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ORIGINAL PAPER

Effect of seawater salinity stress on *Sporobolus pungens* (Schreb.) Kunth, a halophytic grass of the mediterranean embryonic dunes

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Abstract

Sporobolus pungens (Schreb.) Kunth is a perennial rhizomatous grass which develops at several coastal habitats, being relevant in the embryonic dune communities. Considering its importance for dune ecosystem and its fragile situation in the face of changes in sea level derived from global warming, the present study aimed to evaluate *S. pungens* response to increased salinity. One-year-old plants were exposed to different seawater (SW) dilutions (None, 1/16SW, 1/8SW, 1/4SW, 1/2 W and Full-SW). Gas exchange measurements and oxidative stress biomarkers were determined after two months of treatment. Stress conditions were maintained until flowering finished in order to assess the potential effects on the reproductive effort. Strong delay and inhibition of flowering were observed at low salinity levels and full inhibition for further treatments. Gas exchange measurements showed little effect until 1/8SW and a decreased assimilation rate due to mainly stomatal limitations at 1/4SW. Further decreases at higher salinity levels were related to both stomatal and metabolic limitations. As salinity increased, there was a progressive increase in the activity of glutathione peroxidase and glutathione reductase, while catalase activity remained stable. Superoxide dismutase did not vary except for Full-SW, where the activity significantly decreased. The levels of malondialdehyde, a marker of lipid peroxidation, remained unchanged and only increased in Full-SW level. In addition, the concentration of osmolytes, proline and soluble sugars, increased progressively with increasing salinity. In conclusion, *S. pungens* showed strong tolerance to salinity, through physiological adjustments at low levels of salinity, and activating glutathione-dependent enzymes at the highest concentrations. Evidence of oxidative damage was only observed after Full-SW exposure, although no death rate was recorded.

Keywords Halophyte · Sporobolus · Salinity · Oxidative stress · Reactive oxygen species · Seawater

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Abbreviations

SOD	Superoxide dismutase
GPx	Glutathione peroxidase
GRd	Glutathione reductase
CAT	Catalase
MDA	Malondialdehyde
ROS	Reactive Oxygen species
A_n	Net assimilation
g_s	stomatal conductance
C_i	intercellular CO_2
E	Transpiration rate
ETR	electron transport rate

Introduction

Coastal dune systems are dynamic habitats with a distinctive vegetation pattern influenced by several abiotic factors governed mainly by the effect of wind (Maun 2009; Fenu et al. 2013). Salinity is generally considered a minor factor due to high soil permeability (Maun 2009). However, the influence of salinity through the salt spray and periodical inundations may exert a major effect on the more exposed upper beach and fore-dunes where annual vegetation of drift lines and embryonic shifting dunes plant communities develop (Maun et al. 2009, Ruocco et al. 2014). Greaver and Sternberg (2007) gave further evidence for seawater uptake of fore-dune species mainly during the dry season due to salt spray deposition, but also for the underground seawater-freshwater mixture. The Intergovernmental Panel on Climate Change climatic projections indicate that seawater influence will increase both due to sea-level rise and increase of storm and inundation frequency (IPCC 2014). In this sense, dune systems are also directly influenced by human activities and at risk from changes associated with climate change and global warming. In the case of the Mediterranean and, specifically, in first-line plant communities, the removal of *Posidonia oceanica* L., "*banquettes*" imply a direct exposure to the erosive and saline seawater impacts which could aggravate future scenarios regarding sea level rise (Boudouresque et al. 2016).

Salinity exerts its deleterious effects on plants mainly through two processes, the osmotic effect, which reduces the capacity of plants to absorb water, and the ionic effect, which derives from absorption of ions, mainly Na^+ and Cl^-, and their associated toxic effects (Munns 2002). When both effects intervene, plants undergo water stress leading to impairment of photosynthesis. The altered photosynthesis triggers reactive oxygen species (ROS) overproduction and further photochemical impairment entailing a context of stress (Flexas et al. 2004, 2006; Arora et al. 2016). Physiological response by stomatal closure and other metabolic adjustments may partially account for the osmotic effect (Sharma et al. 2012). To maintain water uptake under these conditions, plants accumulate organic osmolytes rich in carbon and nitrogen as sugars and proline to counteract salt influx into the plant (Munns 2011). In addition, and in order to avoid excessive ROS accumulation, plants present specific non-enzymatic and enzymatic antioxidant mechanisms to cope with the excess ROS generated in a situation of physiological stress. Among the main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the members of the ascorbate-glutathione (AsA-GSH) cycle (Hasanuzzaman et al. 2012).

Halophytes have been given several definitions (Grigore et al. 2019). However, the definition given by Flowers and Colmer (2008) is the most generally accepted, describing halophytes as those species capable to complete their life cycle at 200 mM NaCl. The presence of specific mechanisms and structures to confront salinity, such as salt glands, salt compartmentalization or dilution, high water use efficiency coupled with rapid and efficient physiological and biochemical adjustments, and high survival rate are also some of the representative traits for halophytes (Flowers and Colmer 2015). Salinity can also exert a major effect during the reproductive stage, decreasing inflorescence and flower production, and depleting pollen and seed production through flower abortion or sterility (Boscaiu et al. 2005). Reproductive response of halophytes has been considered of great interest, as efficient maintenance of reproductive effort, with stimulatory effect in some cases, can occur under high salinity concentrations (Guo et al. 2019). Since this stage is considered of high ecological significance, its need to be assessed has been regarded as of great interest, and strong evidence of plant tolerance to salinity (Guo et al. 2019).

Sporobolus pungens (Schreb.) Kunth is a rhizomatous grass distributed throughout the coasts of the Mediterranean basin. It develops mainly as part of embryonic dune vegetation near the shoreline jointly with other species in the Ammophilion class (Marcenò et al. 2018). Due to its high ecological flexibility and tolerance to both salinity and waterlogging, it can also be found near salt marshes and rocky coasts (Šegota et al. 2017). Like other species of the same genera, *S. pungens* displays a C4 metabolism (Pyankov et al. 2010) and possess salt glands that allow salt excretion. Taxonomically it is related to *S. virginicus* (L.) Kunth., a widely distributed coastal halophytic grass with a similar ecological role (Peterson et al. 2014). Studies of salt stress on *S. virginicus* have been mainly limited to plant growth, water potential, osmolyte accumulation, ionic content, and salt excretion, and variable and even contradictory results were obtained (Gallagher 1979; Blits and Gallagher

1991; Marcum and Murdoch 1992; Naidoo and Mundree 1993; Naidoo and Naidoo 1998, Bell et al. 2003). The reproductive response has been also assessed with variable results (Blits and Gallagher 1991; Marcum and Murdoch 1992; Naidoo and Mundree 1993). However, to date, there are no studies that analyze the response to salt stress and the enzymatic antioxidant defence mechanisms in *S. pungens*.

Considering the importance of *S. pungens* role in Mediterranean dune systems and the fragility of this habitat in the Mediterranean basin which can be increased by global warming, this study aims to assess the salinity tolerance of *S. pungens* with special emphasis on the antioxidant defensive mechanisms, reproductive success, and physiological response.

Materials and methods

Plant material and experimental design

Sporobolus pungens plants were cultivated using rhizomes collected from Son Serra de Marina (Mallorca, Balearic Islands, Spain, UTM: ETRS89 31 N ED2098), since sexual reproduction (seed production) is rare. Rhizome fragments of equal size (20 cm) were cut and planted in February 2018 and maintained for growth until experimental procedure in 2019. Two months prior to experimentation (prior pre-treatment), plants' aerial and root areas were equally cut to homogenise plant biomass, and fresh substrate was renewed. 60 individuals were then randomly allocated to six seawater treatments (10 plants/treatment) (electric conductivity (EC in mS/cm): Control-Tap Water (1.05), 1/16SW (5.07), 1/8SW (9.30), 1/4SW (16.34), 1/2SW (30.30), Full-SW (55.69). Each treatment was applied for 15 days by progressively watering with increasing seawater concentration starting with 1/8SW level until Full treatment concentration was achieved in each treatment (intervals of 5 days between watering), to avoid osmotic shock (Amor et al. 2005). In this way, 15 days was established as a pre-treatment period and two months of complete treatment before the final measurements were carried out in mid-July 2019. After that, treatments were maintained to assess reproductive effort until the end of the experiment, which was established when flowering ended in mid-late September.

Field conductivity measurements

Soil samples were collected at 22 m distance from the sea, and 5 cm depth (where root system was developing) with a 15-day frequency during the year 2019 on a natural population of *S. pungens* in s'Estanyol de Migjorn (Mallorca, Balearic Islands, Spain, UTM: ETR89 31 N DD9356). Soil conductivity was measured by diluting soil samples in distilled water in a 1:5 ratio with a magnetic shaker for two hours. Samples where then filtered, and conductivity was measured (XS Instruments Cond 51+). Results are indicated in Table 1.

Growing conditions

Plants were cultivated in 3 L pots with culture substrate composed of 61.50% coconut fiber, 33.00% white peat moss, and 5.50% of expanded perlite, fertilized with 4.40 mg/l of Osmocote NPK 19-10-19, a slow-release fertilizer. Plants were maintained through all the growing and experimentation periods under a shade cloth (50% light exclusion) outdoors at the University of the Balearic Islands (Mallorca, Spain). Seawater treatments were established by combining the corresponding proportion of seawater (collected in Sa Ràpita locality) with tap water from the experimental field facility. Watering was done until field capacity with a frequency that varied from weekly to 3 times per week based on soil moisture. Soil conductivity was periodically measured (XS Instruments Cond 51+) to ensure that soil salinity was maintained at the corresponding treatment conductivity value.

Reproductive and growth measurements

Reproductive traits were measured as the number of panicles produced per plant in each treatment. Additional specific measurements were conducted on non-stressed and 1/16SW plants which are indicated in Table 2. No measurements were conducted on 1/8SW panicles since flowering was too scarce for statistical meaningfulness. Growth was

Table 1 Conductivity and temperature values along 2019 in s'Estanyol de Migjorn (Mallorca, Balearic Islands, Spain, UTM: ETR89 31 N DD9356). Conductivity values are mean values of 4 measurements taken every 15 days. Temperature data was collected from Sa Ràpita meteorological station (http://www.balearsmeteo.com/)

Months	Conductivity (mS/cm)	Max Conductivity	Min Conductivity	Mean Temperature (ºC)
January - February	1.75 (0.62)	3.03	0.616	10.07 (0.21)
March - April	4.10 (0.05)	4.217	4.014	13.34 (0.21)
May - June	8.77 (2.76)	15.17	2.102	19.48 (0.40)
July - August	5.51 (1.53)	9.72	2.99	26.14 (0.14)
September - October	2.93 (0.69)	4.5	1.153	21. 59 (0.28)
November- December	2.09 (0.28)	2.93	1.774	13.92 (0.34)

Table 2 Mean (± standard error) of the different panicles measured in control and 1/16SW treatments of *Sporobolus pungens*. (Control: N = 32; 1/16SW: N = 20)

Reproductive trait	Control	1/16 SW	Df.	T	X^2	Z	p-value
Nº spikelets $mm^{-1}$of inflorescence branch (1)	0.78 (0.01)	0.82 (0.02)	200.95	1.48	-	-	0.141
Nº spikelets $mm^{-1}$of inflorescence branch (2)	0.98 (0.02)	1.12 (0.02)	196.92	4.39	-	-	< 0.001 *
Number of branches per inflorescence	16.47 (0.48)	16.70 (0.52)	50	-	20.759	-	0.842
Total Inflorescence branch length (1)	17.35 (0.45)	16.88 (0.49)	226.49	-0.44	-	-	0.662
Flowering Inflorescence branch length (2)	13.77 (0.40)	12.35 (0.41)	-	-	-	-2.151	0.031 *
Panicle Culm length	15.31 (1.84)	32.19 (3.72)	-	-	-	3.668	< 0.001 *
Panicle inflorescence length	55.01 (2.06)	52.16 (2.09)	45.53	-0.88	-	-	0.382
Panicle width	28.77 (0.95)	30.77 (1.47)	34.50	1.39	-	-	0.174

assessed as the elongation of 4 branches per plant from the start of the experiment and after two months of salinity stress. Subsequent Branch Relative Growth Rate (RGR. B) was calculated as indicated by del Vecchio et al. (2013):

$$RGR.B = \frac{In(Lf.) - In(L0.)}{\Delta t}$$

Where L0. Indicates initial branch length, Lf. final branch length, and Δt time elapsed between both measurements.

Gas exchange measurements

Gas exchange measurements were conducted after two months of salt exposure. Plants in each treatment were randomly selected and measured (N = 6–8) using an open gas exchange system with a coupled fluorescence chamber of 2 cm^2 (Li-6400. Li-cor Inc., Lincoln, USA). Measurements were conducted at light saturation of 1150–1300 μmol m^{-2} s^{-1} providing 400 µmol mol^{-1} CO_2 with a flow rate of 300 µmol s^{-1}. Measurements were performed between 10:00 and 14:00 maintaining a 25 ºC temperature and relative humidity of 50–70% inside the leaf chamber. Three leaves were used in each measurement to cover most of the 2 cm^2 leaf chamber. After each measurement, digital images were taken and measured by image analysis (Fiji software; Schindelin et al. 2012) to correct for leaf area. For each measured plant, the following parameters were measured: Net assimilation (A_n), stomatal conductance (g_s), intercellular CO_2 (C_i), Transpiration rate (E), electron transport rate (ETR), and electron transport rate and assimilation ratio (ETR/A_n). Flexas et al. (2002), indicate ETR – Gross assimilation ratio would be a better approach as it accounts for respiration and avoids problems associated with ETR/A_n when A_n is particularly low. Since respiration was not measured, ETR/A_n has been used instead, excluding negative assimilation values in 1/2SW and Full-SW that distort the ratio interpretation.

Sample processing for biochemical assays

After two months of salinity stress, leaf samples were collected and stored at -40ºC. Before testing, leaves were homogenized in 50 mM Tris HCL buffer and 1 mM ethylenediaminetetracetic acid (EDTA) at pH 7.5, in a proportion of 1:5 (weight: volume). Samples were then centrifuged for 10 min at 10000xg and 4ºC to remove cell debris, nuclei, and mitochondria from the supernatant, which was stored for further use. Enzymatic activities and stress biomarkers were determined with a Shimadzu UV-2100 spectrophotometer at 25 ºC and normalized per mg of protein using the colorimetric kit Biorad®, which uses Bovine Albumin Serum (BSA) as a standard. 96-well microplates were read with a Bio-Tek PowerWave XS microplate spectrophotometer.

Organic osmolytes

Total soluble sugars were determined using the method described by Yemm and Willis (1954). Briefly, samples homogenates were added to Pyrex tube containing Anthrone's reagent tube and incubated in a water bath at 100 ºC for 30 min. The reaction was terminated in an ice bath and the absorbance was determined at 630 nm. Proline was determined following the method of Bates et al. (1973). Plant homogenates were deproteinised with 3% sulfosalicylic acid. Samples were then mixed with equal volumes of acid ninhydrin solution and glacial acetic acid at 100ºC for one hour. The reaction was stopped on an ice bath and the chromophore was extracted with toluene and recorded at 520 nm.

Antioxidant enzyme activities

Catalase (CAT) (EC 1.11.1.6) activity was determined according to the method described by Aebi (1984), based on the decomposition of H_2O_2 in phosphate buffer 50 mM at pH 7.0, monitoring the decline in absorbance at 240 nm. Activity is expressed as mK(s^{-1})/mg protein. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by

the decrease of the inhibition of the reduction of cytochrome C by the superoxide anion generated by the xanthine oxidase/hypoxanthine system (Flohé and Otting 1984). The reaction was carried out using 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.8 containing Cytochrome C and xanthine solution. The activity was measured at 550 nm and calculations were based on an absorption coefficient of 28.1 mM^{-1} cm^{-1}. The results are presented as pKat/mg protein. Glutathione reductase (GRd) (EC 1.8.1.7) activity was determined by measuring the oxidation of NADPH 9.6 mM at 340 nm using oxidized glutathione as a substrate and an absorption coefficient of 6.22 nM^{-1} cm^{-1} (Goldberg and Spooner 1984). Results are presented as pKat/mg protein. Glutathione peroxidase (GPx) (EC 1.11.1.9) activity was determined by adapting the method described by Flohé and Gunzler (1984). The activity was measured at 340 nm using H_2O_2 as substrate and GRd as enzymatic and NADPH as non-enzymatic indicators and with NaN_3 as a catalase inhibitor. An absorption coefficient of 6.22 nM^{-1} cm^{-1} was used. Results are presented as nKat/ mg protein.

Lipid peroxidation assay

Malondialdehyde (MDA) levels were used as a lipid peroxidation indicator. MDA concentration was measured by a colorimetric assay based on the specific reaction of MDA with a reagent to produce a stable chromophore with a maximum absorbance at 586 nm. N-methyl-2-phenindole (10.3 mM) was added to samples in acetonitrile: methanol (3:1). After that, HCl 12 N was added and the samples were incubated for 1 h at 45ºC. MDA concentration was calculated using a standard curve of known concentration.

Statistical analysis

Data was tidied, gathered, and analyzed using the statistical software R. Data manipulation and observational data analysis were carried out using the package tidyverse and ggplot2, respectively (Wickham 2016; Wickham et al. 2019). Continuous data, such as growth measurements, were analyzed using linear models (LM), while count and proportional data were modeled using generalized linear models (GLM, with Poisson and Binomial families respectively). Complex correlations among variables were modeled using Generalized Additive Models with the package mgcv (Wood 2017). Model explained variability was evaluated using qqplots (package mgcviz; Fasiolo et al. 2019) d squared. When R squared was not possible to compute, MacFadden pseudoR2 was used (package pscl; Jackman 2020). The effect of the salinity treatment among the response variables was evaluated using the analysis of variance (ANOVA). When not possible, differences were evaluated using the Kruskal Wallis test (McKight and Najab 2010). Differences among treatments were evaluated using the Tukey Honest significance test (Abdi and Williams 2010) or Dunn-test when required (Dinno 2017).

Results

Conductivity values collected in the field along 2019 (Table 1), show steady and low values (equivalent to 1/16SW) between September and April, and increased conductivity during summer (May-August) with mean values equivalent to 1/8SW and maximum values equivalent to 1/4SW. Reproductive effort considered as the number of panicles was negatively affected by salinity treatments (GLM: $X^2 = 129.68$; df = 2; p-value = < 0.001). Panicle production achieved maximum values in non-stressed plants, being strongly reduced at 1/16SW, rare in 1/8SW, and completely absent in 1/4SW onwards (Fig. 1). Flowering (data not shown) was also strongly delayed in stress treatments in comparison to non-stressed plants. Specific measurements of reproductive traits between non-stressed plants and 1/16SW showed mixed differences (Table 2). Reproductive structures displayed an overall increase in 1/16SW treatment compared to non-stressed plants, with significantly higher values in culm length, inflorescence flowering branch length, and number of spikelets per mm of flowering branch length. Other reproductive traits showed a lack of differences between both treatments: panicle width, inflorescence length, total inflorescence branch length, and number of spikelets/total inflorescence branch length.

Plant branching growth (Fig. 1) was affected by seawater treatment ($X^2 = 150.86$; df = 5; p-value = < 0.001). Growth did not show differences at low salinities. At 1/4SW a significant reduction in growth occurred and this negative effect was maintained for the two remaining treatments being all significantly different among them.

Gas exchange was markedly affected by salinity stress (Fig. 2). Effects occurred in assimilation rate (LM: F = 23.44; df = 5; p-value = < 0.001), stomatal conductance ($X^2 = 28.71$; df = 5; p-value = < 0.001) and transpiration rate ($X^2 = 27.79$; df = 5; p-value = < 0.001) with a similar pattern, achieving maximum values in non-stressed plants, small reductions at low and medium salinity levels, and strong reduction with ½SW and Full-SW treatment. Intercellular CO_2 was also affected ($X^2 = 17.09$; df = 5; p-value = < 0.001) with an appreciable increase in ½SW and Full-SW treatment. ETR was also affected with a steady decrease with salinity treatment (LM: F = 18.45; df = 5; p-value = < 0.001). $ETR/A_n ratio$ was also affected by salinity (LM: F = 10.11; df = 5; p-value < 0,001) remaining stable until 1/4SW and increasing slightly at 1/2SW (Fig. 2). Further increase of

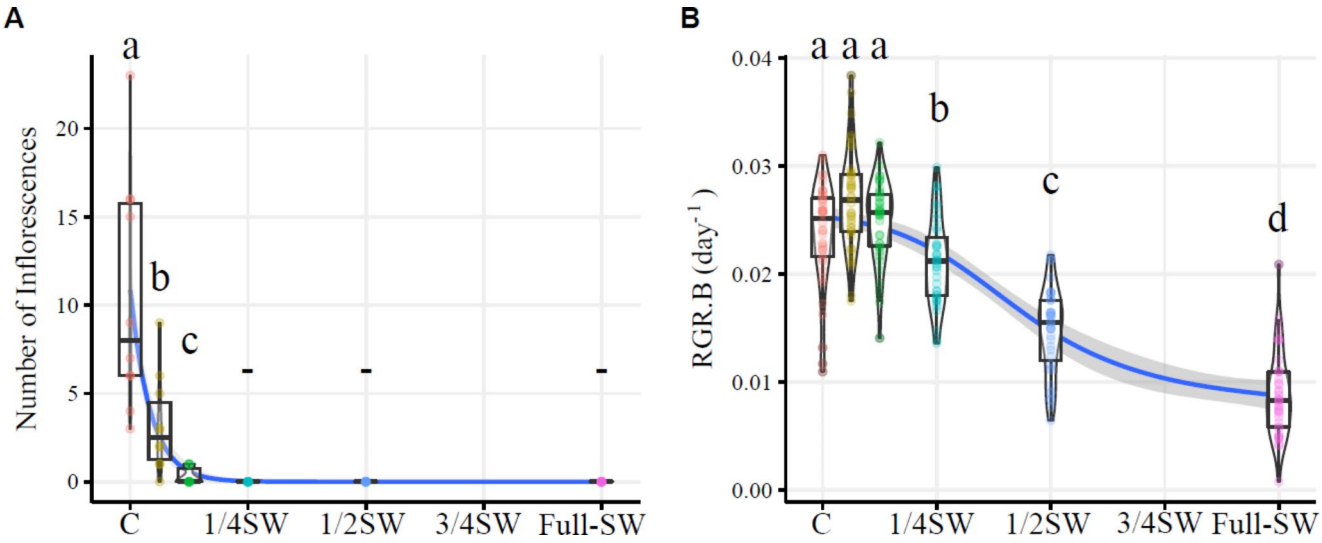

Fig. 1 Number of inflorescences (panicles) (A) and relative growth rate of branching elongation (B). of *Sporobolus pungens* under different seawater concentration treatments (C, 1/16SW, 1/8SW, 1/4SW, 1/2SW, Full-SW). Different letters indicate significant differences among treatments under Dunn test for A and B at alpha 0.05. (A: N = 10; B: N = 37–40)

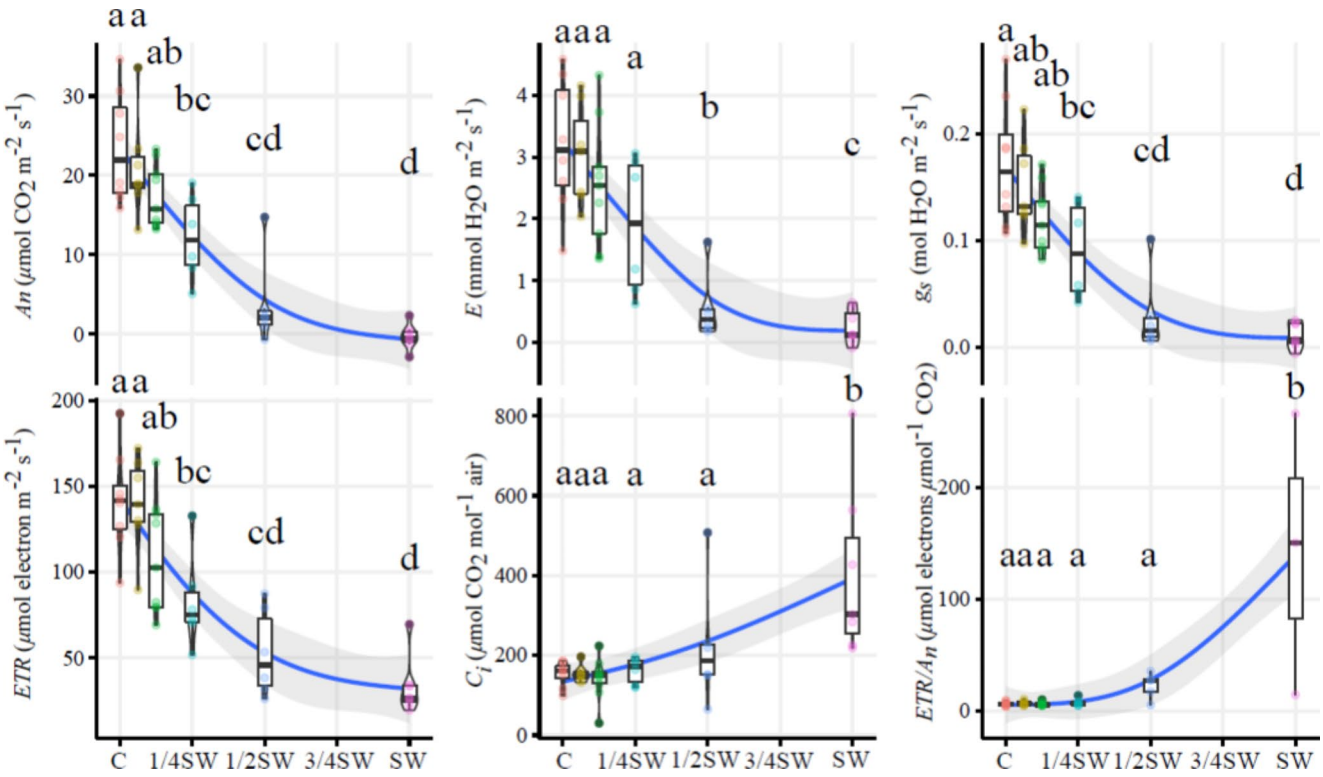

Fig. 2 *Sporobolus pungens* physiological parameters were measured after two months of different seawater concentrations treatments (C, 1/16SW, 1/8SW, 1/4SW, 1/2SW, Full-SW). Different letters indicate significant differences among treatments with Tukey or Dunn test, at alpha 0.05. (N = 6–8)

ETR/A_n ratio could be appreciated in Full-SW treatment (Fig. 2 shows mean of three replicates excluding negative values for Full-SW treatment.)

Osmotic adjustment displayed an increasing pattern (Fig. 3), in 1/8SW maintaining a steady increase in the following treatments. Seawater treatment effect occurred in the case of both studied osmolytes, with a significant impact on soluble sugar content ($X^2 = 19.67$; df = 5; p-value = 0.001), and specially marked effect on proline content (LM: F = 459.35; df = 5; p-value = < 0.001).

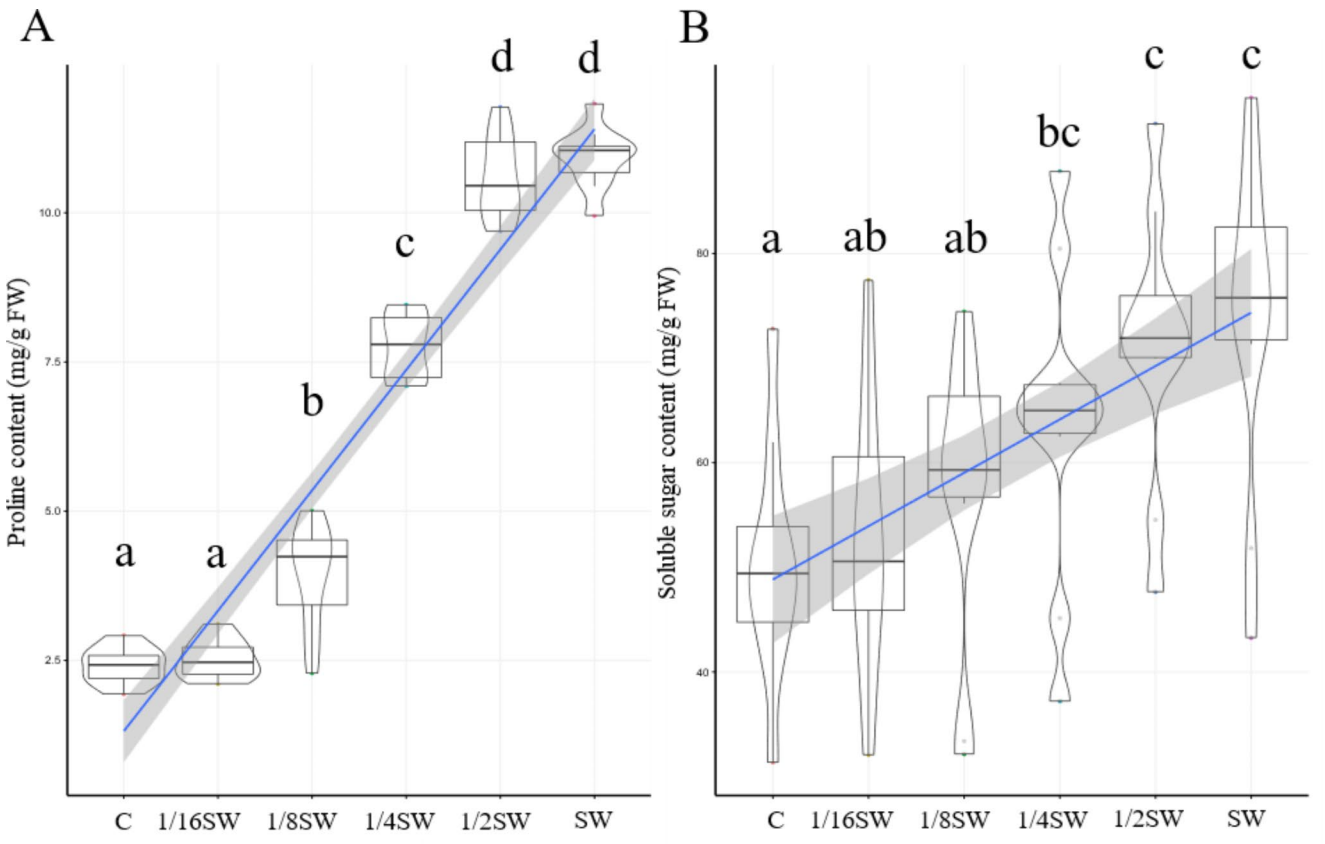

Fig. 3 Proline content (A) and soluble sugar content (B) of *Sporobolus pungens* leaves exposed to two months of different seawater concentrations (C, 1/16SW, 1/8SW, 1/4SW, 1/2SW, Full-SW). The different letter indicates differences among treatments with the Tukey and Dunn Test at alpha = 0.05. (N = 10)

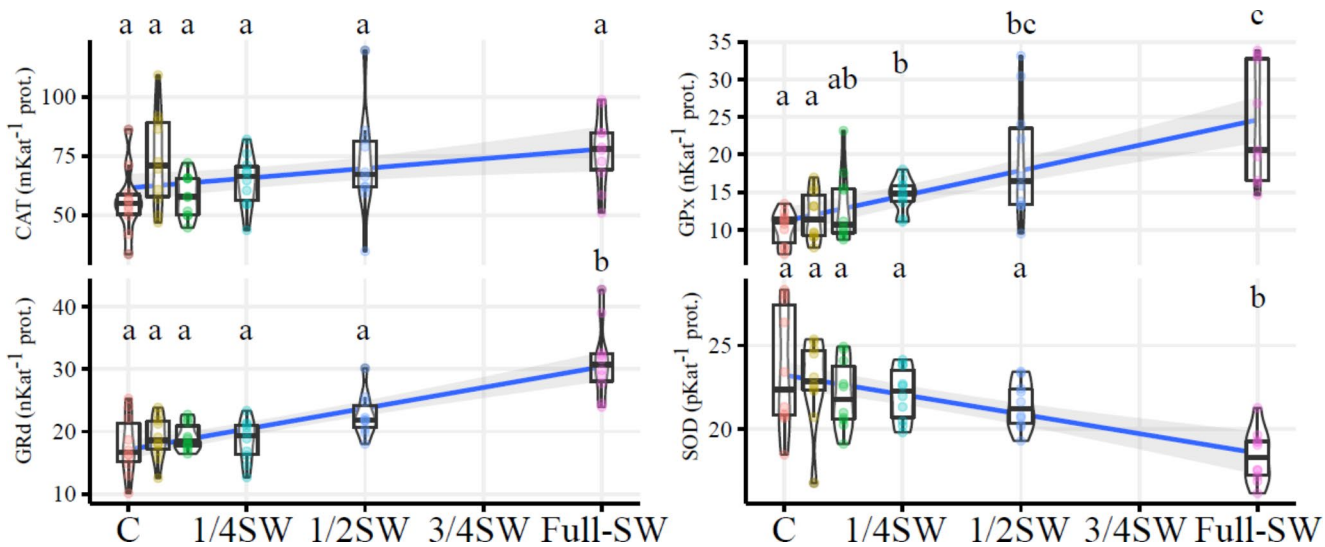

Fig. 4 Antioxidant enzyme activities of leaves of *Sporobolus pungens* exposed to two months of different seawater concentrations (C, 1/16SW, 1/8SW, 1/4SW, 1/2SW, Full-SW). Different letters indicate differences among treatments with Tukey or Dunn test at alpha = 0.05. (N = 10)

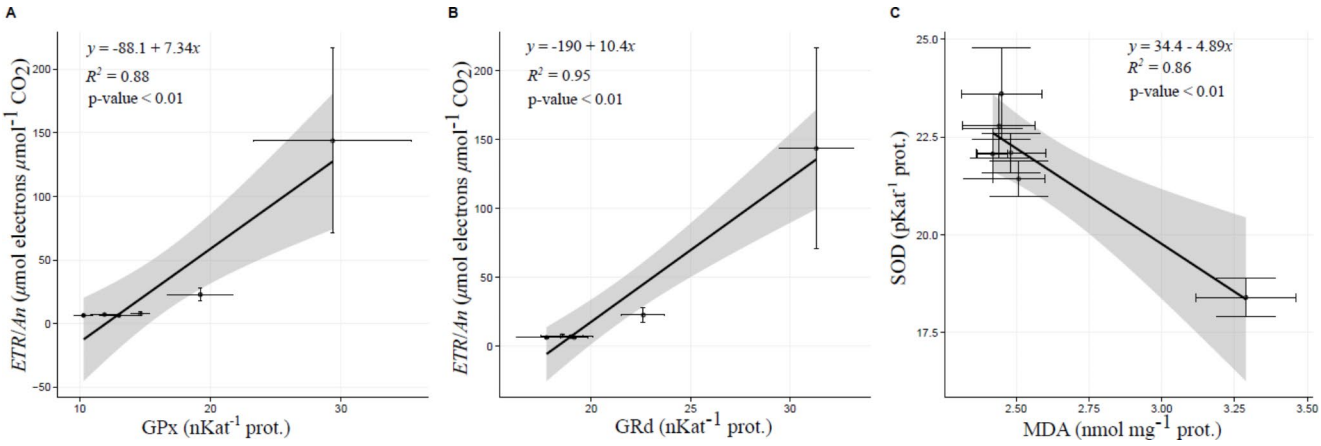

Fig. 5 Linear regression between (a) GPx activity and ETR/A_n ratio (b) GRd activity and ETR/A_n ratio and (c) MDA levels and SOD activity, of plants of *Sporobolus pungens* exposed to 2 months of different seawater concentrations (C, 1/16SW, 1/8SW, 1/4SW, 1/2SW) (N = 6–10). Full-SW values are represented by 3 replicates since negative A_n has been excluded to avoid interpretation difficulties

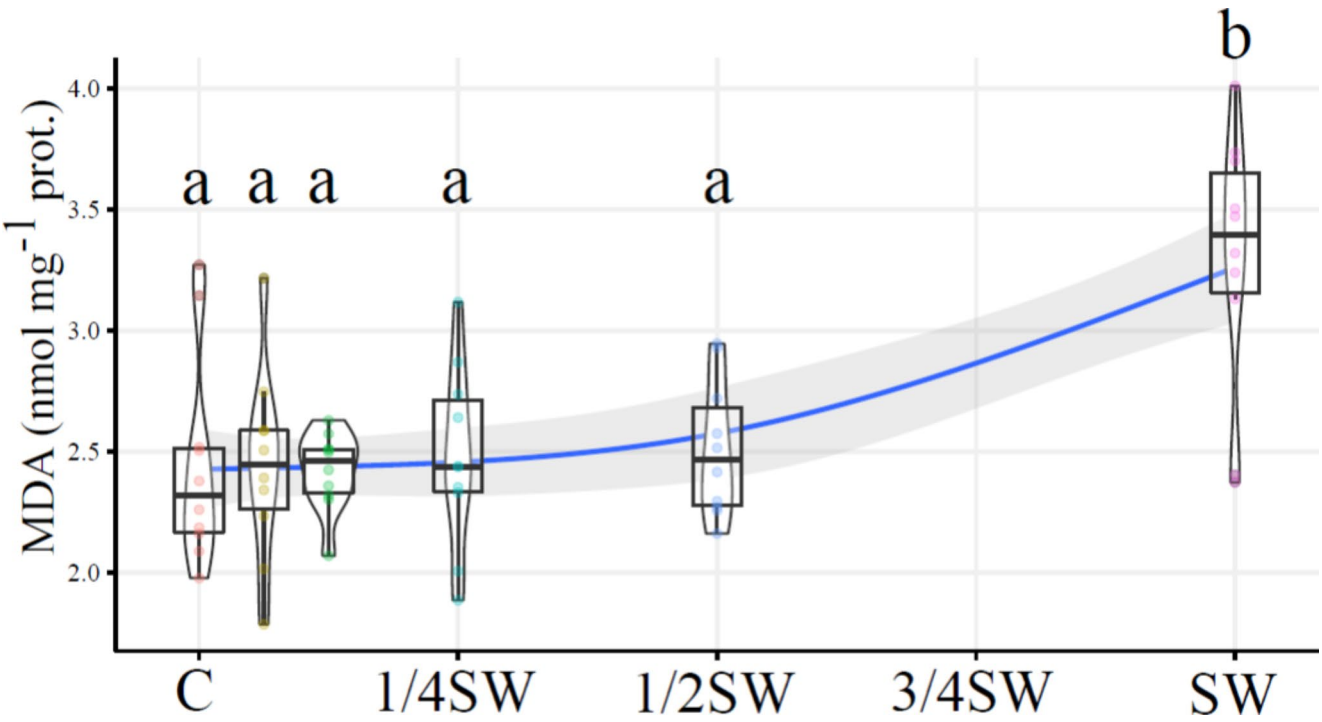

Fig. 6 MDA content of *Sporobolus pungens* leaves exposed to two months of different seawater concentrations (C, 1/16SW, 1/8SW, 1/4SW, 1/2SW, Full-SW). The different letter indicates differences among treatments with the Tukey test at alpha = 0.05. (N = 10)

Biochemical parameters showed a general low variability among treatments which, excluding SOD activity, showed an overall increasing tendency with salinity stress with marked and significant effect in Full-SW plant treatment (Fig. 4). Seawater had a significant effect on CAT activity (LM: F = 2.88; df = 5; p-value = 0.022) with a slight increase along salinity treatments, but no significant differences among treatments. GRd was also affected (LM: F = 16.12; df = 5; p-value = < 0.001), but with non-significant differences until Full-SW treatment. The strongest activity increase could be appreciated with GPx (X^2 = 28.43; df = 5; p-value = < 0.001) where activity began rising significantly on ¼SW treatment and showed a more abrupt increase at Full-SW treatment. SOD activity (LM: F = 6.52; df = 5; p-value = < 0.001) contrasted with the rest of enzymes due to the steady pattern which achieved a significant activity reduction on Full-SW treatment. Further analysis between biochemical and physiological parameters showed significant and positive correlation between GPx enzymatic activity and ETR/A_n ratio (F: 30.63; R^2: 0,88; p-value < 0,001),

and GRd enzymatic activity and *ETR/An* (F: 77.59; R^2: 0,95; p-value < 0,001) (Fig. 5).

MDA levels are presented in Fig. 6. MDA (LM: F = 7.84; df = 5; p-value < 0.001), exhibited a plain absence of concentration variation among treatments until Full-SW treatment. Moreover, MDA increase was significantly correlated with SOD activity decrease (F: 24.23; R^2: 0.86; p-value < 0,001) (Fig. 4).

Finally, there was no mortality reported, with active growth and occasional flowering in stressed treatments (data not shown) when recovery with tap water was conducted in late September.

Discussion

The present study has evaluated the effect of different seawater concentrations on the reproductive, physiological and biochemical response of *S. pungens*. The results showed an overall high tolerance regarding salinity, with a strong regulating response at physiological and reproductive scale from low to medium-high salinity levels (until 1/4SW), and a strong effect at high levels (1/2SW and Full-SW), where biochemical responses and cellular damages are noticeable.

Reproductive and growth response

S. pungens response to salinity stress translates in a strong inhibition and delay of flowering, which is perceptible even at low salinity levels (almost total inhibition at 1/8SW). Considering the conductivity values measured in natural conditions, these results indicate that typical *S. pungens* flowering period could be strongly reduced or locally displaced. Since *S. pungens* flowering period occurs during mid-late summer, mainly after first rains that wash-up soil salinity, the higher influence of seawater under future scenarios, or increased temperatures, could inhibit or strongly displace this flowering process. Moreover, these results contrast with the common perception that halophytes or halotolerant species are capable to tolerate salinity with minor effects on their reproductive capacity (Guo et al. 2019). However, several studies on *S. virginicus* support variable trends for flowering and inflorescence production under saline conditions. Blits and Gallagher (1991) showed variable response depending on plant origin with strong inhibition in dune system ecotypes, while Naidoo and Mundree (1993) reported strong inhibition under salty conditions which are reversed under waterlogging conditions.

By contrast, growth based on morphological branching elongation shows a reduction concomitant to increasing salinity levels beginning at 1/4SW level, and overall stable growth at lower levels. This pattern is in accordance with other studies on *S. virginicus* (Gallagher 1979; Naidoo and Naidoo 1998) where high salinity levels caused a consistent growth reduction. Stimulatory (Marcum and Murdoch 1992, Bell et al. 2003) or lack of effect (Blits and Gallagher 1991; Naidoo and Mundree 1993) has also been argued for *S. virginicus*, although the present results show otherwise. These discrepancies may well be explained because of different growing conditions, experimental procedures (Bell et al. 2003), plant ecotype origin (Blits and Gallagher 1991), or growth stimulation by leaf clipping (Naidoo and Naidoo 1998). With regards to *S. pungens* growth under natural conditions, data collected in the field showed a possible limitation during mid-June and July when conductivity achieves maximum values which resemble values equivalent to 1/4SW. However, caution is required with this comparison, as the conductivity of the soil may reflect a higher conductivity of the water supply. Overall, and considering that field plants are exposed to other factors such as nutrient shortage or drought, it seems that *S. pungens* is capable to tolerate higher salinity values than those observed under natural conditions.

Gas exchange response

Reproductive and growth results show different patterns regarding salinity as salinity exerts its effect differently on both processes. Regarding physiological parameters, a general tendency to reduce with the increase of salinity exposure, resembling the growing pattern, can be observed. Assimilation rate, conductance, transpiration, and ETR maintain small non-significant variation until 1/8SW level indicating low effect and being only appreciable some stomatal limitations. Consequently, decreased photosynthesis cannot be responsible for impaired flowering, although it could be responsible for decreased growth at higher salinity levels. Impaired flowering at low salinity levels may be related to other processes such as developmental adjustments that are mediated by stress hormones and impaired cell expansion and differentiation, processes that typically arise earlier during water stress than impaired photosynthesis (Hsiao 1973; Cho et al. 2017).

At 1/4SW assimilation rate, stomatal conductance, and transpiration decrease, but C_i remain unchanged. Similar patterns have been observed in other C4 grasses as *Aeluropus littoralis* (Barhoumi et al. 2007) and *Desmostachya bipinnata* (Adnan et al. 2016), being indicated that efficient stomatal regulation can be held responsible. But the fact that C_i also remains unchanged indicates that non-stomatal limitations are also operating (Ghannoum et al. 2009, Koyro et al. 2013). In this sense, chlorophyll degradation (Adnan et al. 2016) and mainly biochemical limitations as enzymatic impairment (i.e. phospho-enol pyruvate carboxylase or

Ribulose-1,5-bisphosphate) have been indicated as possible explanations for C4 plants both under drought (Ghannoum et al. 2003; Carmo-Silva et al. 2007), and salinity stress (Maricle et al. 2007). In the subsequent saline levels, 1/2 and Full-SW treatment, C_i values increase. For these levels of salinity, non-stomatal limitations (carboxylation activity) can be considered main responsible rather than diffusional limitations (Koyro et al. 2013) and would be related to a certain degree of plant stress through cellular damage, especially in Full-SW treatment. Moreover, this cellular damage seems especially plausible as ETR/A_n ratio increases for both treatments, indicating strong imbalance between electron flow and CO_2 assimilation, and in term of high probability of ROS production (Geissler et al. 2015).

Organic osmolytes

Osmotic adjustment in halophytic plants can be achieved through the accumulation of inorganic ions and some low molecular weight solutes such as proline, glycine betaine, polyols and soluble sugars (Slama et al., 2015). However, the accumulation of Na^+ and Cl^- ions can reach levels that inhibit enzyme activity, which requires their compartmentalization in the vacuole. Thus, the accumulation of compatible organic solutes that do not alter enzymatic activity is necessary to balance the cytoplasmic osmotic potential with that of the vacuole (Slama et al., 2015). In the present work, a progressive increase in the concentration of proline and soluble sugars is observed with the increase in salinity in the irrigation water. These results agree with those obtained in different species of the *Sporobolus* genus, such as *S. virginicus*, *S. marginatus* and *S. madraspatanus*, where a progressive increase of these solutes is also evidenced as the salinity of the irrigation water increases (Marcum et al., 1992; Naidoo et al., 1998; Joshi et al., 2003; Kumar et al. 2018). In this sense, the accumulation of these solutes can contribute to osmotic adjustment and confer greater tolerance to increased salinity.

Antioxidant response

Impaired photosynthesis under salinity is known to be concomitant with increased production of ROS. In this sense, the enzymatic and non-enzymatic antioxidant systems have been shown to increase their activity and/or levels in order to avoid or minimize possible oxidative damage (Flexas et al. 2004, 2006). In the specific case of *S. pungens*, this translates in enzymatic response variation being mainly evident after Full-SW treatment, salinity level where physiological parameters (especially C_i and ETR/A_n) indicate severe salinity stress.

SOD is considered one of the first lines of defence against ROS production in plants as it is responsible for the detoxification of the highly reactive superoxide anion (O_2^-) to form H_2O_2, a more stable reactive species (Hasanuzzaman et al. 2012; Bose et al. 2014). Several studies on halophytes showed an increase in SOD activity induced by salinity exposure which is considered a major advantage compared to more sensitive species (Bose et al. 2014). However, our results fail to show any increase since SOD activity remains with similar values, and even decreases at Full-SW treatment. Rangani et al. (2016) reported a similar pattern for the halophytic *Salvadora persica* L., arguing the presence of high constitutive SOD activity and justifying the decreased activity at high salinity values due to excessive H_2O_2 production making it more important to allocate resources to activate the defences against this species. Other studies suggested that SOD activity can be strongly influenced by factors such as the interaction between salinity and drought. For example, in the case of *Tamarix chinensis* Lour., where SOD decreases with salinity under mild drought, but increases under severe drought (Liu et al. 2014). Other causes for SOD decrease or lack of variation have been related to variations depending on specific SOD isoenzymes rather than the overall activity (Houmani et al. 2016), and due to the induction of non-enzymatic antioxidants such as proline accumulation (Kartashov et al. 2008). This last possibility might also be a possible explanation for SOD decrease under excessive stressful conditions since proline accumulation has been indicated in the closely related *S. virginicus* (Naidoo and Naidoo 1998) and can be equally observed in *S. pungens* as previously indicated by our data.

H_2O_2 is a central oxygen metabolite, produced as a result of the O_2^- dismutation. The main H_2O_2 source under salinity stress is related to SOD detoxifying activity but also to spontaneous O_2^- dismutation (Hasanuzzaman et al. 2012; Bose et al. 2014; Khan et al. 2020). In this sense, the lack of SOD variation with increased H_2O_2 content found in *Oryza sativa* (Tsai et al. 2005) and *Nitraria retusa* (Boughalleb et al. 2010), are suggested to be linked to other sources such as NADPH oxidase activity followed by spontaneous O_2^- dismutation. Since H_2O_2 also plays a role in the plant signaling system, the control of its levels is important to avoid the potentially damaging effects and to allow the signalling function (Smirnoff and Arnaud 2019). In the case of *S. pungens*, our results showed increased activity of several enzymes related to H_2O_2 scavenging suggesting an elevated H_2O_2 production associated with salt exposure. Catalase activity seems to have limited participation since the increase in its activity is overall small and non-significant. Low catalase activity has been indicated in plants with C4 metabolism since CAT mainly scavenges photorespiratory H_2O_2, a process which is prevented under C4 metabolism (Uzilday et al.

2018). In contrast, glutathione related enzymes, in particular GPx, showed a significant and progressive increase as salinity levels raise which is parallel to the observed decrease in photosynthetic parameters. Especially, the ratio ETR/A_n remained stable at the lowest salinity levels, increasing thereafter, indicating a larger excess of electrons that are not used in photosynthesis, being thus available for ROS production. GPx and specially GRd activity increased in parallel to ETR/A_n, suggesting a likely response to increased ROS production as previously indicated by electron excess. GPx is related to both H_2O_2 and organic peroxides detoxification being a relevant antioxidant enzyme for low levels of oxidative stress in plants (Khan et al. 2020). This would explain the increase in activity observed already starting at medium levels (1/4SW) where saline stress seems to be relevant through both stomatal and non-stomatal limitations. In the case of GRd, the significant increase in its activity in *S. pungens* is limited to high salinity levels (1/2SW and Full-SW) which jointly with SOD decrease indicate that *S. pungens* undergoes strong stressful conditions at these specific levels. GRd activity is considered an important enzyme in the ROS scavenging system of many halophytes (Hasanuzzaman et al. 2012, 2017). It is a major component of the AsA-GSH cycle, which is considered one of the main ROS scavenging mechanisms in plants (Hasanuzzaman et al. 2012). In this sense, *S. pungens* may activate the AsA-GSH cycle as a response to excessive ROS production at high salinity levels (1/2SW and Full-SW). GRd also intervenes jointly with GPx to regulate the GSH/GSSG (oxidized glutathione) ratio by reducing GSSG to GSH, which is essential to maintain the cellular redox state and display a strong protective role under salinity stress (Hasanuzzaman et al. 2017).

MDA levels give further insights on plant stress as they solely increase at the Full-SW level. MDA is widely used as biomarkers of lipid peroxidation and, consequently, of oxidative stress (Hernández and Almansa 2002; Gil et al. 2020). Both physiological parameters and GPx activity show that salinity stress starts to affect *S. pungens* at medium levels. But strong enzymatic variation only can be noticeable at the Full-SW level. This entails that *S. pungens* manages to avoid cellular damage by different physiological and biochemical mechanisms until Full-SW level, where excessive ROS production seems to only occur. These results coincide with other studies on halophytes like *Halopeplis perfoliata* (Forssk.) Buge ex Asch. & Schweinf where MDA levels have been found to increase only under high salinity values (Rasool et al. 2019). However, it must be noted that the activities of GPx and GRd still increase indicating an active response against ROS production. Other halophytes such as *Nitraria retusa* (Forssk.) Asch. and *Atriplex halimus* L. showed a similar pattern of response to salinity, indicating that the antioxidant system, although unable to avoid the increase in cell damage markers, certainly offers enough protection to prevent plant death (Boughalleb et al. 2010).

Conclusions

In conclusion, *S. pungens* tolerates low and medium levels of salinity by mainly displaying physiological and osmotic adjustments, which allows the plant to maintain a high water use efficiency and growth. While flowering is strongly inhibited by low salinity levels, photosynthesis and stem growth are resistant to mild-moderate salinity in *S. pungens*, decreasing thereafter. GSH-related ROS scavenging systems start being activated at medium levels and increase at further salinity levels, showing an efficient scavenging system that jointly with further osmotic adjustments (mainly proline accumulation) translates into low relative cellular damage. Inhibition of flowering together with high salinity tolerance of photosynthesis and vegetative growth indicates the suppression of processes involved in the sexual reproductive effort in favour of maintaining clonal reproduction. Hence, both clonal growth and high salinity tolerance are proposed as key traits that allow *S. pungens* to develop in the embryonic dune system as well as other exposed and saline coastal habitats. In the specific case of embryonic dunes, the results allow us to consider that *S. pungens* is capable of supporting salinity values higher than the natural influence of salinity in this habitat, which may be revealed in the face of possible variations due to global change.

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Author contribution Marcello Cerrato, Jaume Flexas, Antoni Sureda, and Lorenzo Gil contributed to the study conception and design. The methodology was developed by Marcello Cerrato, Antoni Sureda, Jaume Flexas, and Lorenzo Gil. Material preparation, data collection and analysis were performed by Marcello Cerrato, Arnau Ribas-Serra, Pere Miquel Mir-Rosselló, Cyril Douthe and Iván Cortés-Fernández. The first draft of the manuscript was written by Marcello Cerrato, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author and Lorenzo Gil on reasonable request.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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