Physiological Responses of Young Plants of *Carpobrotus edulis* (L.) N.E. Br (Aizoaceae) to Salt and Water Stress

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Abstract: Coastal sand-dune ecosystems have a high conservational value worldwide, but are frequently subjected to the introduction of exotic floral species by man. One of these species, cohabiting successfully with the native flora of Mediterranean-type coastal habitats, is the succulent *Carpobrotus edulis*. This species is native to South Africa and is considered an invasive species due to its capability to compete with the local flora, invading and quickly establishing itself in cleared areas. Studies at the ecological level, on the impact of this species and its hybrids on native flora of different countries, are available in the literature. However *C. edulis* seemingly competitive advantages remain poorly characterized at the physiological level.

To investigate the responses of this species to the main stresses present in sand-dune habitats, *C. edulis* plants, grown from seed under controlled conditions, were subjected to water stress and salt stress. Rapid light response curves, shoot growth and concentration of photosynthetic pigments and soluble protein were determined, and the detection of differentially expressed genes was initiated.

It was found that both types of stress did not negatively affect photosynthetic electron transport rate and shoot concentration of photosynthetic pigments in this species. However, shoot soluble protein concentration was significantly decreased by salt and water stress, in relation to control levels. Both types of stress induced differential gene expression. Although work is still under way, the set of genes affected by water stress appears to be different from the set of genes affected by salt stress.

Keywords: Water stress, salt stress, light response curve, photosynthetic pigments, soluble protein.

INTRODUCTION

The introduction and spread of exotic species is a global phenomenon that is recognized as a major source of environmental change [1]. Invasion of natural ecosystems by alien floristic species modifies the population dynamics of native flora and the structure of plant communities, can accelerate the extinction rate of indigenous species and alter the water and nutrient fluxes [2-4]. In Mediterranean-type climate ecosystems the biological invasion by alien plant species is considered as a determinant factor of environmental alterations [5]. In the Mediterranean or Woodland/ Shrubland biome [6], found in the Mediterranean Basin, California, South Africa, Chile and West coast of Australia, coastal sand-dune ecosystems constitute a considerable part of natural habitats, with a high conservation value. Natural from the North East of South Africa, *Carpobrotus edulis* L., a succulent plant, is a recognized invasive species [7] in those ecosystems. Frequently introduced as ornamental or for stabilization of dune systems, preventing sand movement, *C. edulis* has a vigorous vegetative growth forming long prostrate shoots, rooting at the nodes, that does not seem to be affected by herbivory or competition with the local flora [8,9]. It was reported to reduce soil pH and thus influence nutrient dynamics [10] and to hybridise with its related species [11].

In costal environments plants are frequently subjected to variable levels of water availability as well as salt in the substrate and in the surrounding atmosphere. Hence the establishment of invasive species in those environments depends also on the tolerance to salinity and water stress as competitive advantages in relation to the acclimated local flora. Recent studies have described *C. edulis* production of secondary metabolites such as flavonoids and tannins [12] and its antioxidant, phytochemical and antibacterial properties in different conditions [13, 14]. In contrast, reports on its response to water stress or salt stress have focused mainly on germination rates [15] and the physical components of water potential and photo-synthesis in relation to the apparently inducible Crassulaceae Acid Metabolism (weak CAM-inducible) of *C. edulis* [16-19] and have not addressed metabolic parameters. Although much is discussed about the ecology of *C. edulis*, updated information on its actual physiological performance is scarce.

In view of the information available, the objective of the present study is to characterize the response of young plants of *C. edulis* developing under controlled

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conditions water or salt stress in relation to well-watered, so as to evaluate its physiological performance. This includes a preliminary description of differential expression profile caused by each type of stress. We aim to contribute to the knowledge of this species adaptations and physiological repertoire enabling it to compete advantageously with the native flora of Mediterranean-type habitats.

MATERIALS AND METHODS

Seed Collection and Germination

Dry, indehiscent fruits of *C. edulis* were collected during July, from plants established in sand dunes at Aljezur (Southwest of Portugal). The seeds were separated from the ovary tissues and kept at 4°C until used. For germination purposes, seeds were scarified with the aid of carborundum and soaked in water for two hours, before being placed over wet Whatman paper (90 mm No. 1 filter) in Petri dishes. These were then placed in a walk-in growth chamber at constant temperature (26°C±1.5°C), light intensity of 145 µmol. $m^{-2}.s^{-1}$ and a photoperiod of 12 h.

After germination, seedlings circa 1 cm tall were transferred to substrate consisting of a mixture of autoclaved commercial plant substrate and dune sand (1:1). When circa 3 cm tall and 3 conspicuous leaf nodes, seedlings were transferred to 250 ml plastic pots (2 plants per pot), filled with the same substrate as previously. A total of sixty pots were set up and the plants were allowed to establish in these conditions for one week, with regular watering.

Experimental Design

At the onset of the treatments as well as two days before and every two days after the onset of treatments, all pots were weighted. The pots were randomly separated in three groups of 20. One group was kept as control (C), the second and third goups were subjected respectively to water stress (WS) and to salt stress (SS). In the control group, the difference in weight between consecutive weighting periods was replaced with the corresponding water volume. In the WS treatment it was replaced with the water volume corresponding to only 25% of the weight difference, and in the SS treatment it was replaced with the corresponding volume of a 50 mM NaCl solution.

The experiment lasted for 26 days. On days 0, 5, 12, 19 and 26 after the onset of the experiment (days after treatment, DAT) eight plants of four randomly selected pots per treatment were used for the different determinations. Only shoots were analysed in this study.

Substrate Conductivity

Substrate conductivity was measured on the pots removed from the experiment, on 5 DAT and 26 DAT. For this the method previously described [20] was followed. MilliQ water (Merck Millipore, Darmstadt, Germany) (conductivity of 0.054 $S.cm^{-1}$) was slowly added to each pot, until field capacity was reached. After allowing the substrate to completely soak for 1 hour, 50 ml of MilliQ water were added and the outflow collected from the bottom of each pot. Conductivity was then measured in this solution (CRISON Conductimeter BASIC30, Crison Instruments, S.A, Barcelona, Spain).

Shoot Growth Parameters

At each sampling time 4 pots per treatment were randomly selected and removed from the experiment. The fresh weight of the shoots of eight plants per treatment was recorded. Four of these samples, randomly selected within each group, were frozen in liquid nitrogen, grinded with a mortar and pestle and the powder obtained kept at -80ºC. The other four samples were dried at 65ºC, for at least 48 hours, for dry weight determinations. The mean water percentage determined in these samples was used to calculate pigment and soluble protein concentrations in terms of dry weight to allow comparisons between treatments, thus avoiding the bias introduced by differences in the water status of the plants kept under the different watering regimes.

Photosynthetic Pigments

Photosynthetic pigments were extracted from the samples kept at -80ºC, following the method described by Lichtenhaler and Buschmann [21]. Two hundred and fifty milligrams of grinded plant material were macerated with acetone 100% (0.25:3, w/v) and the extracts were kept at -20ºC until determinations were conducted. After centrifugation at 5000x g for 5 minutes, absorbance was determined with a spectrophotometer (Shimadzu UV 160A, Shimadzu, Japan) at three wavelenghts: 470, 646.8 and 663.2 nm. The equations from Lichtenthaler and Buschmann [21], together with the water percentage previously determined, were used to calculate the concentration of chlorophyll a (c_a), chlorophyll b (c_b) and carotenoids (carotenes and xantophylls, $c_{(x+c)}$) in terms of mg of pigment.g^{-1}dry weight.

Soluble Protein

Soluble protein was extracted from the samples kept at -80°C, following the Bradford method [22]. One hundred milligrams of grinded plant material were macerated with 1 ml of 50 mM Hepes buffer (Thermo Fisher Scientific Inc.) at pH 7.4. The extracts were kept at -20ºC until determinations were conducted. After centrifugation at 5000x g for 5 minutes, 15 µl of supernatant were combined with 785 µl of distilled water and 200 µl of the Bradford reagent (Biorad, California, USA). The reaction was allowed to develop for 30 minutes, after which absorbance was determined at 595 nm (Shimadzu UV 160A, Shimadzu, Japan). The calibration curve was prepared using BSA (Sigma-Aldrich, St. Louis, USA) standards.

Rapid Light Response Curves

The photosynthetic capacity of C, WS and SS plants was evaluated through the determination of rapid light response curves (RLRC), with a fluorometer (Junior PAM Chlorophyll Fluorometer, Walz, Germany) at the temperature in the growth chamber (26°C±1.5°C). This method estimates the rate of electron transport through the leaf photosystem II by sequentially applying 8 levels of consecutively increasing actinic light intensities (photosynthetically active radiation - PAR). In the present case those levels were set to be 66, 90, 125, 190, 285, 420, 625 and 820 µmol.m^{-2}.s^{-1}. The light delivered by the fluorometer and the fluorescence emmited by the sample were conducted by a 1.5 mm-diameter plastic fiberoptic connected to the apparatus.

For the RLRC determinations, which are non-destructive, at least six plants per treatment were used and measurements were taken on both leaves at the third older node from the plant base, in the middle of the light period (6h after lights on). These leaves went through expansion and maturation during the period of the experiment.

Differential Gene Expression Profiling

Total RNA was extracted from the samples kept at -80°C using the E.Z.N.A. plant RNA kit (Omega Bio-Tek, Norcross, USA) according to the manufacturer's instructions. Quality of the extracts was verified through electrophoresis in agarose gel. Quantification of total RNA was done with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.). For detection of differentially expressed genes the Seegene GeneFishing kit (Biogene, Kimbolton, UK) was used, according to the manufacturer's instructions. For this the cDNA obtained from the samples from each treatment at 5 DAT were pooled and the results of the PCR amplification with each primer pair from the kit were compared between the three treatments. Differential bands were extracted from the agarose gels and ligated to the pGEM-T Easy vector (Promega, Mannheim, Germany) according to the manufacturer's instructions. The constructs were used to transform XL1Blue *E. coli* competent cells (Stratagene, Canada). Plasmid extraction from recombinants was done using the NZYMiniprep kit (NZYTech, Lda, Lisboa, Portugal) before commercial sequencing of the clones (StabVida, Setúbal, Portugal). The obtained sequences were visualized and edited with BioEdit [23] prior to the BLAST analysis at the EST database available at GenBank.

Statistical Analysis

Comparisons between group means for statistical significance were done by ANOVA ($P<0.05$) followed by Bonferroni multi comparison tests, using GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS AND DISCUSSION

Shoot Dry Weight Accumulation under Salt and Water Stress

The conditions used to impose water stress (WS) and salt stress (SS) increased the conductivity of the substrate by 5 and 8 times respectively, in relation to control, at the end of the experiment (DAT 26), as shown in Figure **1A**. These conditions also increased the shoot dry weight of the salt stressed (30%) plants in relation to water stressed and control plants (Figure **1B**). Shoot water percentage decreased with time in all treatments and was significantly different between control and water or salt stressed plants at the end of the experiment (Figure **1C**) in circa 30%. A significant interaction between treatment and time ($P<0.05$) was found in a two-way ANOVA for the conductivity and shoot water % but not for shoot dry weight.

Partially dry leaf tips were present in water and salt stressed plants, although this observation was not quantified. At the end of the experiment plants presented 6 pair of leaves on average, in all treatments.

Carpobrotus edulis possess leaves with a peripheral chlorenchyma and colourless internal water storage-tissue [18]. It has been suggested, as hypothetical

adaptive response of succulent plants to water stress, that water is redistributed from old to young leaves, leading to maintenance of metabolic rates in the younger leaves [24]. This situation was not, however, evident in *C. edulis* [19] when, under water stress, the response of whole plants was compared with that of plants with excised older leaves. In the present work although shoot water % of the salt stressed plants tended to be lower than in the other regimes from DAT 12 onwards, this did not seem to affect the capacity for dry weight accumulation.

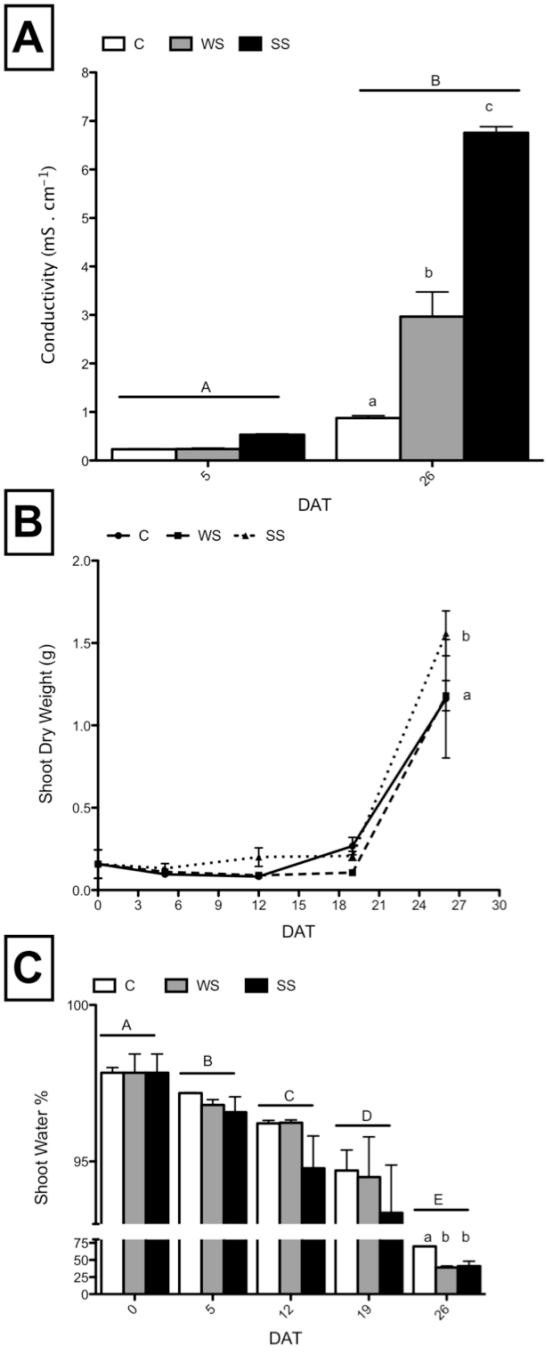

Figure 1: Conductivity of the substrate leachate (mean±SD, n=4) (**A**), shoot dry weight (mean±SD, n=4) (**B**), and shoot water % (mean±SD, n=4) (**C**) of *C. edulis* plants well watered (control - C), subjected to water stress (WS) or to salt stress (SS). DAT - days after treatment. Capital letters indicate significant differences between sampling times. Groups displaying different letters, at each sampling time, are significantly different (P<0.05; Two-Way ANOVA followed by Bonferroni multi comparison test).

It is known that the cellular basis for salt tolerance is the capacity to adjust osmotically to soil salinity by accumulating ions and sequestering the vast majority of these (generally Na^+ and Cl^-) in vacuoles, while in the cytoplasm organic solutes are accumulated to prevent adverse effects on metabolism. Halophytes are suggested to tolerate cytoplasmic Na^+ and Cl^- concentrations of 100-200 mM [25]. Studies at the vegetative stage with *Cakile maritima*, as euhalophyte, showed it requiring the presence of a moderate salt concentration (50-100 mM NaCl) to express its maximal growth potentialities [26]. This species is capable to increase Na^+ concentration of the leaves with increasing NaCl concentration in the medium (up till 500mM), without major effects on growth rate or showing salt-related toxicity symptoms [25]. While we did not measure Na^+ concentration in the leaves of *C. edulis*, it is likely that part of the increase in dry weight in relation to control plants might be due to salt accumulation in leaf cells. However, this would not explain the performance of the water stressed plants. It is possible that in both salt and water stressed plants, the progressive decrease in shoot water %, significantly different from the control plants at the end of the experiment, might have caused related histological changes such as increasing wax accumulation on the leaf surface, increasing cell layers of sclerenchyma and hydrenchyma tissues.

Stress Effect on Photosynthetic Pigments and Soluble Protein

Concentrations of photosynthetic pigments and soluble protein (Figure **2**) were determined in terms of dry weight to correct for differences in water % between the treatments.

A significant interaction between treatment and time was found in the case of chlorophyll a (Chl a, two-way ANOVA, P<0.05), but not in the case of chlorophyll b (Chl b) and carotenoids. Significant differences between treatments were not evident in the case of Chl b (Figure **2B**). Main differences between treatments were seen on DAT 12, when Chl a and carotenoids concentrations increased in water stressed and decreased in salt stressed plants (Figure **2A** and **C**).

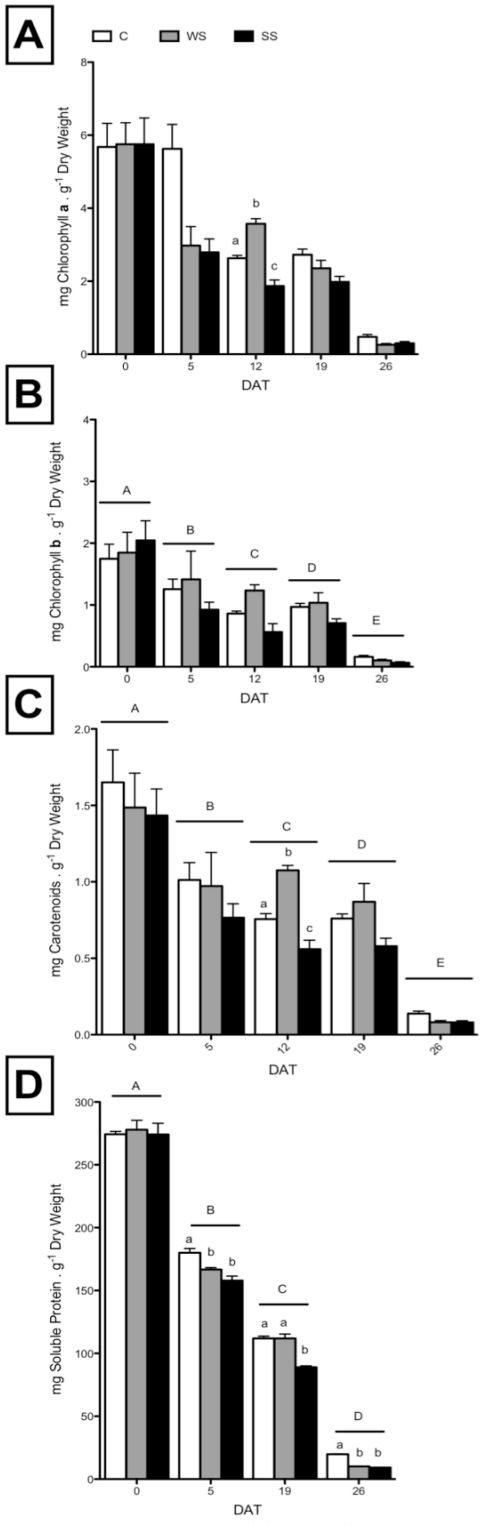

Figure 2: Concentration of Chlorophyll **a** (mean±SD, n=4) (**A**), Chlorophyll **b** (mean±SD, n=4) (**B**), Carotenoids (mean±SD, n=4) (**C**) and Soluble Protein (mean±SD, n=4) (**D**) in the shoot of *C. edulis* seedlings well watered (control - C), subjected to water stress (WS) or to salt stress (SS). DAT - days after treatment. Capital letters indicate significant differences between sampling times. Groups displaying different letters, at each sampling time, are significantly different (P<0.05; Two-Way ANOVA followed by Bonferroni multi comparison test).

This was however a transient effect since at DAT 26 both treatments showed values not significantly different from the control. The effect of time in the concentration of Chl b and carotenoids was found significant (two-way ANOVA, P<0.05), as was the effect of treatment in the case of carotenoids (two-way ANOVA, P<0.05). The ratio between Chl a/Chl b was not affected by treatment or time (data not shown).

A significant effect of the interaction between treatment and time was found (two-way ANOVA, P<0.05) in the case of shoot soluble protein concentration. At the end of the experiment both water stressed and salt stressed plants showed a decrease of 50% in relation to control (Figure **1D**).

Previous studies with water stressed CAM species also did not find major effects in photosynthetic pigments [27, 28] or soluble protein [27] and to the best of our knowledge there are no previous determinations of these parameters in *C. edulis*.

The effect of time in the decrease of photosynthetic pigments and soluble protein concentrations in the shoots was stronger from 19 DAT to 26 DAT. This suggests that during this period the balance between source and sink leaves might have changed, leading to a dilution effect of those metabolites when considering the shoot as a whole. Since this effect was seen across the three regimes, it might also indicate a developmental milestone change in allocation, favouring structural tissues. If this were the case, the difference in dry weight seen at the end of the experiment could partly be due to a stronger ontogenetic drift effect caused by salt stress, leading to an increase in structural dry mass, in advance of that of control and water stressed plants.

Effects on Photosynthesis

The rapid light response curves showed in Figure **3** revealed a higher relative electron transport rate (ETR) in the leaves of plants subjected to salt stress and water stress, in relation to control. This effect is visible for both treatments on 5 DAT (Figure **3A**), and still on 12 DAT (Figure **3B**) for plants under salt stress. The corresponding values of, the ratio between the maximum photosynthetic electron transport rate (ETRm), an analogous to maximum photosynthetic rate in classic light response curves, and the minimum saturation irradiance (Ek), are related to quantum efficiency of photosynthesis and shown in Figure **3E**. A significant effect of time was found for this parameter (two-way ANOVA, P<0.05), and although no significant

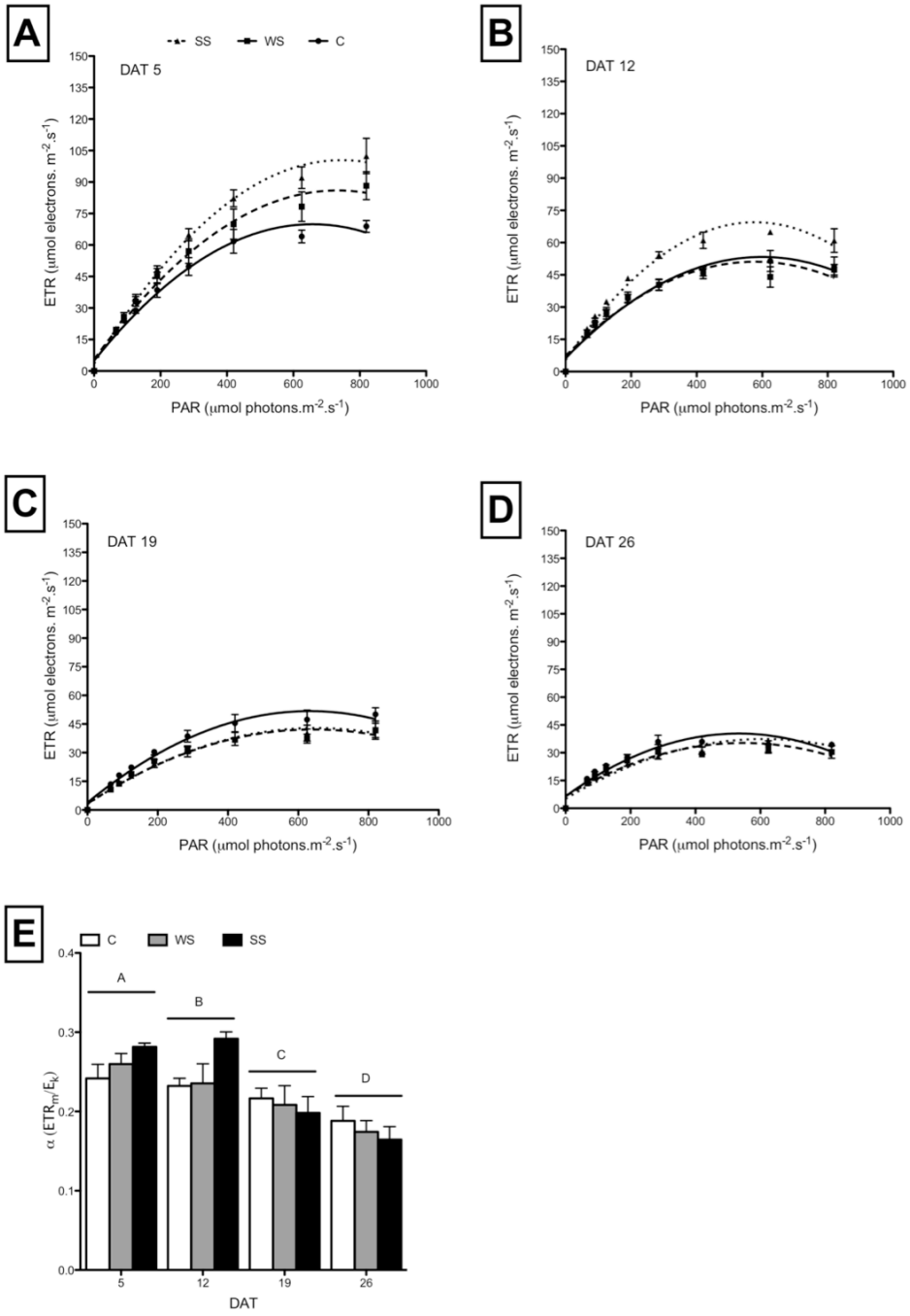

Figure 3: Rapid light response curves (RLRCs) at 5 (**A**), 12 (**B**) 19 (**C**) and 26 (**D**) days after treatment (DAT) and corresponding values of (ETR_m/E_k) (**E**) as a measure of the initial slope of an RLRC, which is related to quantum efficiency of photosynthesis. Mean±SD, n=12. ETR_m - maximum electron transport rate (mol electrons.$m^{-2}.s^{-1}$); E_k - minimum saturating irradiance (mol photons.$m^{-2}.s^{-1}$). DAT - days after treatment, C - Control, WS - Water stress, SS- Salt stress. Capital letters indicate significant differences between sampling times. Groups displaying different letters, at each sampling time, are significantly different ($P<0.05$; Two-Way ANOVA followed by Bonferroni multi comparison test).

differences were found between treatments, a tendency for higher values in salt stressed plants in 5 DAT and 12 DAT is evident.

The measuring program used for the RLRCs determinations exposes the sample to increasing intensities of actinic illumination (or PAR). The time interval of each intensity level is too short for full equilibration of photosynthetic reactions. Therefore these curves provide information on the present state of photosynthesis and should not be confused with classical light response curves in which photosynthetic

rates under steady state conditions are plotted against increasing light intensities, and determined under non-limiting CO_2 concentrations. With increasing PAR the potential potential ETR increases provided that all PSII reaction centres are open (Q_A oxidized). When the rate of quanta absorption exceeds the capacity of carbon fixing dark reactions, electrons will accumulate and back-up at the acceptor side of PSII. Therefore, the Q_A pool will remain partially reduced and ETR will saturate (ETR_m). The results obtained in this study suggest a higher photosynthetic capacity of *C. edulis* plants under salt stress and water stress at DAT 5, which decreases below control levels as the leaf matures.

It has been reported that in inducible CAM, night-time CO_2 assimilation is induced in C3 plants by factors such as drought [29] and salinity [30]. That might be the case with *C. edulis* but this aspect was not addressed in our work. Conversely, the responses obtained in the RLRCs rather suggest different capacities of the photosynthetic apparatus, in relation to treatment, to transport electrons though PSII reaction centres. This would then be related to the levels of oxidized Q_A and proteins involved in charge separation at the P680 reaction centre.

Alterations to Gene Expression under Stress

The preliminary results of gene expression profiling detected in our work (Table **1**) showed that both water and salt stress induced differential gene expression. Five of the six fragments indicated correspond to bands of higher intensity obtained from pooled samples of WS or SS plants at 5 DAT. The results of the BLAST analysis show a consistent correspondence with differential ESTs previously detected in relation to drought stress of *Cicer arietinum* and *Camellia sinensis*, using the SSH (Suppression Subtractive Hybridization) technique. At the present state of knowledge it is not possible to identify the putative genes corresponding to these transcripts. Clearly more work in this area is needed to identify sets of genes related to the response of *C. edulis* and other species to water and salt stress and validate them through quantitative PCR (qPCR).

CONCLUSION

Young plants of *Carpobrotus edulis* show high tolerance to water and salt stress, without diminished shoot dry weight or photosynthetic pigments concentration. Changes in the quality of the biomass accumulated as well as an ontogenetic drift effect is suggested as a result of overcoming salt stress.

In this work we did not analyse the root system. It has been described that some plants have the ability to increase root growth at the early stages of drought stress [31] thus being able to better explore the rizosphere for water. In this context the root/shoot ratio has long been used as a criterion to describe plant capacity for drought resistance [32]. Other studies have shown that changes in the number and size of root cortical cells has substantial effects on the metabolic cost of soil exploration and thereby water acquisition under drought [33]. Clearly the characterization of *C. edulis* root system in future work will add to our knowledge of this species performance under salt and water stress

Overall this work provides new insights about the response of young plants of *C. edulis* to water and salt stress, contributing to understanding the functional processes underlying the success of this species when introduced in coastal sand dunes.

Table 1: Result of the BLAST Analysis for Identification of the Differentially Expressed Genes (DEGs) Detected in *Carpobrotus edulis*. DAT - Days after Treatment

Carbobrotus edulis DEG	GenBank Accession	Homology (%)
A4-1 (297 bp) from Control cDNA at 5 DAT	HO066336 Cicer arietinum cDNA SSH library from drought stressed shoots	90
A9-2 (585 bp) from SS cDNA at 5 DAT	GR409837 Cicer arietinum cDNA library from slow drought stressed roots	99
A15-4 (428 bp) from WS cDNA, 5 DAT	GW317115 Camellia sinensis var. assamica cDNA SSH library from roots subjected to drought stress	90
A15-5 (424 bp) from SS cDNA, 5 DAT	GR409050 Cicer arietinum cDNA library from field drought stressed roots	93
A15-6 (337 bp) from WS cDNA, 5 DAT	GR391501 Cicer arietinum cDNA library from dehydration stressed roots	100
A15-8 (294 bp) from SS cDNA, 5 DAT	GW317130 Camellia sinensis var. assamica cDNA SSH library from roots subjected to drought stress	88

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