Comparative studies on the stress responses of two *Bupleurum* (Apiaceae) species in support of conservation programmes

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Highlights

- *Bupleurum tenuissimum*, a rare, threatened species, behaves like a moderate halophyte
- In contrast, *B. fruticosum* is salt-sensitive but more tolerant to drought
- Changes in biochemical stress markers explain the two species' opposite behaviour
- The obtained results are relevant for site selection in reintroduction programmes

Abstract

Bupleurum tenuissimum is a rare species in Spain, which became extinct in recent decades in many localities, including protected areas. In the study area, the 'Albufera' Natural Park (SE Spain), the species was present in *Limonietalia* grasslands, a priority and endangered habitat, but has not been observed in the last 40 years. At present, it is included in conservation programmes aimed at its reintroduction in this territory. This study's primary objective was to establish the limits of tolerance of this species to salt and water stress under controlled greenhouse conditions. A congeneric species, *B. fruticousm*, was included as comparative material to get insights into its stress response mechanisms. Analysis of changes in growth parameters (stem length, number of leaves, root length, fresh weight and water content of roots and leaves) in response to the applied stress treatments confirmed that *B. tenuissimum* behaves as a facultative (moderate) halophyte susceptible to drought stress, whereas *B. fruticosum* is not severely affected by water stress but is sensitive to salinity, even at low NaCl concentrations. The stress-induced changes in several biochemical parameters (contents of photosynthetic pigments, ions, osmolytes, oxidative stress markers, and non-enzymatic antioxidant compounds, and specific activities of relevant antioxidant enzymes) explained the differential behaviour of the investigated *Bupleurum* species. In addition to the novelty of these findings in a genus that has rarely been studied from this perspective, these results will be relevant for implementing and managing *B. tenuissimum* conservation programmes, guiding the selection of reintroduction sites. The study also provides valuable information on the stress tolerance of *B. fruticosum*, with great potential for the ecological restoration of Mediterranean dry forest vegetation and riparian thickets.

Keywords

Antioxidant systems, drought tolerance, endangered habitats, ion transport, osmolytes, salinity tolerance

Introduction

There is a general agreement that studying the abiotic stress tolerance mechanisms in plants is of enormous practical importance for agriculture. However, the interest of these studies for helping design and implement conservation and regeneration strategies in natural habitats of great ecological value, such as salt marshes, has been less appreciated.

The vegetation of salt marshes includes structural species, which play a fundamental role in the structures of the plant communities; these taxa are relatively abundant and fairly homogeneous in the different territories (Sarika and Zikos, 2020). What provides individuality and added value to salt marsh ecosystems are less abundant, differential species, those on which the uniqueness of each salt marsh depends. Salt marsh communities, particularly coastal lagoons and salt steppes bordering them, are considered priority endangered habitats. In the European Union, their protection is encouraged by the Habitats Directive, endorsing the actions of national and regional governments to safeguard a significant proportion of their surfaces through the Natura 2000 network. In the Valencian Community, these habitats are well characterised (Laguna, 2003), and most of their sites are included in the boundaries of several regional protected areas such as Natural Parks (Ballester et al., 2003), Catalogued Wetlands and Plant Micro-reserves (Laguna et al., 2004). In the Albufera Natural Park, located near the city of Valencia (Eastern Spain) and the reference area of this study, many of these differential species are highly threatened (Ballester et al., 2003); some have even disappeared from this protected area, although they are still present in other Valencian salt marshes, so its future recovery can be performed through reintroduction programmes. However, effective conservation and regeneration programmes of any endangered species require the deepest possible knowledge of the responses of these plants to the environmental stresses affecting them in their natural habitat (Maschinski et al., 2012). The main physiological and biochemical mechanisms of tolerance to drought, salinity and other abiotic stress conditions are known for some of the structural species (Redondo-Gómez et al., 2009; Katschnig et al., 2013; Gil et al., 2011, 2014; Pardo-Domenech et al., 2015; Al Hassan et al., 2016a, 2017; González-Orenga et al., 2019a, 2020a, 2021). However, no or only little information is available on the stress responses of differential species, which are often endemic, rare and/or endangered.

There is overwhelming evidence that all plants use the same basic mechanisms of response to adverse environmental conditions. Generally, however, these responses do not lead to stress tolerance – except for a small percentage of species adapted to different types of abiotic stress in their natural habitats, such as the salt marsh plants (Kumari e al., 2015; Al Hassan et al., 2017). These conserved responses include, amongst others, the control of ion transport, compartmentalisation of toxic ions in vacuoles with the concomitant accumulation of osmolytes in the cytosol to maintain cellular osmotic balance, and the activation of antioxidant systems, both enzymatic and non-enzymatic (Zhu, 2001; Munns, 2002; Volvok, 2015; Vinocur and Altman, 2005; Munns and Tester, 2008; Pollastri and Tattini, 2011; Ozgur et al., 2013; Bose et al., 2014; Kumari et al., 2015). However, the relevance and the relative contribution of these responses to the mechanisms of tolerance of a given species to a particular stress situation are generally unknown.

A rewarding strategy to investigate plants' stress tolerance mechanisms is to perform comparative studies of the stress responses in genetically related taxa with different stress sensitivity. Studies conducted by our research group in recent years have been based on this experimental approach and have provided several interesting and novel results, both in wild plants of the genera *Plantago* L. (family Plantaginaceae) and *Juncus* L. (family Juncaceae) (including halophytes in both cases) and in cultivated plants such as beans and eggplant (Al Hassan et al., 2016 b, c, d, 2017; Plazas el al., 2019).

In the present study, the same strategy was applied to determine the stress tolerance mechanisms of *Bupleurum tenuissimum* L. (Apiaceae), a rare halophyte with high conservation interest, as it became extinct in the Albufera Natural Park and is considered for reintroduction programmes. *Bupleurum fruticosum* L. was included in the study as comparative material.

B. tenuissimum is an annual dwarf herb up to 40 cm in height, distributed in the West, Central and Southern Europe, southern England and coastal areas of the North Sea to southern Sweden, southwestern Asia and northwestern Africa (Neves, 2003). It also has a scarce presence in the Iberian Peninsula and the Balearic Islands, living in marshes, on the borders of salt lagoons and watercourses, on marly, clayey, or brackish soils. Although it is distributed through most Spanish provinces (Neves, 2003), their populations are scattered and often occupying small areas. It can be found between sea level and 800 m in altitude. According to Rivas-Martínez et al. (2002), it is a characteristic species of the phytosociological class *Saginetea maritimi*, although it can be found in other saline vegetation types. In the Valencian region, it grows on terophytic grasslands placed on salty, moist soils (Mateo and Crespo, 2014). According to BDBCV (Biodiversity Data Bank of the Valencian Community), the species has current or recently recorded populations in eight 1×1 km squares – official Spanish grid UTM, datum ETRS89 – so that it can be considered as a very rare species (see Mateo and Crespo, 2014). Besides, the species has not been relocated in seven 10×10 km squares, where it was found during the second half of the 20th century. Amongst those former records, Carretero and Aguilella (1995) cited the species from the 10×10 km squares UTM 30S YJ26 and YJ35, matching the site 'Devesa de El Saler', currently included in the boundaries of the Albufera Natural Park (Ballester et al., 2003). This report is based on an unpublished record of Dr José Mansanet, made in 1978. Thus, this species has not been rediscovered for more than 40 years in this area. The species lived mainly in *Limonietalia* grasslands, an endangered, priority habitat protected by the EU's Directive of Habitats. In addition, some of the habitats where B. tenuissimum grows in Europe have been listed as endangered in the European Red List of Habitats, i.e., the inland saline or brackish helophyte beds, or the temperate inland salt marshes (Janssen et al., 2016). Currently, *B. tenuissimum* is listed as a threatened or close to threatened species at least for Great Britain (Cheffings and Farrell, 2005), particularly for England (Stroh et al., 2014), the Czech Republic and Slovakia (Čerovský et al., 1999; Grulich, 2012; Turis et al., 2014), Sweden (Gärdenfors, 2000), Estonia and the Baltic regions of Poland and Germany (Ingelög et al., 1993), and Turkey (Özhatay et al., 2009). On the contrary, it has been recorded as an exotic invasive species in Argentina and New Zealand (Randall, 2014). In Spain, *B. tenuissimum* was considered an endangered species only for Balearic Islands (Barreno et al., 1984), but it has not been evaluated in the current versions of the national red list (Bañares et al., 2008). At the regional level, it is currently protected as endangered in Murcia (Sánchez Gómez et al. 2002) and Valencia. Indeed, due to its rareness and its ecological requirements, living on endangered habitats, the regional government of the Valencian Community has listed *B. tenuissimum* as strictly protected in the category 'Non-Catalogued Protected Species', through the Valencian Decree 70/2009 (Generalitat Valenciana, 2009); this protection list has been renewed by the Order 6/2013 (Generalitat Valenciana, 2013). The regional Wildlife Service carries out conservation actions from the currently existing populations located outside the Albufera Natural Park (Laguna et al., 2020).

B. fruticosum is a nanophaerophyte up to 200 (300) cm in height, characteristic of Mediterranean shrublands of the phytosociological order *Pistacio-Rhamnetalia alaterni* (Rivas-Martínez et al., 2002), up to 1.200 m.o.s.l. Its behaviour varies depending on the bioclimatic and geographical conditions, but it has been considered a good indicator of slightly acidic and moist soils in the Valencian Community. The species often grows in plant communities dominated by *Arbutus unedo* L. and *Erica arborea* L., or characterises the understory of cork oak (*Quercus suber* L.) and holm oak (*Q. ilex* L.) forests (Costa, 1999), and thermophile pinewoods (*Pinus halepensis* Mill., *P. pinaster* Aiton). *B. fruticosum* frequently joins *Jasminum fruticans* L., *Coriaria myrtiol*ia L., *Myrtus communis* L., *Nerium oleander* L. or *Osyris alba* L., forming sub-riparian shrublands placed on ravines or intermittent riverine sites ('ramblas') in low altitudes (Mateo and Crespo, 2014), often on sandy soils formed after sandstones.

The main objective of this work was to obtain data on the responses to salt stress and water deficit of *B. tenuissimum* under controlled greenhouse conditions. This information could be helpful for conservation and reintroduction programmes, representing a key issue for selecting appropriate sites for reintroductions and other translocations of the plants. On the other hand, comparing its stress responses to those of *B. fruticosum*, a congeneric species adapted to xeric

conditions on non-salty soils, should highlight the relevant mechanisms of stress tolerance in this poorly studied genus. To this aim, the plants were subjected to water stress (complete withholding of irrigation) and salt stress (watering with NaCl solutions of increasing concentration) treatments to analyse their effects on (i) plant growth, (ii) ion transport and accumulation, (iii) osmolyte biosynthesis, and (iv) activation of antioxidant systems.

Material and methods

Plant material

Seeds were collected in the field in two different locations in the Valencia province, those of *B. tenuissimum* from the Plant Micro-reserve Font Amarga in Villanueva de Castellón (geographical provenance: 39º 2' 51.23'' N – 0º 30' 43.06''W) and those of *B. fruticosum* from the Natural Park Sierra de Calderona (provenance: 39º 41' 13.43''N – 0º 30' 42.15'' W). The seeds were provided by the Centre for Forestry Research and Experimentation (CIEF, Generalitat Valenciana). A herbarium voucher of *B. tenuissimum* is kept in the Herbarium of the Botanical Garden of the University of Valencia at VAL, with the barcode VAL202329.

Plant growth and stress treatments

Seeds were sown in seedbeds with a mixture of commercial peat and vermiculite (3:1) and watered twice a week with Hoagland nutrient solution. One month after sowing, the seedlings were transplanted into 12 cm diameter pots filled with 500 g of the same substrate. Treatments were started four months later, when the plants were fully developed, with 7-12 true leaves in *B. fruticosum* and 13-15 in *B. tenuissimum*. Thirty plants with uniform size were selected from each species, to use five individual plants as replicates per treatment. The following treatments were applied: control (irrigation with tap water, twice per week), water stress (complete withholding of irrigation) and salt stress (irrigation, twice per week, with aqueous solutions of 75, 150, 300, and 450 mM NaCl). The plants were maintained in a controlled environment chamber in the greenhouse, under long-day photoperiod (16 h of light), temperatures of 23 °C during the day and 17 °C at night, and 50-80% relative humidity.

The treatments were stopped, and the plants were harvested after 21 days when water-stressed plants showed pronounced wilting symptoms. During the whole period, the moisture and the electrical conductivity (EC) of the substrates were measured twice a week with a WET-2 Sensor (Delta - T Devices, Cambridge, UK). The number of leaves and the plants' height were determined at the beginning and at the end of the treatments, when the plant material was sampled. For each plant, the leaves and the roots were weighed separately (fresh weight, FW), and the root length was measured. A fraction of the material was dried in an oven at 65 °C, until a constant weight was reached (~ 72 h), and weighed again (dry weight, DW), to quantify the water content percentage according to the formula:

$$WC\ (\%) = [(FW\text{-}DW)\ FW^{-1}]\ 100$$

Fresh material was used immediately or flash-frozen in liquid N_2 and stored at -75 °C, and dry material was stored at room temperature in tightly closed tubes, before being used for subsequent biochemical analysis.

Photosynthetic pigments

Leaf contents of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Caro) were determined using spectrophotometric techniques, following the method described by Lichtenthaler and Welburn (1983). Fresh plant material (50 mg) was extracted with one mL of ice-cold 80% acetone. The samples were mixed overnight (12-15 h) in a shaker in the absence of light and then centrifuged at 13,300 *g* for 10 minutes at 4 °C. The absorbance of the supernatants was measured at 470, 646 and 663 nm, and the concentrations of the pigments were calculated

according to equations previously described (Lichtenthaler and Wellburn 1983) and expressed in mg g^{-1} DW.

Quantification of ions

The concentrations of sodium (Na^+), potassium (K^+), chloride (Cl^-), and calcium (Ca^{2+}) were determined in roots and leaves. Samples (50 mg) of ground dry plant material were suspended in 15 mL of deionised water, heated at 95 ºC in a water bath for one hour, followed by cooling on ice and filtration through a 0.45 µm nylon filter, as indicated by Weimberg (1987). The cations were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA) and the anion using a chlorimeter (Sherwood, model 926, Cambridge, UK).

Quantification of osmolytes

Proline (Pro) was quantified from 50 mg of fresh leaf material extracted in 3% aqueous sulphosalicylic acid and subsequently mixed with acid ninhydrin solution, incubated for one h at 95 ºC, cooled on ice and then extracted with two volumes of toluene, following the classical procedure of Bates et al. (1973). The absorbance of the supernatant was read at 520 nm, using toluene as a blank. Samples containing known Pro concentrations were assayed in parallel to obtain a standard curve. Pro concentration was expressed as µmol g^{-1} DW.

Total soluble sugars (TSS) were measured according to the method described by Dubois et al. (1956). Fresh leaf material was ground in liquid N_2 and extracted with 80% (v/v) methanol. After mixing in a rocker shaker for 24 h., the samples were centrifuged at 13,300 *g* for 10 min; supernatants were collected, appropriately diluted with water, and supplemented with concentrated sulphuric acid and 5% phenol. After 20 min incubation at room temperature, the absorbance was measured at 490 nm. TSS concentrations were expressed as equivalents of glucose, used as the standard (mg eq. glucose g^{-1} DW).

The concentration of glycine betaine was determined as described by Grieve and Grattan (1983), with some modifications (Valadez-Bustos et al., 2016). Fresh plant material (0.15 g) was shaken in 1.5 ml miliQ water for 24 hours at 4 ˚C, followed by centrifugation at 13,300 *g* for 10 min. The recovered supernatant was mixed (1:1) with a 2 N solution of H_2SO_4 and incubated on ice for 1 h. Then, 125 µl of the sample were supplemented with 50 µl of an ice-cold KI-I_2 solution (prepared by dissolving 20 g of KI and 15.7 g of iodine in 100 ml of sterile water), which causes the precipitation of glycine betaine in the form of golden crystals. All the remaining steps were performed in the dark. The tubes were stored at 0-4 ˚C for 16 h and then centrifuged at 13,300 *g* for 45 min at 0 ˚C. The supernatant was carefully eliminated and the glycine betaine crystals were dissolved into 1.4 ml of cold 1,2-dichloroethane; the samples were maintained for 2.5 h under dark and cold conditions and finally their absorbance at 365 nm was determined. Reaction mixtures containing known glycine betaine amounts were run in parallel to obtain a standard curve. Glycine betaine concentration was expressed as µmol g^{-1} DW.

Determination of oxidative stress markers and antioxidant compounds

Malondialdehyde (MDA), total phenolic compounds (TPC), and total flavonoids (TF) were measured in extracts prepared in 80% (v/v) methanol from 50 mg ground fresh leaf material. For MDA quantification, the method described by Hodges et al. (1999) was followed, with the modifications introduced by Taulavouri et al. (2001). Extracts were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA) – or with 20% TCA without TBA for the controls – and then incubated at 95 °C for 20 min, cooled on ice and centrifuged at 13,300 *g* for 10 min at 4 °C. The absorbance of the supernatants was measured at 532 nm. The non-specific absorbance at 600 and 440 nm was subtracted, and the MDA concentration was determined using the equations by Taulavouri et al. (2001). MDA contents were expressed as nmol g^{-1} DW.

H_2O_2 was quantified according to the method by Loreto and Velikova (2002), from 50 mg dry leaf material extracted with a 0.1% (w/v) trichloroacetic acid (TCA) solution, followed by centrifuging the extract. The supernatant was thoroughly mixed with one volume of 10 mM potassium phosphate buffer (pH 7) and two volumes of 1 M potassium iodide. The sample's absorbance was determined at 390 nm, and the concentration was calculated against an H_2O_2 standard calibration curve and expressed as μmol g^{-1} DW.

TPCs were measured using the Folin-Ciocalteu reagent according to the method described by Blainski et al. (2013). Methanol extracts were mixed with the reagent and Na_2CO_3 and, after 90 min of incubation in the dark, the absorbance was measured at 765 nm. A standard reaction was performed in parallel using known amounts of gallic acid (GA), and TPC contents were reported as equivalents of GA (mg eq. GA g^{-1} DW).

Quantification of TFs followed the protocol by Zhisen et al. (1999). Methanol extracts of each sample were reacted with $NaNO_2$ and $AlCl_3$ under alkaline conditions, and the absorbance at 510 nm was measured. The concentration of TFs was expressed as equivalents of catechin, used as the standard (mg eq. C g^{-1} DW). This protocol is often claimed to measure 'total flavonoids' in the sample, although this is not strictly true. The method is based on the nitration of aromatic rings containing a catechol group. Several flavonoids, e.g., flavonols and flavanols, and other phenolics, such as caffeic acid and derivatives, react in this way. Nevertheless, phenolic compounds detected in the assay are all potent antioxidants, and there is a good correlation between their levels and the total antioxidant activity of the samples (Zhishen et al., 1999).

Activity of antioxidant enzymes

Crude protein extracts were prepared from the leaf materialand stored frozen at -75 °C, as described by Gil et al. (2014). The protein concentration in extracts was determined according to Bradford (1976) using the Bio-Rad reagent and bovine serum albumin (BSA) as the standard. The specific activities in the protein extracts of the selected antioxidant enzymes were determined by spectrophotometric assays.

Superoxide dismutase (SOD) activity was determined following Beyer and Fridovich (1987) by monitoring spectrophotometrically at 560 nm the inhibition of nitroblue tetrazolium (NBT) photoreduction; the reaction mixtures contained riboflavin as the source of superoxide radicals. One SOD unit was defined as the amount of enzyme to cause 50% inhibition of the NBT photoreduction under the assay conditions, as described in the original protocol.

Catalase (CAT) activity was measured following the consumption of H_2O_2 added to the extracts by the decrease in absorbance at 240 nm (Aebi, 1984). One CAT unit was defined as the amount of enzyme decomposing one mmol of H_2O_2 per minute at 25 °C.

Glutathione reductase (GR) activity was quantified as described by Connell and Mullet (1986), following the oxidation of NADPH − the cofactor in the GR-catalysed reduction of oxidised glutathione (GSSG) – by the decrease in absorbance at 340 nm. One GR unit was defined as the amount of enzyme necessary to oxidise one mmol of NADPH per minute at 25 °C (Connell and Mullet, 1986).

*Statistical analy*sis

Data were analysed using Statgraph v. XVIII (Statgraphics Technologies, The Plains, VA, USA). Significant differences between treatments were tested by one-way analysis of variance (ANOVA) at the 95% confidence level, and post-hoc comparisons were made using Tukey's HSD (honestly significant difference) test ($p < 0.05$). Before the analysis of variance, the Shapiro-Wilk test was used to check for the validity of the normality assumption and Levene's test for the homogeneity of variance. A principal component analysis (PCA) was performed using the means

of all growth and biochemical parameters and final measurements of substrate moisture and electrical conductivity. All the means throughout the text are followed by SE.

Results

Electrical conductivity and moisture of the substrate

The substrate electrical conductivity (EC) in the pots of plants treated with the highest salt concentrations increased considerably, in a time-dependent manner, following a similar pattern for both species. After three weeks of treatment, EC reached values of about 50 dS m^{-1} (in the pots watered with 300 mM NaCl) or 70 dS m^{-1} (for the 450 mM NaCl treatments). Final EC values were much lower for the 75 and 150 mM NaCl treatments (around 7 and 10 dS m^{-1}, respectively) but still above the threshold conductivity for soils to be considered non-saline, which corresponds to 4 dS m^{-1}. EC did not vary significantly in pots from the control or water stress treatments (Figures 1A, B). Soil moisture was maintained constant in the controls during the whole treatment period, whereas it showed some oscillations in the salt treatments, although the final values after three weeks were in all cases similar to those of the control. On the contrary, soil moisture strongly decreased in the water stress treatments by more than 50% already during the first week, reaching final values below 1% in both species (Figures 1C, D).

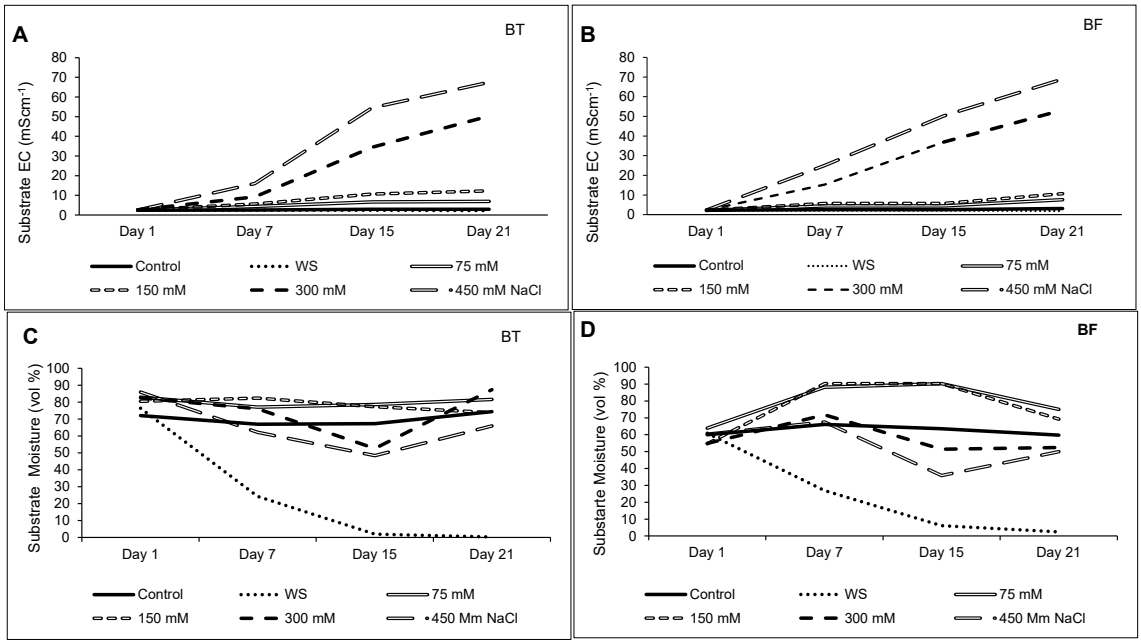

Figure 1. Electrical conductivity (EC) (A and B) and moisture (C and D) of the substrate, measured by a WET sensor twice a week during the 21 days of treatments. *Bupleurum tenuissimum* (BT, panels A and C) and *B. fruticosum* (BF, panels B and D). Values are means of five pots per treatment and species (n =5).

Analysis of plant growth parameters

Both stress treatments inhibited plant growth in the two *Bupleurum* species (Table 1, Figure 2), although they were affected differently. From the observed effects of the water deficit treatment, it appears that *B. tenuissimum* is more sensitive to drought than *B. fruticosum*, as shown by a relatively stronger reduction in some of the measured growth parameters; for example, in the increment on the number of leaves during the treatment period, in relation to the non-stressed controls (Table 1). Similarly, the fresh weight of *B. tenuissimum* plants underwent a reduction of almost 90% in roots and 80% in leaves at the end of the treatment, *versus* ca. 55%, in both organs, in the case of *B. fruticosum* (Figures 2A, B). Water stress caused root dehydration in the two species, but to a lesser extent in the more tolerant *B. fruticosum* (Figure 2C), whereas no water

loss was observed in the leaves of the stressed plants of this species (Figure 2D). On the other hand, the relative reduction of stem growth was the same for *B. tenuissimum* and *B. fruticosum* plants, 50% of the control (Table 1). Root length was the only parameter that increased significantly, albeit slightly, in response to the water stress treatment (Table 1); this result suggests that the plants mimic their behaviour in nature under drought conditions, stimulating root growth searching for deeper and more humid soil layers.

Growth was also inhibited, in plants of the two selected species, in response to salt stress but, in contrast to the higher drought tolerance of *B. fruticosum*, *B. tenuissium* was shown to be the most salt-tolerant, particularly at high external salinities. This difference was observed, for example, in the relative salt-induced reduction of stem length increment and root length during the treatments, as compared to the non-stressed controls, especially in the presence of 300 and 450 mM NaCl (Table 1). Root fresh weight was not affected at all by the salt treatments in *B. tenuissium* plants, whereas in *B. fruticosum*, mean FW values decreased in parallel to the increase in external salinity, although the differences with the control were not statistically significant (Figure 2A). On the contrary, leaf FW was reduced significantly in the presence of high salt concentrations, an effect that was slightly stronger in *B. fruticosum* (Figure 2B). The selected taxa were shown to be highly resistant to salt-induced dehydration; water content did not change at all in roots, in any of the treatments, whereas a slight but significant water loss was observed in leaves, although only at the highest NaCl concentrations tested (Figures 2C, D).

Table 1. Growth responses in the two *Bupleurum* species after 21 days of water stress (complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments. The values shown are means ± SE (n = 5). The same letters within each column indicate homogeneous groups between treatments for each species according to the Tukey test ($p < 0.05$). For each treatment and measured variable, percentages of variation with respect to mean control values (considered 100%) are indicated in parenthesis. Increments in stem length and leaf number were calculated as the difference between values at the end and the beginning of the treatment period.

Species	Treatments	Stem Length Increment (cm)	Number of Leaves Increment	Root Length cm)
B. tenuissimum	Control	32.24 ± 2.08^{c}	40.40 ± 2.31^{c}	22.30 ± 1.46^{ab}
	Water Stress	16.38 ± 0.86^{a} (-50.2%)	13.20 ± 0.66^{a} (-67.3%)	23.24 ± 1.94^{ab} (+4.2%)
	75 mM	31.94 ± 0.12^{c} (-1%)	38.60 ± 1.29^{c} (-4.5%)	18.20 ± 2.17^{a} (-18.4%)
	150 mM	28.14 ± 1.20^{b} (-12.8%)	36.25 ± 4.169^{c} (-10.3%)	25.84 ± 1.13^{b} (+15.9%)
	300 mM	28.00 ± 0.65^{b} (-13.2%)	22.80 ± 1.62^{b} (-43.6%)	20.70 ± 2.87^{ab} (-7.2%)
	450 mM NaCl	18.70 ± 1.54^{a} (-62%)	10.00 ± 0.63^{a} (-75.2%)	19.73 ± 1.71^{ab} (-11.5%)
B. fruticosum	Control	7.60 ± 1.76^{c}	4.80 ± 0.73^{a}	17.00 ± 2.66^{b}
	Water Stress	3.80 ± 0.89^{b} (-50%)	3.00 ± 0.55^{a} (-37.5%)	17.20 ± 3.29^{b} (+1.2%)
	75 mM	4.70 ± 1.26^{bc} (-39%)	6.5 ± 1.29^{b} (+35.4%)	15.00 ± 1.15^{ab} (-11.8%)
	150 mM	1.94 ± 0.51^{ab} (-74.5%)	4.20 ± 1.93^{a} (-12.5%)	13.75 ± 3.56^{ab} (-19.2%)
	300 mM	0.50 ± 0.50^{a} (0.50 -93.5%)	2.80 ± 0.86^{a} (-41.7%)	7.80 ± 2.69^{a} (-54.1%)
	450 mM NaCl	0.10 ± 0.10^{a} (-98.7%)	2.60 ± 1.24^{a} (-54.2%)	10.00 ± 2.09^{ab} (-41.2%)

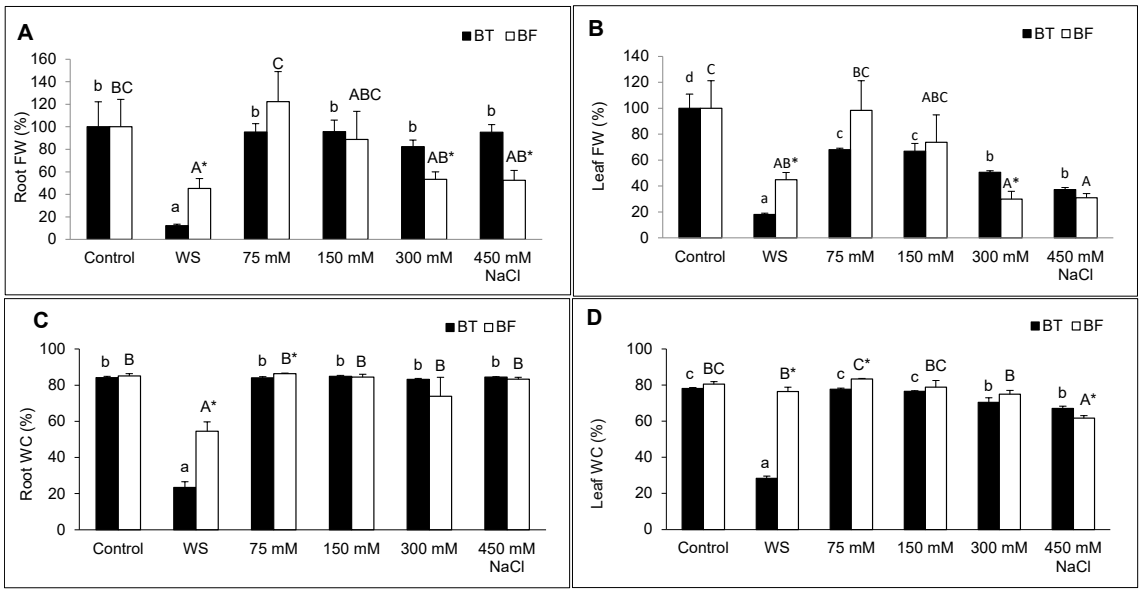

Figure 2. Effect of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments, on fresh weight (FW) of roots (A) and leaves (B), and water content (WC) of roots (C) and leaves (D), in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF, white bars). Mean values ± SE (n = 5) are shown. Same letters (lowercase for *B. tenuissimum* and uppercase for *B. fruticosum*) indicate homogenous groups according to the Tuckey HSD test ($p < 0.05$). An asterisk (*) indicates significant differences between the two species for the same treatment.

Photosynthetic pigments

Stress-induced changes in the concentration of photosynthetic pigments correlated with the relative tolerance of *B. tenuissimum* and *B. fruticosum* to water deficit and salt stress and followed the same pattern for Chl a, Chl b and total carotenoids (Figure 3). Thus, the water stress treatment caused a substantial reduction in the levels of the three pigments in the more drought-sensitive *B. tenuissimum*, whereas no significant differences with the non-stressed controls were found for the more tolerant *B. fruticosum*. Mild and moderate salinity conditions (75 and 150 mM NaCl) did not affect the concentrations of chlorophylls or carotenoids, and only in the presence of high salt concentrations (300-450 mM NaCl) significant reductions in the pigment's contents, as compared with the controls, were observed. This reduction was less pronounced in the more salt-tolerant *B. tenuissimum*, which showed significantly higher values of the three pigments in the 450 mM NaCl treatment (Figure 3). It should be mentioned that *B. tenuissimum* also contained higher levels of chlorophylls (but not of carotenoids) than *B. fruticosum* in control, non-stressed plants (Figures 3A, B).

Quantification of ions

It seemed logical to expect that the water stress treatment, which did not alter the electrical conductivity of the substrates in the pots, should not modify the ion contents in the plants. This expectation was confirmed in our experiments for Na^+, Cl^- and Ca^{2+} concentrations in roots and leaves of stressed plants of the two *Bupleurum* species, which were not significantly different from those in the corresponding controls (Table 2). Changes in K^+ levels in response to water deficit stress, however, followed a different pattern, increasing more than two-fold in leaves of *B. tenuissimum*, whereas a significant reduction of the control values was observed in roots of *B. fruticosum* (Table 2). The treatments with NaCl induced a concentration-dependent increase in root and leaf levels of Na^+, Cl^- and, to a lesser extent, Ca^{2+} in plants of both *Bupleurum* species. Changes in K^+ concentrations in response to increasing external salinity were similar to those detected under water stress conditions: a significant decrease in *B. fruticosum* roots and an increase of about two-fold in *B. tenuissimum* leaves; in both cases, no significant differences were

found between the different salt concentrations in irrigation water (Table 2). It should also be mentioned that, under the same experimental conditions, in roots and leaves of both species, Na^+ generally accumulated to higher levels than K^+ and Cl^-, and Ca^{2+} contents were lower than those of the other ions (Table 2).

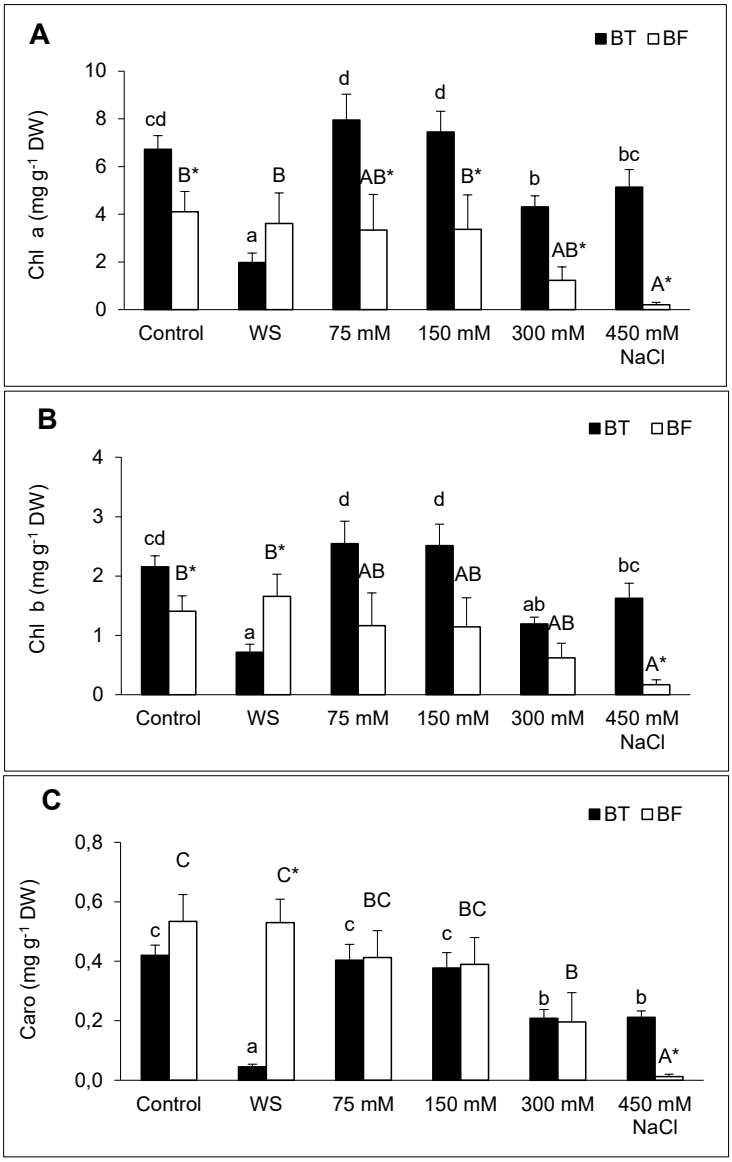

Figure 3. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments on the leaf contents of photosynthetic pigments: chlorophyll a (Chl a, A), chlorophyll b (Chl b, B) and carotenoids (Caro, C), in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF, white bars). Mean values ± SE (n = 5) are shown. Same letters (lowercase for *B. tenuissimum* and uppercase for *B. fruticosum*) indicate homogenous groups according to the Tuckey HSD test ($p < 0.05$). An asterisk (*) indicates significant differences between the two species for the same treatment.

When comparing plants of the two species subjected to the same treatments, although their responses followed the same general patterns, some quantitative differences were observed. For example, leaf Na^+ and Cl^- concentrations were significantly higher in *B. tenuissimum* than in *B. fruticosum*, in the controls and under water stress conditions. However, in the presence of salt, leaf Cl^- contents were lower in *B. tenuissimum* at each NaCl concentration, whereas no differences between species were detected for Na^+ levels (Table 2). Other observed differences refer to leaf

K^+ concentrations in both water- and salt-stressed plants or those of Ca^{2+} under water deficit conditions, which were significantly higher in *B. tenuissimum* than in *B. fruticosum* (Table 2).

Quantification of ion contents in roots and leaves, in response to increasing salinity, also revealed some interesting differences. For example, Na^+ concentrations were significantly higher in leaves than in roots of *B. tenuissimum* plants, in the controls and under water deficit conditions, but higher in roots than in leaves in the presence of 150 and 300 mM NaCl, although not at the highest concentration tested 450 mM; however, no significant differences were observed in *B. fruticosum* for any of the treatments. On the other hand, Cl^- levels were not significantly different in roots and leaves in any of the two selected *Bupleurum* species. Furthermore, contrary to Na^+, Ca^{2+} contents were generally higher in leaves than in roots of *B. tenuissimum* plants. Finally, K^+ concentrations were also significantly higher in leaves but, in this case, in the two species (Table 2).

Accumulation of osmolytes

Proline (Pro) absolute concentrations and relative accumulation patterns in response to stress were different in the two species (Figure 4A). In non-stressed plants, Pro levels were much higher (ca. 12-fold) in *B. tenuissimum* than in *B. fruticosum*. In the former species, Pro increased significantly (1.4-fold) in water-stressed plants but not in response to increasing salinity; in fact, a slight (but significant) reduction was observed in the presence of 450 mM NaCl. In contrast, in *B. fruticosum* plants, mean Pro contents augmented in response to both types of stress, although statistically significant differences with the control were observed only at 150 mM and higher NaCl concentrations. Pro reached a maximum concentration of about 60 μmol g^{-1} DW at the most elevated salinity tested, representing a 12-fold higher value than in the control plants (Figure 4A).

The concentration of total soluble sugars (TSS) in leaves was similar in the control plants of the two species, but their pattern of variation under water stress was different, with a strong reduction (down to about 30% of the control) in *B. tenuissimum*, and a slight, non-significant increase in *B. fruticosum*. No significant change in TSS levels was detected in *B. tenuissimum* plants subjected to salt stress, whereas in *B. fruticosum*, TSS values increased in the presence of 150 and 300 mM NaCl, to decrease significantly at the highest salinity tested (Figure 4B).

Table 2. Ion contents in *Bupleurum tenuissimum* (BT) 0and *B. fruticosum* (BF) after 21 days of water stress (complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments. Values shown are means (in µmol g^{-1} DW) ± SE (n = 5). The same letters within each column indicate homogeneous groups between treatments for each ion and species, according to the Tukey test ($p < 0.05$). Asterisks (*) indicate significant differences between the two species for each treatment, and the plus (+) symbol, significant differences between root and leaf contents of each ion, for the same species and treatment.

Species	Treatments	Na^+ root	Na^+ leaves	K^+ root	K^+ leaves	Cl^- root	Cl^- leaves	Ca^{2+} root	Ca^{2+} leaves
B. tenuissimum	Control	$246.2 \pm 16.1^{a+}$	$405.8 \pm 24.7^{a*+}$	330.8 ± 54.3^{abc}	$334.2 \pm 43.1^{a*}$	$33.0 \pm 0.9^{a+}$	$70.4 \pm 11.0^{a*+}$	$38.7 \pm 2.1^{a+}$	$48.5 \pm 2.2^{a+}$
	Water Stress	$319.1 \pm 21.8^{a+}$	$442.0 \pm 14.4^{a*+}$	$407.2 \pm 77.1^{c+}$	$777.1 \pm 24.4^{d*+}$	63.3 ± 7.8^{a}	$74.0 \pm 7.6^{a*}$	$40.9 \pm 1.4^{a+}$	$55.0 \pm 2.1^{ab*+}$
	75 mM	735.7 ± 77.9^{b}	705.6 ± 53.1^{b}	$198.2 \pm 45.3^{a+}$	$690.3 \pm 55.9^{cd*+}$	148.1 ± 31.5^{b}	168.2 ± 14.3^{b}	60.1 ± 5.7^{b}	64.8 ± 6.3^{bc}
	150 mM	$1239.9 \pm 125.3^{c*+}$	$844.6 \pm 57.8^{b+}$	$283.7 \pm 40.7^{abc+}$	$647.4 \pm 21.4^{bc*+}$	260.7 ± 27.6^{c}	$193.0 \pm 11.2^{b*}$	$73.9 \pm 3.3^{c*+}$	$61.8 \pm 1.6^{abc+}$
	300 mM	$1595.7 \pm 118.2^{d+}$	$1114.6 \pm 100.2^{c+}$	$368.5 \pm 67.1^{bc+}$	$568.6 \pm 23.0^{b*+}$	$488.5 \pm 48.6^{d+}$	$288.0 \pm 25.3^{c*+}$	$98.9 \pm 7.1^{d+}$	$72.9 \pm 1.6^{c+}$
	450 mM NaCl	1628.3 ± 50.1^{d}	1665.2 ± 88.1^{d}	$249.8 \pm 47.0^{ab+}$	$600.2 \pm 29.5^{bc*+}$	$490.3 \pm 28.6^{d*}$	$433.9 \pm 30.3^{d*}$	98.9 ± 5.8^{d}	96.8 ± 9.0^{d}
B. fruticosum	Control	270.9 ± 24.0^{a}	$305.4 \pm 27.4^{a*}$	452.3 ± 44.7^{b}	$458.5 \pm 37.3^{b*}$	41.8 ± 8.8^{a}	$36.2 \pm 7.2^{a*}$	34.32 ± 5.3^{a}	38.2 ± 11^{a}
	Water Stress	259.0 ± 14.6^{a}	$240.6 \pm 14.0^{a*}$	$307.6 \pm 32.3^{a+}$	$471.4 \pm 9.6^{b*+}$	$95.9 \pm 17.0^{ab+}$	$40.4 \pm 3.5^{a*+}$	$48.65 \pm 6.1^{ab+}$	$28.5 \pm 3.7^{a*+}$
	75 mM	563.0 ± 180.0^{a}	461.9 ± 122.7^{a}	$238.2 \pm 9.0^{a+}$	$387.3 \pm 33.2^{ab*+}$	392.7 ± 31.7^{ab}	453.6 ± 48.8^{b}	44.54 ± 5.1^{a}	49.9 ± 6.8^{a}
	150 mM	$651.9 \pm 40.0^{a*}$	791.9 ± 99.2^{a}	$211.0 \pm 25.7^{a+}$	$350.3 \pm 16.2^{a*+}$	442.6 ± 19.1^{b}	$664.3 \pm 97.0^{c*}$	$53.38 \pm 5.1^{ab*}$	98.8 ± 16^{b}
	300 mM	1731.1 ± 149.8^{b}	1272.0 ± 219.1^{b}	291.9 ± 75.5^{a}	$418.8 \pm 28.2^{ab*}$	697.8 ± 79.5^{c}	$825.7 \pm 163.8^{c*}$	84.88 ± 18.2^{ab}	97.6 ± 13^{b}
	450 mM NaCl	1846.3 ± 296.0^{b}	1455.9 ± 237.6^{b}	$193.5 \pm 35.8^{a+}$	$419.6 \pm 47.7^{ab*+}$	$995.6 \pm 199.1^{d*}$	$935.8 \pm 122.6^{c*}$	82.50 ± 17.5^{b}	86.9 ± 10^{b}

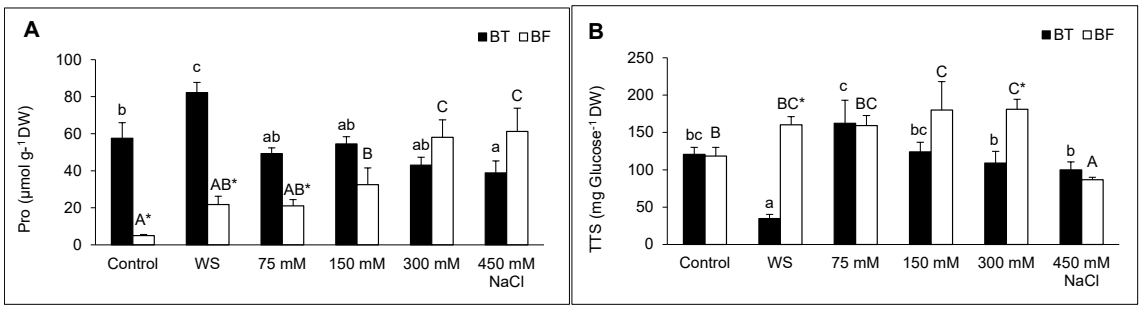

Figure 4. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments on leaf proline (Pro, A), total soluble sugars (TSS, B) and glycine betaine (GB, C) contents, in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF, white bars) plants. The values shown are means ± SE (n = 5). Same letters (lowercase for *B. tenuissimum* and uppercase for *B. fruticosum*) indicate homogenous groups according to the Tuckey HSD test ($p < 0.05$). An asterisk (*) indicates significant differences between the two species for each treatment.

Oxidative stress markers and non-enzymatic antioxidants

The leaf concentration of malondialdehyde (MDA), a widely used oxidative stress marker (Del Rio et al., 2005), increased in *B. tenuissimum* plants subjected to the water stress treatments, reaching values almost double of those of the control, but no significant changes were observed in response to increasing salinity. An opposite MDA accumulation pattern was established for *B. fruticosum* plants: a significant increase in parallel to increasing NaCl concentrations in the irrigation water and no significant differences between the control and water-stressed plants (Figure 5A).

Hydrogen peroxide (H_2O_2) contents did not vary in *B. tenuissimum* plants under water deficit conditions and increased slightly, but significantly, in response to the salt stress treatment; nevertheless, H_2O_2 levels remained relatively low in all assayed samples. In *B. fruticosum*, on the contrary, both stress treatments induced the accumulation of H_2O_2 to relatively high levels, reaching a four-fold increase over control values in the presence of 300 mM NaCl. Interestingly, in plants subjected to the highest salt concentration, 450 mM NaCl, H_2O_2 contents showed a marked reduction (Figure 5B).

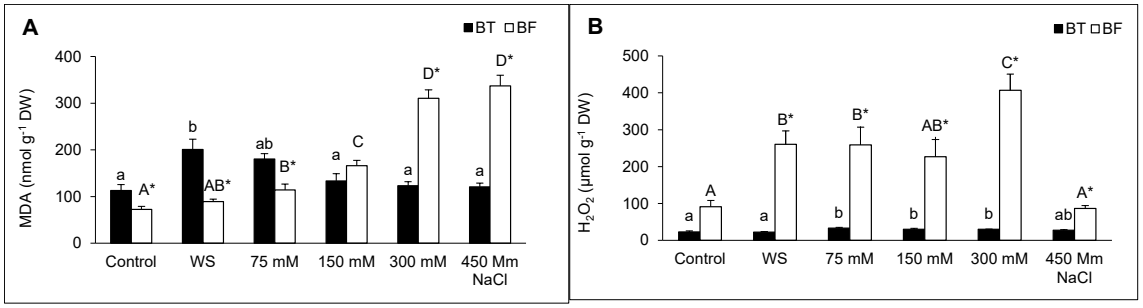

Figure 5. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments, on malondialdehyde (MDA, A) and hydrogen peroxide (H_2O_2, B), in leaves of in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF, white bars). The values shown are means ± SE (n = 5). Same letters (lowercase for *B. tenuissimum* and uppercase for *B. fruticosum*) indicate homogenous groups according to the Tuckey HSD test ($p < 0.05$). An asterisk (*) indicates significant differences between the two species for each treatment.

Concentrations of total phenolic compounds and antioxidant flavonoids

Total phenolic compounds (TPC) and total flavonoids (TF) followed a similar pattern of variation in response to the applied stress treatments (Figure 6). In *B. tenuissiumum*, a significant increase in TPC (Figure 6A) and TF (Figure 6B) contents was found in plants subjected to water deficit conditions; however, no significant differences with the control were observed in salt-stressed plants, except for a slight increase of TF in the presence of 450 mM NaCl (Figures 6A, B). Just the opposite pattern was detected in *B. fruticosum* plants: no significant changes in TPC and TF concentrations under water stress and a significant increase at high salinities (300-450 mM NaCl) (Figures 6A, B).

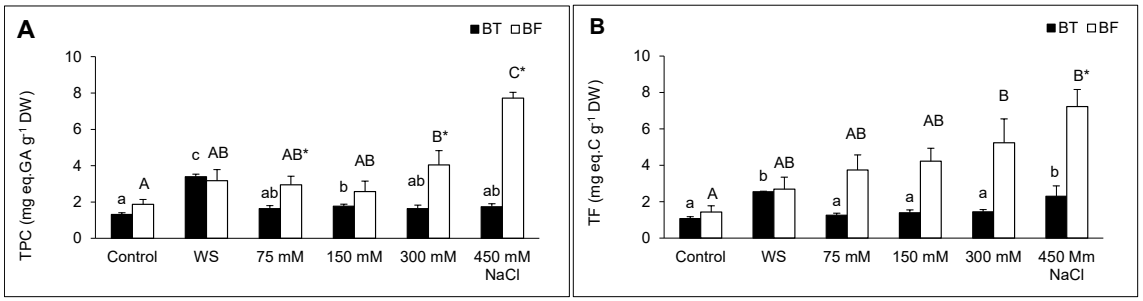

Figure 6. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments, on total phenolic compounds (TFC, A) and total flavonoids (TF, B) contents, in leaves of in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF, white bars) plants. The values shown are means ± SE (n = 5). Same letters (lowercase for *B. tenuissimum* and uppercase for *B. fruticosum*) indicate homogenous groups according to the Tuckey HSD test ($p < 0.05$). An asterisk (*) indicates significant differences between the two species for each treatment.

Activity of antioxidant enzymes

The background activities of the three assayed antioxidant enzymes (SOD, CAT and GR) in non-stressed plants were significantly higher in *B. fruticosum* than in *B. tenuissimum* plants and did not change with respect to the corresponding controls in response to the water stress treatment (Figure 7). In general, these activities increased in salt-treated plants, albeit with different qualitative and quantitative patterns, depending on the specific enzyme and *Bupleurum* species. Thus, SOD specific activity increased in response to salt stress in both species, maintaining higher values in *B. fruticosum* at all tested NaCl concentrations (Figure 7A). CAT was activated in *B. tenuissimum* under mild and moderate salinities (75-150 mM NaCl), and only in the presence of 150 mM NaCl in *B. fruticosum* plants, although reaching a significantly lower activity than in its congeneric species; in both, CAT specific activity decreased to control values at higher salt concentrations, 300-450 mM NaCl (Figure 7B). GR activity was also maximal in plants treated with 150 mM NaCl in both *Bupleurum* species, although in *B. tenuissimum* significant differences with the control were observed at all salt concentrations tested (Figure 7C).

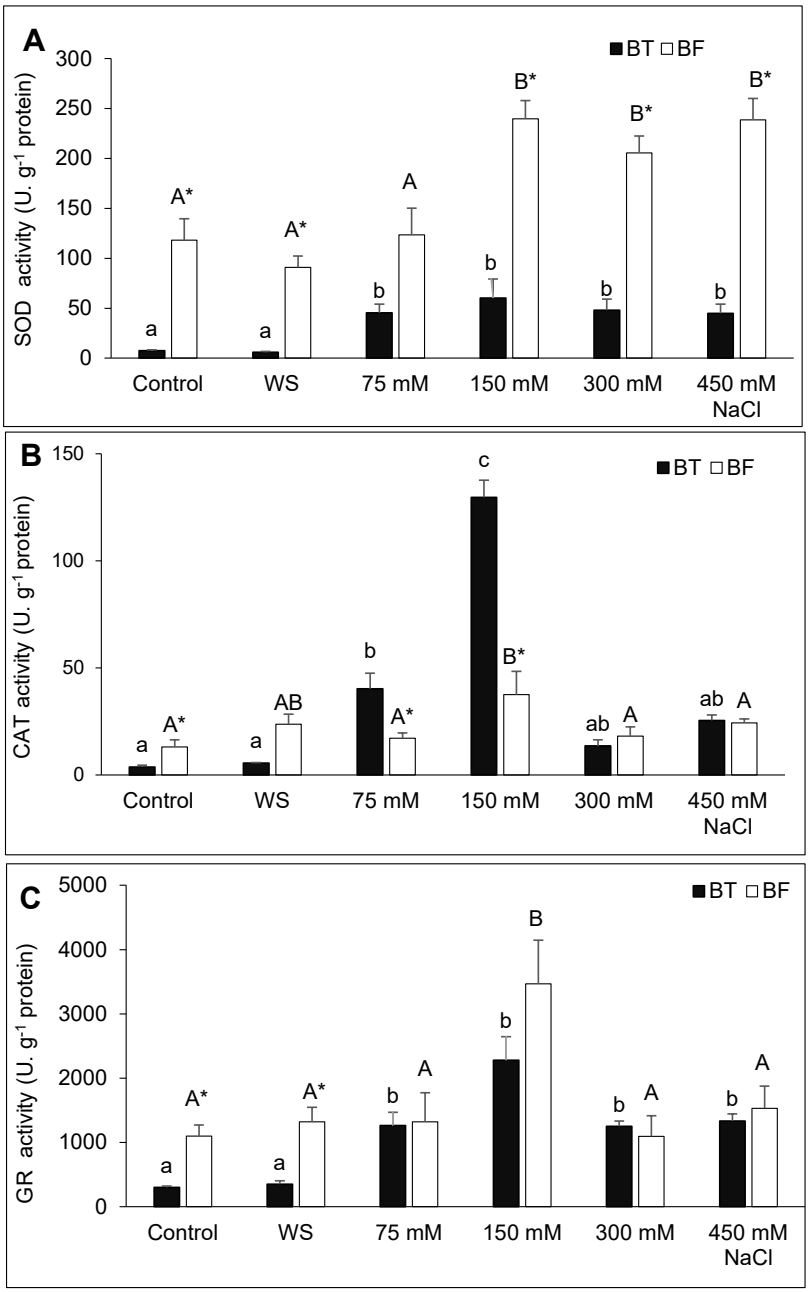

Figure 7. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments on the specific activity of antioxidant enzymes: superoxide dismutase (SOD, A), catalase (CAT, B) and glutathione reductase (GR, C) in leaves of in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF, white bars). The values shown are means ± SE (n = 5). Same letters (lowercase for *B. tenuissimum* and uppercase for *B. fruticosum*) indicate homogenous groups according to the Tuckey HSD test ($p < 0.05$). An asterisk (*) indicates significant differences between the two species for each treatment.

Principal Component Analysis

The PCA performed using the means of all analysed parameters, in the control, water deficit and salt stress treatments of plants of the two species, detected five components with eigenvalues greater than 1, which together explained 90% of the total variability. The first component, which covered 38.4% of the variability, was predominantly related to the salt stress treatments, whereas the second, which explained an additional 21.5%, was more related to the water deficit treatments (Figure 8). Thus, in the positive region of the X-axis, substrate EC clustered with ion contents in leaves and roots, oxidative stress markers (MDA, H_2O_2), antioxidant compounds (TF, TPC) and

enzymes (especially with SOD); all these variables increased in response to salt stress. On the contrary, a negative correlation with salinity was established for growth parameters (root and stem length, leaf number, root and leaf FW) and photosynthetic pigments (Chl a, Chl b and Caro), which decreased in salt-treated plants. In the second component (Y-axis), substrate moisture correlated positively with the water content of roots (RWC) and leaves (LWC) and negatively with Pro, which increased significantly in the water stress treatment (Figure 8A).

The scatter plot of the PCA scores (Figure 8B) indicated a good separation of the different NaCl treatments along with the first component in *B. tenuissimum*, whereas, in *B. fruticosum*, these treatments were separated mostly according to the second component. Water stress values were clearly apart from all other treatments, especially in the most drought-tolerant species, *B. fruticosum* (Figure 9).

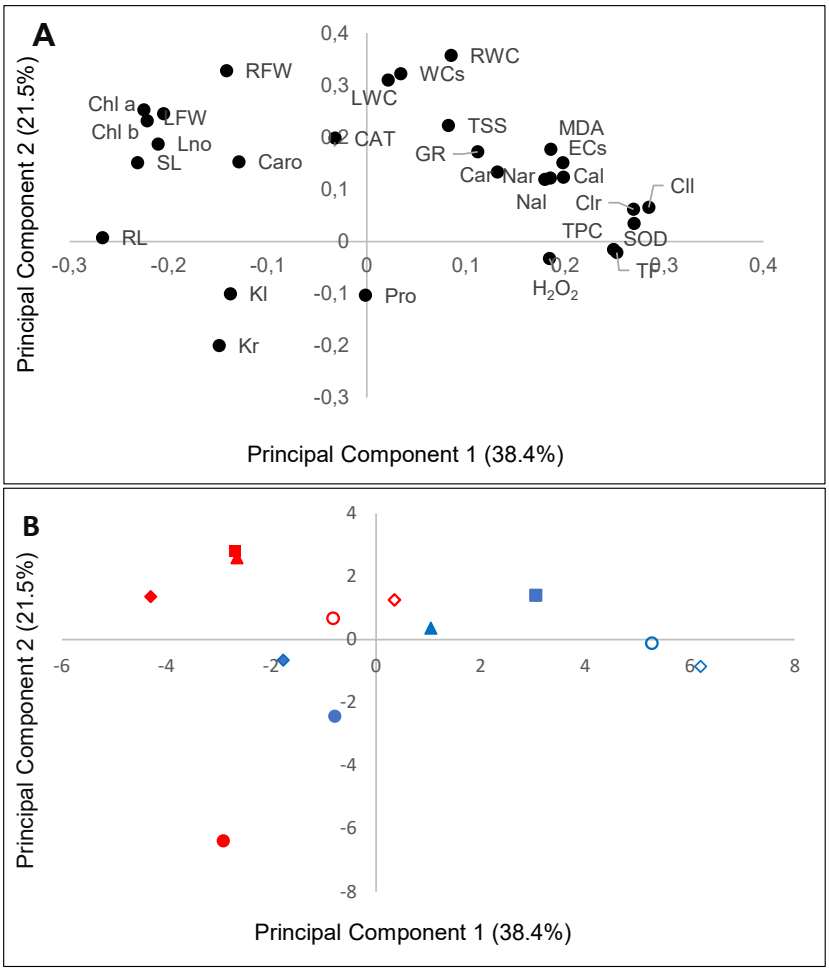

Figure 8. Loading plot (A) of the principal component analysis (PCA), conducted with the analysed substrate and plant parameters, in the control, water deficit and salt stress treatments. Substrate parameters: water content (WCs) and electroconductivity (ECs). Plant parameters: leaf number increment (Lno), stem length increment (SL), fresh weight of roots (RFW) and leaves (LFW), water content of roots (RWC) and leaves (LWC), chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoids (Caro), ions in roots (Nar, Kr, Clr, and Car) and leaves (Nal, Kl, Cll and Cal), proline (Pro), total soluble sugars (TSS), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total phenolic compounds (TPC), total flavonoids (TF), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR). Scatter plot of the PCA scores (B). Different treatments are represented by diamonds (control), circles (water stress), triangles (75 mM NaCl), squares (150 mM), empty circles (300 mM) and empty diamonds (450 mM). Blue symbols correspond to *B. tenuissimum* and red symbols to *B. fruticosum*.

Discussion

The physiology and chemical composition of the *Apiaceae* (=*Umbelliferae*) have been extensively studied because of the broad uses of their species, well known as sources of food, spices and medicines (Heywood, 1998). Specifically, the interest in the genus *Bupleurum* has been chiefly driven by the fact that the roots of *B. sinensis* and several other species are extremely popular in oriental folk medicine (Ashour and Wink, 2011). Accordingly, secondary metabolites have been analysed in more than 50 species of this genus, and around 250 natural compounds from all major phytochemical classes have been identified. However, despite the large number of published articles and reviews on the phytochemistry of *Bupleurum* (Ashour and Wink, 2011; Yang et al., 2017; Zhu et al., 2017; Jiang et al., 2020, amongst others), there is very little information on the responses to drought of these species (Zhang et al., 2007; Yang et al., 2019, 2020) and, to our knowledge, no data are available on their responses to salt stress.

The primary objective of the present work was to explore the limits of salt and drought tolerance in the rare halophyte *B. tenuissimum*, locally extinct in the Albufera Natural Park area, information that could be relevant for the design and implementation of conservation programmes of this endangered taxon. In addition, by comparison with the drought-tolerant congeneric species included in the study, *B. fruticosum*, we expected to gain insight into the main mechanisms conferring tolerance to salt and water stress in this poorly studied genus.

Stress-induced growth inhibition

Bupleurum tenuissimum may be regarded as a facultative halophyte, as it grows on moderately saline soils but can also be found in non-saline habitats (Grigore and Cojocariu, 2020; Tyler et al., 2020), and its seeds do not germinate in the presence of salt (Al-Hawija et al., 2012). *Bupleurum tenuissimum* has been reported to be more drought-tolerant than other species present in the same salt marsh habitats in Central Europe (Čížková et al., 2020). However, when compared to *B. fruticosum* – a Mediterranean species popular as a moderately drought-tolerant ornamental – it was by far more susceptible to water deficit, imposed under greenhouse conditions, as indicated by the stronger growth inhibition observed after the three-week water stress treatment. In contrast, the determination of several growth parameters showed that *B. tenuissimum* is more salt-tolerant than *B. fruticosum*, being relatively less affected in the presence of salt and only by high NaCl concentrations. The degradation of photosynthetic pigments, a common effect of abiotic stress in plants, also correlated with the relative tolerance of the two species. In response to water stress, chlorophylls a and b and carotenoid levels decreased significantly in *B. tenuissimum* compared to the controls, but did not vary in *B. fruticosum* plants. In contrast, the reduction in pigments contents was more pronounced in *B. fruticosum* than in *B. tenuissimum* in the presence of salt, especially at the highest concentration tested, 450 mM NaCl. The observed behaviour under the artificial greenhouse conditions also corresponded to the apparent ecological preferences of the two analysed species regarding the characteristics of their natural habitats.

Salt tolerance mechanisms based on the control of ion transport and accumulation

One of the basic salt tolerance mechanisms, which differentiates halophytes from glycophytes, is based on ion uptake and transport control. Glycophytes are generally salt-excluders; their primary mechanism of salt stress resistance is to prevent the foliar accumulation of toxic ions, either by reducing their uptake by the roots or by blocking their transport to the aerial parts of the plant (Maathuis et al., 2014; Volvok, 2015). On the other hand, most dicotyledonous halophytes, particularly highly tolerant succulent species, are salt-includers, actively transporting toxic ions from roots to shoots and storing them in the leaf vacuoles to avoid their deleterious effects in the cytoplasm (Flowers et al., 1977; Flower and Colmer, 2008; Maathuis et al., 2014; Grigore and Flowers, 2021). As a species only moderately tolerant to salinity, *B. tenuissimum* does not fit precisely within any of the two categories mentioned above, showing an intermediate behaviour between typical glycophytes and highly salt-tolerant halophytes. The analysis of the observed differences in ion accumulation in response to the applied stress treatments between *B. tenuissimum* and the more salt-sensitive *B. fruticosum* provides some hints on the mechanisms underlying the higher tolerance of the former species.

Under control conditions, in *B. tenuissimum* plants, the levels of Na^+ and Cl^- (and also Ca^{2+}) were significantly higher in leaves than in roots, whereas no differences were observed in non-stressed plants of *B. fruticosum*, suggesting the presence of active ion transport to the aerial part of the plant at low external salinity in the more salt-tolerant species. Accumulation of inorganic ions in the leaves, which will contribute to osmotic balance, can be considered a constitutive stress tolerance mechanism in *B. tenuissimum*. When plants of the two investigated species were subjected to increasing NaCl concentrations, significant, concentration-dependent increases in Na^+ and Cl^- contents were observed in both, roots and leaves. In *B. fruticosum*, Na^+ and Cl^- levels were not significantly different in leaves and roots for each salt concentration. In contrast, in *B. tenuissimum*, lower ion concentrations were measured in leaves than in roots at intermediate salinities (150-300 mM NaCl), indicating the existence of a mechanism blocking ion transport to the aboveground organs, as reported in other species, such as *Juglans regia* L., where more tolerant cultivars had lower foliar Na^+ concentrations than the salt susceptible ones (Lofti et al. 2009). This blocking mechanism, which seems to be overcome at an even higher NaCl concentration (450 mM), can partly explain the higher relative salt tolerance of this species.

It is generally assumed that Na^+ is more toxic than $Cl^–$, as the cation competes with K^+ for the same binding sites in proteins, interfering with K^+ transport and inhibiting many enzymes that require K^+ (Maathuis, 2009; Maathuis et al. 2014). Consequently, most studies attributed the ionic component of salt stress to Na^+ toxicity, largely overlooking the effects of $Cl^–$; only recently, its role in salt toxicity has been reconsidered (Wu and Li, 2019). $Cl^–$ is required at low levels as a micronutrient for plants, but higher concentrations are toxic, impairing photosynthesis and growth (Tavakkoli et al., 2010, 2011). Furthermore, $Cl^–$ toxicity is also related to its antagonism with $NO_3^–$, resulting in a limited nitrogen supply (Wu and Li, 2019). In our experiments with the two *Bupleurum* species, the patterns of variation in response to the salt treatments were the same, qualitatively, for the two ions, Na^+ and Cl^-. However, the absolute values of Cl^- contents were consistently lower than those of Na^+ for each salt concentration, organ, and species, suggesting the existence of exclusion mechanisms for this anion. Interestingly, Cl^- levels were generally lower in *B. tenuissimum* than in *B. fruticosum*, especially in the leaves and at high external salinities, which could also contribute to the higher salt tolerance of the former species. Similar results have been previously obtained when comparing some related taxa with different degrees of salt tolerance. For example, foliar $Cl^–$ contents in salt-treated plants of the glycophyte *Juncus articulatus* were substantially higher than those in the halophytes *J. maritimus* and *J. acutus* at the same salinity levels (Al Hassan et al., 2016d). Similarly, salt-sensitive bean cultivars accumulated Cl^- in leaves at a higher concentration than more tolerant ones (Bayuelo-Jiménez et al., 2012; Al Hassan et al., 2016b). In this species, the levels of $Cl^–$ were higher than those of Na^+, as also reported for other legumes and woody species, which exclude Na^+ from the leaf blades more efficiently than $Cl^–$ (Wu and Li, 2019).

Accumulation of Na^+ is generally accompanied by a decrease in K^+ levels since, as mentioned above, both cations compete for the same protein transporters (Flowers et al. 1977; Greenway and Munns, 1980). Besides, Na^+ also causes plasma membrane depolarisation and the further loss of cellular K^+ by activating outward rectifying K^+ channels (Greenway and Munns, 1980). K^+ is an essential nutrient, playing critical roles in cell elongation and maintenance of membrane integrity and stability, the activity of many enzymes, protein synthesis, photosynthesis, osmoregulation, stomatal movement, energy transfer, phloem transport, cation-anion balance, and stress resistance (Marschner, 2012). Under drought and salt stress conditions, K^+ is essential in osmotic adjustment and turgor regulation within guard cells during stomatal movement, and low K^+ is often related to increased oxidative stress (Wang et al., 2013). Therefore, mechanisms allowing the maintenance of high leaf K^+ concentrations in the presence of salt, such as the activation of its transport to the shoots of the plants, are essential for salt tolerance and are common in many halophytes (Al Hassan et al., 2016c,d). This is also the case in *B. tenuissimum*, which showed significantly higher K^+ levels in the leaves of salt-treated plants than the non-treated controls, whereas no differences were observed in the salt-sensitive *B. fruticosum.*

The role of calcium counteracting the harmful effects of salt stress has been known for a long time (Bressan et al., 1998). Calcium is required to maintain the structure and functional

integrity of plants, stabilise membranes and cell wall structures, regulate ion transport and selectivity, and control cell wall enzyme activities (Vahdati and Lotfi, 2013). Under stress conditions, Ca^{2+} plays an essential role as a signalling molecule, mediating the main tolerance mechanisms, such as osmoprotectant accumulation, activation of antioxidant systems, or stress responses involving polyamines and nitric oxide (NO) (Seifikalhor et al., 2019). A significant, concentration-dependent increase of Ca^{2+} contents, in response to increasing salinity, has been observed in roots and leaves of the two analysed *Bupleurum* species and may contribute to salt resistance mechanisms, as reported in other species (Vahdati and Lotfi, 2013). However, Ca^{2+} is not responsible for the differences in salt sensitivity since its accumulation patterns were similar in *B. tenuissimum* and *B. fruticosum*.

Stress-induced accumulation of osmolytes

Avoiding cellular dehydration and maintaining osmotic balance under different abiotic stress conditions generally requires the accumulation of compatible solutes, or osmolytes, in the cytosol. Besides their primary function in osmoregulation, osmolytes play multiple roles in stress tolerance, acting as low-molecular-weight chaperones, ROS scavengers or signalling molecules (Ashraf and Foolad, 2007; Sanders and Arndt, 2912; Slama et al., 2015; Sing et al., 2015). One of the most common osmolytes in plants is proline (Szabados and Savouré, 2010). Apart from its function as a compatible solute, Pro acts as a potent antioxidant, directly quenching ROS, such as O_2, H_2O_2, and $OH^{\bullet}$, maintaining a low $NADP^+$ to NADPH ratio, or stabilising ROS-scavenging and mitochondrial respiration enzymes (Szabados and Savouré, 2010; Gupta and Huang, 2011; Bose et al., 2014). Pro represents an excellent stress biomarker for those species that accumulate this osmolyte in response to different stress treatments. However, Pro may or may not have a direct role in tolerance mechanisms, depending on the species (Mansour and Ali, 2017). When comparing genetically related taxa, in some cases, there is a positive correlation between the levels of Pro and the relative degree of tolerance, i.e., Pro accumulates to higher concentrations in the more tolerant plants (Al Hassan et al., 2016d, Lofti et al., 2010, 2019). However, in other cases, the correlation is negative, and the highest Pro content is measured in the most sensitive genotype (e.g., Al Hasan et al. 2016b). The *Bupleurum* species selected for this work clearly fit the latter scenario. In *B. tenuissiumum*, which is relatively more tolerant to salinity and more sensitive to drought, leaf Pro contents increased significantly, compared to the non-stressed controls, in water-stressed plants but did not vary in response to the salt treatments. The opposite was true for the more drought-tolerant and salt-sensitive *B. fruticosum*: Pro levels gradually increased in parallel to increasing NaCl concentrations but did not change significantly with respect to the control in plants subjected to the water deficit treatment. It should also be noticed that, in non-stressed plants, Pro levels were higher in *B. tenuissiumum* than in *B. fruticosum*, probably conferring to the former a somewhat increased degree of constitutive resistance to stress. These results indicate that the increase in Pro concentration, in relation to the values determined in control plants, can be used as a reliable indicator of the level of stress suffered by the *Bupleurum* plants.

The specific involvement of TSS in cellular osmoregulation under stress is more difficult to assess, due to the multiple biological functions of soluble sugars, as direct products of photosynthesis, metabolic precursors and energy sources, involved in many physiological processes. TSS have been reported to play a specific role as osmolytes and osmoprotectants in stress tolerance mechanisms in different species (e.g., Gil et al., 2013; Al Hassan et al., 2016d). However, no clear pattern of variation of TSS levels, in response to the water deficit and salt stress treatments, could be found in *Bupleurum*, except for a pronounced reduction in *B. tenuisssimum* plants subjected to water stress. This can be explained by the drastic inhibition of photosynthesis in this drought-sensitive species, shown by the substantial decrease in photosynthetic pigments observed in these water-stressed plants.

Oxidative stress and activation of antioxidant systems

Abiotic stress is associated with the increased production of reactive oxygen species (ROS). ROS were initially considered just as toxic by-products of aerial metabolism that caused oxidative stress, but at present, their essential role as signalling messengers in several key physiological processes is well established (Apel and Hirt, 2004; Foyer and Noctor, 2005; Das and Roychoudhury, 2014). ROS, including, for example, molecular O_2, O_3, H_2O_2 and highly reactive free radicals, when in excess, have damaging effects on nucleic acids, lipids and proteins, inducing severe dysfunctions and, eventually, cell death (Apel and Hirt, 2004). Several ROS cause lipid peroxidation, which affects the selectivity and permeability of membranes, leading to leakage of ions and other metabolites (Ozgur et al., 2013). Hydrogen peroxide (H_2O_2) along with malondialdehyde (MDA), a marker of membrane lipid peroxidation, are widely used to estimate the level of oxidative stress experienced by plants and their degree of sensitivity to a particular type of stress (e.g., Chakraborty and Pradhan, 2012, Moussouraki et al., 2019, Zhu et al. 2020). Although the reliability of MDA as a marker of oxidative stress has recently been questioned, due to methodological aspects or misinterpretation of the results (Morales and Munné-Bosch, 2019), in the two *Bupleurum* species analysed here, its levels matched perfectly with the other measured parameters. MDA contents increased significantly only under those experimental conditions stressful for each species: in the water-stressed plants of the halophyte (and drought-sensitive) *B. tenuissimum*, and salt-stressed plants of the more salt-sensitive *B. fruticosum*, especially al high salinities (300 and 450 m NaCl).

Hydrogen peroxide is considered as a moderately reactive ROS formed when the superoxide radical ($O_2^{-\bullet}$) is dismutated into O_2 and H_2O_2, either non-enzymatically or, mainly, in the reaction catalysed by superoxide dismutase (SOD). As for other ROS, at low concentration $O_2^{-\bullet}$ has an important signalling function in essential physiological processes, but when in excess, it inactivates many enzymes, oxidising cysteine and methionine residues (Das and Roychoudhury, 2014). In the present work, H_2O_2 increased significantly in *B. fruticosum* plants subjected to both stress treatments, water deficit and increasing salinity (up to 300 mM NaCl). In *B. tenuissimum*, H_2O_2 contents were much lower than in *B. fruticosum* under all tested conditions and showed only a slight increase in the salt stress treatments.

Plants activate two main mechanisms in the defence against toxic levels of ROS, a series of antioxidant enzymes and the synthesis of non-enzymatic, low-molecular-weight antioxidant compounds, such as ascorbic acid (AA) or phenolic compounds, particularly the subgroup of flavonoids, amongst others (Gill and Tuteja, 2010; Miller et al., 2010; Oprică and Vochița, 2020). Some halophytes possess constitutive antioxidant activities, more effective than in glycophytes (Ozgur et al., 2013). For example, higher constitutive levels of catalase (CAT), peroxidases (POX, APX) and glutathione reductase (GR) activities were found in the salt-tolerant *Hordeum marinum*, as compared to *H. vulgare* (Seckin et al., 2010). Also, higher Pro concentrations, both in control and salt-stressed plants, and higher levels of SOD gene expression under stress, were measured in the halophyte *Thellungiella salsuginea* than in the glycophyte *Arabidopsis thaliana* (Taji et al., 2004).

Superoxide dismutase (SOD) is considered the first line of defence against oxidative stress in plants (Alscher *et al.*, 2002). It catalyses the removal of $O^{2\bullet-}$ by dismutating it into H_2O_2 and O_2 (Das and Roychoudhury, 2014). Halophytes tend to possess intrinsically higher levels of SOD, a trait that can be considered an adaptive advantage over glycophytes, and the activity of this enzyme generally increases in the presence of salt, whereas in glycophytes, both increases and reductions of SOD activity have been reported (Bose et al. 2014). Catalase (CAT) catalyses the conversion of H_2O_2 to H_2O (Willekens et al., 1997). The enzyme has a high affinity for H_2O_2, a very fast turnover rate and is very efficient in decomposing the peroxide (Bose et al., 2014). Most reports on salt- and drought-tolerant species indicate CAT activation under stress conditions (Bose et al., 2014; Laxa et al., 2019). GR is an oxidoreductase with the primary function of maintaining the intracellular levels of reduced glutathione (GSH), which is involved in a wide range of essential functions and has a strong reducing potential (Couto et al., 2016). According to published reports, GR activity may increase, decrease or remain unchanged in response to stress treatments, depending on the species and the experimental conditions, but non-tolerant plants

appear to predominantly activate the glutathione-dependent scavenging system (Laxa et al., 2019).

All three enzymes appeared to be involved in the responses of the two *Bupleurum* species to salt stress, as their specific activities increased in the presence of NaCl, albeit with different qualitative and quantitative patterns. In *B. tenuissimum*, GR and, to a lesser extent, SOD activities increased significantly over control values at all salt concentrations tested, but the strongest activation was observed in CAT activity, although only at low and moderate (75 and 150 mM NaCl) salinities. In *B. fruticosum*, the activities of the three enzymes in control, non-stressed plants were much higher than in *B. tenuissimum*. Consequently, their relative increase in response to the salt treatments was weaker. Furthermore, the activation of CAT and GR in *B. fruticosum* was detected only in the presence of 150 mM NaCl, not at lower or higher concentrations. Taken together, these data indicate that the salt treatment induced a more substantial increase in the enzymatic antioxidant activity in *B. tenuissimum* than in *B. fruticosum*, in agreement with the higher salt tolerance of the former species. On the other hand, the antioxidant enzymes do not seem to participate in tolerance mechanisms against drought, as their specific activities remained unchanged with respect to the controls, in plants of the two species, after the water deficit treatment. In another species of the genus, *B. chinense*, SOD, CAT and peroxidase activities have decreased under water stress conditions (Yang et al., 2019).

Not all ROS are eliminated by antioxidant enzymes; several highly toxic species can be scavenged only by strong non-enzymatic antioxidants, such as phenolic compounds and, especially, flavonoids (Bose et al., 2014). The latter category is considered a secondary ROS scavenging system in plants suffering damage to the photosynthetic apparatus due to excess excitation energy (Fini et al., 2011). In many halophytes, an increase in phenolics and flavonoids concentrations in response to stress has been reported (Ksouri et al., 2012; Ozgur et al., 2013; Gil et al., 2014); however, in many others, no significant variation has been observed, for example, in several *Limonium* species (González-Orenga et al., 2019a, 2020a). It is very likely that the more salt-tolerant species do not need to activate antioxidant systems since they possess efficient mechanisms to prevent excessive ROS production, for example, by limiting Na^+ accumulation in the cytosol (Bose et al., 2014). This is not the case for the investigated *Bupleurum* species, and we did observe a stress-induced increment in phenolics and flavonoids levels, in agreement with their relative tolerance and the MDA accumulation data. Thus, in *B. tenuissimum*, more tolerant to salinity, TPC and TF contents increased in plants subjected to water stress but did not vary in salt-treated plants – except for a small increment of TF in the presence of 450 mM NaCl. In the more drought-tolerant *B. fruticosum*, the opposite behaviour was observed: an increase in the levels of these antioxidant compounds in response to the salt treatments, especially at high salinities, and no change in water-stressed plants.

Implications for conservation

Overall, all the parameters analysed allowed clear separation of the two species and their responses to the two different stress types. *Bupleurum tenuissimum*, a moderate halophyte, is more susceptible to drought, whereas, on the contrary, *B. fruticosum* is not seriously affected by drought but by salinity, even at low NaCl concentrations. Considering this differential behaviour can be relevant for future plantations of these species, particularly for *B. tenuissimnum*, in the frame of conservation and regeneration programmes. This species has been traditionally linked to the phytosociological class *Saginetea maritimae*, including taxa that usually grow on coastal and subcoastal shallow sandy soils. To live under those ecological conditions, *B. tenuissimum* should be more resistant to drought. On the contrary, its local behaviour in the Valencian Community is closer to plants living in moister salty soils (i.e., phytosociological orders *Limonietalia* or *Juncetalia maritimae*), often impermeable and clayey. For instance, the presence of the most demanding species of halophytes, in terms of soil moisture, such as *Limonium albuferae* (see González-Orenga et al. 2019b) or *Thalictrum maritimum* (González-Orenga et al., 2020b), can serve as good indicators for *B. tenuissimum* reintroduction sites in the Albufera Natural Park.

Regarding *B. fruticosum*, it is an underutilised species for ecological restoration, still missing in lists of valuable Valencian species for dry-forest vegetation (i.e. García-Fayos, 2001), and riparian scrubs (Prada & Arizpe, 2008).

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