Elemental localisation and a reduced glutathione redox state protect seeds of the halophyte Suaeda maritima from salinity during over-wintering and germination

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Camille-Madeleine Szymansky[ab], Adele Muscolo[c], Margaret Yeo[b], Louise Colville[b], Innes Clatworthy[d], Tobias Salge[d] and Charlotte E. Seal[b*] **Elemental localisation and a reduced glutathione redox state protect seeds of the halophyte *Suaeda maritima* from salinity during over-wintering and germination** Environmental and Experimental Botany 190 (2021) 104569. **which has been published in final** **https://doi.org/10.1016/j.envexpbot.2021.104569**

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Abstract

Saltmarshes are increasingly at risk from sea level rise through climate change. Their vegetation is comprised of halophytes, salt-tolerant plants, that naturally regenerate from seed, yet the combination of functional traits associated with salinity tolerance in seeds is still poorly characterised. Combining approaches in microscopy, biochemistry and physiology, we focussed on elucidating two mechanisms that are commonly utilised by adult halophyte plants: ion accumulation and elemental localisation, and antioxidant protection against oxidative stress, in seeds of the saltmarsh halophyte *Suaeda maritima* during over-wintering and germination.

Fresh seeds were dormant, and required 19 weeks of submergence in artificial sea water (ASW) at 5 °C, mimicking over-wintering in the field, to release dormancy. Seed ion contents were quantified, along with the redox state of glutathione and low-molecular weight thiol intermediates of its synthesis (Cys, Cys-Gly, γ-Glu-Cys), the production of the reactive oxygen species H_2O_2 and electrolyte leakage to indicate ion movement. Once dormancy was released, seeds were germinated in solutions of ASW and iso-osmotic solutions of polyethylene (PEG) for comparison. Temporal changes in the glutathione and low-molecular weight thiols redox state, H_2O_2 and electrolyte leakage were measured during germination. In addition, spatial localisation of the seawater elements Na, Cl, Mg, Ca, K and S within the seed was resolved, and seed moisture content measured to indicate the extent of osmotic adjustment.

Fresh and pre-treated seeds had an abundance of Na^+ and Cl^-, as well as H_2O_2 and thiol disulphides (i.e., oxidised thiols) that are indicative of oxidative stress, but this was accompanied by a large pool of reduced glutathione and thiols suggesting a potentiated antioxidant system in preparation for germination. During germination in ASW, the glutathione and thiol redox state was highly reduced, with no consistent production of H_2O_2. Na and Cl localised in the seed testa and tegmen, while Mg and K were only found in the embryo, and Ca and S had a broad distribution across the seed. Small differences in seed moisture content did not account for a higher maximum threshold to germination of 2.22 times the concentration of ASW compared to 1.76 times ASW-equivalents for PEG. Moreover, θ_{Halo} was higher (i.e., slower germination) in the presence of ASW than PEG, suggesting that slower germination may give more time to initiate tolerance mechanisms in response to salinity that promote germination. Seeds of *S. maritima* appear to utilise seawater ions to their benefit during germination in salinity, using elemental localisation to protect the embryo from potential toxicity of Na and Cl, while maintaining a highly reduced cellular redox state.

Keywords

Glutathione redox state, functional traits, halophyte, ion localisation, seed germination, halotime model.

1. Introduction

Coastal wetlands provide a natural buffer against coastal erosion and salt water intrusion, providing a unique habitat for plants and animals, and make a huge contribution to global carbon sequestration (Hulme, 2005; Duarte et al., 2013). However, with sea level rise projected to increase by up to 2 m by 2100 (Bamber et al., 2019), coastal habitats and the plants which inhabit them, including salt tolerant halophytes, are under threat from climate change.

Whereas salt-sensitive plants, that include most of the world's economic crops, suffer osmotic and ionic stress even under low salinity levels, halophytes complete their life cycle under high saline conditions of around 200 mM NaCl or more (Flowers and Colmer, 2008; Munns and Tester, 2008). Nearly all species of halophyte naturally regenerate from seed and therefore rely on germination to establish the next generation of plants (Sealand Dantas, 2020). Tolerance to salinity during germination is variable, and can range from species whose seeds only germinate in fresh water to those that can tolerate over 1M NaCl (Gul et al., 2013). By exploiting the linear relationship of germination rate with salinity concentration, the maximum threshold of salt for germination to occur has been estimated for a handful of halophytes, such as *Atriplex prostrata* (505 mM NaCl at 15 °C), *Suaeda maritima* (1280 mM NaCl at 5 °C) and *Tecticornia indica* (2473 mM NaCl at 15 °C) (Allen et al. 2000; Seal et al., 2018; Seal and Dantas, 2020). Yet the combination of functional traits underlying salt tolerance during germination is still largely unknown.

One of the first challenges is for a seed to imbibe enough water for the germination process to conclude, since a saline solution may have a lower osmotic potential than that of the seed (Seal and Dantas, 2020). The seeds of some species are unable to do this, and may only complete their germination once the salt is removed and replaced by fresh water (Gul et al., 2013). Of the limited studies available, some species have overcome this gradient in osmotic potential by accumulating Na^+ within the whole seed (e.g., *Suaeda maritima*), which for some has been resolved further as localisation into the seed coat (e.g., *Suaeda physophora* and *Salicornia pacifica* var. *utahensis*), to aid osmotic adjustment under saline conditions (Khan et al., 1985; Song et al., 2005; Seal et al., 2018). Localisation of Na^+ and Cl^- away from the embryo is likely to be advantageous since high concentrations can be toxic to metabolism (Flowers et al., 2015).

Another consequence of exposure to salinity is the increased production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide and the hydroxyl radical, originating mostly from respiration in germinating seeds (Kranner and Seal, 2013; Bose et al., 2014). At low levels, ROS can have a range of beneficial roles. During the imbibition of dry seeds, an oxidative burst of ROS, such as H_2O_2, may occur as metabolic activity resumes (Wojtyla et al., 2016). H_2O_2 is an important messenger and regulator of key developmental processes such as dormancy release, cell-wall loosening and reserve mobilisation (Wojtyla et al., 2016) and is present in germinating seeds of the halophytes *Arthrocnemum macrostachyum*, *Arthrocnemum indicum*, *Suaeda fruticosa* and *Limonium stocksii* (Hameed et al., 2014; Nisar et al., 2019). However, ROS levels require careful control by ROS-processing enzymes and antioxidants, to avoid oxidative damage to macromolecules and cellular structures (Kranner and Seal, 2013; Bose et al., 2014). For example, peroxidation of membrane lipids leads to a loss of membrane integrity and an increase in electrolyte leakage. Eventually, the accumulation of damage may be too great to repair and can result in seed death (Kranner and Seal, 2013).

Glutathione (γ-glutamyl-cysteinyl-glycine) is a major water-soluble antioxidant. In unstressed cells, glutathione is present in a reduced form (GSH) and can donate electrons to detoxify free radicals, forming glutathione disulphide (GSSG), as part of the ascorbate-glutathione cycle (Kranner and Seal, 2013; Bose et al., 2014). The glutathione redox state has been linked to seed viability across a range of species, where seed viability loss is associated with a switch towards more oxidising cellular conditions (Kranner et al., 2006). In halophytes, glutathione has frequently been identified as an important component of the antioxidant response to salinity exposure. For example, in plants of *Suaeda maritima*, a higher concentration of GSH, lower GSSG concentration and more reducing cellular conditions were observed when grown in seawater with permanently flooded conditions than in drained conditions, suggesting a heightened glutathione antioxidant system in response to waterlogging (Alhdad et al., 2013). During germination, *Suaeda fruticosa* and *Limonium stocksii* showed an increasing GSH:GSSG ratio during germination in 200 and 400 mM NaCl (Hameed et al., 2014). However, in seedlings of *Suaeda maritima* germinated in 0 – 400 mM NaCl, no significant differences were observed in the concentration of GSH, GSSG or the reduced and disulphide forms of other low-molecular weight thiols such as cysteine (Boestfleisch et al., 2014). Recently, it has been proposed that H_2O_2 levels were closely linked to temporal changes in the GSH/GSSG redox state during seed germination (Gerna et al., 2017), further supporting the notion that cellular redox changes are intricately involved in the germination process.

In this study, we investigated the combination of traits that underlie tolerance to salinity in halophyte seeds. Our focus is on seeds of the saltmarsh halophyte *Suaeda maritima* (Amaranthaceae) that grows in the northern hemisphere, as well as in tropical habitats as an introduced species (Seal et al., 2018). It frequently occupies the lower saltmarsh and is adapted to temporary flooding and hypoxic conditions in the soil (Alhdad et al., 2013; Behr et al., 2017). As an annual plant, mature seeds are dispersed each autumn (typically October) in a dormant state, and over-winter in cold-saline conditions in the field until they germinate in the spring (March-April; Wetson et al., 2008). *S. maritima* is a colonising species and germinates well in bare patches resulting from disturbance (Chapman, 1947; Tessier et al., 2000), which is important in establishing new saltmarsh habitats such as created by sea level rise (Hulme, 2005).

Taking a multidisciplinary approach combining physiology, biochemistry and microscopy, we first investigated the role of ion accumulation and oxidative stress (through the glutathione redox state, production of the ROS H_2O_2 and electrolyte leakage) in protecting fresh (dormant) and pre-treated (mimicking over-wintering in the field to release dormancy) seeds from salinity. Then, once dormancy was released, seeds were germinated in solutions of artificial seawater (ASW) and iso-osmotic solutions of polyethylene (PEG), to simulate the osmotic constraint of salinity without ion toxicity, for comparison. The halotime model (Seal et al., 2018) was adapted to estimate, for the first time, the maximum concentration of seawater for germination to occur and the halo time constant (i.e., seawater accumulation over time at a specific temperature for germination to complete). Temporal changes in the redox state of glutathione and low-molecular weight thiol intermediates of its synthesis (Cys, Cys-Gly, γ-Glu-Cys), as well as H_2O_2 production and electrolyte leakage were also measured during germination. Finally, the spatial localisation of the seawater elements Na, Cl, Mg, Ca, K and S within the seed was resolved, and seed moisture content measured to indicate the extent of osmotic adjustment.

2. Materials and Methods

2.1. Seed collection and pre-treatment of seeds

Mature fruits of *Suaeda maritima* (Amaranthaceae) were collected from the saltmarsh at Cuckmere Haven, Seaford, East Sussex, United Kingdom (50° 45′ 39.24″ N, 0 08′ 57.71″ E) in October 2018. Seeds were removed from the fruits by hand. The relative humidity (RH) of fresh seeds was measured with a hand-held Rotronic (Hygrolog-D, Rotronic Instruments, Crawley, UK) and corresponded to 100 % RH at 20 °C. Seeds were stored at 75 % RH at 15 °C until experiments commenced.

Seeds of *S. maritima* correspond to a perispermic seed morphology (Fig. 1). The coiled embryo, which is chlorophyllous, is placed towards the centre of the seed. There is a layer of perisperm between the outer edge of the embryo and the testa inner surface located symmetrically on both sides, and a tegmen between the testa and the perisperm (Fig. 1c and d; Martin, 1946; Shepherd et al., 2005; Sukhorukov et al., 2018). The embryo almost completely occupies the internal area of the seed. The storage material is mostly nucellus-derived perisperm, consisting of "thin-walled, non-living cells densely packed with starch grains" (Shepherd et al., 2005).

Fresh seeds of *S. maritima* are dormant and require cold-wet stratification for a period of up to 20 weeks to break dormancy (Wetson et al., 2008). Following the protocol of Wetson et al., (2008), seeds were submerged in full-strength ASW (1.0 ASW; containing 411 mM NaCl, 53.52 mM $MgCl_2$, 10.16 mM $CaCl_2$, 9 mM KCl and 28 mM Na_2SO_4 (Harvey, 1966)) at 5 °C, within 12 days of seed collection. Germination was monitored after 9, 14, 16 and 18 weeks of submergence on subsets of 10 seeds sown onto one sheet of germination paper (90 mm diameter, Fisherbrand; Fisher Scientific, Loughborough, UK) moistened with 3.5 mL of dH_20 in Petri dishes at 15/5 °C (12/12h light/dark, with the higher temperature corresponding to a light intensity of 11 W/m^2), reflecting the temperature of spring germination in the natural habitat (Wetson et al. 2008). Based on the data obtained (see Results), 19 weeks of submergence was selected as sufficient time to release dormancy.

2.2. Ion content of fresh and pre-treated seeds

Cations and anions were extracted from seeds and analysed by ion chromatography (DIONEX ICS-1100, Waltham, MA, USA). Fresh and pre-treated (submerged in 1.0 ASW at 5 °C for 19 weeks) seeds were rinsed in dH_20 for 10 seconds and then dried at 103 °C for 17 h (ISTA, 2019). For quantification of anions, 0.5 g of dried material was extracted in 50 ml of anion solution (3.5 mM $Na_2CO_3/NaHCO_3$) under stirring for 20 min and filtered. For quantification of cations, 1 g of dried material was ashed at 550 °C for 5 – 6 h in a porcelain capsule. The ash was then mineralized for 30 min at 100 °C using 1M HCl solution and filtered. Analysis by ion chromatograph used the eluent 20 mM methane-sulfonic acid and ions were quantified using a standard curve. All solvents and reagents were purchased from Panreac (Barcelona, Spain).

2.3. Germination

All germination tests were conducted on pre-treated seeds (i.e., non-dormant) as described in section 2.1. Four replicates of 25 were sown onto two layers of germination test paper moistened with 7 mL of each treatment solution. The Petri dishes were placed in plastic bags to prevent evaporation during the test and incubated at 15/5 °C. Seeds were germinated in the solutions dH_2O (0 ASW) and five different concentrations of ASW ranging from two times full-strength ASW (2.0 ASW) to quarter-strength ASW (0.25 ASW). Iso-osmotic solutions of PEG 8000 were also prepared to correspond to each of the ASW solutions (0.25 ASW = -0.643 MPa; 0.5 ASW = -1.277 MPa; 1.0 ASW = -2.614 MPa; 1.5 ASW = -3.954 MPa; 2.0 ASW = -5.508 MPa) using the equation (Michel, 1983; Eq. 4):

$$\Psi = 0.130[\mathrm{PEG}]^2\, T - 13.7[\mathrm{PEG}]^2$$

where Ψ = osmotic potential and T = temperature (10 °C, i.e. the average of 15/5 °C with a 12 h photoperiod). A freezing point osmometer (Roebling Micro-Osmometer Autocal Type 13 Osmometer, Camlab, Cambridge, UK) was used to confirm the osmotic potentials. 7 mL of PEG was also used to moisten two layers of germination test papers, since the ratio of solution volume to germination test paper weight was > 12 (Hardegree and Emmerich, 1990).

Germination was scored daily as radicle emergence of at least 1 mm. Experiments were conducted for up to 30 days where no further germination was observed. All non-germinated seeds were rinsed five times in dH_2O, blotted dry, and transferred to new germination test papers moistened with 7 mL of dH_2O and incubated at 15/5 °C. This recovery test was conducted for up to 25 days where no further germination was observed. For any remaining non-germinated seeds, a small proportion of the seed coat was removed and seeds were submerged in 1 % 2,3,5-triphenyl tetrazolium chloride for 48 h in the dark (ISTA, 2019) at 25 °C. Seeds were rinsed for 10 seconds with dH_2O and cut longitudinally to assess the embryo for staining (viable seeds stain red and non-viable seeds are unstained), as well as to identify if seeds were mouldy or empty.

2.4. Germination data analysis

For each treatment, cumulative germination percentages were plotted over time and a Boltzmann curve was fitted. From this, the time to achieve 10 - 90 % germination was calculated. A halotime model was created by regressing the reciprocal of time to achieve each percentile ($1/t_g$) against the concentration of ASW or the osmotic potential of PEG (in units of

ASW, termed ASW equivalent units or ASW_{equiv}), according to the equation:

$$1/t_g = ([ASW] - [ASW]_{max\ g}) / \theta_{Halo\ g}$$

where $[ASW]_{max\ g}$ is the threshold or base [ASW] of percentile g, above which there is no germination. $\theta_{Halo\ g}$ is the hydro time constant which can be calculated from the inverse of the slope for percentile g (Seal et al., 2018).

A mean of $\theta_{Halo\ g}$ was taken to estimate θ_{Halo} for the seed population. The statistical difference in θ_{Halo} between ASW and ASW_{equiv} was assessed using a Student's test (*t*-test) ($P < 0.05$). The x-intercepts, representing $[ASW]_{max\ g}$ and $[ASW_{equiv}]_{max\ g}$ were estimated for 50 % germination ($[ASW]_{max50}$; $[ASW_{equiv}]_{max50}$).

2.5. Preparation of seeds for elemental microscopy and temporal analyses

Two contrasting ASW solutions, 1.0 and 1.5 ASW, were selected as imbibition treatments along with their corresponding iso-osmotic PEG solutions and dH_2O (0 ASW) for physiological and biochemical analyses. These dilutions of ASW were chosen to reflect differences in germination rate (based on t_{50}) while > 80 % germination was still achieved (see Fig. 2a). Prior to the start of all biochemical experiments, non-dormant seeds were imbibed for 20 % and 80 % of the time to achieve at least 80 % germination, hereafter referred to as the early (EI) and late (LI) imbibition time points, respectively, in the five imbibition treatments at 15/5 °C (Table 1). In addition, fresh (i.e., dormant) and pre-treated (i.e., non-dormant, to represent the start of imbibition) seeds were also analysed for comparison.

2.5.1. Seed moisture content

Seed moisture content (MC) of five replicates of 5 seeds was measured. Following imbibition, seeds were rinsed with dH_2O water for 10 seconds and transferred to an oven at 103 °C for 17 h (ISTA, 2019). MC was calculated gravimetrically and expressed on a fresh weight basis:

$$MC\ (\%) = [(FW - DW) / FW] \times 100$$

where FW is the fresh weight and DW is the dry weight.

2.5.2. Glutathione and low-molecular-weight thiols

Seeds were freeze-dried for five days. Four replicates of 30 mg of freeze-dried seeds were ground using a pestle and mortar to a fine powder in liquid nitrogen. The extraction from seed powder was carried out on ice in 1 mL of 0.1 M HCl with 30 mg polyvinylpolypyrrolidone (PVPP) and 0.5 % (v/v) Tween. The mixture was centrifuged for 40 min at 4 °C and 15000 g, and the resulting supernatant was divided in two, one part for the determination of total GSH content and the other part for the determination of GSSG content, following the protocol of Seal et al. (2010). Glutathione and the intermediates of glutathione metabolism, cysteine (Cys), cysteinyl-glycine (Cys-Gly) and γ-glutamyl-cysteinyl (γ-Glu-Cys), were separated by reversed HPLC (Agilent 1260; Cheshire, UK) on a Poroshell 120 EC-C18 column (150 × 3.0 mm i.d., 2.7 µm particle size; Agilent, Cheshire, UK), and detected fluorometrically (excitation: 380 nm; emission: 480 nm) with a gradient elution of 0.25 % (v/v) acetic acid in distilled water at pH 3.9/methanol. Standards of these low-molecular-weight thiols (Sigma Aldrich, UK) at different concentrations were prepared to construct calibration curves for quantification.

2.5.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) production was indirectly quantified via the formation of indamine, which is catalysed by the H_2O_2-dependent horseradish peroxidase (HRP) using a modified method of Visscher et al., (2018). Briefly, three replicates of 100 ± 10 mg FW of seed per treatment were ground in an ice-cold mortar and homogenized in 1 mL of 0.2 M perchloric acid. After centrifugation at 13000 g for 15 min at 4 °C, 500 µL of the resulting supernatant was collected and neutralized to pH 7.5 with 1 M and 0.1 M KOH and then centrifuged again at 1000 g for 5 min at 4 °C. The supernatant obtained was immediately used for spectrophotometric assay using a WPA Lightwave s2000 UV/Vis spectrophotometer. Two technical replicates for each biological replicate were measured. Absorbance values at 590 nm were quantified using a standard curve of known concentrations of H_2O_2 within a 0 - 500 µM range.

2.5.4. Electrolyte leakage

The electrical conductivity of three replicates of 20 seeds was measured. Seeds were rinsed with dH_2O for 10 seconds and transferred into 15 mL of dH_2O. Conductivity was measured using a meter (4520 Jenway; Fisher Scientific, Loughborough, UK) after two minutes (to remove any electrolytes on the seed surface) and then after 5 h at room temperature (20 °C; Marin et al. (2018)). The measurements at 2 min were subtracted from those at 5 h and expressed per 20 seeds (µS).

2.5.5. Spatial localisation of seawater elements within the seed

Seeds were imbibed in 0 ASW, 1.0 ASW and 1.5 ASW for the LI time point only (Table 1). Seeds were then sectioned (longitudinally and transversely) with a scalpel and freeze-dried. Sections were coated with 10 nm carbon (Cressington 208; Watford, UK). Sections were analysed using scanning electron microscopy (FEI Quanta 650 FEG, Thermo Fisher Scientific; Loughborough, UK) and mapped for elemental concentration (Na, Cl, Mg, Ca, K and S) using an energy-dispersive X-ray detector (9 kV accelerating voltage, spot size of 2, 1.5h scan time; Quantax annular flatQUAD, Bruker; Coventry, UK) equipped with ESPIRIT 2.1 software.

2.6. Additional data analysis

Differences of means between two groups was performed using a Student's t-test. The normality of residues and the homogeneity of variances were verified using a Shapiro-Wilk test (*shapiro*-test) and Fisher's test (*var*-test). To compare data for pre-treated seeds with ASW and PEG treatments, and to compare the effect of the iso-osmotic solutions 1.0 ASW and 1.0 PEG, and 1.5 ASW and 1.5 PEG, a one-way ANOVA with post-hoc t-test was used. The assumption of normal distribution was verified by analysis of quantile-quantile plots and the normality of residues and the homogeneity of variances were verified using Shapiro's test (*shapiro*-test) and Levene's test (*var*-test). When the assumption of a normal distribution was not met, data were arcsine-transformed and the analysis repeated. If the assumption of normal distribution was still not met, a Kruskal-Wallis test with post-hoc Dunn's test was used. Statistical analyses were performed in R (version 4.0.3) or Origin 8.6 (Origin Lab Corporation, Northampton, MA, USA).

3. Results

3.1. Seed pre-treatment and germination

Fresh seeds were dormant and did not germinate at 15/5 °C. Periodic sampling during submergence in 1.0 ASW at 5 °C revealed the following germination: 40 % after 9 weeks, 50 % after 14 weeks, 80 % after 16 weeks and 100 % after 18 weeks of submergence. Since each test was run for at least 7 d, there was a minimum of a one-week lag to obtain the germination results and therefore 19 weeks was selected for the dormancy breaking pre-treatment.

Concentrations of Na^+ and Cl^- were the highest among the salt ions detected in fresh seeds and significantly decreased after 19 weeks pre-treatment (Table 2). Concentrations of Mg, Ca and

K significantly decreased during pre-treatment except for SO_4^{2-} that slightly increased.

The germination of pre-treated seeds varied with ASW concentration and their corresponding iso-osmotic PEG solutions (Fig. 2a). The percentage of germination was highest in 0.25 PEG and 0.5 PEG at 97 % and 96 % respectively, and was over 88 % in 0 ASW, 0.25 ASW and 0.5 ASW. Germination was similarly high in 1.0 ASW (85 %), 1.0 PEG (88 %) and 1.5 ASW (82 %), three of the imbibition treatments selected for further analyses. In contrast, germination was lower in 1.5 PEG (52 %) and 2.0 ASW (33 %), with no germination occurring at 2.0 PEG. Following recovery of non-germinated seeds on water, all treatments reached a total of 86 - 100 % germination (Fig. 2b). The few seeds that remained non-germinated after recovery were mostly mouldy, with very few empty or non-viable seeds (Supplementary Table 1).

Halo time parameters were estimated by regressing germination rate against ASW concentration and iso-osmotic solutions of PEG (calculated as ASW equivalent units, ASW_{equiv}) for different percentiles (10 - 90 %) of the seed population (Fig. 3). Germination rate decreased linearly with increasing concentration of ASW and ASW_{equiv}. Germination in ASW was the slowest (θ_{Halo} = 6.43 ASW d) but had the highest 50 % maximum threshold of 2.22 x ASW ($[ASW]_{max50}$; Fig. 3a). This was in contrast to germination in iso-osmotic solutions of PEG that was associated with the fastest germination (θ_{Halo} = 5.06 ASW_{equiv} d) but a lower $[ASW_{equiv}]_{max50}$ of 1.76 (Fig. 3b).

3.2. Seed moisture content and spatial localisation of seawater elements

The MC of fresh seeds was 17 % and pre-treated seeds was 40 % (Fig. 4). Compared to pre-treated seeds, MC significantly increased to 45 % in seeds imbibed in 1.0 ASW at EI, and decreased to 35 % MC in seeds imbibed in 1.0 PEG at LI and at both imbibition time points in 1.5 ASW and 1.5 PEG. There were significant differences between ASW and iso-osmotic solutions of PEG, with a 5 % higher MC of seeds imbibed at LI in 1.0 ASW than in 1.0 PEG. At EI, seeds imbibed in 1.5 ASW had a significantly lower MC than in 1.5 PEG, but by LI, MC was the same between these treatments.

Microscopic analysis of seeds imbibed in 1.5 ASW revealed that Na accumulated in the testa and Cl in the tegmen (Fig. 5b). Moreover, K and Mg were concentrated in the embryo (Fig. 5c,e) but Ca and S were more broadly distributed within the seed (Fig. 5d,f). Localisation of Na, Cl and K was also observed in seeds imbibed in 1.0 ASW and to a lesser extent in seeds imbibed in 0 ASW (Supplementary Fig. 1).

3.3. Temporal changes in glutathione, other LMW thiols and indicators of oxidative stress

Compared to pre-treated seeds, GSH was significantly higher in seeds imbibed in all treatments except 0 ASW at EI. The concentration of GSH was significantly lower in seeds imbibed in ASW than in iso-osmotic solutions of PEG at EI (for example, 2187 and 2492 nmol g^{-1} DW in 1.5 ASW and 1.5 PEG respectively). However, by LI, concentrations of GSH were significantly higher in seeds imbibed in ASW (for example, 2960 and 2533 in 1.5 ASW and 1.5 PEG respectively). GSSG was present in fresh seeds at 424 nmol g^{-1} DW but was not detected in pre-treated seeds. Except for seeds imbibed in 1.0 PEG at EI, seeds imbibed in all other treatments showed an increase in GSSG concentration. This was especially apparent in seeds imbibed in 1.0 PEG at LI, and in 1.5 ASW and 1.5 PEG, where the highest concentration of 816 nmol g^{-1} DW was detected in seeds imbibed in 1.5 PEG at LI. Comparing seeds imbibed in iso-osmotic solutions of ASW and PEG revealed that the concentration of GSSG was significantly lower in seeds imbibed in ASW than in PEG except in 1.0 ASW/1.0 PEG at EI. Consistent with this, the percentage of GSSG of the total glutathione (GSH + GSSG) was lower in seeds imbibed in ASW than in iso-osmotic solutions of PEG (Table 3). The highest percentage of GSSG was found in seeds imbibed in 1.5 PEG (24.4 % at LI), over four-times higher than present in seeds imbibed in iso-osmotic 1.5 ASW (5.7% at LI).

The low molecular weight thiols Cys, Cys-Gly and γ-Glu-Cys were also detected in seeds. Cys was the second most abundant thiol present after GSH, with up to 469 nmol gDW^{-1}. The concentration of Cys was significantly higher than in pre-treated seeds at the start of germination in nearly all of the imbibition treatments (Fig. 6b). However, Cys, Cys-Gly and γ-Glu-Cys did not change in such a predictable pattern as GSH or GSSG during imbibition and with treatment (Fig. 6 b,c,d). Where differences were observed in seeds imbibed in iso-osmotic solutions of ASW and PEG, reduced thiol concentrations were typically higher in seeds imbibed in ASW and the concentration and percentage of thiol disulphides were higher in seeds imbibed in PEG (Table 3). Thiol disulphides were not detected in seeds imbibed in every treatment, particularly for Cys-bis-Gly (Table 3).

The highest concentration of H_2O_2 was 2.4 μM g DW^{-1} in fresh seeds (Table 4). Compared to pre-treated seeds, there was a significant production of H_2O_2 only in seeds imbibed in 1.5 ASW, however values never exceeded 2.15 μM g DW^{-1}.

The electrical conductivity was assessed to measure the leakage of solutes from seeds (Table 4). Fresh seeds had the highest conductivity of 40 µS and was lower at less than 12.46 µS in all other imbibition treatments. The conductivity of seeds did not significantly change from pre-treated seeds in any of the imbibition treatments, but was higher in seeds imbibed in 1.5 ASW than in 1.5 PEG.

4. Discussion

Saltmarshes are a hostile environment for any plant to survive, as salinity imposes a plethora of osmotic and toxic effects on plant growth and development (Flowers and Colmer, 2008, Munns and Tester 2008). Whereas the different strategies for plants to tolerate these conditions are reasonably well identified, such as the accumulation of salt ions in leaf vacuoles to aid osmotic adjustment and a potentiated antioxidant system to quench the excessive formation of ROS (Flowers and Colmer, 2008; Kranner and Seal, 2013), the functional traits underlying salt tolerance in seeds are less resolved. Here, evidence is presented for ion accumulation and a reduced cellular redox state in response to early signs of oxidative stress, in freshly matured seeds of *S. maritima* that over-winter in cold-saline conditions, and elemental localisation, the maintenance of a reduced cellular redox state and beneficial effects of seawater ions during germination.

4.1 Tolerance strategies of fresh seeds and seeds over-wintering in cold-saline conditions

The seeds of saltmarsh halophytes in the northern hemisphere are often shed in the autumn and require an over-wintering period of cold-wet conditions for dormancy to be released, so that germination can begin in the spring when temperatures start to rise but the concentration of salinity is low (Ungar, 1978, Wetson et al., 2008). The conditions experienced during such periods not only impact on the timing of these events (Wetson et al. 2008), but exposure to potentially toxic ions may have permanent cellular damage. In dormant seeds of *S. maritima* collected fresh from the field, Na^+ and Cl^-, the most abundant ions in seawater, were prevalent along with a mixture of other ions such as K^+. When seeds were transferred to water to test for electrolyte leakage, the data suggests that these ions readily moved out of the seeds down an osmotic gradient. Their presence was accompanied by biochemical changes. In mature, dry seeds, a high proportion of total glutathione is present as GSSG (e.g., 24 % GSSG in pea seeds), which typically accumulates during seed maturation and is reduced to GSH within the first hours of imbibition (Colville and Kranner, 2010; Gerna et al., 2017). Although fresh seeds of

S. maritima are desiccation tolerant (RBG Kew, 2021), and therefore undergo maturation drying, the saltmarsh environment is not dry and seed MC reflected this at 17% MC. Nonetheless, 13.6 % GSSG was present, and these seeds had one of the highest concentrations of GSH, Cys-Gly and γ-Glu-Cys of all the treatments. This was also associated with high concentrations of the ROS H_2O_2. After 19 weeks of pre-treatment, ion concentrations were significantly lower for all ions except SO_4^{2-}, and with this, GSSG was not detected, the proportion of other disulphides were < 1 % and H_2O_2 was barely detectable. This implicates ion accumulation within the seed and oxidative stress before the event of germination, and highlights the importance of maintaining a reduced cellular redox state that will likely contribute towards the persistence of seeds to over-wintering in cold-saline field conditions.

4.2 The beneficial effects of seawater ions on germination

Non-dormant seeds of *S. maritima* germinated readily in ASW, with a maximum threshold of 2.22 times full strength ASW. This is consistent with a previous study where the maximum germination threshold was around 1M NaCl (where there is 411 mM NaCl in 1.0 ASW) at temperatures between 5 and 15 °C (Seal et al., 2018). This ranks seeds of *S. maritima* among some of the most tolerant to salinity during germination, similar to other coastal halophytes such as *Suaeda japonica*, *Spartina alterniflora* and *Arthrocnemum indicum* (Gul et al., 2013). However, caution is needed when making inter-specific comparisons since temperature can have a large effect on tolerance to salinity during germination (Allen et al., 2000; Zhang et al., 2012; Seal et al., 2018; Seal and Dantas, 2020). In temperate habitats, seeds of *S. maritima* germinate during the spring when temperatures are mild (15/5 °C) and salinity levels are low (Wetson et al., 2008). This is further reflected in the near full recovery of non-germinated seeds of *S. maritima* in water once salinity is removed, which is typical of many halophyte species (Gul et al., 2013). Thus, although seeds of *S. maritima* show a broad physiological tolerance to salinity *ex situ*, germination timing in nature will be governed by a combination of environmental factors.

The localisation of Na and Cl into testa and tegmen, respectively, suggests that these tissues formed a physical barrier to protect the embryo against their accumulation and toxicity. This has also been observed in the testa for Na^+ and Cl^- in the halophytes *Salicornia pacifica* var. *utahensis* and *Atriplex canescens* (Khan et al., 1985) and for Na^+ *Suaeda physophora* and *Haloxylon ammodendron* (Song et al., 2005). During germination, the accumulation of Na^+ within the seed has been linked to the maintenance of seed fresh weight in several halophyte species (Song et al., 2005) and the maintenance of MC in seeds of *S. maritima* (Seal et al., 2018), suggesting a role in osmotic adjustment. Although we observed slight, yet significant, differences in MC (no more than 5%) in seeds imbibed in 1.0 ASW compared to those imbibed in 1.0 PEG, this was not reflected in the final germination percentage (85 and 88 % respectively). However, the MC of seeds imbibed in 1.5 ASW and 1.5 PEG was the same by LI, at 5 % lower than in pre-treated seeds, yet final germination was higher in seeds imbibed in ASW (82 %) than in PEG (52 %). As germination will only complete (i.e., the emergence of the radicle) above a critical water threshold (Seal et al., 2018), it can be assumed that enough water was available within the seed to achieve this in the 1.5 ASW and 1.5 PEG imbibition treatments.

Na and Cl were not the only elements to be detected within the seed. In addition, K and Mg localised in the embryo. There are mixed reports as to whether K^+ and Mg^{2+} promote germination in salinity, although both can be more toxic than Na^+ (Manzoor et al., 2017; Tobe et al., 2002), and their beneficial effects on germination may only be seen when in combination with Ca^{2+} (Tobe et al., 2002; Gao et al., 2018), which appeared to have a broad distribution across the seed. Nonetheless, both K^+ and Mg^{2+} and are considered plant macronutrients. K^+, fundamental to plant growth and metabolism, may experience efflux from the cytosol through ROS mediated channels in response to NaCl, making cytosolic K^+ retention an important trait of salt tolerance in plants (Wu et al., 2018). The localisation of K in the embryo could also be an important trait of salt tolerance in seeds. Mg^{2+} has multiple roles in plants especially in relation to photosynthetic carbon fixation (Hauer-Jákli et al., 2019), which could be important for *S. maritima* since the embryo is chlorophyllous. Mg^{2+} deficiency has also been linked to a lower GSH:GSSG ratio (i.e., more oxidised state) in leaves of Arabidopsis, as impairment of CO_2 fixation causing ROS production (Hermans et al., 2010), implicating an association between Mg^{2+} and the cellular redox state.

During germination, evidence is presented for the synthesis of GSH and GSSG in seeds imbibed in nearly all of the treatments. The accumulation of GSH was slower in seeds imbibed in ASW than in PEG, so that although seeds imbibed in PEG had a higher concentration than in seeds imbibed in ASW at EI, this was reversed by LI. Moreover, the concentration and percentage of GSSG was higher in seeds imbibed in PEG than in ASW at LI. Thus, it appears that seawater ions had a beneficial effect on germination that was not osmotic. This was further apparent when halotime parameters were considered. Although θ_{Halo} was higher (i.e., slower germination) in the presence of ASW than PEG, the maximum threshold to germination was also higher in ASW ($[\mathrm{ASW}]_{\mathrm{max50}}$ = 2.22) than PEG ($[\mathrm{ASW}_{\mathrm{equiv}}]_{\mathrm{max50}}$ = 1.76). Slower germination may give more time to initiate tolerance mechanisms in response to ASW that promote germination.

During GSH synthesis, the bond formation between GSH and Cys is the rate-limiting step, and may explain why the intermediates Cys-Gly and γ-Glu-Cys were not abundant during germination (Zagorchev et al., 2012). In contrast, Cys was abundant in seeds. Cys has been associated with stress tolerance, including as an osmoprotectant acting as a sulphur donor for the amino-acid methionine (Hesse et al., 2004) and as a ROS scavenger (Soares et al., 2019). In addition, CySS can form complexes with alkali-metals such as Na^+, suggesting a possible role in Na^+ ion sequestration (Zagorchev et al., 2012), which could explain why CySS was more abundant in seeds imbibed with PEG than ASW.

Even though there was a partial conversion of reduced thiols to their disulphides, especially in seeds imbibed in 1.0 PEG, 1.5 PEG and 1.5 ASW (of up to 25% for GSSG, 13 % for CySS, 25 % for Cys-bis-Gly and 27 % for bis-Glu-Cys), the cellular redox state remained highly reduced. This is in contrast to seedlings of *S. maritima* germinated in NaCl that contained about 50 % GSSG at ca. 35 % germination (Boestfleisch et al., 2014) and NaCl-stressed cells of *Dactylis glomerata* with 40 % GSSG during cell differentiation (Zagorchev et al., 2012). It is likely that our treatments selected for biochemical analyses were not "stressful" to reflect a period where the antioxidant mechanisms were insufficient to cope with any stress, characterised by a major shift towards an oxidised cellular redox state (Kranner et al., 2006), as seeds subjected to all treatments achieved at least 80 % germination. There was also no obvious indication of damage to cellular membranes during imbibition, with higher electrolyte leakage from seeds imbibed in 1.5 ASW than in 1.5 PEG most likely reflecting the movement of salt ions out of the seed down an osmotic gradient, since electrolyte leakage was tested in water. Nonetheless, differences in glutathione in seeds imbibed in PEG and in ASW were evident during germination. In contrast, there was no consistent evidence of involvement of H_2O_2 during germination, unlike H_2O_2 that was observed to increase during the germination of the subtropical halophytes *Suaeda fruticosa* and *Limonium stocksii* in NaCl (Hameed et al., 2014).

In conclusion, seeds of *S. maritima* were highly adapted to saline conditions, through a combination of strategies involving ion accumulation and localisation, a potentiated glutathione and low-molecular weight antioxidant system to prevent oxidative stress and the maintenance of seed moisture content to levels sufficient for germination to complete. This culminated in a higher θ_{Halo} in seeds imbibed in ASW than in iso-osmotic solutions of PEG, that was tolerant to over twice the concentration of ASW. The localisation of potentially toxic Na and Cl away from the embryo may be paramount to achieving germination under salinity, yet it remains to be explored whether it is a common feature of all halophyte species or specific to certain habitats or lineages.

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Declaration of Interest

None.

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Author Contributions

CES and CMS conceived the study and designed the experiments. CMS conducted the experiments and statistical analyses. MY carried out the seed cleaning/pre-treatment and provided training of some biochemical analyses. AM conducted the ion analysis. IC and TS conducted the scanning electron microscopy with elemental analysis. CMS, CES and LC drafted the manuscript, and all authors contributed towards critically revising the manuscript and give their final approval.

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Figure legends

Fig. 1. *Suaeda maritima* seed morphology and anatomy. (a) and (b) External views of the seed; (c) longitudinal and (d) cross-section of the seed. Scale bars represent 0.50 mm.

Fig. 2. (a) Cumulative germination of pre-treated seeds in solutions of ASW and iso-osmotic PEG at 15/5 °C. Data points are mean values of four replicates of 25 seeds, and the lines are the fitted Boltzmann curves. (b) Recovery of non-germinated seeds from Fig. 2a at the end of the germination test. Seeds were recovered on dH_2O and incubated at 15/5 °C.

Fig. 3. Germination rate ($1/t_g$) in solutions of (a) ASW and (b) iso-osmotic PEG (calculated as ASW equivalent units, ASW_{equiv}). Values are the mean halo time (θ_{Halo}) ± standard deviation for the population, and the maximum concentration of ASW and ASW_{equiv} for 50 % germination ($[ASW]_{max50}$; $[ASW_{equiv}]_{max50}$). Lower case letters show significantly different values using a Student's test ($P < 0.05$).

Fig. 4. Seed moisture content of fresh (F) and pre-treated (PT) seeds exposed to different treatments during imbibition in ASW and PEG (PT = start of imbibition, EI = early imbibition, LI = late imbibition; see Table 1 for details). Values are the means of five replicates ± standard deviation. * indicates significant differences from pre-treated seeds; # indicates significant differences between corresponding EI or LI of 1.0 ASW and 1.0 PEG, and † of 1.5 ASW and 1.5 PEG (one-way ANOVA, $F(11,44) = 60.88$, $P < 0.0001$; post-hoc t-test at $P < 0.05$).

Fig. 5. (a) Scanning electron micrograph of a seed, showing localisation of (b) Na and Cl, (c) Mg and Cl, (d) Ca and Cl, (e) K and Cl, and (f) S, O, Na and Cl. Seeds were imbibed in 1.5 ASW for LI time point. Scale bars represent 50 μm.

Fig. 6. Concentration of low molecular weight thiols of fresh (F) and pre-treated (PT) seeds imbibed in different concentrations of ASW and PEG (PT = start of imbibition, EI = early imbibition, LI = late imbibition; see Table 1 for details). (a) Concentration of glutathione (GSH) and glutathione disulphide (GSSG); (b) cysteine (Cys) and cystine (CySS); (c) cysteinyl-glycine (Cys-Gly) and cystinyl-bis-glycine (Cys-bis-Gly); and (d) γ-glutamyl-cysteinyl (γ-Glu-Cys) and bis-γ-glutamyl-cystine (bis-γ-Glu-Cys). Data are mean values of four replicates ± standard deviation. * indicates significant differences from pre-treated seeds; # indicates

significant differences between corresponding EI or LI of 1.0 ASW and 1.0 PEG, and † of 1.5 ASW and 1.5 PEG (one-way ANOVA with post-hoc t-test or Kruskal-Wallis test with post-hoc Dunn’s test; $P < 0.05$; see Supplementary Table 2).