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Different tolerance to salinity of two populations of *Oenothera drummondii* with contrasted biogeographical origin

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Abstract

Oenothera drummondii is a native species from the coastal dunes of the Gulf of Mexico that has nowadays extended to coastal areas in temperate zones all over the world, its invasion becoming a significant problem locally.

The species grows on back beach and incipient dunes, where it can suffer flooding by seawater, and sea spray. We were interested in knowing how salinity affects this species and if invasive populations present morphological or functional traits that would provide greater tolerance to salinity than native ones. To this end, we conducted a greenhouse experiment where plants from one native and from one invading population were irrigated with five salinity treatments. We measured functional traits on photosynthetic, photochemical efficiency, water content, flowering, Na^+ content, pigment content, and biomass.

Although *O. drummondii* showed high resistance to salinity, the highest levels recorded high mortality, especially in the invasive population. Plants exhibited differences not only in response to time under salinity conditions, but also according to their biogeographic origin, the native population being more resistant to long exposure and high salt concentration than the invasive one. Native and invasive populations showed different response to salt stress in photosynthesis and transpiration rates, stomatal conductance, water use efficiency, carboxylation efficiency, electron transport rate, electron transport efficiency, energy used in photochemistry, among others. The increasing salinity levels resulted in a progressive reduction of photosynthesis rate due to both stomatal and biochemical limitations, and also in a reduction of biomass and number and size of flowers, compromising the reproductive capacity.

Keywords: Biomass; coastal dunes; flowers; invasive species, Na+ content; photosynthesis; pigments; salt stress

1. Introduction

Coastal dunes are terrestrial ecosystems whose formation and functioning is the result of the interaction of terrestrial and marine processes (Martinez and Psuty, 2004). They provide important ecological services, including the protection of interior areas against the action of the sea, recreation, and preservation of biodiversity (Everad et al., 2010). These ecological services are currently threatened because coastal dunes are one of the types of ecosystems most severely affected by the invasion of alien species (Campos et al., 2004; Castillo and Moreno-Casasola, 1996; Giulio et al., 2020). The heterogeneity of habitats in coastal dunes, the strong environmental stress, and the occurrence of intense disturbances, are considered factors that promote the invasion by alien species (Acosta et al., 2008; Lortie and Cushman, 2007; Santoro et al., 2012).

The habitat heterogeneity causes coastal dune plant communities to show a marked zonation pattern (Cowles, 1899, Doing, 1985) driven by abiotic and biotic factors (Hesp, 1991). It is considered that the main factors that condition the installation, growth, and reproduction of plants are sand movement and salinity, which act more strongly near the coast (Maun, 2009). The harshest environmental conditions occur on the back beach and incipient dunes (García Mora et al., 1999; Hesp, 1991; Maun, 2009), where plants have to cope with salinity from both aboveground by the sea saline spray and belowground by soil salinity (mainly due to occasional seawater flooding, but also by the marine intrusion in groundwater) (Du and Hesp 2020). The effect of salinity on the establishment of plant species in these habitats has important consequences in the development of incipient dunes and foredune (Hesp, 2002; van Puijenbroek et al., 2017). Although coastal dunes are reported as one of the most invaded ecosystems in Europe (Chytrý et al., 2008), habitats closer to the sea tend to be less susceptible to invasion, due to the harsh environmental conditions that act as environmental filters (Carboni et al., 2010; Gallego-Fernández and Martinez, 2011).

Several studies dealing with the effects of saline conditions on beach and dune plant species have already been published (see Du and Hesp, 2020 for a recent review). Although it is evident that an accumulation of salts in soil affects plant fitness, mismatching metabolic processes and causing inhibition of growth and a reduction of the photosynthesis rate (Loreto et al., 2003; Munns, 2002, Paranychianakis et al., 2005; Zhu, 2001), the results achieved have been uneven. This fact has been motivated mainly because plants tolerance to salinity is species-specific, but also because it depends on the degree of salinity experienced or the environmental conditions of the site as irradiation, atmospheric CO_2, water availability, or soil characteristics (Fini et al., 2014; Pérez-Romero et al., 2019). This is especially relevant for

Mediterranean plant species and particularly for coastal dune plants. In these regions, salinity is concomitant with high irradiation levels, summer hot temperatures, high wind, and seasonal water deprivation that is aggravated by the low field water capacity of sandy soils. All of them are factors that enhance the effects of salinity on plants inhabiting Mediterranean coastal dunes.

In any case, the studies carried out so far have proven that salinity-stress induced osmotic imbalance and leaf dehydration affecting plant performance at all levels. In numerous studies, it has been demonstrated that salt stress reduced plant growth (Akinci et al., 2004, Akram et al., 2002; Zunzunegui et al. 2017), retarded flowering and fruit ripening, and reduced fruits size and number per plant (Koffi et al., 2019). Also, it modified the content of bioactive compounds (Prasad et al., 2014) and it caused the accumulation of potentially toxic ions in organs (Zunzunegui et al., 2017). So, plants that have evolved in salty environments commonly display a set of morpho-physiological attributes developed during their phylogenetic adaptation that allow them to cope with high salinity stress. In summary, plants show a species-specific strategy to avoid or to control the entry and accumulation of toxic ions and to limit the water loss to prevent leaf dehydration. For example, one of the first mechanisms to cope with elevated salt concentrations and to avoid leaf dehydration is stomatal closure, but that turns in a reduction of the photosynthetic rate (Chaves et al., 2009; Flexas et al., 2004) that is unbearable / non sustainable at long-term (Hasegawa et al., 2000). Other options are to exclude Na^+ ions from its tissue or on the contrary to tolerate its presence within the cells.

Oenothera drummondii subsp. *drummondii Hook.* (Onagraceae) is a short-lived perennial species, native from coastal dunes of the Gulf of Mexico, under tropical and subtropical climate. Nowadays this species has extended on coastal dunes all over the world and its invasion has become a significant problem in many coastal areas. In south Spain, where the species was first recorded in 1957 (Silvestre 1980), it has been shown that the species produces a high impact on the diversity and function of the invaded dune systems (Gallego-Fernández et al., 2019; García de Lomas et al., 2015) and that its rapid expansion has been facilitated by its high fecundity and because its seeds can be dispersed by marine currents and by endozoochory (Gallego-Fernández et al., 2021). In addition, this species is well acclimated to the Mediterranean dunes, Zunzunegui et al. (2020) observed a higher photosynthetic rate and better water status in *Oenothera drummondii* than in the native species *Achillea maritima*. Also, Díaz Barradas et al. (2020) found that under water-stress south Spain population of *O. drummondii* showed better physiological performance than the

other three studied populations, natives and non-natives, which gives this species a great invasive capacity in this area. The individuals of *O. drummondii* are typically found in the foredune and inland dunes, where they contribute to sand stabilization, and thus the species can be considered an early colonizer (Gallego-Fernández et al., 2019). But the species also grows on the back beach and incipient dunes, where, as indicated previously, the impact of high salinity by sea spray or seawater flooding is stronger, making these areas less susceptible to invasions.

Studies carried out by Gallego-Fernandez et al. (2021) have shown that *O. drummondii* seeds can be dispersed by marine currents, since 0.64 % of the seeds are able to float in water for at least 8 days and then germinate, under low and medium salinity soil conditions (< 200 mM NaCl). However, once the species establishes on the back beach and incipient dunes, it would also be important to determine the degree of tolerance to salinity of mature individuals (Lum and Barton, 2020). We can assume that tolerance to salinity may be of major importance determining the invasiveness of plant species, specifically in the case of a coastal dune species as *O. drummondii*. Recognising the factors that facilitate the introduction, establishment and expansion of invasive species is particularly relevant for designing management plans, as well as for evaluating the degree of invasion and alteration caused in native ecosystems.

The present study assesses how salinity affects mature individuals of *O. drummondii* populations on the photosynthetic response, flowering, and biomass allocation. We set the following specific objectives: I) Which functional traits give the species *O. drummondii* greater tolerance to salinity, II) Does the invasive population possess functional traits providing greater tolerance to salinity than the native population? and III) Which physiological mechanisms can explain better the reproductive response and biomass allocation pattern of our study species? To this end, a greenhouse experiment was conducted where one native and one alien population of young plants were irrigated with different salinity concentrations. Based on the knowledge of this species and its invasive behaviour, our starting hypothesis is that the species will present a high tolerance to salinity both in the duration of exposure and in the salt concentration. In addition, based on the fact that both populations came from coastal dune environments subjected to the influence of salinity by sea spray and by flooding by seawater or marine intrusion, we do not expect to find differences between populations.

2. Methodology

2.1 Study species

Oenothera drummondii subsp. *drummondii* Hook (*Onagraceae*), known as beach evening primrose, is a short-lived perennial herb, with hairy leaves, yellow flowers, and dry fruits. Flowers are self-compatible, outcrossing, and pollinated by hawkmoths in their native habitats (Wagner et al., 2007). The flowers produce numerous small-sized seeds with a high germination rate (≈90%) (Gallego-Fernández et al., 2021). Its stems are from erect to procumbent, ascending to about 50 cm in height with a strong taproot. It grows across the entire beach-dune gradient of coastal dune habitats, but its cover and biomass increase landwards (Gallego-Fernández et al., 2019). Although native from Eastern Mexico and South-eastern USA areas with humid subtropical and wet tropical climates, the species acclimation to Mediterranean climate has been very successful (Zunzunegui et al., 2020). Studies of Moreno-Casasola (1988) and Gallego-Fernández and Martinez (2011) have proven that the species is relatively scarce in its native range, while it is abundant in non-native areas of South Spain (Gallego-Fernández et al., 2021). *O drummondii* is considered invasive in Spain, Israel, and China (Dufour-Dror, 2012; Gallego-Fernández et al., 2019; Xu et al., 2012) but has also been introduced into Australia, South Africa, Egypt, and New Zealand (ALA, 2014; Frean et al., 1997; Heenan et al., 2002; Shaltout et al., 2016).

2.2 Experimental design

A greenhouse experiment with a factorial design of two factors, population (native and invasive) and salinity (five levels) was carried out. Two-month-old plants were irrigated with different saline solutions and the physiological performance was examined measuring gas exchange, photochemical efficiency, relative water content (RWC), and leaf dry matter content (LDMC) at 7, 14, 21, 29, 42 and 55, days after irrigation with different NaCl concentrations. After this period, we collected leaves for pigment content measurement, and plants were harvested, separated into leaves, stems, and root, dried, weighted, and sodium (Na^+) content in the different organs measured.

2.2.1 Plant material and growth conditions

We collected seeds of *O. drummondii* in two different areas of the world with contrasting climatic conditions: a native population in Texas (the USA, 29° 30' N and 94° 30' W) with subtropical climate (Cfa, annual precipitation 1100 mm, and mean annual

temperature 20.8 ºC) and an invasive non-native population in Huelva (Spain, 37° 09'N 06° 54'W) with Mediterranean climate (Csa, annual precipitation 473 mm, and mean annual temperature 17.8ºC). The seeds were germinated in seedbeds filled with peat at the greenhouse of the University of Seville under optimal conditions. Following germination, seedlings were transplanted into 3-L pots (one seedling/pot) with a mixture of commercial composts, perlite, and washed sand (1:1:1) and then fertilized to ensure enough nutrients for plant growth (universal fertilizer, Flower, NPK, 6-4-6.) and pots were placed in shallow trays (5 pots per tray). Trays were watered twice a week and the positions of the pots in the tray, as well as the trays on the table, were randomly changed once a week. Greenhouse conditions were set at a 14/10-hr photoperiod with 21-25°C night/day temperature cycles, while relative humidity was maintained at 40-60%. Natural daylight irradiances of 300 to 1500 μmol $m^{-2}s^{-1}$ were supplemented on cloudy days with 400-W metal halide lamps.

After two months, 50 similar-sized healthy plants of each population were selected for the salinity experiment (N = 10 plants x 5 treatments x 2 populations). The salinity treatments ranged from freshwater (control plants, 5mM) to seawater (600 mM). Ten plants were used per salinity treatment (5 mM, 100 mM, 200 mM, at 300 mM and 600 mM). To obtain the salinity solutions, NaCl was added to tap water (0.5 g, 5.9 g, 11.8 g, 17.6 g, 26.5 g, and 35.3 g of NaCl per L, respectively). On experimental day 1, two litres of each salinity solution were added to every tray to a depth of 1.5 cm (two trays for each salinity treatment). The water level of the trays was checked every two or three days and when necessary, topped up with distilled water at the marked level. Besides, every week the trays were washed with tap water and new solutions were added.

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2.3 Physiological measurements

We measured physiological traits in 5 pots per treatment and population (5 plants x 5 treatments x 2 populations = 50 pots), under a controlled period of time. Measurements were taken between 11:00 and 14:00 hours solar time, Gas exchange variables, photochemical efficiency of photosystem II, relative water content, and leaf dry matter content were measured before the begging of the experiment (day 0) and at days 7, 14, 21, 29, 42, 55, from the beginning of the salinity conditions.

2.3.1 Gas exchange

Photosynthetic capacity and photochemical efficiency of photosystem II were measured utilizing a CO_2/H_2O analyzer LI-6400 (LI-COR Inc., Neb., USA). The equipment

was connected with the leaf chamber fluorometer (Li-COR 6400-40), which is designed with a uniform, integrated actinic LED light and connected to a fluorometer that enables simultaneous measurement of chlorophyll fluorescence and gas exchange over the same leaf area. Measurements conditions inside the leaf-chamber were 1500 μmol m^{-2} s^{-1} PPFD and 400 μmol mol^{-1} CO_2. Measurements were carried out in two healthy mature leaves per plant before the midday depression in assimilation rates, from 9:00 h to 11:00 h (solar time). The mean value per plant was calculated and used in the statistical analysis. In total 2 leaves x 5 plants x 5 salinity treatment x 2 populations were measured in each sampling day and the following variables were provided: net photosynthetic rate (A_n, μmol CO_2 $m^{-2}s^{-1}$), transpiration rate (E, mmol H_2O $m^{-2}s^{-1}$), stomatal conductance (g_s mol H_2O $m^{-2}s^{-}$) and internal CO_2 concentration (C_i, vpm). From these variables, we calculated: instantaneous water use efficiency (WUE) as the ratio of CO_2 uptake per H_2O transpired (A_n/E), intrinsic WUE as the ratio of CO_2 uptake per H_2O potentially transpired (A_n/g_s) and instantaneous carboxylation efficiency as the ratio of CO_2 uptake per intercellular CO_2 concentration (A_n/C_i).

Simultaneously, on the same light-adapted leaves, the leaf chamber fluorometer with the actinic light provided values of steady-state fluorescence (F_s), maximal fluorescence (F_m') after a short (0.8 sec) saturating light flash (8,000 μmol photons $m^{-2}s^{-1}$) and minimal fluorescence (F_0') with a short pulse of weak far-red light. With these variables we calculated intrinsic maximum photochemical efficiency of PSII ($F_v'/F_m' = (F'_m-F'_0)/F'_m$), operating photochemical efficiency of PSII ($\Phi_{PSII} = (F'_m-F_s)/F'_m$), the coefficient for photochemical quenching or the fraction of the maximum photochemical efficiency of PSII that is achieved under actinic irradiance ($qP = (F_m'-F_s)/(F_m'-F_0')$). Also, we estimated the apparent photosynthetic electron transport rate ($ETR = \Phi_{PSII} \times PAR \times PSII/PSI \times ah$), where PAR denote the photosynthetic active radiance intensity, PSII/FSI is the proportion of light energy involved in the photoexcitation assigned to PSII and was kept as 0.5 considering an equal energy distribution between PSI and PSII (Krall and Edwards, 1992), and ah is the irradiance that is absorbed by photosystems, which was assumed to be 0.84 % that consider than 16% of photosynthetic active radiation is not absorbed by leaves (Baker, 2008; Björkman and Demming, 1987; Genty et al., 1989). The ratio of ETR to the carbon assimilation rate (ETR/A_n) was also calculated.

As dark-adapted leaves were not measured, following Demmig-Adams et al. (1996), we used the diurnal values of photochemical efficiency of open photosystem II centres to estimate the percentages of absorbed light going to thermal energy dissipation and the one

utilized to photosynthetic electron transport. The light energy absorbed by PSII is dissipated in three fractions, the one absorbed by the PSII antennae used in photochemistry ($\Phi_P = \Phi_{PSII}$), the one dissipated thermally (Φ_D), and the remaining fraction, not going into either of them, known as excess (Φ_E). Excess energy is linearly correlated with the rate of photoinactivation of PSII (Kato et al., 2002). Thermal energy dissipation fraction was calculated as $\Phi_D = 1- F'_v/F'_m$; while the light energy used in photochemistry for PSII antennae was estimated as $\Phi_P = F'_v/F'_m \times qP$. These variables allow an assessment of changes in the rate of thermal energy dissipation, non-photochemical, and photochemical chlorophyll fluorescence quenching. The residual fraction of energy knows as excess was calculated from $\Phi_E = F'_v/F'_m \times (1- qP) = 1- \Phi_P - \Phi_D$ (Demming-Adams et al., 1996).

Relative water content (RWC) represents a good index of the leaf hydric status and was measured as follows: RWC = (FM - DM) × 100 /(SM – DM). One leaf of each plant (n = 100) was collected every sampling day and weighted (FM). Saturated mass (SM) was determined after hydrating the leaves in distilled water for 24h in dark refrigeration. The samples were then dried in an oven at 70°C for 48h and weighted to obtain dry mass (DM).

Leaf Dry Matter Content (LDMC) is related to tissue density and plant productivity and was calculated as the ratio dry leaf mass to fully water-saturated leaf mass (mg g^{-1}) following Garnier et al. (2001).

2.4 Flowers number/size

For each treatment, flowers were collected and counted from their appearance at the beginning of the experiment, day 1, to the end, day 55. Before the salinity treatment, neither of the two-month-old plants had produced any flower. The date of appearance of the first flower was noted for each plant, as well as the diameter of 10 flowers per treatment when possible, weekly.

2.5 Biomass

At the end of the experiment, the 100 plants used in the experiment were harvested and separated into three fractions: roots, stems, and leaves (dead and alive). The roots were gently removed from the substrate by washing them. After that, the different organs were oven-dried at 70 ºC for 2 days and the following variables were calculated: leaf biomass (B_l), stem biomass (B_s), aerial biomass ($B_a = B_l + B_s$), root biomass (B_r), and plant total biomass ($B_t = B_l + B_s + B_r$).To assess the effect of salinity on biomass allocation, we also calculated the ratios: aerial to root biomass (B_a/B_r), aerial to plant total biomass (B_a/B_t) and root to plant total

biomass (B_r/B_t). All leaves cut for RWC, LDMC, and pigment analyses were weighed and added to the total aerial biomass.

2.6 Pigment content in leaves

Green leaves fresh tissue (0.3 g) was collected from each plant at the end of the experiment (pigment content was not measured in plants from the highest salinity treatment due to high mortality and lack of healthy leaves in both populations). The material was ground and photosynthetic pigments were extracted using 100% acetone. The samples were kept at -18 ºC during the 24h extraction, to prevent pigment degradation, and then centrifuged at 4000 rpm for 15 min. Pigment content was quantified using a spectrophotometer using the Gauss-Peak Spectra method following Küpper et al. (2007). With this method, the quantification of pigment profile is faster and less expensive than with HPLC, and nonetheless, it has proved to be efficient for higher plants (Duarte et al., 2015, Repolho et al., 2017). The absorbance spectrum of each sample was measured from 350 nm to 700 nm, every 1 nm, and then it was fitted by a linear combination of these Gauss Peak Spectra including corrections for wavelength inaccuracy, baseline instability, or sample turbidity (Küpper et al., 2007). This enabled the quantification of the following chlorophylls and carotenoids, using SigmaPlot Software (Systat Software, Inc., USA): chlorophylls a (Chl a) and b (Chl b),) antheraxanthin (A), β-carotene (βcar), lutein (L), violaxanthin (V), and zeaxanthin (Z). Several indexes were calculated from the content of these pigments: the ratio chlorophyll a/b (Chl a/b); the ratio of total carotenoids to total chlorophylls (Car/Chl); the de-epoxidation index (DEI = AZ/VAZ) to evaluate photo-protection mechanisms (Kuwabara et al. 1998) as the ratio between antheraxanthin + zeaxanthin to total pigments from the xanthophyll cycle (VAZ cycle). This cycle is a light-dependent conversion of three xanthophylls in a cyclic reaction that allows dissipating energy as heat. The DEI denotes the quantity of de-epoxidised residues in one molecule of the VAZ cycle, and hence, $0 < DEI < 2$. Also, based on the fact that one of the main mechanisms of photoprotection in plants is transferring energy from Chl a to L (Jahns and Holzwarth 2012), we calculated the ratio between lutein and total chlorophylls (L/Chl).

2.7 Sodium content in leaves, stem, and roots

Na^+ content in the three fractions was measured to study the role of ionic stress in explaining the effect of salinity (Munns and Termaat, 1986). At the end of the experiment when all the plants were separated into organs, dried and weighted for biomass calculations,

0.5 g of leaves, stem, and root tissues from each plant were ground and ashed at 550 °C for 8 h, followed by acid digestion. Na^+ ion concentration was determined by atomic absorption spectrophotometry on an ICE 3500 Atomic Absorption Spectrophotometer (Thermo) at the Servicio de Investigación Agraria of the Universidad de Sevilla.

2.8 Statistical analysis

All the physiological variables were tested for significant differences using repeated-measures ANOVA with time (0, 7, 14, 21, 29, 42, 55 days) as the within-subject effect, and populations and salt concentration as the between-subject effect. As Mauchly's sphericity was not assumed in all the variables, we applied the conservative Greenhouse-Geisser statistical correction when necessary. Pairwise post hoc comparisons (between treatments, between times of exposure) were detected using the Tukey's test.

Statistical differences among salt concentration and populations for the variables measured at the end of the experiment -biomass, flower size, and pigment contents- were contrasted using a two-way ANOVA (with salt concentration and populations as independent factors). Pairwise differences between the five salt treatments were detected by a Tukey's test. The number of flowers was contrasted by means of χ^2 test. To address whether salinity correlates with physiological or biomass variables, Pearson correlations were performed with salinity concentration as predictors. Kolmogorov–Smirnov non-parametric test was used to determine whether the variables studied met the assumption of normality. All statistical analyses were performed with SPPS 26 software package (Chicago, IL, USA).

3. Results

3.1 Physiology

Although almost all plants survived to high NaCl concentrations, the highest salinity levels (600 mM) recorded high mortality, especially in the invasive population with 90% mortality in contrast to the native one with a 40% ($\chi^2 = 5.49$, $p = 0.05$). No mortality was recorded in any of the other treatments.

Table 1: repeated-measures ANOVA results for photosynthesis and photochemistry variables, as well as RWC and LDMC for *Oenothera drummondii*. Abbreviations: net photosynthetic rate (A_n), transpiration rate (E), stomatal conductance (g_s), intrinsic WUE (A_n/g_s), instantaneous carboxylation efficiency (A_n/C_i), apparent photosynthetic electron transport rate (ETR), light dissipated thermally

(Φ_D), light used in photochemistry (Φ_P), light excess (Φ_E), relative water content (RWC), leaf dry matter content (LDMC).

	F	df	**dF**	**P**
time	12.09	90	1242	0,001
site	1.244	15	26,0	0.303
treatment	5.82	60	116	0.001
time*site	2.71	90	1242	0.001
site* treatment	1.75	60	116	0.030
time*treatment	3.4	360	3240	0,001
time*site*treatment	1.6	270	3240	0.001

	time			**time*population**			**time*treatment**			**time*popul*treatment**		
Variables	**dF**	**F**	**P**	**dF**	**F**	**P**	**dF**	**F**	**P**	**dF**	**F**	**P**
A_n	3.65	311.3	**0.001**	3.652	8.240	**0.001**	14.60	31.74	**0.001**	10.95	1.267	0.251
g_s	3.31	141.0	**0.001**	3.316	4.186	**0.006**	13.26	17.22	**0.001**	9.949	4.628	**0.000**
ETR	4.66	142.3	**0.001**	4.660	4.022	**0.002**	18.64	13.51	**0.001**	13.98	2.058	**0.017**
E	3.48	222.1	**0.001**	3.481	5.055	**0.001**	13.92	23.37	**0.001**	10.44	3.963	**0.000**
A_n/g_s	2.09	17.81	**0.001**	2.096	3.763	**0.026**	8.383	10.66	**0.001**	6.287	1.752	0.117
ETR/A	3.24	17.43	**0.001**	3.249	5.743	**0.001**	12.99	6.582	**0.001**	9.748	.458	0.910
A/C_i	4.0	100.9	**0.001**	4.066	2.435	**0.049**	16.26	10.94	**0.001**	12.19	2.363	**0.008**
qP	2.61	56.8	**0.001**	2.617	1.187	0.317	10.46	5.725	**0.001**	7.850	1.409	0.204
Φ_D	3.92	41.90	**0.001**	3.926	1.686	0.158	15.70	5.373	**0.001**	11.77	2.081	**0.022**
Φ_P	4.7	138.4	**0.001**	4.729	3.015	**0.014**	18.9	12.72	**0.001**	14.1	1.840	**0.036**
Φ_E	4.34	46.30	**0.001**	4.342	.920	0.460	17.36	5.044	**0.001**	13.02	2.428	**0.005**
C_i/C_a	2.09	16.88	**0.001**	2.099	3.772	**0.026**	8.395	10.57	**0.001**	6.296	1.706	0.128
RWC	3,57	85.00	**0.001**	3,570	7.60	**0.001**	14,278	10.8	**0.001**	14,28	1.76	**0.042**
LDMC	4,65	77.8	**0.001**	4,651	6.4	**0.001**	18,60	3.8	**0.001**	18,606	2.73	**0.001**

According to the repeated measures ANOVA, plants exhibited significant differences not only in response to time under salinity conditions (Table 1) but also between the population's origin in several of the measured traits as shown by the within-subject differences for the interaction time*population (Table 1). This time*population interaction evidenced that native and invasive populations had different temporal patterns in response to salt stress for the variables A_n, g_s, E, A/g_s, A/C_i, ETR, ETR/A_n, Φ_P, C_i/C_a. In contrast, the interaction time*treatment was significant for all the measured variables since not all salinity treatments evolved in the same way. To finish, the third-order interaction, time*population*treatment, was significantly different for the variables g_s, E, ETR, A/C_i, Φ_D, Φ_P, Φ_E, revealing that the studied populations presented a different response pattern to increasing salt concentrations for these variables (Table 1a, b).

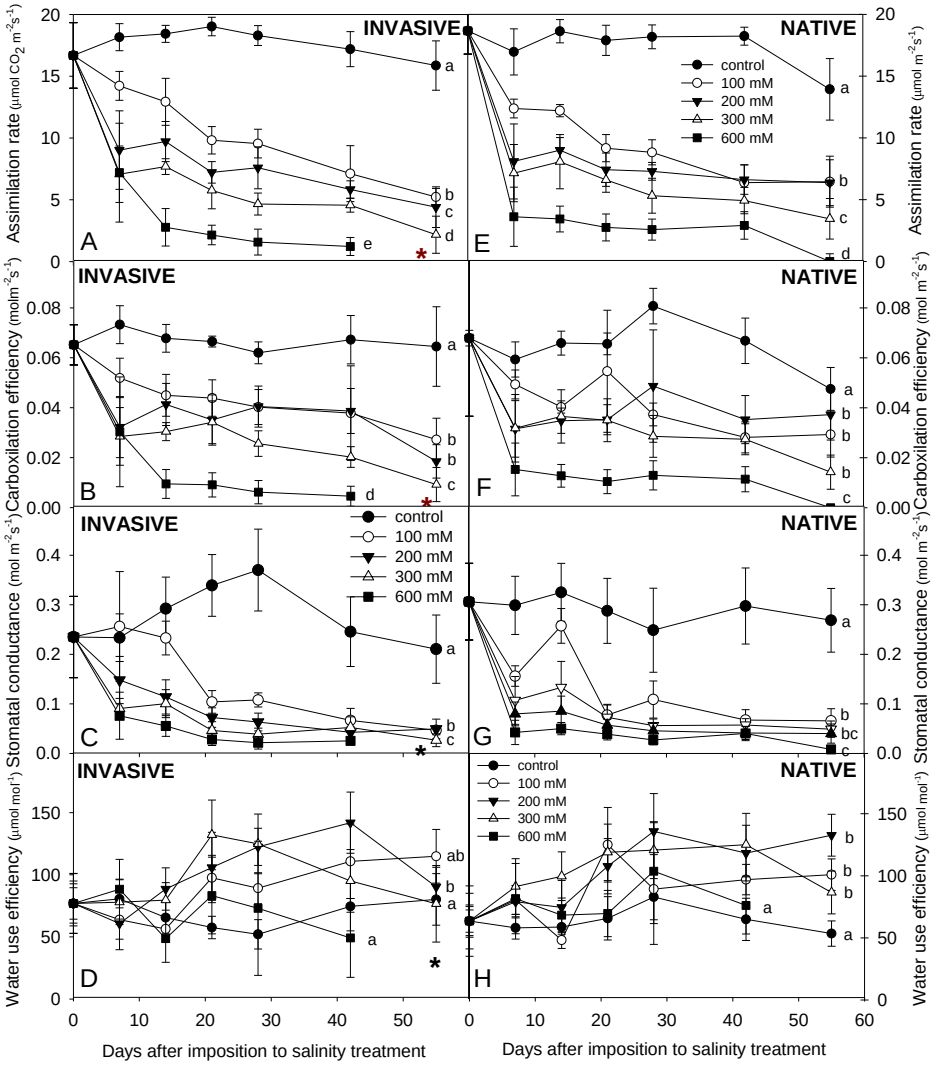

Figure 1: Mean values ± sd of one invasive (A-D) and one native populations of *O. drummondii* (E-H) for 5 salinity treatments from day 0, before the beginning of the experiment, to the end of the experiment on day 55 (n = 25 for each population and sampling period). Assimilation rate (A, E), instantaneous carboxylation efficiency (A_n/C_i: B, F), stomatal conductance (C, G), intrinsic water use efficiency (A_n/g_s D, H). Different letters at the end of the lines indicate significant differences between treatments for the total period at $p < 0.05$.

The growth of salt-stressed plants resulted in a slow progressive reduction of the photosynthetic rate, transpiration, stomatal conductance, and an increase in the use of water. The first week under salinity conditions A_n values decreased in a range between 20 and 60% (100 mM to 600 mM respectively) in both populations, while at the end of the experiment this decrease reached values between 54%, for 100 mM, and 100%, for 600 mM (Fig 1A, E). The g_s values dropped by 75% to 97% at the end of the experiment (100 mM to 600 mM respectively), while A_n/C_i values decreased by 45% to 100%, correspondingly to the lowest and the highest concentrations in both populations (Fig 1 B, C, F, G). On the contrary, WUE

increased significantly in response to salinity in a different way in the two populations (Fig 1D, H). The increase was recorded from the first week (40%) in the native population, while for the invasive one it was recorded from the third week (70%-120%).

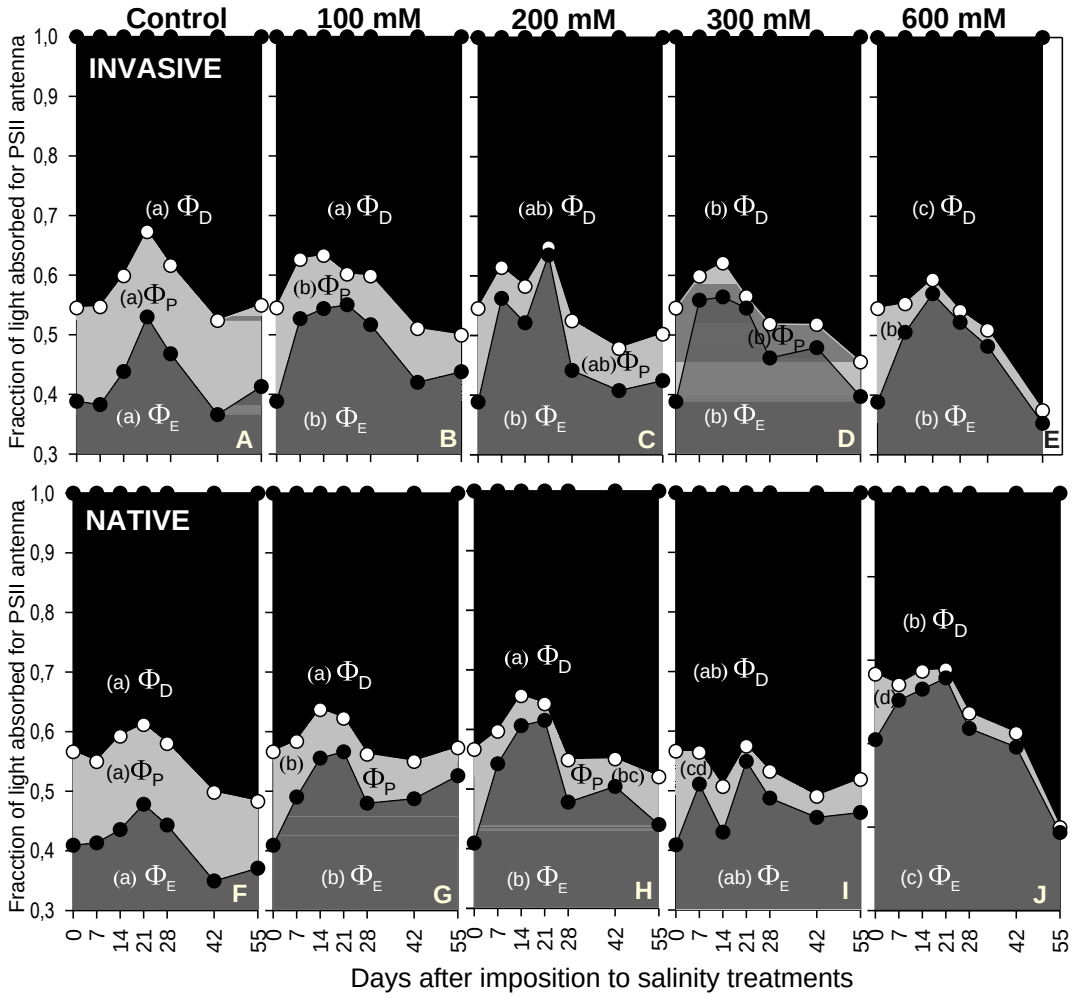

Figure 2: Fractions of the light energy absorbed in PSII antenna that is dissipated thermally (Φ_D, black), used in photochemistry (Φ_P, light grey), and the remaining light energy excess (Φ_E, dark grey) in one invasive (A-E) and one native population (F-J) of *Oenothera drummondid* for 5 salinity treatments (5, 100, 200, 300, 600 mM) from day 0 before the beginning of the experiment to the end of the experiment in day 55 (n = 25 for each population and sampling period). The letters in parentheses next to the name of the variables indicate significant differences between treatments for the total period at $p < 0.05$.

After 55-d of exposure to salt stress, the changes in the fractions of light energy absorbed by PSII antennae allocated to photochemistry versus dissipation, as shown in figure 2, indicating that energy thermal dissipation increase along with increasing levels of salinity, but also with the time of exposure. In the same way, the energy allocated to PSII photochemistry decreased significantly with salinity treatment and time of exposure.

Additionally, differences between populations in response to the salt stress were found. Although in both populations the energy assigned to photochemistry decreased with salinity, in the native population this descent is mainly due to an increase of energy excess, while in the invasive population it is a result of a thermal dissipation raise.

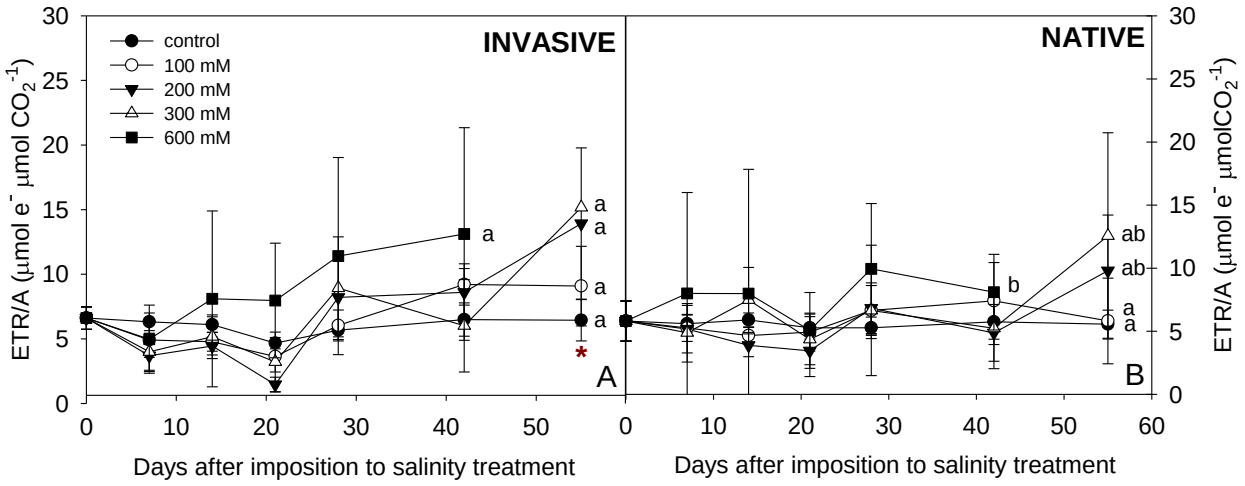

Figure 3: Mean values ± sd for ETR/A_n (μmol electrons µmol CO_2^{-1}) in one invasive (A) and one native populations (B) of *Oenothera drummondii* for 5 salinity treatments from day 0 before the beginning of the experiment to the end of the experiment on day 55 (n = 25 for each population and sampling period). Different letters at the end of the lines indicate significant differences between treatments for the total period at $p < 0.05$.

Plants under salt stress showed ETR/A_n values higher than the control plants, but significant differences among treatments were only found in the invasive population.

Table 2: One-way ANOVA result for pigment, Na^+ content, and biomass at the end of the study. Abbreviations: Biomass (B), Na content (Na^+), ratio aerial biomass to root biomass (B_a/Br), ratio aerial biomass to total biomass (B_a/B_t) and ratio root biomass to total biomass (B_r/B_t), total chlorophylls (Chl), total carotenoids (Car), β-carotene (βcar), lutein (L), xanthophylls (VAZ), ratio chlorophyll a/b (Chl a/b); ratio carotenoids to chlorophylls (Car/Chl), de-epoxidation index (DEI: AZ/VAZ), ratio lutein to total chlorophyll (L/Chl).

		INVASIVE		**NATIVE**				**INVASIVE**		**NATIVE**	
Variables	**dF**	**F**	**p**	**F**	**p**	Variables	**dF**	**F**	**P**	**F**	**p**
B_{Leaf}	4	16.0	**0.001**	3.7	**0.010**	**Chl**	3	0.883	0.459	3.6	**0.024**
B_{Stem}	4	59.8	**0.001**	24.7	**0.001**	**Car**	3	2.7	0.063	1.4	0.249
B_{Root}	4	36.3	**0.001**	12.8	**0.001**	**L**	3	1,.5	0.235	2.5	0.075
B_{Total}	4	60.6	**0.001**	23.8	**0.001**	**β car**	3	1,.9	0.140	8.5	**0.001**
B_a/B_r	4	17.4	**0.001**	3.9	**0.009**	**VAZ**	3	1,.5	0.224	1.1	0.343
B_l/B_t	4	13.5	**0.001**	8.1	**0.001**	**Chl a/b**	3	2,.0	0.127	2.8	0.052

B_r/B_t	4	18.3	**0.001**	3.9	**0.009**	**Car/Chl**	3	7,.2	**0.001**	0.9	0.451
Na^+ Leaf	4	22,2	**0.001**	37.2	**0.001**	**AZ/VAZ**	3	1.6	0.199	1.4	0.259
Na^+ Stem	4	40,6	**0.001**	93.3	**0.001**	**L/Chl**	3	0.9	0.421	1.7	0.185
Na^+ Root	4	82,9	**0.001**	107.9	**0.001**						

Salinity induced a progressive increase in pigment content. A positive correlation was found between total chlorophyll, carotenoids, lutein, or β carotene contents with increasing levels of salinity: Chl total ($r = 0.348$, $p = 0.002$), Car ($r = 0.345$, $p = 0.002$), β car ($r = 0.487$, $p = 0.001$, L ($r = 0.355$, $p = 0.014$). However, no pattern was found in the pigment relative composition between populations, neither differences between salt treatments, nor in DEI, or L/Chl.

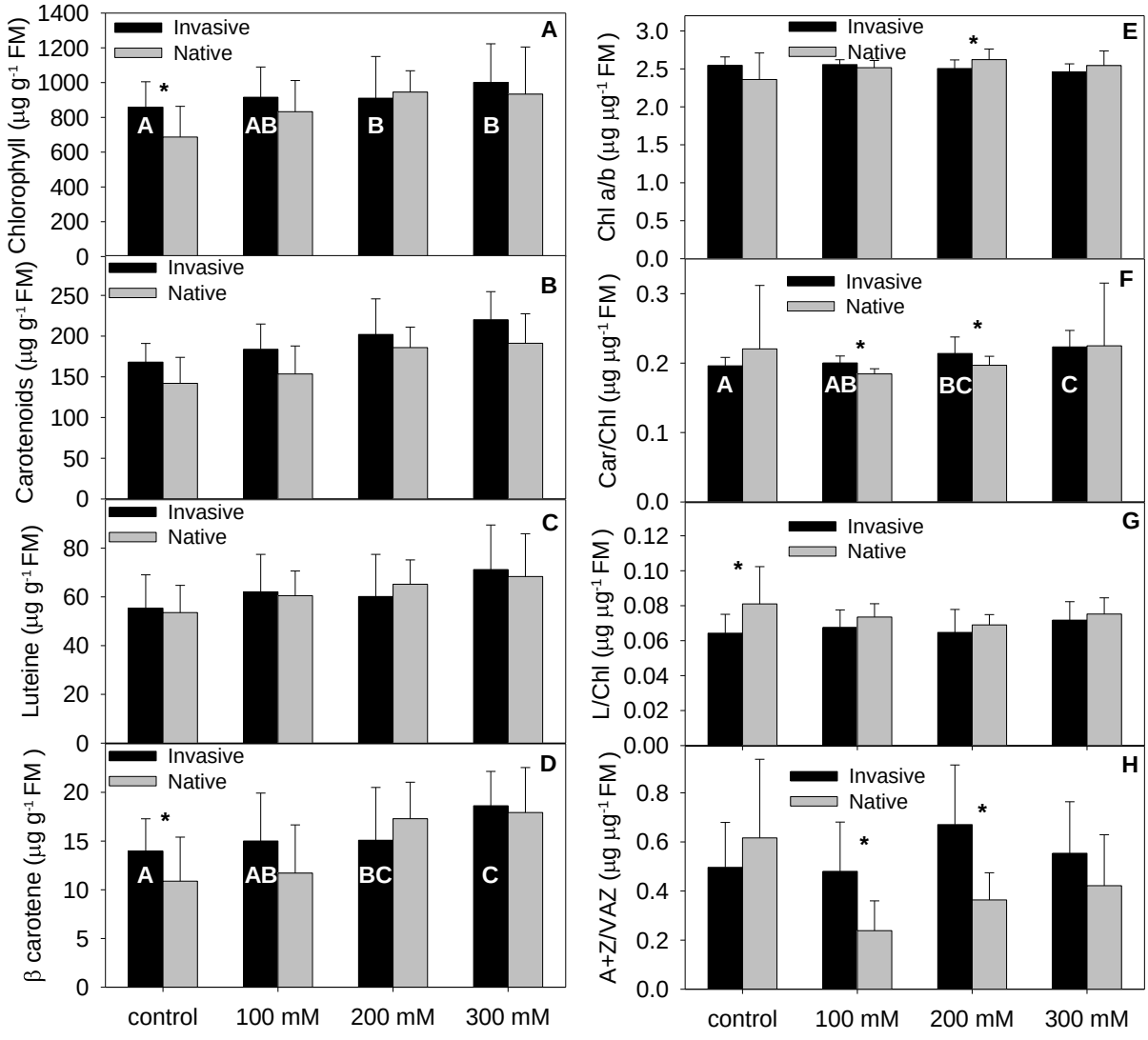

Figure 4: Mean values ± sd for total chlorophyll (A), carotenoids (B), Lutein (C), carotene (D), Chlorophyll a/b ratio (E), Car/Chl ratio (F), L/Chl ratio (G) and AZ/VAZ = De-epoxidation index (H) in one invasive and one native populations of *Oenothera drummondii* for 4 salinity treatments (FM:

fresh mass). A: antheraxanthin, Car: total carotenoids, Chl: chlorophyll, L: lutein, V: violaxanthin, Z: zeaxanthin. Columns marked with different letters indicate a significant difference between treatments for that population, while asterisks indicate significant differences between populations both at $p < 0.05$. Values are the mean of 10 plants for each population and treatment (40 plants for population). Pigment content was not measured in plants from the highest salinity treatment (600 mM) due to high mortality and lack of healthy leaves in both populations.

Salinity induced progressive dehydration of the leaves in all the treatments during the study period, although this was more evident in the invasive population. No differences were found in LDMC between populations, neither between salinity concentrations.

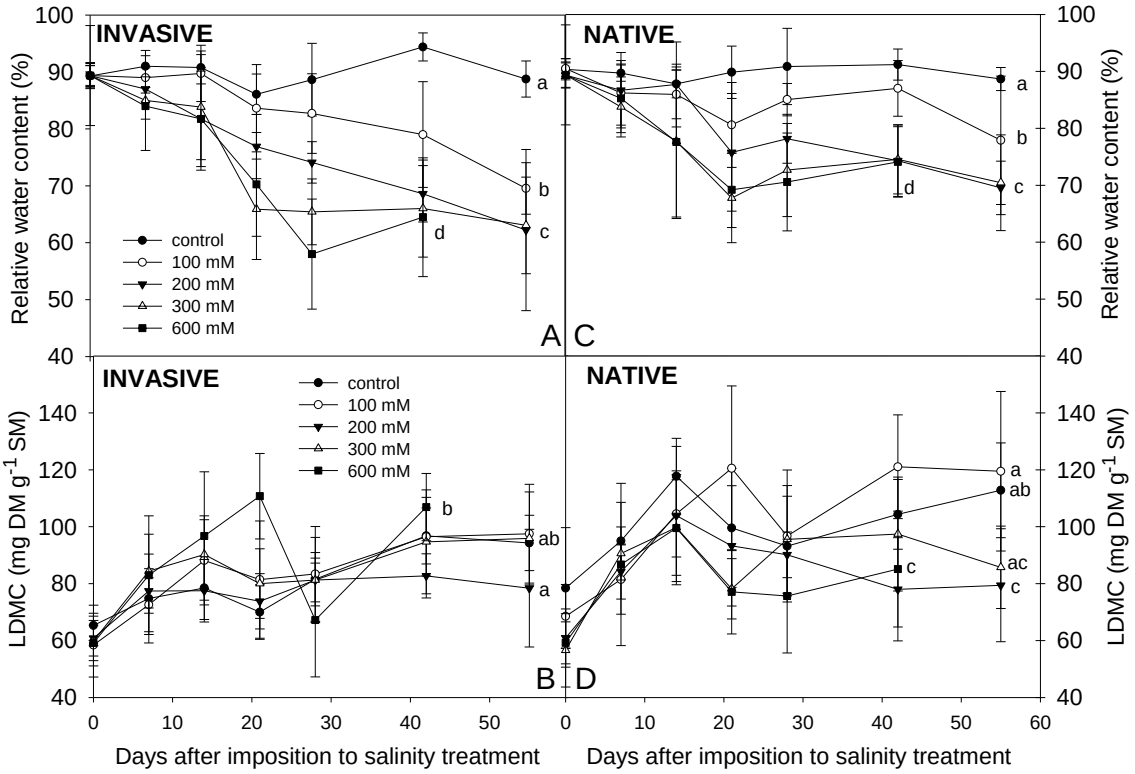

Figure 5: Mean values ± sd for relative water content and leaf dry matter content (LDMC, DM: dry mass; SM: saturated mass) in one invasive (A, B) and one native populations (C, D) of *Oenothera drummondii* for 5 salinity treatments from day 0 before the beginning of the experiment to the end of the experiment on day 55 (n = 25 for each population and sampling period). Different letters at the end of the lines indicate significant differences between treatments for the total period at $p < 0.05$.

3. 2 Biomass and flowers

Progressive salt stress was accompanied by the reduction of biomass and by changes in the biomass allocation to the different organs in both populations (Fig 6). Biomass of the different organs linearly decreased with salinity: leaves ($r = -0.447$, $p = 0.001$), stems ($r = -0.736$, $p = 0.001$), roots ($r = -0.715$, $p = 0.001$), B_T ($r = -0.777$, $p = 0.001$). Similarly, the allocation to the different organs was also correlated with salinity, B_a/B_r ($r = 0.481$, $p = 0.001$), B_l/B_t ($r = 0.557$, $p = 0.001$), B_r/B_t ($r = -0.461$, $p = 0.001$). Conversely, *Oenothera* plants did show a different growth pattern associated with the populations. For example,

biomass allocation to leaves (B_L/B_T) increased more in plants from the invasive population than those of the native one under salinity stress, while the opposite happened in biomass allocation to stems, which was higher in the native population. Also, differences among populations in allocation to roots or root biomass were found at 300 and 600 mM respectively.

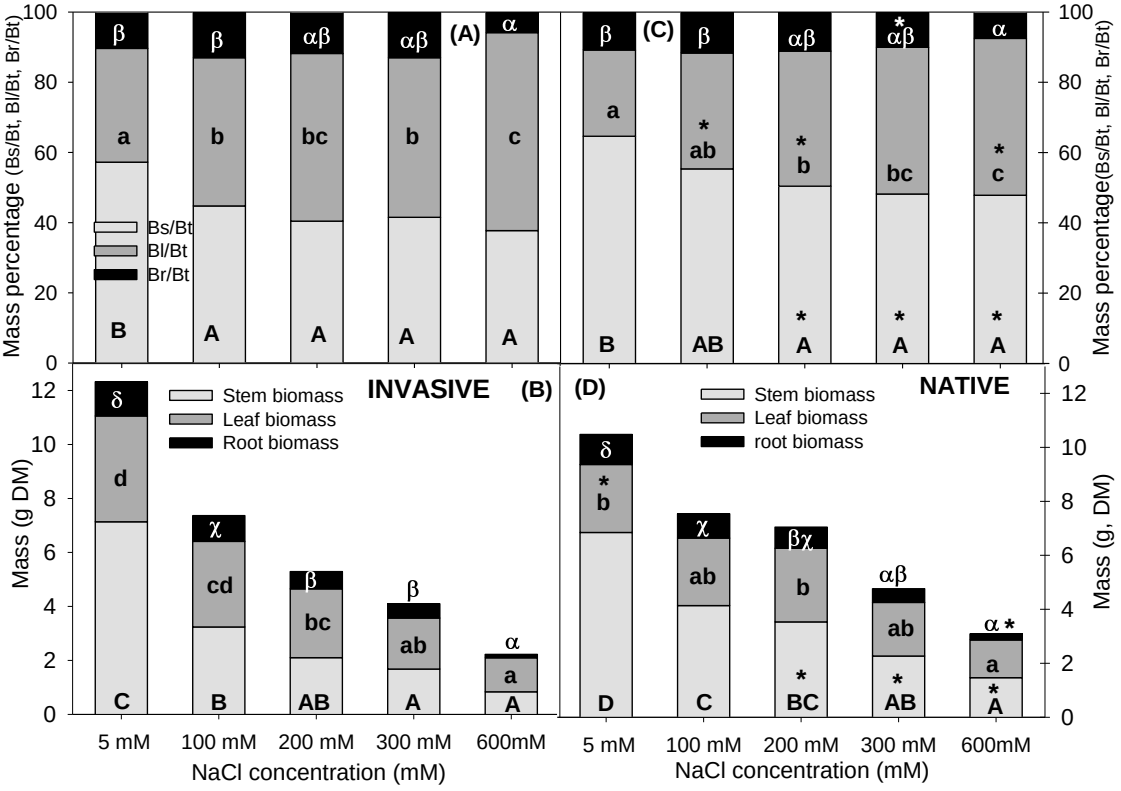

Figure 6: Biomass of leaves, stems, and roots of *Oenothera drummondii* at the end of the experiment (B, D) for the 5 salinity treatments and relative biomass allocation (A, C) expressed in %. Columns marked with different letters indicate significant difference between treatments in each population (uppercase for B_s/B_t, lowercase for B_l/B_t, and Greek letter forB_r/B_t)), while an asterisk indicates significant difference between populations both at $p < 0.05$. Stem Biomass: Bs, leaf Biomass: Bl and root biomass: Br, DM: dry mass. Values area the mean of 10 plants for each population and treatment (50 plants for population).

Salinity stress markedly affected flowering performance, reducing the number of flowers (native: $\chi^2 = 401.7$, invasive $\chi^2 = 590.3$ $p < 0.001$) but also the size of flowers ($F_{native}= 39.6$; $F_{invasive}= 76.4$; $p < 0.001$) and their mass ($F_{native}= 62.6$; $F_{invasive}= 114.0$; $p < 0.001$) in both populations (Fig 7A, C). While in the control treatment the highest number of flowers was recorded in the invasive population, under salinity conditions the opposite situation occurred, with the highest number of flowers recorded in the native population ($\chi^2 = 13.73$ $p < 0.01$). This suggests a different flowering strategy in response to the saline environment. On the

other hand, our data revealed that increasing salt stress applied to the young *O drummondii* individuals did not delay plant flowering (Fig 7B, D).

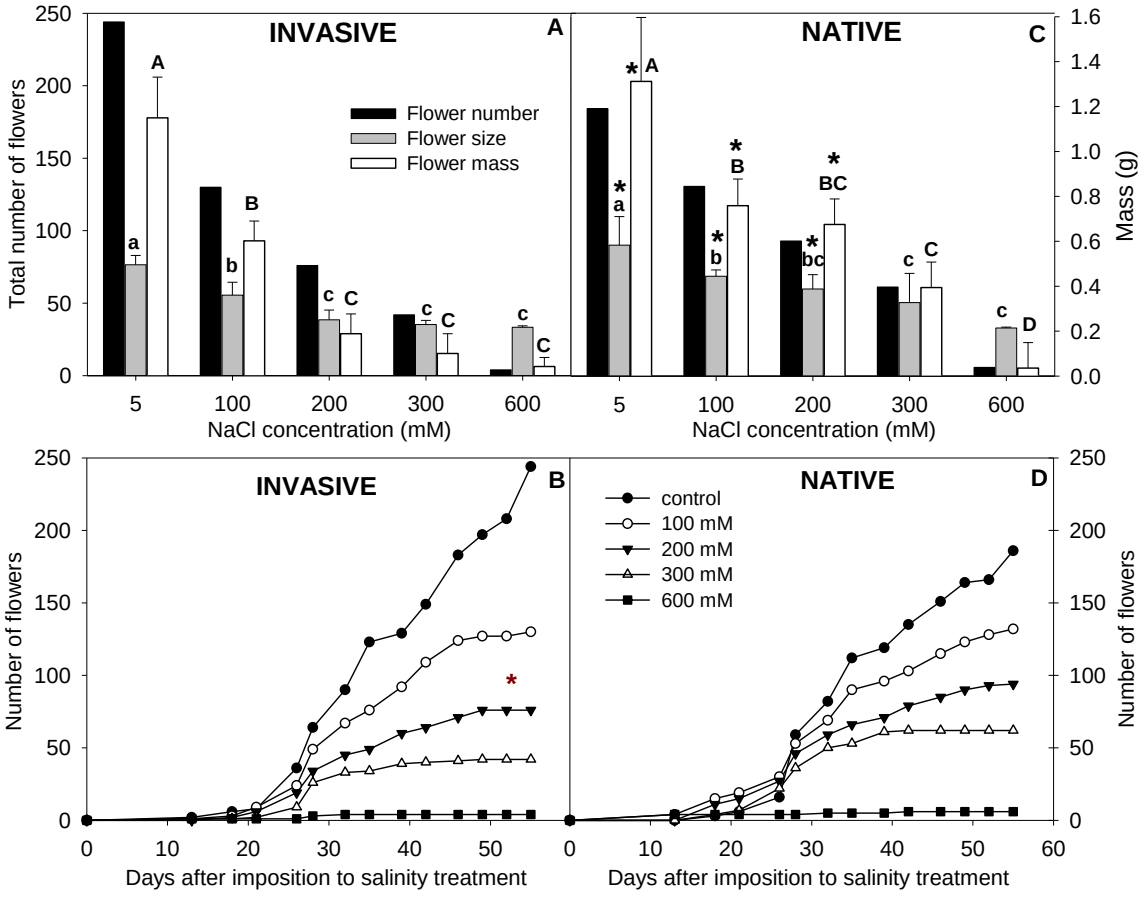

Figure 7: Total number of flowers of 10 plants per treatment and mean ± sd of, size, and mass of flowers of *Oenothera drummondii* for the 5 salinity treatments in one invasive (A, B), and one native (C, D) population. Values represent mean of 50-10 flowers, except for the number of flowers, where accumulated values of 10 plants per treatment are shown. Columns marked with different letters indicate significant difference between treatments in each population (lowercase for flower size and uppercase for flower mass), while asterisk indicates significant differences between populations for each variable and treatment; both at $p < 0.05$.

3.3 Na^+ Content

Increasing levels of salinity led to increasing Na^+ accumulation in all tissues. Leaves were the organs where Na^+ accumulated first. No differences were found between populations in response to high salinity conditions, except at 600 mM concentrations, where root tissues of the native population accumulated more Na^+ than in the invasive one.

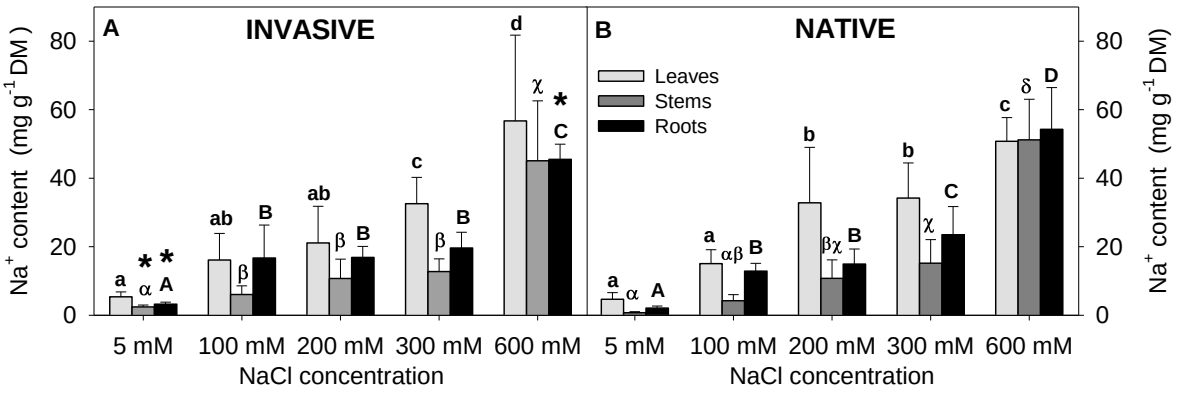

Figure 8: Na+ content (mean ± SD) of *Oenothera drummondii* (mg g^{-1} dry mass) at the end of the experiment for the 5 salinity treatments. Asterisks indicate statistical differences between populations (Invasive, A, and Native, B) at each salinity treatment and organs ($p < 0.05$). Different letters represent statistical differences ($p < 0.05$) between salinity treatments in each organ (lowercase for leaves, uppercase for roots and Greek letter for stems).

4. DISCUSSION

The data recorded in this study proved that, even although resistant to salinity, *O. drummondii* is sensitive to sustained high salinity levels (600 mM), which affected plant performance at all levels. The salt-induced ionic toxicity effects resulted in a slow and progressive reduction of photosynthesis (gas exchange as A_n, g_s, E, A_n/C_i, and photochemistry as Φ_P, ETR, and ETR/A_n) related to both stomatal and biochemical limitations. The increasing levels of salinity also resulted in a reduction of the biomass and number and size of flowers, hence compromising the reproductive capacity of the species, particularly at salinity levels similar to seawater. The biogeographical origin of the populations affected the intensity and timing of physiological, reproductive, and morphological responses to salinity, the native population being more resistant to long exposure, and to high salt concentration than the invasive one. Under no-salt toxicity, plants from the invasive population (Mediterranean origin) presented higher biomass, number of flowers, and chlorophyll and carotenoids content than native ones (subtropical origin). However, under salt toxicity conditions, these differences disappeared, and even under intermediate salinity concentrations (200 mM), plants from the native population surpassed the invasive population in biomass and number of flowers. Contrarily to what we expected, the invasive population did not show functional attributes that improved its salt-tolerance in comparison to the native one.

The subtropical origin of the native population, where disturbance events such severe tropical storms and hurricanes are common (Keim et al., 2007), may be the underlying factor to explain their greater tolerance to salinity. The intensity and frequency of these events

629 determine the degree of water intrusion and flooding or overwash through waves that
630 facilitate the entry of seawater into dune community changing species composition and
631 abundance (Gornish and Miller, 2010). Contrastingly, severe storms are less frequent in the
632 southwestern Spanish coast (Rodríguez-Ramírez et al., 2003) so that, investment in defence
633 mechanisms in response to saline conditions would be less necessary for *O. drummondii*
634 individuals from the invasive population.

635 The inhibition of the photosynthetic capacity under salinity conditions might be due
636 to stomatal closure, which reduces the capacity of CO_2 uptake and is often associated with
637 osmotic stress (Munns, 1993; van Puijenbroek, 2017). However, the differences found
638 between treatments in E or g_s were smaller than in A_n, which suggests that there may be other
639 mechanisms in addition to osmotic stress controlling the photosynthetic capacity, different
640 from stomatal closure and low intercellular CO_2 concentration.

641 Although stomatal closure is generally one of the principal causes of declining photosynthetic
642 rates, metabolic limitations such as a reduction in the activity or content of the Rubisco (Parry
643 et al., 2002) can also limit photosynthesis. In our study, the decrease in the carboxylation
644 efficiency (A/C_i) under high salinity conditions, with the consequent increase in intercellular
645 CO_2 concentration, would also indicate non-stomatal limitations of the photosynthesis but
646 metabolic impairment mainly in biochemical carboxylation. These limitations would probably
647 be associated with a decrease of Rubisco carboxylase activity as a consequence of the
648 accumulation of Na^+ ions in leaf tissues (López-Climent et al., 2008; Silva et al., 2011) as
649 shown by Na^+ tissue data recorded in the study, where leaves were the organs that
650 accumulated Na^+ first. Accordingly, the reduction of photosynthesis under salt stress would
651 be caused by two processes: stomatal and metabolic limitations by the effect of Na^+ ions
652 accumulation, osmotic and ionic stress respectively.

653 *Oenothera drummondii* plants subjected to salt treatments showed an imbalance
654 between photosynthesis and photochemistry. In plants in optimal conditions, photosynthetic
655 assimilation and electron transport rate are usually correlated. In our study, plants under salt
656 stress showed ETR/A_n ratio higher than the controls. The increase in ETR/A_n ratio represents
657 an imbalance between e^- flow and the CO_2 assimilation during photosynthesis, which together
658 with a decrease in A_n/C_i might indicate loss of photosynthetic efficiency. This impairment
659 between CO_2 assimilation and photochemistry due to the elevated salinity conditions might

indicate an e- flow to other e- consuming physiological processes, rather than to CO_2 assimilation, such as photorespiration. (Baker et al., 2007; Ribeiro et al., 2009).

Na^+ accumulation in plant tissues has consequences also at the cellular level. Concerning photochemistry effects, for example, it causes damage in the thylakoids in the chloroplast (Wang et al 2009), it reduces photosystem I and II activity, it affects photosystem II repair which enhances photoinhibition and it reduces photosynthetic efficiency (Murata et al., 2017). The indexes used to estimate energy going to thermal energy dissipation, Φ_D, and the shift in photochemistry to photosynthetic electron transport, Φ_P represent along with the remaining excess fraction (Φ_E) the total energy allocation of the PSII reaction center. Reductions in Φ_P along with increasing levels of Φ_D and Φ_E have been shown to be responses to salt stress (Tsai et al., 2019; Wu et al., 2018,). Consequently, the reduction of the Φ_P from the first week in all salt concentrations implies that the photosynthetic efficiency of *O. drummondii* plants under salt conditions is compromised. The increase in thermal dissipation, especially after 4 weeks at high salt treatments, suggests greater activity of the xanthophyll cycle. Although our pigment content data are not conclusive on the role of the xanthophyll cycle (significant differences in the DEI were only obtained in the native population), they did prove the existence of a positive correlation between carotenoid content and salinity and therefore suggest that these pigments may have a significant role in the thermal dissipation of light energy in response to salinity.

The triple interaction time*population*treatment, reveal that the native and invasive populations presented different temporal responses to salt stress for variables related to photochemistry ETR, Φ_D, Φ_P, Φ_E. The light absorbed by PSII is dissipated in three pathways, photosynthetic electron transport (Φ_P), thermal dissipation (Φ_D), and excess energy (Φ_E). In our study, invasive plants enhanced Φ_D, while native plants enhanced the excess fraction particularly in 600 mM salt treatment. Following Kato et al. (2003, 2002), electron transport (photosynthesis, photorespiration, or the water-water cycle) and Φ_D operate as mechanisms of photoprotection, while Φ_E determines the photoinactivation rate of PSII. The different capacities of photoprotective mechanisms of plants may determine their susceptibility to photoinactivation which is linearly correlated to Φ_E. Consequently, with the data obtained, we suggest the existence of different strategies in the dissipation of light between the two populations. Thus, in the native population, the decrease in photochemical efficiency is due to the inactivation of PSII, while in the invasive population the protective mechanisms of heat dissipation would predominate. The strategy of the native population resulted to be more

efficient than the one of the invasive, where the mortality in the highest salinity treatment reached 90 % in contrast to only 40 % in the native.

Regarding pigment content, it is noticeable that total chlorophyll content and carotenoids were higher in plants under salt stress compared with controls, suggesting that these stressful conditions stimulated pigment production. This was expected, as mentioned above, in the case of carotenoids, as these pigments act also as antioxidants (Edge et al., 1997), and have been described by several authors to increase in response to salt stress for their protective role (Gomes et al., 2017). Salinity, among many other factors, leads to oxidative stress in plants, which affects numerous biological processes that conduct to changes in the physiological, biochemical, and molecular processes of cellular metabolism. The higher content in carotenoids may be the plant's response to oxidative stress induced by salinity conditions (Falk and Munné-Bosch, 2010). The indexes calculated to study the relative composition of pigment were only significant for the ratio Car/Chl in the invasive plants, indicating the important role of these protective pigments, but also the different strategies used in the two populations regarding pigment composition. Saline conditions often induce a reduction in chlorophyll content in sensitive species (Sudhir and Murthy, 2004). In contrast, our target species increased Chl (and carotenoids) may be to promote an increase in A_n proving to have protective mechanisms against salinity.

Although plants avoid salts from being transported from soil to roots, this defensive mechanism is not very efficient at high salt levels. In our study, differences in Na+ concentration in the different organs appear to be significant at least from salinities greater than 200 mM. As NaCl concentration increased, biomass, size, and number of flowers declined sharply. This decrease in response to increasing levels of salinity is unlikely to be due to a nutrient deficit since each pot was fertilized during the transplant and the substrate used contained one-third of compost (that not only provides nutrients but also increases the microbial activity releasing nutrients that are already in the soil). The high accumulation of Na^+ recorded in leaves would indicate passive and active transports from roots to leaves. This transport is a common salt tolerance mechanism in dicotyledonous halophytes, as leaves can be shed so as to remove the tissues where toxic ions accumulated. However, this is an energy consuming process (ATP is required to move ions across a semipermeable membrane) that would explain the decrease in biomass, (stem, leaves, root, flowers), suggesting a tradeoff between growth and the defensive mechanism. Additionally, the increase in Na^+ content in leaves, stems, and roots was also linked with a fast decrease in photosynthesis (A_n, E, and g_s)

and photochemistry (Φ_P) performance. These would support the results discussed above which would point out both types of stress, ionic, and osmotic (Munns and Tester, 2008) as responsible for the response of *O. drummondii* to salinity. We are aware that Na+ content values alone do not allow to assess the ionic components of salt stress, as the ionic balance also depends on reducing the uptake of other cations (mostly K^+), or the entry of other anions, (Cl^-) in response to net Na^+ accumulation (Tyerman et al., 1997). Nevertheless, high Na^+ content in the aerial part of *O. drummondii* plants, such as those observed in our study, would cause high Na^+/K^+ ratios that would inactivate enzymes and alter metabolic processes in the plants (Sudhir and Murthy, 2004).

Biomass reduction was observed in aerial and underground organs in both populations, however, the allometric response to salt stress was different between populations. Although a higher allocation to leaves was a common pattern to increasing levels of salinity, this was significantly lower in the individuals of the native population, which showed similar B_l/B_t and B_s/B_t ratios. Additionally, the reduction in leaf biomass occurred from a concentration of 200 mM in plants of the invasive population, while in the native one it occurred at a concentration of 600 mM, which reinforces the idea of a greater resistance of the native individuals to salinity stress. Moreover, while the invasive population recorded a higher number of flowers in control plants than the native one, under salt stress higher number of flowers was recorded in the native population. This suggests not only a different flowering strategy but a better response of the native population to the saline environment.

Coastal habitats are already considered to be between the most vulnerable habitats of the world (Du and Hesp, 2020; Levinsh, 2006). Which added to the consequences of climate change (sea-level rise, coastal erosion, higher salt spray production, and transport among others) (Du and Hesp, 2020; Young and Ribal 2019), can result in significant changes in dune morphology and vegetation of these areas. This scenario will enhance species tolerant to sand burial, low water availability, poor soils, salt spray deposition, and sea overwash as it is the case of the invasive species *Oenothera drummondii*. These results along with the previous ones obtained by Gallego-Fernandez et al. (2021) indicate that *O. drummondii* manifested a good tolerance to salinity, even if increasing levels of salinity resulted in decreasing biomass, reproductive capacity, and physiological performance. Only, high levels of salinity maintained over time compromised the reproductive capacity of this species and lead to mortality. For this reason, even though the impact of high salinity is stronger in these areas,

the efforts aimed at eradicating this invasive species must include the foredune front and the back beach among their actions to achieve the objective.

We can conclude that *O. drummondii* exhibits a high resistance to salinity that varies with the biogeographical origin of the populations, the native population being more resistant to long time exposure and high salt concentration than the invasive one. This greater tolerance to salinity of the native population from Texas may be related to its biogeographic origin, since the area where *O drummondii* population is located is subjected to a great frequency of strong storms and hurricanes that facilitate the entry of seawater. The tolerance to continued exposure to salinity might be one of the factors explaining the high expansion rate of this species in coastal areas, which can be enhanced in the future as a consequence of climate change. Among the effects of climate changes, stated by IPPC (Intergovernmental Panel on Climate Change) in dune habitats, are the rising sea-levels, and the increase in salinity, both favouring spreading of tolerant species, such as *O. drummondii.*

Acknowledgments

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