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Stress tolerance mechanisms in *Juncus*: Responses to salinity and drought in three *Juncus* species adapted to different natural environments

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Responses to salinity and drought were analysed in three rush species with different degrees of salt tolerance. The most tolerant species, sea rush and spiny rush, inhibit more efficiently the transport of toxic ions to the aerial part of the plants, activate potassium transport at high external salt concentrations, and accumulate much higher levels of proline as osmoprotectant. These findings contribute to elucidate relevant stress tolerance mechanisms in *Juncus* species.

Abstract. Comparative studies on the responses to salinity and drought were carried out in three *Juncus* species, two halophytes (*J. maritimus* Lam. and *J. acutus* L.) and one more salt-sensitive (*J. articulatus* L.). Salt tolerance in *Juncus* depends on the inhibition of transport of toxic ions to the aerial part: in the three taxa, Na^+ and Cl^- accumulated to the same extent in the roots of salt treated plants; however, ion contents were lower in the shoots and correlated with the relative salt sensitivity of the species, with the lowest levels measured in the halophytes. Activation of K^+ transport at high salt concentration could also contribute to salt tolerance in the halophytes. Maintenance of cellular osmotic balance is mostly based on the accumulation of sucrose in the

three species. Yet, neither the relative salt-induced increase in sugar content, nor the absolute concentrations reached can explain the observed differences in salt tolerance. Proline, on the contrary, increased significantly in the presence of salt only in the salt-tolerant *J. maritimus* and *J. acutus*, but not in *J. articulatus*. Similar patterns of osmolyte accumulation were observed in response to water stress, supporting a functional role of proline in stress tolerance mechanisms in *Juncus*.

Keywords: abiotic stress; drought tolerance; halophytes; ion transport; proline accumulation; salt tolerance.

Introduction

Salinity, together with drought, is one of the most severe environmental stress factors which shape the distribution of plant species in nature, and is also responsible for large losses in crop production worldwide: accumulation of salts dissolved in irrigation water is leading to the progressive 'secondary' – of anthropic origin – salinisation of arable land, mainly in arid and semi-arid regions; this problem will worsen in the near future due to the effects of climate change (Boyer 1982; Bartels and Sunkar 2005; Watson and Byrne 2009; IPCC 2014; Fita *et al*. 2015). While all major crops and most wild species are relatively sensitive to salt stress, some plants – the halophytes – have evolved different mechanisms that allow them to withstand high salinity levels in their natural habitats.

Studies on the responses to salt stress have provided overwhelming evidence that plants react to increased soil salinity by activating a series of basic, conserved response mechanisms, including the control of ion transport, maintenance of cellular osmotic balance, the synthesis of 'protective' metabolites and proteins, or the activation of antioxidant systems (Zhu 2001; Vinocur and Altman 2005; Hussain *et al*. 2008; Ozgur *et al*. 2013; Bose *et al*. 2014; Kumari *et al*. 2015; Volkov 2015). Activation of these mechanisms counteracts, at least partly, the deleterious effects of high salinity in the soil, which are the result of the two components of salt stress: osmotic (water) stress, leading to cellular dehydration, and salt (ion) toxicity, causing inhibition of metabolic processes and affecting mineral nutrition (Schulze *et al.* 2005; Munns and Tester, 2008). The osmotic effect is not specific for salt stress: other environmental conditions, such as drought, cold, or high temperatures, also cause dehydration in plant cells; therefore, one of the commonest mechanisms of response to different stressful conditions is based on the

biosynthesis and accumulation of organic compatible solutes or osmolytes – such as proline, glycine betaine, soluble sugars or polyalcohols – for osmotic adjustment (Munns and Termaat 1986; Chen and Murata 2008; Flowers and Colmer 2008; Munns and Tester 2008; Szabados and Savouré 2010; Gil *et al.* 2013).

These basic responses against salinity are shared by all plants, and their activation does not necessarily lead to salt tolerance; in fact, as mentioned above, most plant species are glycophytes; that is, salt sensitive. Therefore, salt tolerance, which varies widely in different species, must depend on the relative efficiency of the aforementioned mechanisms of response (Pang *et al*. 2010; Kumari *et al*. 2015). Moreover, there is no single halophytic 'model species', as different salt tolerant plants use different mechanisms to efficiently cope with the deleterious effects of high soil salinity. Yet, the relative contribution of different salt stress responses to salt tolerance in a given species – or in a group of related taxa – remains largely unknown.

In agreement with these ideas, we believe that performing comparative studies on the responses to salt stress of genetically related taxa with different degrees of tolerance – such as congener wild species adapted to distinct habitats – will help to elucidate relevant salt tolerance mechanisms. Our working hypothesis is that, if a specific response to salt stress contributes significantly to salt tolerance, it should be more efficiently activated in the more tolerant taxa. Therefore, our proposed experimental approach is based on the correlation of the relative salt tolerance of the species under study with salt-induced changes in the levels of biochemical markers associated to particular response pathways.

The genus *Juncus* seems to be appropriate for this kind of comparative studies. It includes more than 300 species, salt-sensitive and salt-tolerant (Wilson *et al*. 1993), growing over a wide geographic range covering all continents (except Antarctica), and a spectrum of ecological habitats extending from salt marshes for the most tolerant species, to humid non saline areas where more sensitive species of the genus flourish.

Three species adapted to different natural habitats were chosen for this study. *J. maritimus* Lam. is a halophyte, common in temporarily flooded wetlands in the temperate regions of the world, including the Mediterranean basin. *J. acutus* L. is a sub-cosmopolitan species, that often coexist with *J. maritimus* but is common also on dunes, where water is the main limiting ecologic factor; it has been reported as less salt tolerant than *J. maritimus* (Boscaiu *et al*. 2011; 2013). *J. articulatus* L. seems to be a much more sensitive species, generally growing in fresh water environments; it is frequent in the northern hemisphere and in Australia,

in different humid areas such as wetlands, and along the margins of drains, irrigation channels, creeks and rivers (Albrecht 1994; Chambers *et al*. 1995). However, to our knowledge, no previous study has been carried out on the stress tolerance of this *Juncus* species under controlled conditions.

Regarding the taxonomic relation of the three *Juncus* species, *J. acutus* and *J. maritimus* are recognised as close taxa, belonging to the same subgenus (*Juncus*), whereas *J. articulatus* was classified within the subgenus *Septati* Buchenau, section *Ozophyllum* Dumort (Fernádez-Carvajal 1981); these relationships within the genus have been confirmed by molecular systematic studies (Drábkóva *et al*. 2006; Jones *et al*. 2007).

The major aim of this work was to correlate the relative salt tolerance of the aforementioned *Juncus* species – established from their distribution in nature and by measurements of salt-induced growth inhibition under controlled experimental conditions – with specific responses based on the control of ion transport and the accumulation of different osmolytes. Since the responses to drought and salinity partly overlap, the analysis was extended to plants subjected to water stress treatments, to check whether the same mechanisms were responsible for the relative resistance of the analysed *Juncus* species to both stresses. In line with the ideas discussed above, the results of this study should contribute to our knowledge on the general mechanisms of stress tolerance in plants and, particularly, should help to distinguish those stress responses that are relevant for tolerance in *Juncus*, from those that are not.

Material and methods

Plant material and experimental design

Seeds of *J. acutus* and *J. maritimus* were harvested in a salt marsh located in 'La Albufera' Natural Park (Province of Valencia, Spain), and those of *J. articulatus* in a non-saline area of the same Natural Park. Seeds were sown directly into a moistened mixture of peat (50%), perlite (25%) and vermiculite (25%), in 1 L pots (Ø = 11 cm) placed in 55 x 40 cm plastic trays (12 pots per tray). Three weeks after sowing, seedlings were transferred to individual pots with the same substrate and grown for additional three weeks. During the entire course of germination and seedling growth, the substrate was kept moist, by adding 1.5 L of Hoagland nutritive solution to each tray, twice a week. Water and salt stress treatments were then started, six weeks after sowing, selecting five individual pots with seedlings of the same size for each species and treatment (control, different salt concentrations and water stress). The control plants were

maintained under the same conditions as before, watering them twice a week with 1.5 L Hoagland nutritive solution per tray. Salt stress treatments were performed by adding to each tray the same volume of nutritive solution, but containing NaCl at the final concentrations of 100, 200 or 400 mM; these solutions were freshly prepared by dissolving the required amount of solid NaCl in the standard Hoagland solution. Artificial drought treatments were initiated at the same time, by completely ceasing irrigation of the plants, which otherwise were maintained under the same conditions as the controls. All experiments, from germination of the seeds to the stress treatments, were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 hours of light), temperature fixed at 23^{o}C during the day and 17^{o}C at night, and a CO_2 level of ca. 300 ppm, measured with a Vaisala GMD20 duct mounted carbon dioxide transmitter. Humidity in the growth chamber was monitored with a Testo humidity data logger (model 174H), and ranged between 50 and 80%. After eight weeks of treatment, all salt-stressed, water-stressed and control plants (5 replicas per treatment and per species) were harvested and plant material used for further analyses.

Soil analysis

Electrical conductivity ($EC_{1:5}$) of the substrate was measured after eight weeks of treatment. Soil samples were taken from five pots of each treatment, air-dried and then passed through a 2-mm sieve. A soil:water (1:5) suspension was prepared in deionised water and mixed for one hour at 600 u/min, at room temperature. Electric conductivity was measured with a Crison Conductivity meter 522 and expressed in dS m^{-1}(Gil *et al*. 2011).

Plant growth parameters

The following growth parameters were determined at the end of the stress treatments: length of the longest shoot, fresh weight (FW), dry weight (DW) and water content (WC %) of the shoots. To obtain the water content, part of the fresh material was weighed (FW), dried for four days at 65°C, until constant weight, and then weighed again (DW); the water content percentage was calculated by the following formula: WC (%) = [(FW – DW)/ FW] x 100 (Gil *et al*. 2014).

Ion content measurements

Contents of potassium, sodium and chloride were determined in shoots and roots of the plants sampled after the stress treatments. Measurements were performed according to Weimberg

(1987), in aqueous extracts obtained by incubating the samples (0.15 g of dried and ground plant material in 25 mL of water) for 1 h at 95ºC in a water bath, followed by filtration through a filter paper (particle retention 8-12 µm). Sodium and potassium were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, USA) and chlorides were measured using a Merck Spectroquant Nova 60® spectrophotometer and its associated test kit (Merck, Darmstadt, Germany).

Osmolyte quantification

Proline (Pro) content was determined in fresh plant material by the ninhydrin-acetic acid method described by Bates *et al*. (1973). Pro was extracted in 3% aqueous sulfosalicylic acid, the extract was mixed with acid ninhydrin solution, incubated for 1 h at 95ºC, cooled on ice and then extracted with two volumes of toluene. The absorbance of the organic phase was measured at 520 nm, using toluene as a blank. Pro concentration was expressed as µmol g^{-1} DW.

Glycine betaine (GB) was determined in dried plant material, according to Grieve and Grattan (1983). The sample was ground with 2 mL of Mili-Q water, and then extracted with 1, 2-dichlorethane; the absorbance of the solution was measured at a wavelength of 365 nm. GB concentration was expressed as µmol g^{-1} DW.

Total soluble sugars (TSS) were quantified according to the method described by Dubois *et al*. (1956). Dried material was ground and mixed with 3 mL of 80% methanol on a rocker shaker for 24–48 h. Concentrated sulphuric acid and 5% phenol was added to the sample and the absorbance was measured at 490 nm. TSS contents were expressed as ‘mg equivalent of glucose’ per gram of DW.

HPLC analysis of carbohydrates

The soluble sugar fraction (mono and oligosaccharides) was analysed using a Waters 1525 high performance liquid chromatography system coupled to a 2424 evaporative light scattering detector (ELSD). The source parameters of ELSD were the following: gain 75, data rate 1 point per second, nebulizer heating 60%, drift tube 50ºC, and gas pressure 2.8 Kg/cm^2. Analysis was carried out injecting 20 µL aliquots with a Waters 717 auto-sampler into a Prontosil 120-3-amino column (4.6 x 125 mm; 3 µm particle size) maintained at room temperature. An isocratic flux (1 mL/min) of 85% acetronitrile (J.T. Baker - Avantor Performance Materials) during 25 minutes was applied in each run. Glucose, fructose and sucrose were identified and quantified with the

Waters Empower Pro software by co-injection of the authentic standard compounds (purchased from Sigma Aldrich). Identification of sugars in the plant extracts was performed by spiking the samples with known amounts of glucose, fructose and sucrose.

Statistical analysis

Data were analysed using the programme Statgraphics Centurion XVI. Before the analysis of variance, the Shapiro-Wilk test was used to check for validity of normality assumption and Levene's test for the homogeneity of variance. If ANOVA requirements were accomplished, the significance of the differences among treatments was tested by one-way ANOVA at a 95% confidence level and post hoc comparisons were made using the Tukey HSD test. All means throughout the text are followed by SD.

Results

Effects of salt stress

Electrical conductivity of substrates

Electrical conductivity ($EC_{1:5}$) was recorded in samples of the pot substrates after eight weeks of salt and water stress treatments. For all species, a similar increase in $EC_{1:5}$ was detected in parallel to the increase of NaCl concentrations, reaching about 14 dS m^{-1} in the pots watered with nutritive solution containing NaCl at a final concentration of 400 mM (data not shown); this confirms the high correlation between $EC_{1:5}$ and the concentration of the saline solutions used in the treatments. As expected, the water stress treatments did not modify the electrical conductivity of the substrates in the pots, for any of the three studied *Juncus* species, as compared with the corresponding controls (data not shown).

Growth parameters

Salt treatments inhibited growth of *Juncus* plants, in a concentration-dependent manner, as shown by determination of several growth parameters (Fig. 1). For example, the length of the longest shoot was reduced in *J. articulatus* and *J. acutus* by nearly twofold in the presence of 400 mM NaCl, with respect to the control, non-stressed plants. A slightly smaller relative reduction in shoot length (about 1.5-fold) was observed in *J. maritimus* under the same conditions (Fig. 1a). Plant mass accumulation also decreased in response to salt stress; the relative reduction of fresh weight in the 400 mM NaCl treatment, when compared with the

corresponding controls, was similar for *J. acutus* and *J. maritimus* (65% and 70%, respectively) but of more than 90% in *J. articulatus* (Fig. 1b), thus confirming that this species is the most sensitive to salinity of the analysed *Juncus* taxa, as suggested by its distribution in nature. Water contents decreased with increasing external salt concentrations, from about 80% in control plants to 65%, approximately, in plants treated with 400 mM NaCl, without significant differences detected in the three *Juncus* species under study (Fig. 1c). Therefore, the observed salt-dependent reduction of fresh mass accumulation is indeed due mostly to growth inhibition, and not simply to loss of water under salt stress conditions.

Ions contents in roots

Na^+ levels increased in the roots of the three *Juncus* species, in parallel to increasing salt concentrations in the nutritive solution (Fig. 2a), reaching similar levels – between 3000 and 3500 µmol g^{-1} DW – in plants of the three taxa treated with 400 mM NaCl. A nearly identical pattern of salt-induced Cl^- accumulation in roots was also observed in all species, reaching about 3300 µmolg^{-1} DW at the highest NaCl concentration tested (400 mM NaCl) (Fig. 2b).

In general, K^+ levels in roots did not vary significantly in response to the salt treatments applied (Fig. 2c), although the concentrations measured in *J. articulatus* were about half of those determined in *J. acutus* and *J. maritimus*. K^+/Na^+ ratios in the roots of the control plants were much higher in *J. acutus* and *J. maritimus* (> 2) than in *J. articulatus* (about 0.5), and these values decreased in the presence of NaCl, in the three *Juncus* species (Fig. 2d).

Ions contents in shoots

Contrary to what was observed in the roots, where similar concentrations of Na^+ and Cl^- were measured in the three *Juncus* species, accumulation of these ions in the shoots differed quantitatively in the three taxa, depending on their relative degree of salt tolerance. Although Na^+ and Cl^- levels increased in response to salt, in a concentration-dependent manner, the highest contents were measured in *J. articulatus*, the most salt-sensitive of the analysed taxa, while the lowest levels were detected in the most tolerant, the halophyte *J. maritimus* (Figs. 3a, b). It should be pointed out that, in all cases, the Na^+ and Cl^- concentrations reached were significantly lower in the shoots than in the roots of the plants, especially those of Na^+, with the largest differences observed in the most tolerant *Juncus* species (compare Figs. 2a, b with Figs. 3a, b).

Accumulation of K^+ in shoots, in the presence of increasing NaCl concentrations, also showed different patterns depending on the relative tolerance of the species under study. In *J. articulatus*, K^+ concentrations were higher than in the other taxa – and also almost three-fold higher than in *J. articulatus* roots – but did not change significantly with the different salt treatments (Fig. 4c). In the halophytes *J. maritimus* and *J. acutus*, on the other hand, K^+ contents in shoots decreased at low salinity levels, with reference to non-treated control plants, but increased again in the presence of high external NaCl concentrations (Fig. 3c). K^+/Na^+ ratios in the shoots of the control plants were relatively high, between 10 and 20, but dropped below 0.5 in the presence of NaCl (Fig. 3d).

Osmolyte contents

The levels of common osmolytes – proline, glycine betaine, total soluble sugars – were determined in shoots of the three *Juncus* species, after treatment with increasing NaCl concentrations (Fig. 4). A significant, salt-induced accumulation of these compatible solutes (which were present at similar concentrations in all control plants), was observed in all cases, although with quantitative differences in the different taxa. Thus, a large increase in Pro contents was detected in the halophytes *J. acutus* and *J. maritimus* upon the salt treatments, reaching nearly 60-fold over the non-treated controls in the presence of 400 mM NaCl; under the same conditions, Pro levels remained very low, increasing only 2-fold in the less tolerant *J. articulatus* (Fig. 4a). This clearly different behaviour of the salt tolerant and salt sensitive *Juncus* species was not observed for the other tested osmolytes, GB and TSS, which showed similar salt-dependent accumulation patterns in the three taxa. Salt-treated *J. acutus* and *J. maritimus* plants accumulated somewhat higher concentrations of GB and TSS, respectively, and their levels were slightly lower in *J. articulatus* than in the halophytes (Fig. 4b, c), but these differences were by far smaller than those observed in Pro contents.

HPLC fractionation of the extracts revealed three major peaks of soluble carbohydrates, corresponding to glucose, fructose and sucrose (Fig. 5). All three sugars accumulated in the shoots of salt-treated *J. articulatus* plants, reaching similar concentrations (approximately 150 µmol g^{-1} DW) in the presence of 400 mM NaCl, the highest concentration tested. In the halophytes *J. acutus* and *J. maritimus* a large increase in sucrose contents – but not in those of glucose or fructose – was observed in response to the salt treatments (Fig. 5).

Effects of drought stress

The same parameters measured in salt-treated plants were determined as well in *Juncus* plants subjected to a water stress treatments – eight weeks after they were watered for the last time. Drought also inhibited growth, as indicated by the reduction in the length of the longest shoot of the plants (Fig. 6a) and, more clearly, by a strong relative reduction in the fresh weight of the water-stressed plants as compared to the non-stressed controls (Fig. 6b). According to this criterion, the less salt-tolerant *J. articulatus* is also the taxon most sensitive to drought, showing a FW reduction of 97% after eight weeks without water (the corresponding values for *J. maritimus* and *J. acutus* were 88% and 83%, respectively) (Fig. 6b). These data suggested that the effect of water stress on plant growth was stronger than that of salt stress at the highest NaCl concentration tested. However, in this case the reduction of fresh mass was partly due to loss of water, which ranged between 70% (in *J. maritimus*) and 90% (in *J. acutus*) (Fig. 6c), values much higher than those observed in salt-treated plants (Fig. 1). In any case, the relative drought tolerance of the three *Juncus* species was maintained when growth inhibition was calculated in terms of dry weight reduction as compared to the corresponding controls (data not shown).

As it should be expected, ions contents (sodium, chloride, and potassium), showed no significant changes in roots or shoots of the three studied *Juncus* species under water stress (see 'supplementary material', Fig. S1).

Concerning osmolyte contents under water stress conditions, the accumulation patterns of Pro, GB and TSS were similar to those observed in the presence of NaCl. Thus, drought induced a strong increase in Pro levels in the halophytes, between 50 and 70-fold higher than in the controls, reaching almost 200 µmol g^{-1} DW in the most tolerant *J. maritimus*; in *J. articulatus*, the most stress-sensitive taxon, Pro levels remained very low, with only a ca. twofold increase in the shoots of the water-stressed plants (Fig. 7a). Water stress also induced the accumulation of GB (Fig. 7b) and TSS (Fig. 7c), but to a much lesser extent, between 2- and 3-fold over the controls, and without large differences between the three *Juncus* species.

The drought-dependent increase in the levels of soluble sugars detected in all three *Juncus* taxa was due to accumulation of sucrose, as demonstrated after the carbohydrates were separated and quantified by HPLC. Sucrose contents strongly increased in water stressed plants, reaching values of 160 – 180 µmol g^{-1} DW, without clear differences in the different species (Fig. 7f). Contrary to what was observed in salt-treated plants, water stress treatments did not induce the accumulation of glucose or fructose in *J. articulatus*; in fact, there was a significant

reduction in the levels of these two sugars after the drought treatment. In the halophytes *J. maritimus* and *J. acutus*, either no significant changes or only small reductions in the contents of glucose and fructose were detected (Fig. 7d, e).

Discussion

The most general effect of stress on plants is inhibition of growth, as the plants redirect their resources – metabolic precursors and energy – from primary metabolism and biomass accumulation to the activation of specific defence mechanisms (Munns and Tester 2008; Gupta and Huang 2014). Accordingly, growth inhibition in the presence of salt has been reported for all investigated species, halophytes and glycophytes alike, although extremely salt-tolerant dicotyledonous halophytes may show a slight stimulation of growth at low or moderate salt concentrations (Flowers *et al*. 1986). Some previous studies have been published on the responses to salt stress of *Juncus* species, regarding seed germination, vegetative plant growth or ion accumulation in the plants (Clarke and Hannon 1970; Rozema 1976; Partridge and Wilson 1987; Espinar *et al*. 2005; 2006; Naidoo and Kift 2006; Vicente *et al*. 2007), but very few including different taxa of the genus (e.g., Rozema 1976; Boscaiu *et al*. 2011; 2013). To the best of our knowledge, no comparative analyses on the responses to both, salinity and drought have been carried out on *Juncus* species adapted to different natural habitats, such as those reported here.

Reduction of fresh weight in parallel with increasing external salinity – in relation to the corresponding non-stressed controls – appears to be a reliable criterion to assess the relative salt tolerance of *Juncus* species, as previously suggested (Rozema 1976). According to our results, *J. maritimus*, considered as a typical halophyte, is the most tolerant of the studied species, slightly more than *J. acutus*, which is also a salt-tolerant species, often reported as subhalophyte (Boscaiu *et al*. 2011; 2013). Both taxa are much more tolerant than *J. articulatus*, a species not investigated before. Thus, the responses to salt stress under controlled artificial conditions closely correspond to the species natural distribution and their ecological optima. In the presence of salt, the decrease in water content of the aerial part of the plants was small, and almost identical for the three species; therefore, the relative reduction of fresh weight was mostly due to growth inhibition, indicating that the *Juncus* plants possess efficient mechanisms to limit salt-induced dehydration, independently of their relative degree of salt tolerance. Water stress, on the other hand, caused a stronger dehydration of the shoots, but the relative resistance of the

investigated taxa to drought and salinity followed similar patterns, with *J. acutus* and *J. maritimus* showing higher tolerance than *J. articulatus*. Irrespective of the relative tolerance of the species under study, which was clearly established, the high resistance of all of them – even *J. articulatus* – to quite harsh stress conditions should be pointed out. The plants survived eight weeks in the presence of 400 mM NaCl, or in the absence of water, even though they were strongly affected, could not develop further and eventually died shortly afterwards.

Several previous studies, in which ion contents in different species growing in the same saline habitat were measured, indicated that monocotyledonous halophytes are able to exclude toxic ions (Na^+ and Cl^-) from the aerial parts of the plants, while in dicotyledonous salt-tolerant plants, the ions are efficiently transported to the leaves and are supposed to be stored at high concentrations in the vacuoles, according to the 'ion compartmentalisation hypothesis' (e.g., Albert and Popp 1977; Wyn Jones *et al*. 1977; Gorham *et al*. 1980; Flowers *et al*. 1986; Rozema 1991; Glenn *et al*. 1999). Our results in *Juncus* are in agreement with those data. In the three analysed species, Na^+ and Cl^- contents increased in response to increasing NaCl concentrations in the soil, both in roots and shoots, but reaching higher absolute values in the roots, in all cases. Most important, accumulation of the ions in the shoots closely correlated with the relative sensitivity to salt stress of the three *Juncus* species: the lowest levels were measured in the most tolerant species, *J. maritimus*, followed by *J. acutus*, also a halophyte, whereas the highest were determined in the less tolerant *J. articulatus*. Therefore, inhibition of ion transport to the aerial parts is not a mere response to salinity in *Juncus*, but must be relevant for salt stress tolerance in this genus. This process is not controlled by differential ion uptake from the soil, but clearly at the level of transport from the roots to the shoots – since ion contents in the roots are similar in the three species – and could be mediated by ion transporters of the HKT gene family, which seem to play an essential role in these Na^+ exclusion mechanisms (Munns and Tester 2008; Hamamoto *et al*. 2015).

Sodium accumulation in plants is usually accompanied by a reduction in the endogenous concentrations of potassium, as both ions compete for the same membrane transporters (Niu *et al*. 1995; Rodriguez-Navarro 2000). This general reaction to salinity does not seem to take place in *Juncus*, as no significant decrease in K^+ levels was detected in the roots of any of the three taxa, or in *J. articulatus* shoots. The capacity to maintain K^+ concentrations despite the progressive accumulation of toxic Na^+ ions was considered by Rozema (1976) as the basis of salt tolerance in halophytic species of this genus. Our results indicate, on the contrary, that this

mechanism cannot be relevant for tolerance, as it has been observed also in the more sensitive species, *J. articulatus*. The pattern of variation in K^+ contents in the shoots of the halophytes *J. maritimus* and *J. acutus*, in response to increasing salinity, is also worth mentioning: K^+ decreases at low external NaCl concentration, as compared to the control, non-stressed plants, to increase again in the presence of higher salt concentrations. It seems, therefore, that in the salt-tolerant *Juncus* taxa accumulation of Na^+ at high levels activates transport of K^+ from the roots to the shoots of the plants, to limit the reduction of K^+/Na^+ ratios. This mechanism most likely contributes significantly to salt tolerance in *Juncus* and, in addition, appears to be ecologically relevant. In a previous study carried out in the field, in a littoral salt marsh near the city of Valencia (Gil *et al*. 2014), we observed that K^+ levels in shoots of *J. maritimus* and *J. acutus* were higher in summer than in spring, in parallel with a higher accumulation of Na^+ (and Cl^-). In summer – normally the most stressful season in the Mediterranean climate – we determined much higher soil salinity (based on electric conductivity measurements), and Na^+ and Cl^- levels than in spring, while K^+ contents in the soil remained very low and practically constant throughout the year.

Osmolyte accumulation in the cytosol is also a general response to abiotic stress in plants, and it is generally assumed that it contributes significantly to tolerance by counteracting, at least partly, cellular dehydration caused by different stress conditions, including salinity and drought. In addition to their function in osmotic adjustment, compatible solutes may play other important roles in the mechanisms of stress tolerance, as low-molecular weight chaperones, ROS scavengers or signalling molecules (Smirnoff and Cumbes 1989; Zhu 2001; Ashraf and Foolad 2007; Chen and Murata 2008; Szabados and Savouré 2010; Grigore *et al*. 2011; Gil *et al*. 2013). It has been reported that monocotyledonous halophytes accumulate preferentially soluble carbohydrates (sugars and polyols) for osmotic balance (Gorham *et al*. 1980; Briens and Larher 1982). We have indeed detected a concentration-dependent increase in total soluble sugars in response to the NaCl treatments, but reaching roughly the same levels in the three *Juncus* species, irrespective of their relative salt tolerance. Similarly, TSS also increased in the shoots of *Juncus* plants subjected to water stress, again without large differences between the three taxa. HPLC fractionation allowed the identification of glucose, fructose and sucrose as the major sugars present in all *Juncus* plants, as reported for *J. maritimus* and *J. acutus* grown in nature (Gil *et al*. 2011). However, the *Juncus* halophytes and their less tolerant congener (*J. articulatus*) showed different patterns of sugar accumulation. Significant salt- and water stress-dependent

increases in sucrose contents were detected in all three taxa, while *J. articulatus* showed distinct responses to salinity and drought: the latter treatment significantly decreased the shoot levels of glucose and fructose, whereas these compounds increased in the presence of salt.

Contrary to other osmolytes – such as proline, glycine betaine or some polyalcohols – which are present in the plants at very low levels unless their biosynthesis is activated under stress conditions, soluble sugars are components of primary metabolism that play different functional roles in the cell, as precursors of other metabolites, major energy source or signalling molecules. The concentrations of sugars must be controlled by many different inputs and mechanisms and it is much more difficult to assess their specific roles in stress defence (see Gil *et al*. 2013, for an extended discussion). Therefore, some of the changes in sugar levels observed in *Juncus* shoots might not be directly related to specific stress responses. Nevertheless, the high sugar concentrations measured should clearly contribute to osmotic adjustment in the presence of NaCl, or in the absence of irrigation, thus protecting the plants against the effects of salt and water stress. Yet, here again, it is important to point out that there is no positive correlation between sugar contents and the relative degree of tolerance of the *Juncus* taxa – actually, in the salt treatments the combined concentrations of glucose, fructose and sucrose were somewhat higher in the most salt-sensitive species, *J. articulatus*, than in the halophytes. Therefore, differences in salinity or drought tolerance within the genus *Juncus* do not seem to be due to differential accumulation of soluble carbohydrates.

Proline is not generally considered as a preferential functional osmolyte in monocotyledonous salt-tolerant plants, and the concentrations of free Pro measured in control plants – around 2 µmol g^{-1} DW – were much lower than those of sugars. In salt-treated plants, however, a large increase in Pro content was observed, up to 50 to 60-fold over the controls in the presence of the highest NaCl concentration tested (400 mM), but only in the halophytes *J. maritimus* and *J. acutus*. In the salt sensitive *J. articulatus* Pro levels increased only about 2-fold under the same conditions. The pattern of Pro accumulation in response to water stress was almost identical, with large increases detected only in *J. maritimus* and *J. acutus.* The differential accumulation of this osmolyte in the shoots of *Juncus* plants, depending on the relative tolerance of the studied species, clearly supports a functional role of Pro in the mechanisms of salt and drought tolerance in this genus. Pro probably participates significantly in cellular osmotic adjustment under stress conditions, although it reached maximum absolute levels somewhat lower than those of soluble sugars. Yet its contribution to salt tolerance mechanisms is most

likely mediated, to a large extent, by its additional activities as 'osmoprotectant' – low-molecular-weight chaperon and ROS scavenger (Szabados and Savouré 2010).

Conclusion

Salt tolerance in *Juncus* depends to a large extent on the partial inhibition of transport of toxic ions (Na^+ and Cl^-) from the roots to the plant aerial parts and on the activation of K^+ transport at high external salt concentrations (to limit the reduction of K^+/Na^+ ratios). In addition, the accumulation to relatively high levels of Pro in the shoots of the plants is also important for tolerance to both, salt and water stress, since it contributes to osmotic adjustment but also because of the 'osmoprotectant' roles of this osmolyte. The efficiency of these processes correlated positively with the relative tolerance of the investigated species, and could be distinguished from other stress responses, such as accumulation of soluble sugars, that were activated to a similar extent in the three *Juncus* taxa, and therefore could not be directly involved in their mechanisms of tolerance to stress.

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