (NPQ) suggesting that proline protected chloroplast structures from the toxic effects of salt stress. In addition, the accumulation of endogenous proline may be an adaptive strategy of seeds to avoid germination under stressful conditions and thus ensure good seedlings' growth (Hosseinifard et al., 2022).

Proline is not only an osmolyte contributing to maintaining cell turgor under osmotic constraints, but it is also considered as a multifaceted molecule. This amino acid acts as a molecular chaperone and an antioxidative defence molecule, scavenging reactive oxygen species (ROS). It is also a signalling molecule that activates specific genes with crucial functions for plant stress response and recovery. Furthermore, it is a source of nitrogen and carbon (Ghosh et *al*., 2022). The over-accumulation of proline is positively correlated with stress tolerance in plants, indicating that maintaining cellular homeostasis, water uptake, osmotic adjustment,

and redox balance, are key processes to protect cell structures and mitigate oxidative damage (Hosseinifard *et al*., 2022).

Proline priming activates physiological and molecular pathways enabling the seed or plant to respond more quickly and/or more vigorously after exposure to salinity. Yet, it has to be mentioned that despite the advantageous effects of proline applications, negative effects may occur at high doses (Liu Hua-Long *et al.,* 2014). Consequently, it is crucial to determine the most appropriate concentration of proline when using priming approach for a successful improvement of the germination process and seedling growth.

In this context, the present study aims to assess the effects of different concentrations of proline on germination and seedling growth by focusing on ecophysiological and biochemical traits related to germination, seedling water status, osmotic adjustment capacity (osmotic potential and accumulation of compatible solutes) and functional integrity of Photosystem II (PSII) under salt stress conditions.

Materials and Methods

Seed material and treatments

The seeds of the oleaginous halophyte *C. maritima* used in this study were harvested in August 2017 in Raoued (20 km Northeast from Tunisia, 36°58'00.5"N, 10°12'35.5"E). 1800 seeds were used, with six replicates for each treatment and twenty seeds per replicate (n= 6 replicates, for each treatment 20 seeds per replicate). For sterilization, the seeds were disinfected for 10 min in a 2% sodium hypochlorite solution followed by washing with distilled water. Proline-priming solution (0.2 L) was prepared for each priming application. Seeds were soaked for 12h at room temperature in dark conditions. For seed priming, four proline concentrations [Pro] were used: 0, 1, 5, or 20 mM. After priming, seeds were washed with distilled water and dried between two filter papers. Unprimed seeds were used as the control treatments. For each treatment, 20 seeds were germinated in square Petri dishes (12 cm x 12 cm x 1.5 cm), double-lined with filter paper, with six replicates. Unprimed and primed seeds were moistened with 10 mL of the following NaCl concentrations (0, 100, or 200 mM) for 10 d. Germination and seedling growth took place in a climate chamber at 25 °C.

Germination parameters

The number of germinated seeds was recorded daily for up to 10 d of the experiment. Radical extension of 2 mm was measured as germination. Besides, the following parameters were determined:

Germination kinetics

The kinetics of germination was measured per unit of time, represented by three phases: the latency duration when seeds assimilate the capacity to germinate. Secondly, an exponential phase showed an increase in the cumulative germination percentage of seeds and finished with a stationary stage, where germination percentage remained maximum and constant with time.

Germination percentage (GP)

The germination percentage was calculated by the following formula, described by Ashraf and Foolad (2005). GP (%) = (A/B) × 100. Where 'A' is the total number of seeds germinated in saline solution of varying concentrations (0, 100, 200 mM NaCl) after 10 days and 'B' is the total number of seeds.

Speed of germination (SG)

This parameter evaluates the dynamics of the germinative capacity and was calculated as described by Wardle et al. (1991) as follows:

$SG = (N1\times1) + (N2-N1) \times 1/2 + (N3-N2) \times 1/3 + \ldots (Nn-Nn\text{-}1) \times 1/n.$

With N1, N2, N3, Nn-1, Nn: proportion of germinated seeds observed at first, second, third… (n – 1), (n) days or hours.

Recovery percentage (RP)

After 10 days, ungerminated seeds in salt were transferred to distilled water for another 7 days to assess their viability. The experimental conditions were the same as described above. The recovery germination percentage (RP) was calculated by using the given formula:

RP (%) = C/D × 100.

Where 'C' is the number of germinated seeds in recovery tests and 'D' is the total number of seeds transferred to distilled water (Zhang *et al.,* 2014).

The total germination percentage (TGP)

This parameter was determined by the following formula:

TGP (%) = (A + D)/B × 100 (Zhang *et al.,* 2014).

Extraction and assay of α-Amylase

Primed and unprimed *C. maritima* seeds were germinated in presence of different salt concentrations (0, 100, and 200 mM NaCl).

After 72 h, 400 mg of seeds were homogenized with 50 mM Tric-HCl, pH 7.0. The homogenate was centrifuged at 30,000 *g* for 30 min. α-amylase was assayed using heat-treated crude extracts (75 °C for 15 min in presence of $CaCl_2$ (3 mM)) to neutralize β-amylase and α-glucosidase (Sun and Henson, 1991; Guglielminetti *et al.,* 1995). The assay buffer contained 50 mM Na-acetate (pH 5.2) and 10 mM $CaCl_2$. The substrate was 2.5% boiled soluble starch (incubation lasted for up to 15 min). An aliquot (100 µL) was taken from the assay mixture and treated with 250 µL of DNS solution (40 mM DNS, 400 mM NaOH, 1 M K-Na tartrate, heated at 50 °C, and filtered through filter paper) for 5 min at 105 °C. After dilution with distilled water (up to 5 mL), the absorbance at 530 nm (A530) was read and reducing power was evaluated using a standard curve obtained with glucose (0-9 µmol).

Seedling growth and morphological analysis:

The morphological parameters were performed on 10-day-old seedlings. The seedlings were carefully removed from Petri dishes, washed in distilled water and dried on filter paper. Cotyledons (the first leaves), hypocotyls and roots of each seedling were separated and data for fresh biomass were recorded.

Dry Weight (DW) was determined after placing seedlings in the oven to dry at 65 °C for 72 h. The measured parameters were: Seedling Length (SL) and Seedling Dry Weight (SDW).

Seedling Vigour Index (SVI): Seedling weight and length vigour indices were determined by the following formula of Abbasian and Moemeni (2013):

Seedling Length Vigour Index (SLVI) = (mean shoot length + mean root length) × TGP

Seedling Weight Vigour Index (SWVI) = mean seedling weight × TGP

(Shoot length and root length: Data not shown were used to calculate SLVI and SWVI).

Relative cotyledon water content (RCWC)

RCWC was calculated in the primary cotyledons using the following equation:

RCWC (%) = 100 × (FCW – DCW/(TCW - DCW), where turgid cotyledons weight (TCW) was obtained by immersing fresh cotyledons (FC) in distilled water for 24 h, and dry cotyledons weight (DCW) was measured after oven drying samples at 65 °C for 48 h.

Cotyledons osmotic potential (COP)

Frozen cotyledon samples (100 mg) were ground and centrifuged at 15,000 *g* for 10 min and cell sap was collected. The osmotic potential was measured using the vapour pressure osmometer (Osmomat 030, Genotec, Berlin, Germany), and converted from mOsmol kg^{-1} to MPa using the following formula:

ψs (MPa) = - c (mOsmol kg^{-1}) × 2.58 × 10^{-3}, according to the Van't Hoff equation (Nobel 1991).

Chlorophyll fluorescence

Chlorophyll fluorescence was monitored using a pulse modulated chlorophyll fluorescence meter (OS1-FL) as described by Laifa *et al.* (2021). After a dark-adaptation period of 30 min, the minimal fluorescence yield of the dark-adapted state (F0) was determined by a weak red-light pulse (6 s). Maximal fluorescence yield of the dark-adapted state (Fm) was measured during a subsequent saturating pulse of white light (8,000 µmol photon m^{-2} s^{-1} for 0.8 s).

Chlorophyll fluorescence was measured following the procedure described by Genty et al. (1989). The quantum yield of photochemical energy conversion in PSII was calculated as Y(II) = (Fm'-Fs)/Fm', where Fs and Fm' are steady-state and maximum fluorescence yields of the light-adapted state, respectively. The quantum yield of regulated nonphotochemical energy dissipation in PSII (Y(NPQ) = Fs/Fm' - Fs/Fm) and the quantum yield of nonregulated nonphotochemical energy dissipation in PSII (Y(NO) = Fs/Fm) were determined according to Lazár (2015).

Endogenous proline and soluble sugar contents

Proline content was determined according to the method of Bates *et al.* (1973). The content of total soluble sugars (TSS) in the studied samples was determined according to Yemm and Willis (1954) using glucose as a standard. The absorption was determined at 640 nm. TSS was expressed as µmol g^{-1} DW.

Lipid peroxidation (MDA)

Lipid peroxidation was estimated by measuring the concentration of malonyldialdehyde (MDA). Fresh samples were homogenized in 0.1% (w/v) TCA solution. The homogenate was centrifuged at 15,000 *g* for 10 min. An aliquot of the supernatant was added to 0.5% TBA in 20% TCA. The mixture was heated at 90 °C for 30 min in a shaking water bath, and then was cooled in an ice bath. The samples were centrifuged at 10,000 *g* for 5 min, and the absorbance of the supernatant was read at 532 and 600 nm (Ben Amor et *al.,* 2005). The MDA concentration was calculated according to the molar extinction coefficient of MDA (155 mM cm^{-1}) method according to Yemm and Willis (1954).

Statistical analysis

The obtained data were subjected to analysis of variance (One-way ANOVA) using the statistical software package SPSS version 16.0, and means were compared according to Duncan's multiple-range test at 5% level of significance. Correlation analysis and Principal Component Analysis (PCA) were done using XLSTAT software version 2014 (Addinsoft, Paris, France, www.xlstat.com), considering variables centred around their means and normalized with a standard deviation of 1. The first two principal components, which accounted for the highest variation, were then used to plot two-dimensional scatter plots.

Results

Effect of proline priming on seed germination and α-amylase activity

In salt-free conditions, proline seed priming increased markedly the final percentage of germination (Figure 1A). However, increasing salinity triggered a delay in this process as shown by the longer latency phase

(Figure 1A, 1B) and the lower speed of germination (Figure 1B). The effect of proline seed priming was both salt and proline concentration-dependent. Indeed, the strongest improvement of priming occurred at 1-, 5-, and 20-mM proline, respectively under 0, 100, and 200 mM NaCl treatments indicating faster responsiveness to salt. In particular, 20 mM proline shortened the latency phase and improved the germination capacity of *C. maritima*, especially at 200 mM NaCl. At this salinity level, the final percentage germination was ca. 38% as compared to unprimed seeds (ca. 22%) (Figure 2).

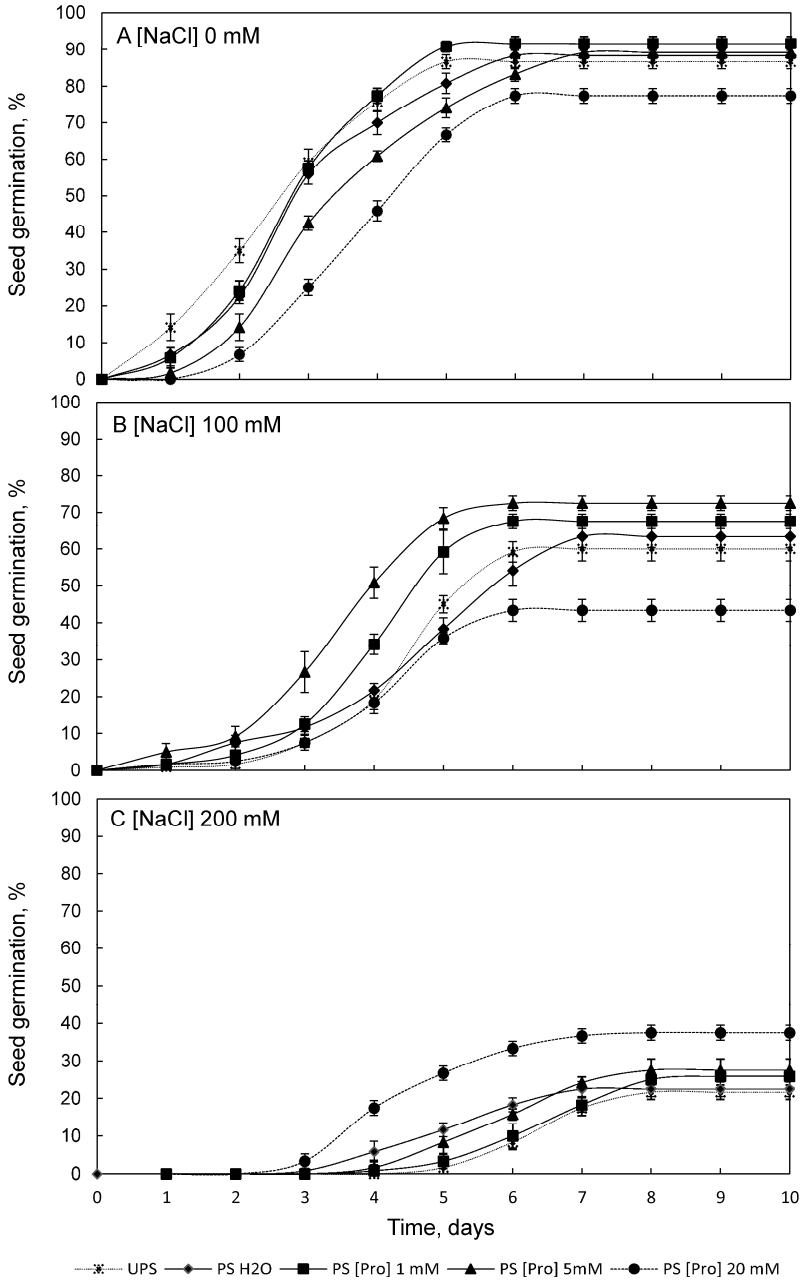


Figure 1. Effect of priming with proline on the kinetic of *C. maritima* seed germination over 10 d under different salinities: (A) 0 mM NaCl, (B) 100 mM NaCl, and (C) 200 mM NaCl
Values are means ± S.E. (n = 6).

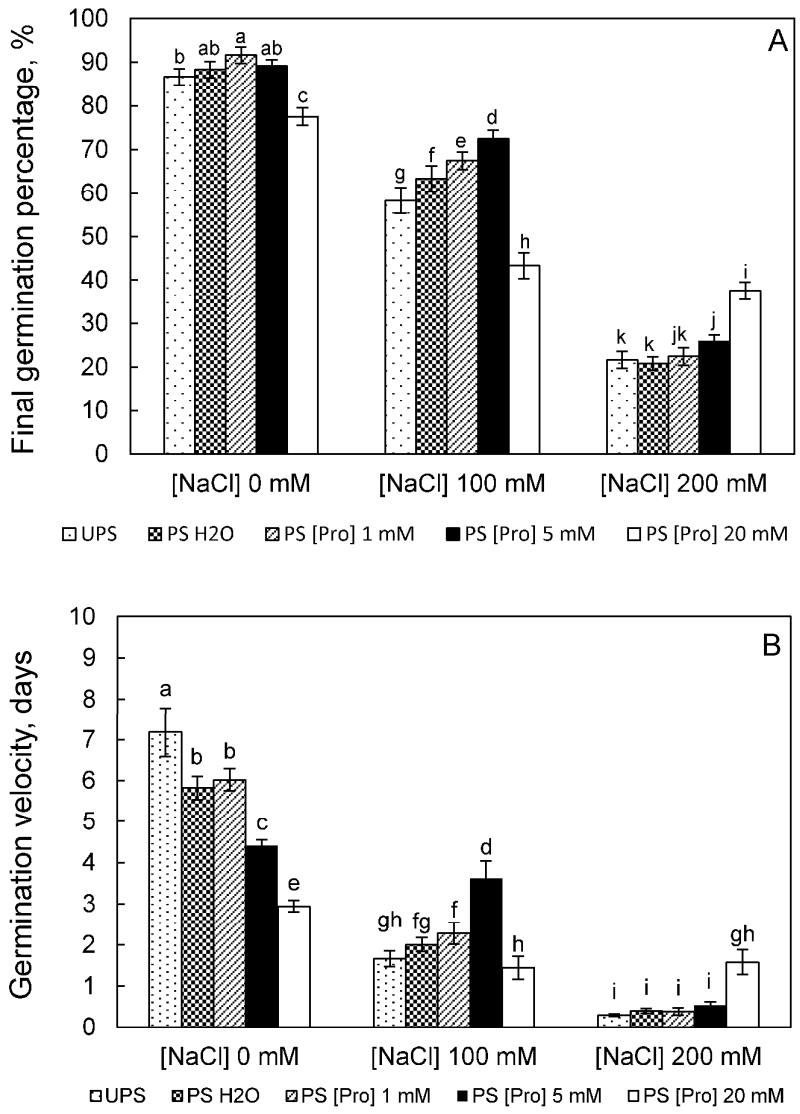


Figure 2. Effect of priming with proline on germination parameters of *C. maritima* seeds under salinity stress; 0, 100 and 200 mM NaCl: (A) Germination Percentage, (B) Speed of Germination
Values are means ± S.E. (n = 6). Bars labeled with the same lower-case letters in each panel are not significantly different at $p \leq 0.05$ (Duncan's test).

α-amylase activity decreased significantly with increasing salinity at 72 h of seed imbibition. This parameter declined from 0.156 U. µmol glucose min-1 in control to 0.115 U. µmol glucose min-1 in 200 mM NaCl (Figure 3). Proline seed priming significantly enhanced α-amylase activity under saline conditions. Thus, proline accelerated the germination process by enhancing the α-amylase activity, which ultimately led to higher final germination percentage under high salinity. Nevertheless, the higher proline concentration (20 mM) was not effective in the control condition. Clearly, the activities of α-amylase were improved by appropriate proline concentration.

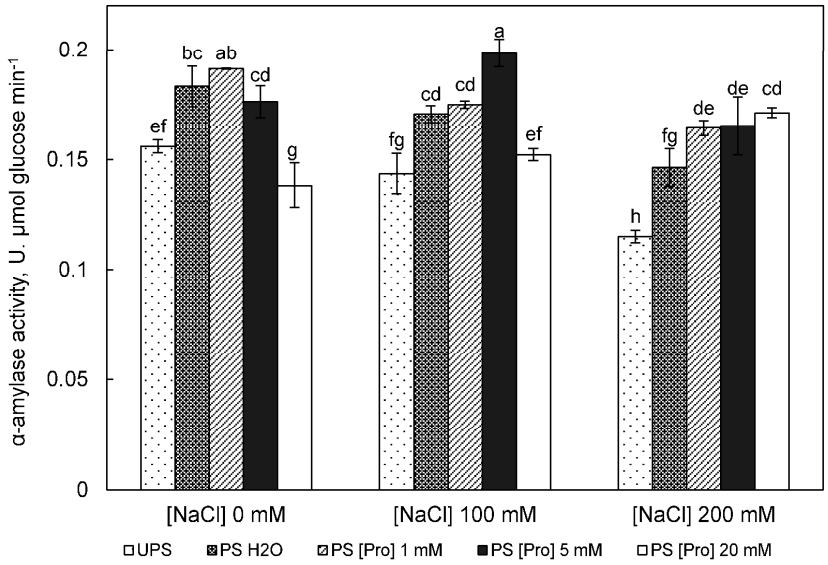


Figure 3. Effect of priming with proline on α-amylase activity at 72h during germination of *C. maritima* seeds under different salinities: 0, 100, and 200 mM NaCl

Values are means ± S.E. (n = 3). Bars labeled with the same lower-case letters are not significantly different at $p \leq 0.05$ (Duncan's test).

Effect of proline priming on seed recovery percentage

C. maritima seeds that did not germinate in NaCl were transferred to distilled water to test their recovery ability. After 7 days, high recovery capacity of germination for un-germinated seeds was observed after alleviation of 200 mM NaCl (Figure 4).

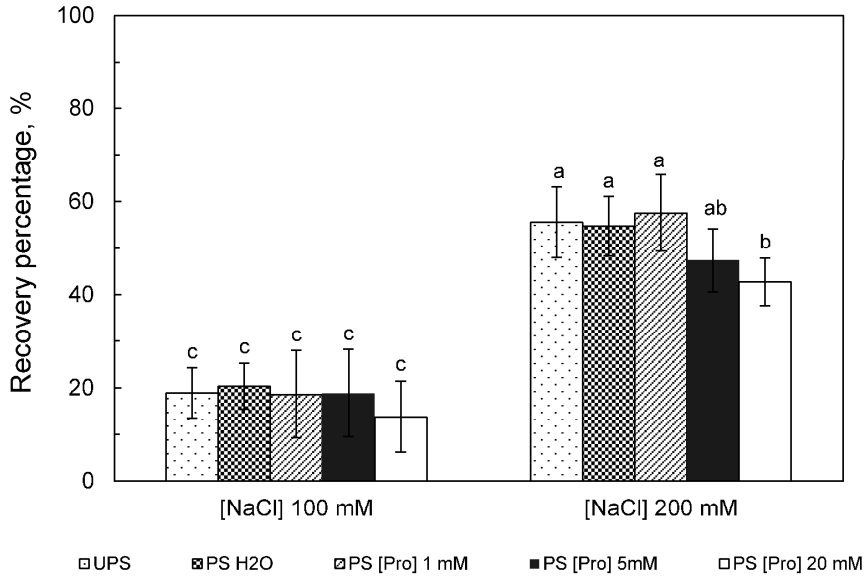


Figure 4. Effect of priming with proline treatments on the recovery percentage of *C. maritima* seeds under salinity stress: 100 and 200 mM NaCl

Values are means ± S.E. (n = 6). Bars labeled with the same lower-case letters are not significantly different at $p \leq 0.05$ (Duncan's test).

Effect of proline seed priming on chlorophyll fluorescence

The maximum quantum yield of PSII (Fv/Fm) was close to 0.8 in proline-primed plants exposed to 0 and 100 mM NaCl (Figure 5A). This parameter declined significantly in 200 mM NaCl-treated seedlings, whereas proline pre-soaking resulted in an increase of Fv/Fm. However, priming (0, 1, 5, 20 mM Pro) led to a significant improvement in the quantum yield of photochemical energy conversion in PSII [Y(II)] (Figure 5B) compared to the unprimed treatment. By contrast, we observed a decrease in quantum yield of regulated nonphotochemical energy loss in PSII [Y(NPQ)] and the quantum yield of nonregulated nonphotochemical energy loss in PSII[Y(NO)] (Figure 5C, D).

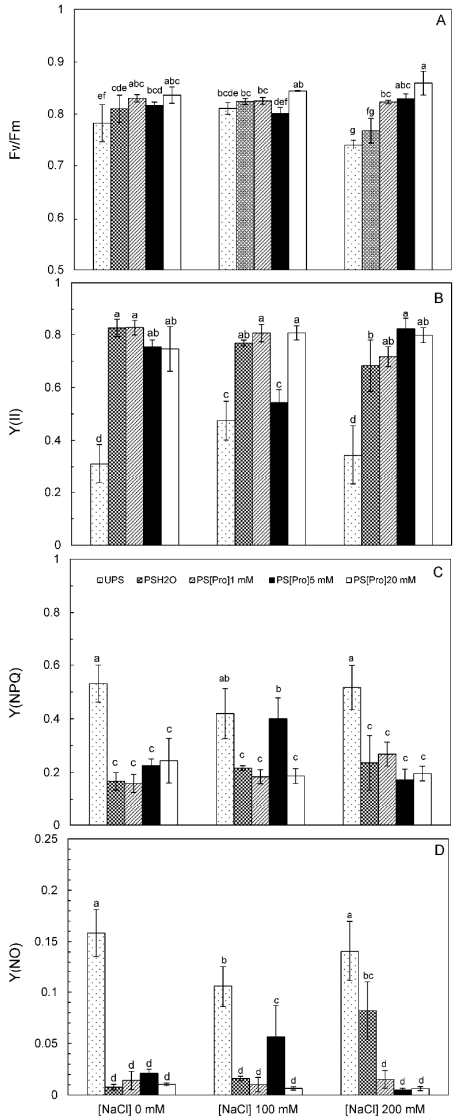


Figure 5. Variation of maximum photochemical efficiency of PSII (Fv/Fm) (A), efficient quantum yield of PSII [Y(II)] (B), regulated nonphotochemical quenching [Y(NPQ)] (C), and nonregulated nonphotochemical quenching [Y(NO)] (D) in *C. maritima* seedlings grown for 10 days on salty medium: 0, 100, and 200 mM NaCl after seed priming with proline

Values are means ± S.E. (n = 3). Bars labeled with the same lower-case letters in each panel are not significantly different at $p \leq 0.05$ (Duncan's test).

Effect of proline seed priming on seedling vigour, water, and osmotic status

Seedling Dry Weight (SDW) was significantly decreased by salinity but was substantially improved by proline seed priming treatment (Table 1). Maximum dry weight of seedlings was observed at 1 mM Pro, 5 mM Pro, and 20 mM Pro, respectively under 0, 100, and 200 mM NaCl. At the highest salinity, the greatest SWVI was attained at 20 mM Pro, as shown in Table 1. The seedling length was also reduced by salt but was enhanced with different pre-soaking treatments (Table 1). As a result, the priming significantly increased the SLVI.

Table 1. Effect of priming with proline on seedling growth rate: Seedlings Dry Weight (SDW), Seedling Weight Vigour Index (SWVI) (n = 4), Seedling Length (SL), and Seedling Length Vigour Index (SLVI) (n = 12) of *C. maritima* seedlings under NaCl stress. For each parameter, values followed by different letters are significantly different according to Duncan's test at $p \leq 0.05$.

NaCl level	Seed Priming Level	Seedling DW, mg/six plants	Seedling Length, mm	SWVI	SLVI
[NaCl] 0 mM	**UPS**	43.15 ± 0.26 c	67.67 ± 1.14 c	3.92 ± 0.02 b	614.64 ± 10.35 d
	PSH$_2$O	43.03 ± 1.55 c	70.42 ± 1.34 bc	4.02 ± 0.14 b	657.22 ± 12.47 c
	[Pro] 1 mM	51.03 ± 0.41 a	82.83 ± 1.77 a	4.93 ± 0.03 a	800.72 ± 17.13 a
	[Pro] 5 mM	43.20 ± 2.19 c	73.83 ± 1.77 b	4.03 ± 0.20 b	689.11 ± 43.38 b
	[Pro] 20 mM	42.63 ± 1.14 c	73.00 ± 4.44 b	3.48 ± 0.09 d	596.17 ± 36.29 d
[NaCl] 100 mM	**UPS**	37.50 ± 0.66 e	56.33 ± 3.08 e	2.53 ± 0.04 g	380.25 ± 10.35 g
	PSH$_2$O	40.28 ± 0.67 d	62.17 ± 4.16 d	2.85 ± 0.05 f	440.35 ± 29.49 f
	[Pro] 1 mM	43.28 ± 0.46 c	58.92 ± 2.87 de	3.17 ± 0.03 e	432.05 ± 21.05 f
	[Pro] 5 mM	47.53 ± 1.34 b	71.42 ± 1.87 bc	3.68 ± 0.10 c	553.48 ± 14.48 e
	[Pro] 20 mM	37.95 ± 0.41 e	47.75 ± 2.03 f	1.93 ± 0.03 hi	242.73 ± 10.31 j
[NaCl] 200 mM	**UPS**	23.13 ± 0.63 i	34.75 ± 1.88 g	1.50 ± 0.04 j	225.88 ± 12.21 j
	PSH$_2$O	24.98 ± 0.79 h	46.83 ± 2.38 f	1.62 ± 0.05 j	304.42 ± 15.45 hi
	[Pro] 1 mM	27.60 ± 0.82 g	48.00 ± 1.33 f	1.89 ± 0.06 i	328.00 ± 9.07 h
	[Pro] 5 mM	33.28 ± 1.31 f	45.58 ± 1.77 f	2.05 ± 0.08 h	281.10 ± 10.89 i
	[Pro] 20 mM	38.85 ± 1.61 de	56.08 ± 2.12 e	2.49 ± 0.10 g	359.87 ± 13.62 g

Relative Cotyledon Water Content (RCWC) decreased markedly upon salt exposure but was significantly enhanced by seed pre-soaking (Figure 6A). In addition, a significant lowering in Cotyledon Osmotic Potential (COP) with the increase of NaCl, proline supply, and their combined effect was observed as compared to the control (Figure 6B).

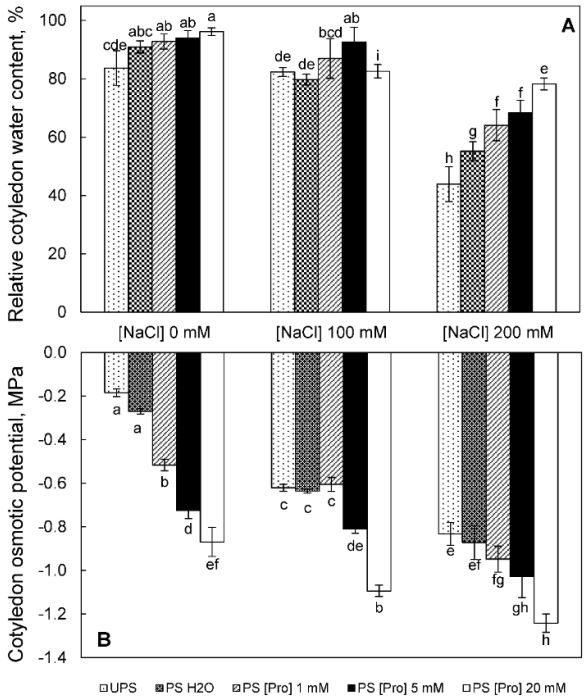


Figure 6. The influence of priming with proline on Relative Cotyledon Water Content (A) and Cotyledon Water Potential (B) of 10-day-old *C. maritima* seedlings grown at 0, 100, and 200 mM NaCl

Values are means ± S.E. (n = 3). Bars labeled with the same lower-case letters in each panel are not significantly different at $p \leq 0.05$ (Duncan's test).

Effect of proline seed priming on proline, total soluble sugars, and MDA accumulation

Proline seed priming positively affected the contents of osmolytes (proline and total soluble sugars) in cotyledons of *C. maritima* seedlings exposed to salt stress (Figure 7A, 7B). Endogenous proline content increased significantly with the increase of proline concentration supplied. The same tendency was observed for total soluble sugars. In contrast, priming treatments significantly reduced the malondialdehyde (MDA) accumulation in cotyledons under the salt stress conditions compared to the control (Figure 7C).

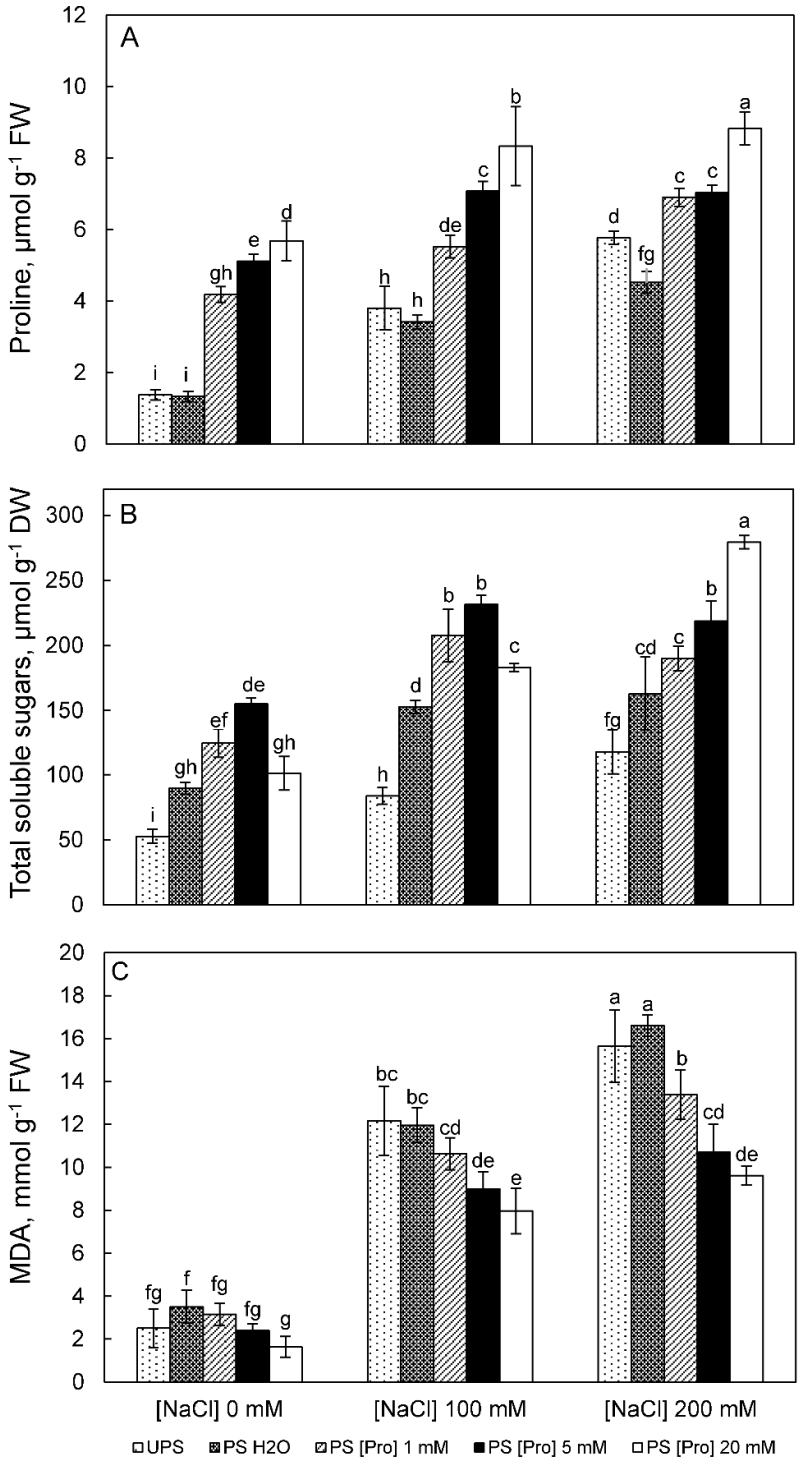


Figure 7. Effect of priming with proline on endogenous proline (A), total soluble sugars (B), and MDA levels (C) in cotyledons 10-day-old *C. maritima* seedlings grown at 0, 100, and 200 mM NaCl

Values are means ± S.E. (n = 4). Bars labeled with the same lower-case letters in each panel are not significantly different at $p \leq 0.05$ (Duncan's test).

Discussion

Seed priming is commonly considered a cost-effective and efficient technique to enhance both the germination process (in terms of percentage, rate, and reserve utilization) and seedling emergence and vigour under harsh environmental conditions (Ellouzi *et al.,* 2017; Feghhenabi *et al.,* 2020; Farsaraei *et al.,* 2021; Ben Youssef et *al.,* 2022; Ellouzi *et al.,* 2023a; Ellouzi *et al.,* 2023b; El Moukhtari *et al.,* 2023).

Our data show that unprimed seeds of *C. maritima* reached their maximal germination percentage in a salt-free medium, but were adversely affected especially at the higher concentration of NaCl (200 mM) which also increased the latency phase. This is in agreement with previous reports on halophytes, such as *Limonium cassonianum* (Giménez Luque et *al.,* 2013) and *L. tabernense* highlighting their salt sensitivity at the germinative stage (Delgado Fernandez *et al.,* 2016). Interestingly, when non-germinated seeds were transferred to distilled water, the full capacity of seeds, and hence, viability was to a large extent preserved. This suggests that the inhibition of the germination process under stress may be due to secondary or conditional dormancy state of seeds caused essentially by the limitation of water supply (osmotic effect). This acclimation strategy inhibited cell division and differentiation and consequently radicle protrusion. On the other hand, we observed a delayed germination (as shown by the decrease in germination rate) by the time requisite for seeds to adjust their osmotic pressure with stress (Ben Miled *et al.,* 2000).

Seed priming alleviates salt-induced seed dormancy by hastening germination (Singh *et al.,* 2015). This delay for a longer period might be due to the achievement of the maximum effects of metabolic and physiological preparations on seeds for germination, *via* controlling the water absorption that guaranteed good seedling establishment (Ruttanaruangboworn *et al.,* 2017). In our study, salt impact is likely due to a lower seed inhibition as well as the accumulation of toxic ions (Baskin and Baskin, 1989; Khan *et al.,* 2017). This effect was slightly attenuated by 1 mM proline supply under high salt. In addition, we noted that proline (20 mM) broke seed dormancy and promoted seed germination of *C. maritima*. Similar results were shown for *Opuntia streptacantha* under moderate salinity when using priming with low proline concentrations (1, and 5 mM) (Ochoa-Alfaro *et al.,* 2008). Proline-primed seeds showed better desiccation tolerance and significantly better imbibition (Kavi Kishor and Sreenivasulu, 2014; Aloui *et al.,* 2014). This initiates the pre-germination metabolic processes much earlier and prepared for effective radicle protrusion (Farooq *et al.,* 2017), as shown in our study. On the other hand, the addition of proline improved seed hydration and stimulated the activity of α-amylase protein molecules (Figure 3), which in turn might have improved the mobilization of stored carbohydrate reserves (Kata *et al.,* 2014; Liu Hua-long *et al.,* 2014) and protein degradation enzymes (Khatami *et al.* 2017). α-amylase has a major role in initial fast growth and development of the embryo (Singh *et al.,* 2015; Sghayar et al., 2023). So, this process was efficient in improving the speed and uniformity of seedling emergence (Farooq et al., 2020). Statistically significant positive correlations were observed between proline doses and parameters related to the germination (Table 2): SG (r = 0.84; 5 mM Pro at 100 mM NaCl) and (r = 0.61; 20 mM Pro at 200 mM NaCl); GP (r = 0.47; 1 mM Pro at 0 mM NaCl), (r = 0.55; 5 mM Pro at 100 mM NaCl); (r = 0.41; 20 mM Pro at 200 mM NaCl).

Proline priming might also lead to the modification of the seed hormonal status during germination, by stimulating abscisic acid (ABA) activity without allowing radicle protrusion in non-stress conditions (Lutts *et al.,* 2016). On the other hand, proline accumulation induces replacement of the $NADP^+$ pool, which activated the oxidative pentose phosphate pathway (OPPP) in the plastids (Hare *et al.*, 2003). The secondary functional link between enhanced proline synthesis and stimulation OPPP activity, as well as the coupling of these two pathways, are clearly important for the germination process.

The relationship between proline seed priming and inducing salt tolerance was clearly highlighted by Principal Component Analysis (PCA) which correlated the proline concentration with seed germination

parameters. PCA data indicate that the effective dose of proline is positively correlated with α-amylase activities and GP and that the effect was dependent on the level of salt.

Salt stress severely affected seedling growth in *C. maritima*, disturbed cotyledon water status and increased the levels of free proline, total sugars and MDA. Proline is known as an effective free radical scavenger with antioxidant capacity, stimulates growth and protecting against stress (Messedi *et al.* 2016). The obtained data showed a significant improvement in vegetative growth parameters (seedling DW, seedling length, SWVI, SLVI) of *C. maritima* either in control or salt conditions following seed priming (Figure 8).

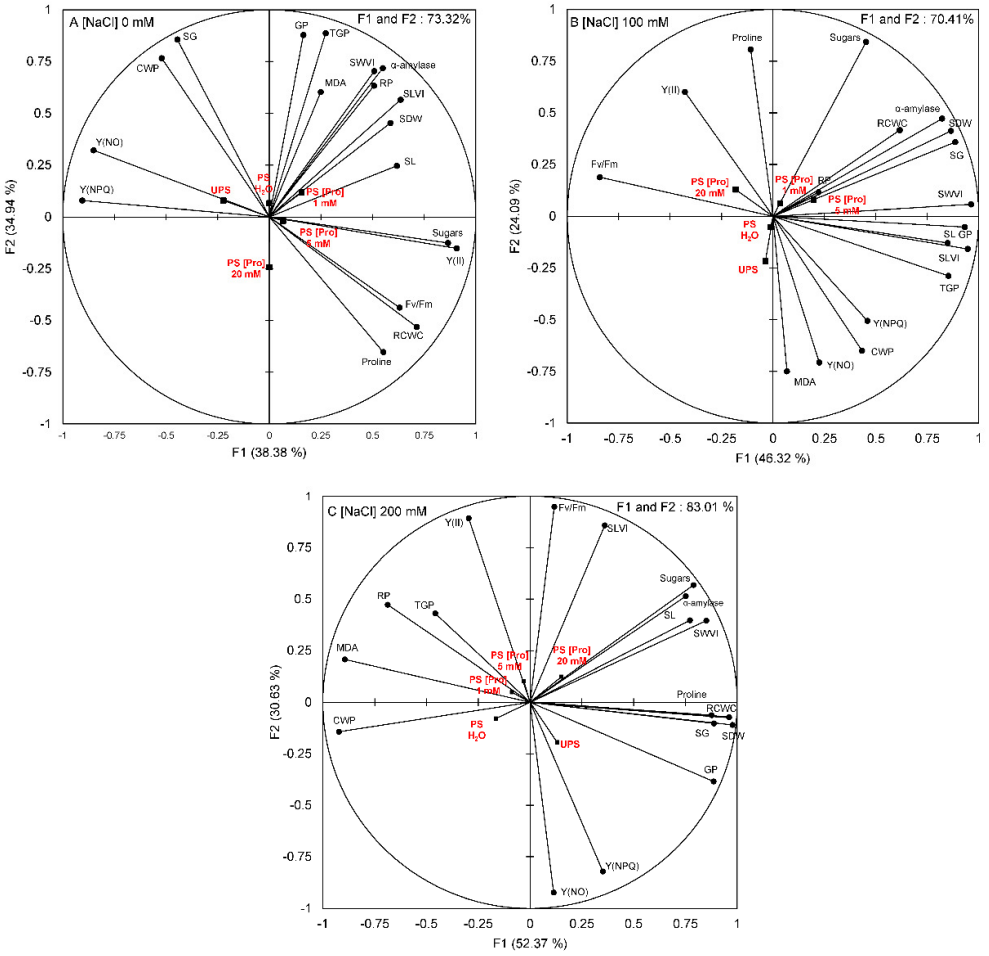


Figure 8. Principle Component Analysis. The significance of parameters used to study the response of proline-primed seeds of *C. maritima* at the germination and early vegetative stages under salt stress conditions. Panel A: Response to non-stress condition. Panel B: Response to moderate salt stress (100 mM NaCl). Panel C: Response to high salt stress (200 mM NaCl). A notable response was observed in the integrated PCA analysis, showing a significant correlation of plantlet response and the proline priming treatments under high salinity. F1, F2: Factorial planes. α-Amylase: α-Amylase Activity; CWP: Cotyledon Water Potential; Fv/Fm: Maximum photochemical efficiency of PSII; GP: Germination Percentage; MDA: MDA content; Proline: Proline content; RCWC: Relative Cotyledon Water Contents; SDW: Seedlings Dry Weight; SG: Speed of Germination; SL: Seedling Length; SLVI: Seedling Length Vigor Index; Sugars: Total sugars content; SWVI: Seedling Weigh Vigor Index; Y(II): Efficient quantum yield of PSII; Y(NO): Nonregulated nonphotochemical quenching; Y(NPQ): Regulated nonphotochemical quenching.

Table 2. Correlation matrices (Pearson(n)) illustrating the influence of proline priming on physiological and biochemical parameters under varying salinity (0, 100, and 200 mM NaCl)

[NaCl] 0 mM					
Variables	UPS	PS H_2O	PS [Pro] 1 mM	PS [Pro] 5 mM	PS [Pro] 20 mM
SL	**- 0.39 ***	- 0.21	**0.62**	0.02	- 0.04
SLVI	**- 0.33 ***	- 0.08	**0.74**	0.10	**- 0.43**
GP	0.00	0.16	**0.47**	0.23	**- 0.85**
SG	**0.63 ***	0.18	0.25	- 0.29	**- 0.77**
TGP	- 0.03	0.19	**0.48**	0.19	**- 0.83**
α-amylase	- 0.32	0.35	**0.55**	0.18	**- 0.75**
RP	- 0.20	0.16	**0.51**	0.02	**- 0.49**
SDW	- 0.21	- 0.23	**0.92**	- 0.20	- 0.28
SWVI	- 0.16	- 0.06	**0.88**	- 0.05	**- 0.61**
RCWC	**- 0.76 ***	- 0.06	0.12	0.24	0.45
CWP	**0.62 ***	0.47	- 0.01	- 0.41	**- 0.68**
MDA	- 0.07	**0.47**	0.28	- 0.13	**- 0.55**
Proline	**- 0.58 ***	**- 0.59**	0.17	0.42	**0.58**
Sugars	**- 0.76 ***	- 0.18	0.35	**0.64**	- 0.05
Fv/Fm	**- 0.61 ***	- 0.10	0.28	0.03	0.40
Y(II)	**- 0.95 ***	0.33	0.34	0.15	0.13
Y(NPQ)	**0.92 ***	- 0.34	- 0.37	- 0.14	- 0.07
Y(NO)	**0.98 ***	- 0.29	- 0.24	- 0.18	- 0.27
[NaCl] 100 mM					
Variables	UPS	PS H_2O	PS [Pro] 1 mM	PS [Pro] 5 mM	PS [Pro] 20 mM
SL	- 0.16	0.15	- 0.02	**0.65**	**- 0.62**
SLVI	- 0.14	0.14	0.10	**0.67**	**- 0.78**
GP	- 0.13	0.11	0.31	**0.55**	**- 0.84**
SG	- 0.32	- 0.12	0.05	**0.84**	**- 0.45**
TGP	0.02	0.14	0.24	**0.46**	**- 0.86**
α-amylase	**- 0.62**	0.07	0.18	**0.77**	- 0.40
RP	0.04	0.12	0.02	0.04	- 0.22
SDW	**- 0.50**	- 0.14	0.26	**0.82**	- 0.44
SWVI	- 0.25	0.02	0.29	**0.71**	**- 0.76**
RCWC	- 0.22	- 0.44	0.18	**0.67**	- 0.20
CWP	0.36	0.31	0.39	- 0.15	**- 0.91**
MDA	**0.46**	0.41	0.07	- 0.34	**- 0.60**
Proline	**- 0.48**	**- 0.58**	- 0.03	0.38	**0.71**
Sugars	**- 0.84**	- 0.17	0.39	**0.55**	0.06
Fv/Fm	- 0.19	0.15	0.18	**- 0.77**	**0.63**
Y(II)	**- 0.70**	0.30	0.43	- 0.46	0.43
Y(NPQ)	**0.59**	- 0.28	- 0.42	0.51	- 0.40
Y(NO)	**0.82**	- 0.28	- 0.35	0.22	- 0.40
[NaCl] 200 mM					
Variables	UPS	PS H_2O	PS [Pro] 1 mM	PS [Pro] 5 mM	PS [Pro] 20 mM
SL	0.07	**- 0.78**	0.01	0.09	**0.61**
SLVI	**- 0.45**	**- 0.60**	0.11	**0.32**	**0.61**
GP	**0.72**	**- 0.50**	**- 0.41**	- 0.23	**0.41**
SG	**0.51**	**- 0.42**	- 0.34	- 0.36	**0.61**
TGP	**- 0.71**	0.15	0.15	0.32	0.10
α-amylase	0.06	**- 0.88**	- 0.08	0.36	**0.54**
RP	**- 0.83**	0.29	0.27	0.34	- 0.06
SDW	**0.56**	**- 0.55**	- 0.41	- 0.22	**0.62**
SWVI	0.06	**- 0.55**	- 0.38	0.00	**0.87**
RCWC	**0.60**	**- 0.71**	- 0.33	- 0.02	0.45
CWP	- 0.30	**0.52**	0.40	0.15	**- 0.77**
MDA	**- 0.66**	0.43	**0.56**	0.10	- 0.43
Proline	**0.45**	- 0.34	**- 0.73**	0.01	**0.60**
Sugars	- 0.05	**- 0.65**	- 0.17	0.03	**0.84**
Fv/Fm	**- 0.70**	- 0.39	0.21	0.28	**0.60**
Y(II)	**- 0.90**	0.03	0.12	0.41	0.34
Y(NPQ)	**0.87**	- 0.15	- 0.03	- 0.38	- 0.30
Y(NO)	**0.81**	0.29	- 0.31	- 0.40	- 0.39

- 1	- 0.9	- 0.8	- 0.7	- 0.6	- 0.5	- 0.4	- 0.3	- 0.2	- 0.1	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1

The values indicate correlation coefficients (r). Bold values indicate statistically significant correlations at $p \leq 0.05$ level. The red gradient signifies positive correlations, while the blue gradient denotes negative correlations. The colour intensity corresponds to the strength of correlation (refer to the scale on the left side of the correlation matrices). α-Amylase: α-Amylase Activity; CWP: Cotyledon Water Potential; Fv/Fm: Maximum photochemical efficiency of PSII;

GP: Germination Percentage; MDA: MDA content; Proline: Proline content; RCWC: Relative Cotyledon Water Contents; SDW: Seedlings Dry Weight; SG: Speed of Germination; SL: Seedling Length; SLVI: Seedling Length Vigour Index; Sugars: Total sugars content; SWVI: Seedling Weigh Vigour Index; Y(II): Efficient quantum yield of PSII; Y(NO): Nonregulated nonphotochemical quenching; Y(NPQ): Regulated nonphotochemical quenching.

In *Arabidopsis thaliana*, the oxidation of proline generated NADPH, which could be utilized to support root elongation (Ueda *et al.,* 2008). A close relationship was also reported between growth and photochemistry (Jamil *et al.* 2014). In our study, chlorophyll fluorescence measurements were used to evaluate photosynthetic efficiency of leaves and Fv/Fm. It is commonly known that Fv/Fm serves as an indicator of stress (Maxwell and Johnson, 2000) or photoinhibition. Our results demonstrated that priming increased both Fv/Fm and Y(II) and concomitantly decreased malondialdehyde (MDA) contents. This supports the assumption that proline contributed to maintaining the functional integrity of PSII by delaying oxidative stress. This especially at high salinity (200 mM NaCl). Most recent research suggests that exogenous phytoprotectants increased plant tolerance by regulating photosynthetic electron flux (Yang *et al.* 2018), wherein the mechanism might involve beneficial effects of these compounds in redox regulation of photosynthetic activities under stress.

For all primed plants (hydro and proline priming), ACP showed a significant negative relationship between salinity and seedling growth parameters, Fv/Fm and Y(II). Similar trends were reported by Jamil *et al.* (2014) and Lazár (2015), indicating an increase in the proportion of radiation absorbed by chlorophyll associated with PSII that is used in photochemistry. On the other hand, the decrease in both Y(NPQ) and Y(NO) depends on the regression of energy excitation loss highlighting that proline seed priming had a beneficial impact on the photochemical reaction by absorbing light energy distribution under salt stress. We observed an accumulation of free proline and carbohydrates in tissues. These components are primarily involved in osmotic adjustment in cells (Prida and Das, 2005). Thus, osmotic adjustment is an important mechanism during salt tolerance. Proline promoting the non-enzymatic antioxidant defence (Kubala *et al.,* 2015) and a negative correlation was observed in our study between malondialdehyde (MDA) contents and proline levels in primed seedlings (Figure 8) confirms that the proline-priming technique improved membrane protection of seedlings and protein structures under salt stress condition (Messedi *et al.,* 2016). The removal of ROS by proline supply that enhances the amount of carbohydrates might be a result of enzymes activation in early germination such as α-amylase (Sultana *et al.,* 2000) or/and avoiding inhibition of key enzymes as those controlling the Calvin cycle in chloroplasts (Pan *et al.,* 2006). This hypothesis requires verification in a future study.

Proline is typically considered one of the key substances that acts as a stress signal molecule modulating adaptive functions (Szabados and Savouré, 2009). It also ensures osmotic adjustment and non-enzymatic antioxidative protection which is believed to promote species under biotic stresses (Ben Rejeb *et al.,* 2014). It is known that proline homoeostasis in tissues must be preserved during development and growth, otherwise potentially leading to several aberrations (Kavi Kishor and Sreenivasulu, 2014). Excessive accumulation of proline could harm the cellular membrane system and inhibit growth and cell division (Maggio *et al.,* 2002).

In our study, the supply of this osmoticum at high doses (20 mM) decreased the rate and speed of germination of *C. maritima* under non-stress and moderate salinity conditions, as shown in Figure 3, Table 1, and Table 2. Hare *et al.* (2003) mentioned that in *A. thaliana*, high accumulation of endogenous proline led to a decrease in the rate of radicle emergence. Moreover, a feedback inhibition of P5CS (strongly transcribed throughout embryogenesis) at high proline level revealed an important role in proline synthesis of glutamate in the regulation of embryo growth (Hare et *al.,* 2001). In addition, the imbalance in proline accumulation can be toxic for certain tissues if it is partially catabolized, generating toxic levels of P5C, elevating ROS content, and leading to apoptosis (Miller *et al.* 2009). Furthermore, destabilizing the DNA helix, lower the DNA melting point, increase susceptibility to S1 nucleases and increase insensitivity to DNAase1, are consequences of proline supply at high concentrations (Miller *et al.* 2009).

Intriguingly, the effect of proline priming is contingent on both the dose of proline and the level of salinity. More generally, this effective and straightforward priming technique could be used to mitigate the impact of environmental stress on plants, owing to proline high penetration ability and rapid mobilization capacities. These properties might boost seed performance and foster effective defence mechanisms in seeds and seedlings against salinity stress, which is further supported by higher antioxidant response and osmotic adjustment. The mechanisms of 'priming memory' developed in seeds can be harnessed during subsequent salinity stress exposure, thereby inducing greater stress resilience in seedlings and plants (Figure 9).

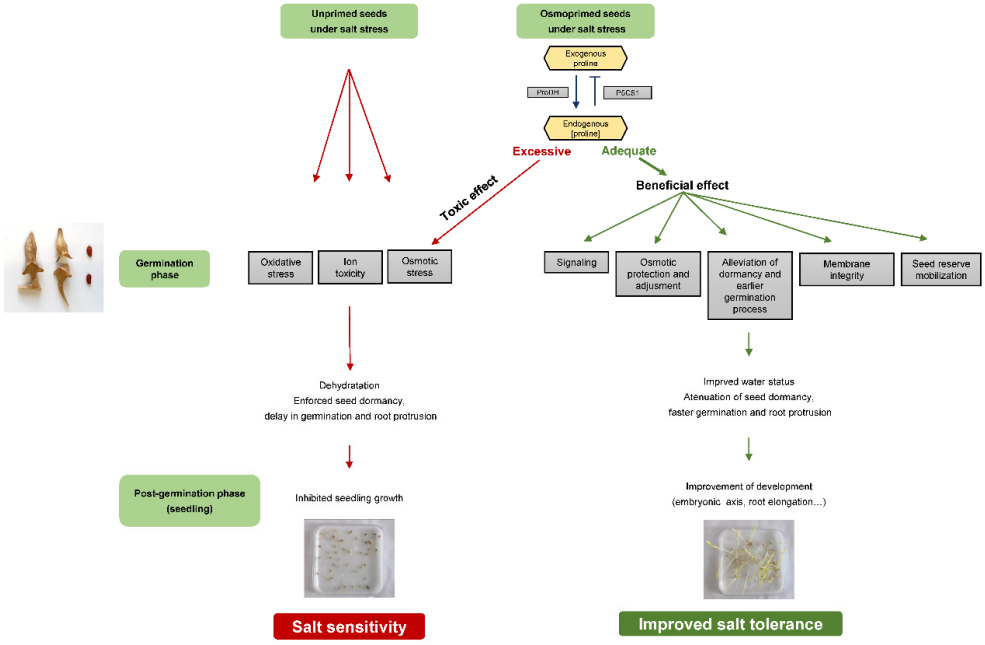


Figure 9. Summary illustration comparing salt stress responses in *C. maritima* seedlings after seed priming with proline. In the depiction, the red colour indicates adverse effects, while green highlights beneficial effects

Conclusions

In conclusion, the present investigation indicates that the lower doses of proline (1 mM and 5 mM) used in priming enhanced seed vigour, supported earlier radicle protrusion, and promoted seedling establishment under control and favourable conditions (0 and 100 mM NaCl, respectively). Consequently, proline priming could be a pertinent approach for conferring better tolerance, restoring the full germination potential of *C. maritima* seeds, inducing physiological and biochemical mechanisms, such as hydrolysis, activating enzymes (e.g., amylase), and improving seedling growth activity through non-enzymatic oxidative defence under salinity.

Authors' Contributions

Conceptualization: DM and CA; Supervision: DM; Experimentation: FF and DH; Experimentation - Chlorophyll fluorescence measurements: FZ. Data analysis: FF, DH, and DM; Data analysis - Principal

Component Analysis (PCA) and correlation analyses: WZ; Writing - Original draft: FF, DM, and WZ; Review and editing: AD. Final manuscript approval: All authors read and approved the final manuscript.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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