Monica Boscaiu; Cristina Lull; Llinares Palacios, JV.; Oscar Vicente; Boira Tortajada, H. (2013). Proline as a biochemical marker in relation to the ecology of two halophytic Juncus species. Journal of Plant Ecology. 6(2):177-186. doi:10.1093/jpe/rts017.

Proline as a biochemical marker in relation to the ecology of two halophytic *Juncus* species

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Running title: Proline accumulation in *Juncus*

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Abstract

Aims

Osmolytes, used for maintaining osmotic balance and as 'osmoprotectans', are synthesised in plants as a general, conserved response to abiotic stress, although their contribution to stress tolerance mechanisms is still unclear. Proline, the most common osmolyte, accumulates in many plant species in parallel with an increase in external salinity, and is considered as a reliable biochemical marker of salt stress. We have measured proline levels in two halophytic, closely related *Juncus* species, under laboratory and field conditions, to assess the possible relevance of proline biosynthesis for salt tolerance and therefore for the ecology of these two taxa.

Methods

Proline was quantified in plants treated with increasing NaCl concentrations and in plants sampled in two salt marshes located in the provinces of Valencia and Alicante, respectively, in south-east Spain. Electrical conductivity, pH, Na^+ and Cl^- concentrations were measured in soil samples collected in parallel with the plant material.

Important Findings

Treatment with NaCl inhibited growth of *J. acutus* plants in a concentration-dependent manner, but only under high salt conditions in the case of *J. maritimus*. Salt treatments led to the accumulation of proline in both species, especially in the more salt-tolerant *J. maritimus*. The results obtained under laboratory conditions were confirmed in plants sampled in the field. In all samplings, proline contents were significantly lower in *J. acutus* than in the more tolerant *J. maritimus* growing in the same area. A direct correlation of soil salinity and proline levels could not be established, but a seasonal

variation was detected, with an increment of proline contents in conditions of accentuated water deficit. Our results suggest that proline biosynthesis is not only an induced, general response to salt stress, but also an important contributing factor in the physiological mechanisms of salt tolerance in *Juncus*, and it is therefore correlated with the ecology of the two species.

Key words

Halophytes, *Juncus acutus*, *Juncus maritimus*, osmolytes, salt stress

INTRODUCTION

Salt marshes are highly interesting ecosystems, which have been extensively studied from multiple points of view. Such habitats constitute a good example of stressful environments, for the well-kwon deleterious effects of high soil salinity on plants (Flowers *et al*. 1986; Serrano 1996), where only adapted, salt tolerant species – the halophytes – can survive. The genus *Juncus*, with more than 300 species, includes both, salt sensitive (glycophytes) and salt tolerant taxa. Two halophytes of this genus, *Juncus acutus* L. and *J. maritimus* Lam., are common in littoral salt marshes in the south-east of the Iberian Peninsula. Both are perennial plants, belonging to the subgenus *Juncus*, distributed on humid soils, temporally flooded, and with a high amount of alkaline carbonates (Fernadez-Carvajal 1982). They often share the same habitats, and are frequent in communities of the class *Juncetea maritimae* Bolos, but have different ecological optima. *J. acutus* is extremely competitive on sandy soils with low and

72 moderate salinity, or even gypsicolous (Boira 1988, 1995), and tolerates well the
73 summer drought typical in Mediterranean ecosystems; *J. maritimus*, on the other hand,
74 is associated with higher humidity, and can be frequently found in communities of the
75 class *Arthrocnemetea* Br.-Bl. and R. Tx. 1943 (Costa and Boira 1981), which are typical
76 of very saline habitats in SE Spain. Therefore, according to their ecology, *J. maritimus*
77 appears to be more salt tolerant than *J. acutus*; the latter taxon behaves more as
78 sabulicolous, as it is frequent in soils with a sandy texture. The two species coexist in
79 conditions of increased soil humidity, representing an ecotonal situation for *J. acutus*,
80 but in strongly saline areas of salt marshes, only *J. maritimus* is present.
81 The adaptations of halophytes to saline environments are multiple, involving
82 complex interactions at the physiological, biochemical and molecular levels (Zhu 2001).
83 One of the fundamental aspects of the response of these plants to soil salinity is their
84 ability to compensate the high external osmotic pressure, thus avoiding the
85 physiological drought characteristic of such environments. A general, conserved
86 mechanism of response to salt stress – as well as to other environmental conditions
87 causing cellular dehydration, such as drought, cold, high temperatures or heavy metals –
88 is the synthesis and accumulation in the cytoplasm of compatible solutes, the so-called
89 'osmolytes'. Osmolytes are very soluble, low-molecular-weight organic compounds,
90 which are not inhibitory to the metabolism even at high concentrations. Besides their
91 direct function in osmotic adjustment, they act as 'osmoprotectants', by directly
92 stabilizing proteins, membranes and other macromolecular structures under dehydration
93 conditions, and by protecting the cell against oxidative stress as scavengers of reactive
94 oxygen species (Flowers and Colmer 2008; Hare *et al.* 1998; Szabados and Savouré
95 2010). Osmolytes are very diverse from the chemical point of view, including, for

example, polyols (glycerol, sorbitol, mannitol), sugars (trehalose, sucrose) or some amino acids and derivatives (proline, glycine betaine) (Flowers *et al*. 1986; Flowers and Colmer 2008; Serrano 1996). Osmolyte accumulation is not restricted to salt tolerant plants, but common for glycophytes and halophytes; in fact, osmolyte biosynthesis represents a striking case of convergent evolution in solving osmotic problems by all organisms, ranging from microorganisms to plants (Burg *et al.* 1996; Yancey *et al*. 1982).

Proline (Pro) is probably the most common compound accumulated by plants as a response to salt, water, or cold stress (Chu *et al*. 1978; Grigore *et al*. 2011; Murakeözy *et al*. 2003; Szabados and Savouré 2010; Verbruggen and Hermans 2008). There are many published reports showing that, under controlled laboratory conditions, the concentration of Pro in plants increases in parallel with an increase of the external salinity level, and there are also some studies on Pro contents in plants collected from the field (see reviews by Marcum 2002; Munns 2002, 2005; Parvaiz and Satyawati 2008; Sen *et al*. 2002; Tester and Davenport 2003). Some of the earliest data regarding Pro accumulation in halophytes are those recorded by Stewart and Lee (1974), who found that Pro levels in *Triglochin maritima* were low in the absence of salt, but increased as the salinity was raised. Afterwards, many authors identified relatively high levels of this amino acid in a large variety of halophytic taxa (e.g., Flowers and Hall 1978; Tipirdamaz *et al*. 2006; Youssef 2009). The use of Pro as osmolyte in *Juncus* was first shown by Cavalieri and Huang (1979) in *J. roemerianus*. More recently, Naidoo and Kift (2006) reported a significant increment of Pro in plants of *J. kraussii* treated with NaCl.

In summary, it is well established that Pro biosynthesis is a general response to salt stress in all those species that use it as the major osmolyte. What is not so clear is the relative contribution of Pro accumulation to the mechanisms of salt resistance in a particular species, i.e. whether or not it is important for tolerance. When comparing the levels of Pro in plants with different degrees of salt sensitivity, they are often higher in the more resistant ones; however, there are also many examples in which there is no positive correlation between Pro contents and tolerance (e.g., Ashraf and Foolad 2007; Chen *et al.* 2007; Guerrier 1998; Lutts *et al.* 1996).

We have measured Pro levels in *Juncus acutus* and *J. maritimus* plants treated with different NaCl concentrations under controlled growth chamber conditions, and in samples of the two species collected from two salt marshes in two successive seasons. Our aim was not only to check whether Pro contents increased with increasing salt concentrations, but also to try and correlate them with the degree of salt tolerance of the two species. If, according to our working hypothesis, Pro accumulation is an important factor contributing to salt tolerance in *Juncus*, Pro levels should be relatively higher in *J. maritimus*. These two taxa represent an ideal material for this kind of comparative studies, since they are closely related from a taxonomic point of view – and therefore, most likely, also genetically – and often sharing the same habitat, but differ in their tolerance to soil salinity, their ecological requirements and local distribution.

MATERIAL AND METHODS

Sampling design

142 Proline levels were determined in adult plants collected from the field as well as in
143 young plants obtained by seed germination and maintained in a growth chamber under
144 controlled conditions. *J. acutus* and *J. maritimus* plant material was sampled at 'Clot de
145 Galvany', a littoral salt marsh located near Elche, in the Province of Alicante
146 (39.12ºN/0.20ºE), as well as in a second salt marsh in the Natural Park of 'La
147 Albufera', in El Saler, near the city of Valencia (38º15N/0.42ºW). In both salt marshes,
148 two neighbouring sites were selected, with different salinity levels – according to their
149 vegetation and to the electrical conductivity of the upper layer of the soil, measured
150 with a field conductivity-meter. In the sites with lower soil salinity, located towards the
151 border of the two marshes (Clot 1 and Saler 1), both species were present; in the central
152 part of the marshes (Clot 2 and Saler 2), with higher salinity, only *J. maritimus* was
153 found, and was considerably more abundant than in the plots with lower salinity. In
154 both areas, samples were collected twice in 2010, in the middle of July and in the
155 middle of November, respectively.
156
157 **Plant material and salt treatments**
158 From each site, culm fragments of five plants were collected separately, cooled on ice
159 and transported to the laboratory, where part of the plant material was weighed and
160 stored frozen at -75ºC; the rest was dried in the oven at 65º for 3-4 days until constant
161 weight, to calculate the percentage of dry weight of each plant . Sampling was repeated
162 in two successive seasons (summer and autumn 2010) from the same individual plants,
163 which had been labelled at the time of the first sampling.
164 *J. acutus* and *J. maritimus* seeds were collected in summer 2008 in the Natural
165 Park of 'La Albufera', and stored at room temperature for several months previous to the

166 experiments. Seeds were sown and germinated in seed trays containing a mixture of
167 peat and vermiculite (3:1). Three months after sowing, young plants were transferred to
168 individual plastic pots of 12 cm diameter with the same substrate, and grown for
169 additional three weeks. Salt treatments (75, 150, and 300 mM NaCl) were then started,
170 and carried out by adding 150 mL of salt solutions (or distilled water, for the control
171 treatments) to the pots, once per week. This volume was enough to maintain the
172 moisture of the substrate throughout the experiment. All procedures were carried out in
173 a growth chamber (Infraca), fitted with three 58 W Philips Master TL-D fluorescent
174 lamps per shelf, providing a PAR of approximately 150 $\mu E\ m^{-2}\ s^{-1}$ during the light time
175 of a 12 h photoperiod. The temperature was kept at 25ºC in the light and 15ºC in the
176 dark. After three months of salt treatments, plants were harvested, weighed on a
177 precision balance, and then frozen and stored at -75ºC, except for three randomly
178 selected plants per treatment, which were used for determination of the mean dry
179 weight, as indicated above for the material collected in the field. The increment in
180 length was calculated by measuring the length of culms at the beginning and at the end
181 of the salt treatments.
182
183 **Soil analysis**
184 Three soil samples were collected at 20 cm depth from each of the selected zones in the
185 two salt marshes, simultaneously with the plant material. Soil samples were air-dried,
186 and then passed through a 2 mm sieve. Textural analysis was performed using the
187 hydrometer method (Bouyoucos 1962). Electrical conductivity and pH were measured
188 in saturate soil paste extracts in a Crinson Conductimeter Basic 30 and a Crinson pH-
189 meter Basic 20+, respectively (Schofield 1942; USSL Staff 1954). Sodium was

190 determined in the saturate soil paste extracts with a Flame Photometer Jenway PFP7
191 (Schuhknecht 1963) and chloride by the precipitation/titration method Mohr (Ayres
192 1970). The same analyses were carried out on the substrate used to grow the plants in
193 pots in the growth chamber, at the end of the salt treatments.
194
195 **Climate analysis**
196 Climatic data were obtained from the nearest meteorological stations – Elche for 'Clot
197 de Galvany' and Benifayó for El Saler – provided by the Agroclimatic Information
198 System for Irrigation (SIAR), Spanish Ministry of Environment, Rural and Marine
199 Affairs (MARM). Water deficit was calculated according to the cumulative rainfall and
200 evapotranspiration during the four months previous to the collections of plant material.
201 This period was chosen since it represents the interval between the two samplings.
202
203 **Proline quantification**
204 Frozen plant material (250 mg), collected in the field or from plants grown in the
205 climate chamber, was ground to a fine powder in a mortar, in the presence of liquid
206 nitrogen, and Pro content was determined according to the method of Bates *et al*. (1973)
207 with minor modifications, as described in Vicente *et al.* (2004). Pro content was
208 expressed in µmol gr^{-1} DW.
209
210 **Statistical analysis**
211 Data were analysed using SPSS, v. 16. Levene and Cochran tests were applied to check
212 whether the requirements of the analysis of variance are accomplished. Significance of
213 differences among treatments and among species was tested by applying one-way

ANOVA. When the ANOVA null hypothesis was rejected, post-hoc comparisons were performed using the Tukey test. A relation of Pro levels and NaCl concentrations, in plants subjected to salt treatments in the growth chamber, was established by applying the optimal correlation. Effect of plot salinity and of seasonal variation was checked for plants sampled in the field, and additionally a two way ANOVA was applied to check their interaction.

RESULTS

Soil and substrate analysis

Soil characteristic were determined in samples collected in the field at 20 cm depth, in summer and autumn 2010, and are summarised in Table 1. The texture of the soil in Clot 1 is loam, in Clot 2 is silty loam and in Saler 1 and 2 is sandy. The pH is more alkaline in El Saler than in Clot de Galvany, but the salinity of the latter is by far higher. In Clot 2 and Saler 2, the two plots with more silt, higher Na^+ and Cl^- levels were measured, showing therefore also higher EC values. Accumulation of silt material in the central depression of salt marshes determines a significant difference of salt concentration and a selective habitat for the studied species. Clot 1, located at the border of the salt marsh, includes a higher percentage of sand and shows very low EC_{SE}, and Na^+ and Cl^- contents, whereas Clot 2, the second plot located in the central part of the same salt marsh, has higher amount of silt, and is extremely saline. In the salt marsh in El Saler, the differences between the two plots are not so extreme; still, EC_{SE} is about 4-fold higher in Saler 2 than in Saler 1. When comparing seasonal variations, the only significant differences were registered in Clot 2, which is the most saline of all

238 experimental plots. Here, average EC_{SE} was almost 40 dS/m in July, increasing to 97
239 dS/m in November. Mean Na^+ levels ranged from 349 to 696 mM, and those of Cl^- from
240 580 to 1433 mM, in summer and autumn 2010, respectively.
241 Concerning the plants grown in the climate chamber, the analysis of the substrate
242 indicated that there is a gradual increment of EC_{SE}, Na^+ and Cl^- with the increase of
243 NaCl concentrations in the salt treatments (Table 2); after three months of watering the
244 plants with 0.3 M NaCl, the electric conductivity of the substrate in the pots surpassed
245 95 dS/m. The measured pH values decreased with increasing salinity.
246
247 **Climate analysis**
248 As they are located near the sea, both salt marshes belong to thermomediterranean
249 thermotype, characterised by warm temperatures (yearly mean of 16 – 18ºC) and by
250 mild winters. The ombrotype in El Saler is dry, but in Clot is semi-arid, as indicated by
251 their aridity indexes of 10.1 and 18.87, respectively (Rivas-Martínez and Rivas Saénz
252 2009). Mean temperatures are also higher in Clot, with a thermicity index of 408 *vs*. 378
253 in El Saler.
254 In the Mediterranean climate, summers are generally hot and dry, and autumns
255 rather wet, but the autumn of 2010 was exceptionally dry, as can be seen from the
256 monthly values of rainfall and evapotranspiration in Fig. 1. The four months previous to
257 each collection of plant samples were dry in both locations, but the water deficit was
258 notably higher in autumn in Clot de Galvany (-469.33 mm before the autumn sampling,
259 as compared to -378.80 mm before the summer sampling). In El Saler, the water deficit
260 was similar in both periods: -325 mm in summer and -306 mm in autumn.
261

Effect of controlled salt treatments on plant growth and proline accumulation

Salt treatments negatively affected the growth of *J. acutus* in a concentration-dependent manner, as shown by a reduced increment of plant length (Fig. 2a) and a decrease of fresh weight (Fig. 2b). A reduction of more than 83% in the length, and more than 87% in the weight was detected in the plants treated with 300 mM NaCl, as compared with the non-treated controls. Growth of *J. maritimus* was also inhibited by salt, but to a lower extent than in *J. acutus* and only at high concentrations. In fact, low salinity levels (75 mM NaCl) stimulated growth of *J. maritimus* plants, in terms of both, increase in culm length and biomass accumulation (Fig. 2).

Treatments with increasing salt concentrations led to a significant and progressive accumulation of Pro in the aerial part of both species; the correlation between the applied NaCl concentrations and the increase of Pro levels was not linear: this increment was more accentuated at higher salinities (150 and 300 mM) than in the 75 mM NaCl treatment (Fig. 3). When comparing the two species, the more salt tolerant, *J. maritimus*, was shown to accumulate relatively higher levels of Pro in response to salt stress. Thus, except for the control treatments, significant differences were detected in plants subjected to the same NaCl concentration; these differences increased with increasing salinity, so that, at 300 mM NaCl, Pro content in *J. maritimus* was about 2.4-fold higher than in *J. acutus* (Fig. 3).

Proline contents in plants collected from their natural environments

Levels of Pro were also determined in *J. acutus* and *J. maritimus* plants collected from the two experimental sites in each salt marsh, in two successive seasons (Fig. 4). Comparing the two species, mean Pro contents were significantly lower in *J. acutus*

than in *J. maritimus* , within the two plots where they were present together (Saler 1 and Clot 1), and in summer as well as in autumn; the difference was especially pronounced – about six fold – in the autumn samples from Clot 1. When comparing the experimental plots defined in the two salt marshes, and despite their large differences in soil salinity, estimated from EC measurements (Table 1), no clear correlation with Pro contents in the plants could be established. For example, no significant differences in Pro levels were detected in the samples of *J. maritimus* collected in summer from all experimental zones, whereas in autumn the highest values were observed in Clot 1, the plot with the lowest electrical conductivity (Fig. 4).

One-way ANOVA, however, detected a significant seasonal variation of Pro contents in *J. maritimus*. Average values of Pro increased in autumn in Saler 2 and, especially, in Clot (Fig. 4). Interestingly, the strongest increment was found in Clot 1, which is the least saline but the driest of the experimental zones selected for this study. When applying two-way ANOVA the interaction between the two factors – plot and season – was significant at the 99% confidence level for both salt marshes.

Discussion

The observed growth responses of *J. acutus* and *J. maritimus* to NaCl treatments, under controlled laboratory conditions, clearly supported the notion that the latter species is more salt tolerant than the former. The higher tolerance to salinity of *J. maritimus* has also been observed in a previous study on seed germination of the two *Juncus* species; although germination percentages and germination rates decreased in both taxa with increasing NaCl concentrations following a similar pattern, recovery of the germination

capacity of the seeds, after removal of salt stress, was enhanced only in *J. maritimus* (Boscaiu *et al.* 2011), a behaviour common for many halophytes (Keiffer and Ungar 1997; Ungar 1978). These findings are in agreement with the local distribution of the two taxa in the studied salt marshes, and also with their general pattern of distribution in littoral areas in soth-east Spain. *J. acutus* is more competitive on sandy soils, with moderate electrical conductivity, whereas *J. maritimus* is much extended on soils with a high amount of fine soil fraction in the deeper horizon, which generates a good retention of water (Boira 1988); since such marshes function as small endorheic basins, salinity is very much increased by accumulation of salts washed from neighbouring zones. The two species often grow in the same plant communities in the area of study, but *J. maritimus* is much more frequent and competitive on strongly saline soils, as indicated by the phytosociological relevé of the association *Puccinelio festuciformis – Arthrocnemetum fruticosi* Br.-Bi. 1931 en. nom. J.M. Géhu 1976 (Costa and Boira 1981).

Optimal growth of *J. acutus* was observed in the absence of salt, with a gradual reduction in the length and weight of the plants as the concentration of NaCl increased. A similar behaviour has been reported for other *Juncus* species (Naidoo and Kift 2006; Rozema 1976) and is considered as a common response to salt stress in monocotyledonous halophytes; growth of many dicotyledonous salt tolerant plants, on the other hand, is stimulated at low or moderate salt concentrations (Glenn and O´Leary 1984; Rozema 1991; Yeo and Flowers 1980). Interestingly, this also happened in our experiments with *J. maritimus*, which reached the highest culm length and fresh weight at 75 mM external NaCl. There are additional reports showing that moderate salt concentrations, such as 100 mM NaCl, stimulated the growth of this species (Clarke and

334 Hannon 1970; Partridge and Wilson 1987). However, this is not necessarily an
335 invariable response to salinity in *J. maritimus*; in similar, independent experiments from
336 our laboratory using plants obtained form a different batch of seeds, we found optimal
337 development in plants grown in the absence of salt (Boscaiu *et al*. 2011). The apparent
338 lack of reproducibility in these experiments may be due to differences in genetic,
339 developmental or environmental factors; in particular, the responses to salinity are
340 dependent to a large extent on the age and the developmental stage of the plants
341 (Vicente *et al*. 2004).

342 In both *Juncus* species, treatment with increasing salt concentrations led to a
343 parallel accumulation of Pro, used for osmotic balance and as osmoprotector. This is in
344 agreement with the overwhelming available evidence indicating that Pro biosynthesis is
345 a reliable marker of salt stress – at least in those species that use Pro as the major
346 osmolyte (Cavalieri and Huang 1979; Liu *et al*. 2008); glycine betaine or other
347 compatible solutes would fulfil a similar function in species which are not Pro
348 accumulators. Moreover, there is also a good correlation between Pro contents and the
349 degree of salt tolerance: the more halophytic species, *J. maritimus*, accumulated Pro to
350 higher levels than *J. acutus* in response to the same NaCl treatments, although the two
351 taxa showed no significant differences in background levels of the osmolyte in the
352 absence of salt; these results suggest that Pro biosynthesis, induced as a response to salt
353 stress, is an important contributing factor in the mechanisms of salt tolerance in *Juncus*.

354 The data mentioned above were obtained using *Juncus* plants grown under
355 controlled experimental conditions, far different from those of the natural habitats of the
356 plants, regarding for example soil characteristics such as nutrient availability or the pH
357 of the substrate (Lidón *et al*. 2009). Therefore, to confirm that the laboratory results had

ecological meaning, we considered important to determine Pro levels also in plant material collected from the field, in areas with different soil salinity and in different seasons. By comparing samples of *J. maritimus* collected in summer and autumn 2010, we confirmed the stress-induced accumulation of Pro in this species. The salinity of plot 2 in Clot de Galvany, characterised by a more arid climate than El Saler, considerably increased in autumn 2010 in relation to the strong water deficit registered in this period. Accordingly, mean Pro contents in plants of *J. maritimus* growing in this area significantly increased in autumn, but the highest Pro values were registered in plants from plot 1 in Clot. Interestingly, Clot 1 is the least saline of the four zones analysed, but also the driest, due to the climatic conditions and to its texture, which includes a higher percentage of sand. Therefore, apart from the effects of salt stress – with its two components of osmotic stress and ion toxicity – the plants are simultaneously subjected to water stress; both environmental conditions will cause cellular dehydration and the induction of osmolyte production. Our data suggest that the major trigger of Pro biosynthesis in *Juncus* is the water deficit rather than soil salinity *per se*, although these two stress factors are obviously related; this would partially explain the lack of correlation between salinity of the soil and Pro contents of the plants. There are several studies showing that osmolyte levels in plants from natural environments vary along the year, increasing in the most stressful periods (e.g., Murakeözy *et al*. 2003). In Mediterranean habitats, the most stressful season is generally summer, characterised by high temperatures and lack of rain. In salt marshes, in addition to drought, salinity is increased because of evaporation of water and concentration of salts. In 2010, the year this study was carried out, in the salt marsh in Clot de Galvany, atypically, the most stressful period was autumn, due to the high rate of evapotranspiration and very low

382 precipitation. In El Saler, the water deficit in autumn was not so intense, since the level
383 of previous precipitation was somehow higher than in Clot; therefore, smaller seasonal
384 differences in Pro levels were detected.

385 Comparing field and laboratory data, there seems to be a discrepancy between
386 plants subjected, apparently, to a similar degree of salt stress – as indicated by electrical
387 conductivity measurements – concerning accumulation of Pro, whose levels are much
388 higher in those plants treated with NaCl in the growth chamber than in plants collected
389 in the wild. However, it is not possible to make direct quantitative comparisons between
390 the two sets of data, for several reasons. First, the environmental conditions of the plants
391 and, most important, their developmental stage – young plants grown from seeds in the
392 lab *vs*. fully-grown adult individuals in the wild – are very different. The substrate used
393 for the pots is organic, facilitating an increased absorption of NaCl, which explains the
394 high values of electric conductivity registered. In addition, the roots of the potted plants
395 are found in a limited and reduced environment with homogeneous salinity, whereas in
396 the field roots can explore a more heterogeneous and considerably larger volume of soil.
397 The EC measurements were performed in samples from the upper soil layer, the first 20
398 cm from the surface, where dissolved salts accumulate by water transport from the
399 shallow water table, but most of the root system of each plant is probably spread
400 through less saline soil; therefore, the degree of salt stress must be actually much lower
401 for the plants growing in the field than for potted plants.

402 As discussed above, a direct correlation between soil salinity and Pro levels in
403 the plants present in the selected salt marshes could not be established. However,
404 concerning the relative salt tolerance of the two *Juncus* species, the data obtained with
405 plant material sampled in the field supported the conclusions of the experiments carried

406 out in the laboratory, since the more halophytic taxon, *J. maritimus*, accumulated
407 significantly higher Pro levels than *J. acutus* under the same environmental conditions –
408 plants growing in the same experimental plot, and in both sampling seasons.
409 Pro, as other osmolytes, seems to fulfil several roles in the mechanisms of stress
410 tolerance in plants (Szabados and Savouré 2010). Although relatively low Pro
411 concentrations might be required for its function as direct 'osmoprotectant' of proteins
412 and cellular structures, or as scavenger of ROS, much higher levels are probably
413 necessary for the maintenance of osmotic equilibrium under salt stress conditions. In the
414 present study, we have not measured ion contents in the plants, and a quantitative
415 assessment of the role of Pro biosynthesis in osmotic adjustment cannot be made.
416 However, cation levels have been determined in salt-treated plants of both species in
417 previous experiments (Boscaiu *et al*. 2011), which showed, for example, accumulation
418 of Na^+ to 85 µmol gr^{-1} DW in *J. acutus* and to 116 µmol gr^{-1} DW in *J. maritimus*, in the
419 presence of 300 mM external NaCl; comparison of these data with the results of the
420 present work would suggest that Pro accumulation, by itself, is not sufficient to
421 compensate the osmotic pressure due to uptake and compartmentalization in the vacuole
422 of toxic ions – not only Na^+, but also Cl^-. Nevertheless, Pro may not be the only
423 compatible solute involved in osmoregulation in the investigated species. We have
424 recently shown that plants of *J. acutus* and *J. maritimus*, growing in El Saler, also
425 contained relatively high levels of sucrose (around 100 µmol gr^{-1} DW, as average) as
426 well as glucose and fructose (30 – 40 µmol gr^{-1} DW), and how changes in the
427 concentration of these sugars correlated positively with the intensity of environmental
428 stress affecting the plants during the course of the year (Gil *et al*. 2011). Therefore, in

addition to Pro, some soluble carbohydrates can also contribute to osmoregulatory mechanisms in *Juncus*.

ACKNOWLEDGEMENTS

This work has been supported by grants from the Spanish Ministry of Science and Innovation – with contribution from the European Regional Development Fund – to O. Vicente (project CGL2008-00438/BOS), and from the Polytechnic University of Valencia to M. Boscaiu (programme PAID-06-09).

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561

562 Figure legends

563

564 **Figure 1**. Water deficit during 2010, in 'Clot de Galvany' and 'El Saler', as a function
565 of monthly precipitation (Pp) and evapotranspiration (ETP). Gray areas correspond to
566 the periods considered for calculation, during the four months before the samplings of
567 plant material, in the middle of July and in the middle of November. Data were
568 registered by the nearest meteorological stations, located in Elche, province of Alicante
569 and Benifayó, province of Valencia, respectively.

570

571 **Figure 2**. Growth responses of *J. acutus* and *J. maritimus* plants, treated for three
572 months with the indicated concentrations of NaCl under controlled conditions in a
573 growth chamber. Percentages of culm length increments (a), and of fresh weight (b)
574 were calculated with respect to the values of non-treated controls, which were
575 considered as 100% (12.05 cm length increment and 308 mg fresh weight in *J. acutus*,
576 and 7.12 cm and 224 mg in *J. maritimus*) (means ± SD, n = 10). Different lower case
577 letters, latin for *J. acutus* and greek for *J. maritimus*, indicate significant differences
578 between treatments; asterisks indicate significant differences between the two species
579 for a given NaCl concentration ($\alpha = 0.05$).

580

581 **Figure 3**. Proline accumulation upon salt stress treatments in *J. acutus* and *J. maritimus*.
582 Plants were treated for three months with NaCl at the indicated concentrations; the
583 figure shows the experimental data of Pro contents (means ± SD, n = 5). Different lower
584 case letters, latin for *J. acutus* and greek for *J. maritimus*, indicate significant

differences between treatments; asterisks indicate significant differences between the two species for a given NaCl concentration ($\alpha = 0.05$).

Figure 4. Proline contents in *J. acutus* and *J. maritimus* plants growing in their natural habitats under different environmental conditions. The values shown are Pro contents (means ± SD, n = 5) determined in plant material sampled in experimental plots with different soil salinity level (1 < 2), defined in two littoral salt marshes, in Clot de Galvany (C1 and C2) and El Saler (S1 and S2), and in two successive seasons (summer and autumn, 2010). Different lower case letters indicate significant differences between experimental plots, and asterisks between the summer and autumn samples from the same plot ($\alpha = 0.05$).

597 **Table 1**: Soil characteristics of the experimental zones, as indicated (Mean values and SD, n = 6, three samples collected in summer and

598 three in autumn 2010)

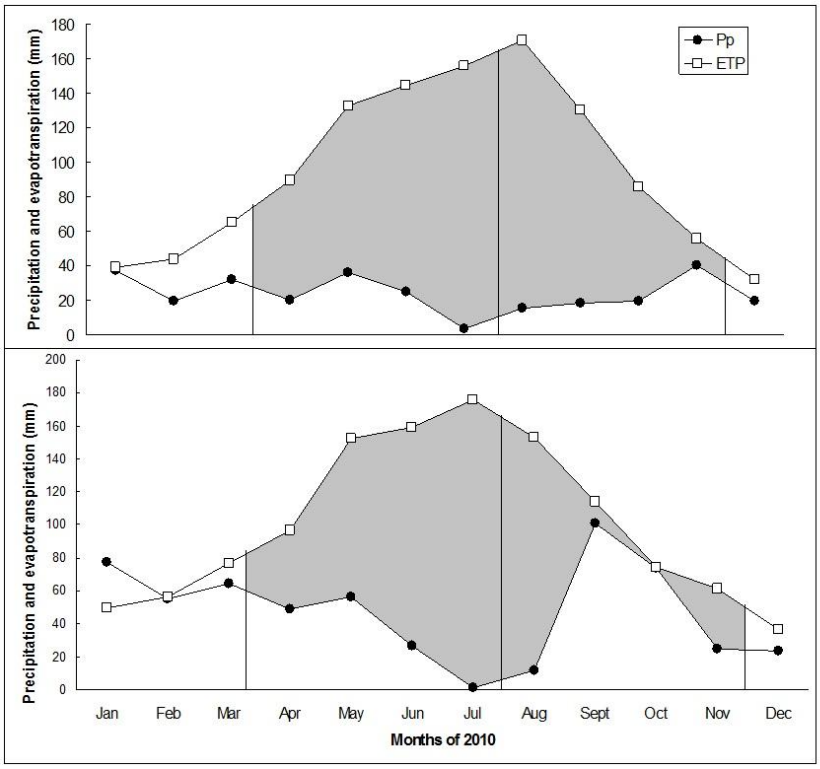
599

Zone	Sand (%)	Silt (%)	Clay (%)	pH	EC_{SE} (dS m^{-1})	Na^+(mM)	Cl^- (mM)
Clot 1	44 ± 0.8	42.3 ± 1.2	13.6 ± 0.9	8.1 ± 0.2	1.2 ± 0.8	3.6 ± 2.6	13.5 ± 9.7
Clot 2	30 ± 0.8	64.3 ± 1.4	5.6 ± 0.4	7.5 ± 0.2	68.1 ± 31.9	522.2 ± 194	1006.6 ± 491
Saler 1	96 ± 0.7	1.2 ± 0.2	2.7 ± 0.1	8.3 ± 0.1	3.5 ± 0.9	11.2 ± 1.9	42.5 ± 17.5
Saler 2	93 ± 0.9	3.4 ± 0.4	3.5 ± 0.2	8.5 ± 0.1	15.0 ± 4.0	190.5 ± 34	266.6 ± 7.3

600
601

Table 2: Electrical conductivity (EC_{SE}), pH, and Na^+ and Cl^- contents in the saturation soil paste extract of the pots, after three months treatment with increasing NaCl concentrations (mean values ± SD, n = 4)

Treatment	pH	EC_{SE} (dS m^{-1})	Na^+ (mM)	Cl^- (mM)
Control	7.63 ± 0.25	1.07 ± 0.26	6.01 ± 0.88	8.75 ± 1.44
75 mM NaCl	6.49 ± 0.16	28.05 ± 3.17	329.00 ± 5.51	449.75 ± 30.30
150 mM NaCl	6.22 ± 0.14	54.75 ± 3.05	524.82 ± 28.85	850.00 ± 57.73
300 mM NaCl	5.88 ± 0.18	95.45 ± 9.95	746.64 ± 68.73	1400.00 ± 230.94



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610 Fig. 1

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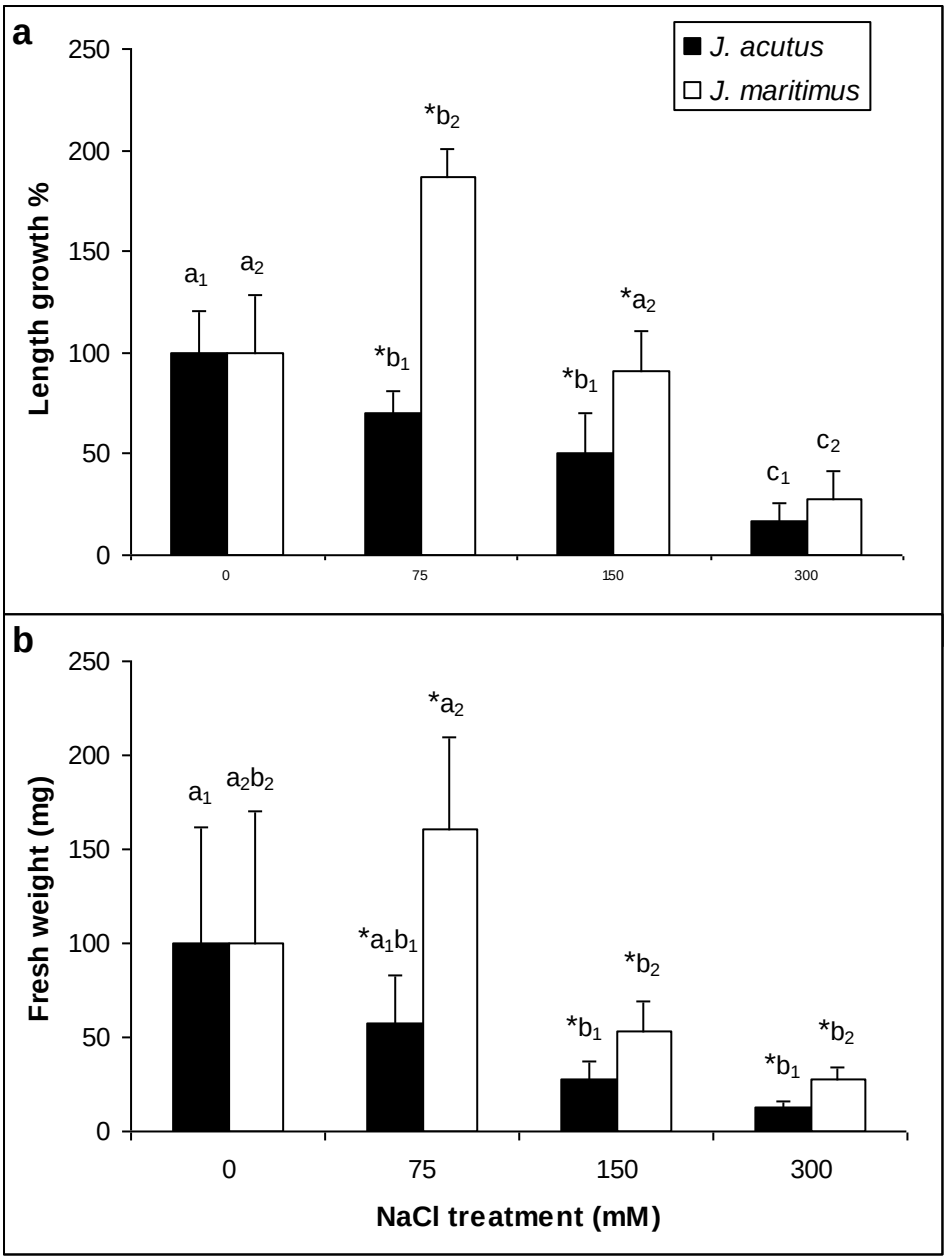

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615 Fig. 2

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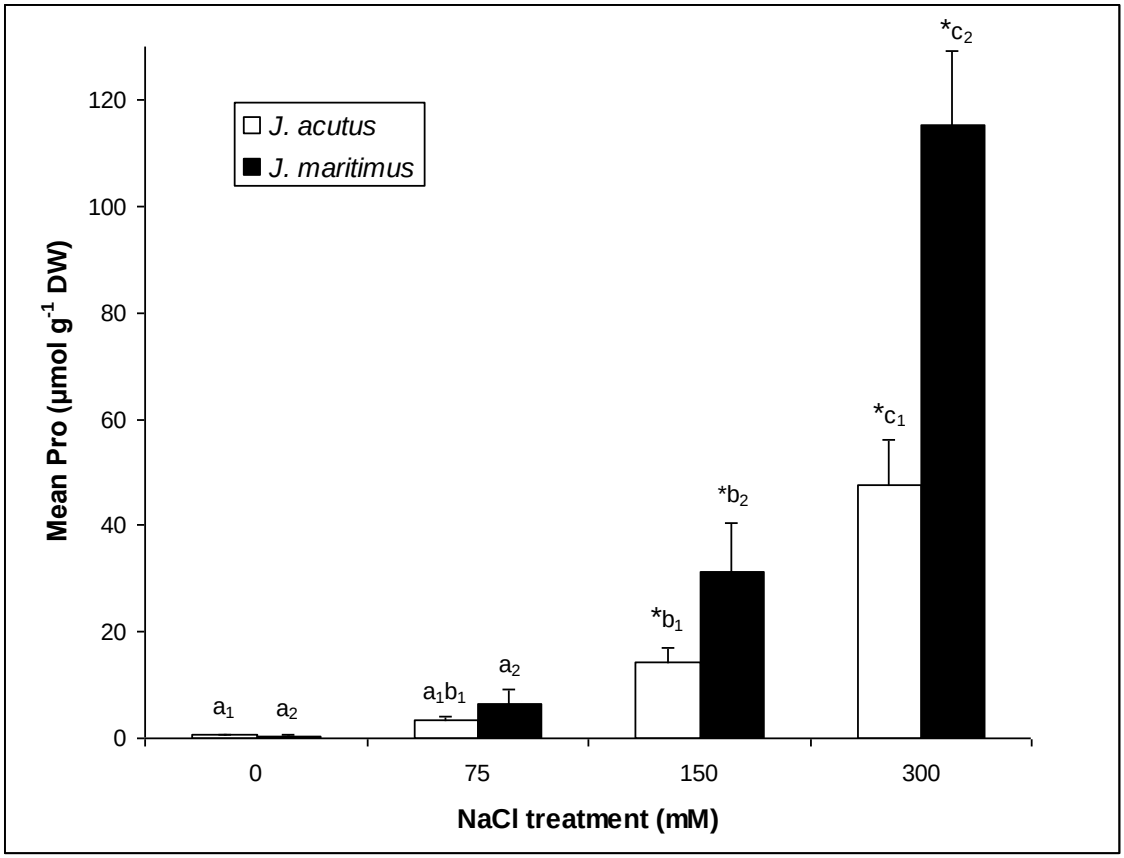

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619 Fig. 3

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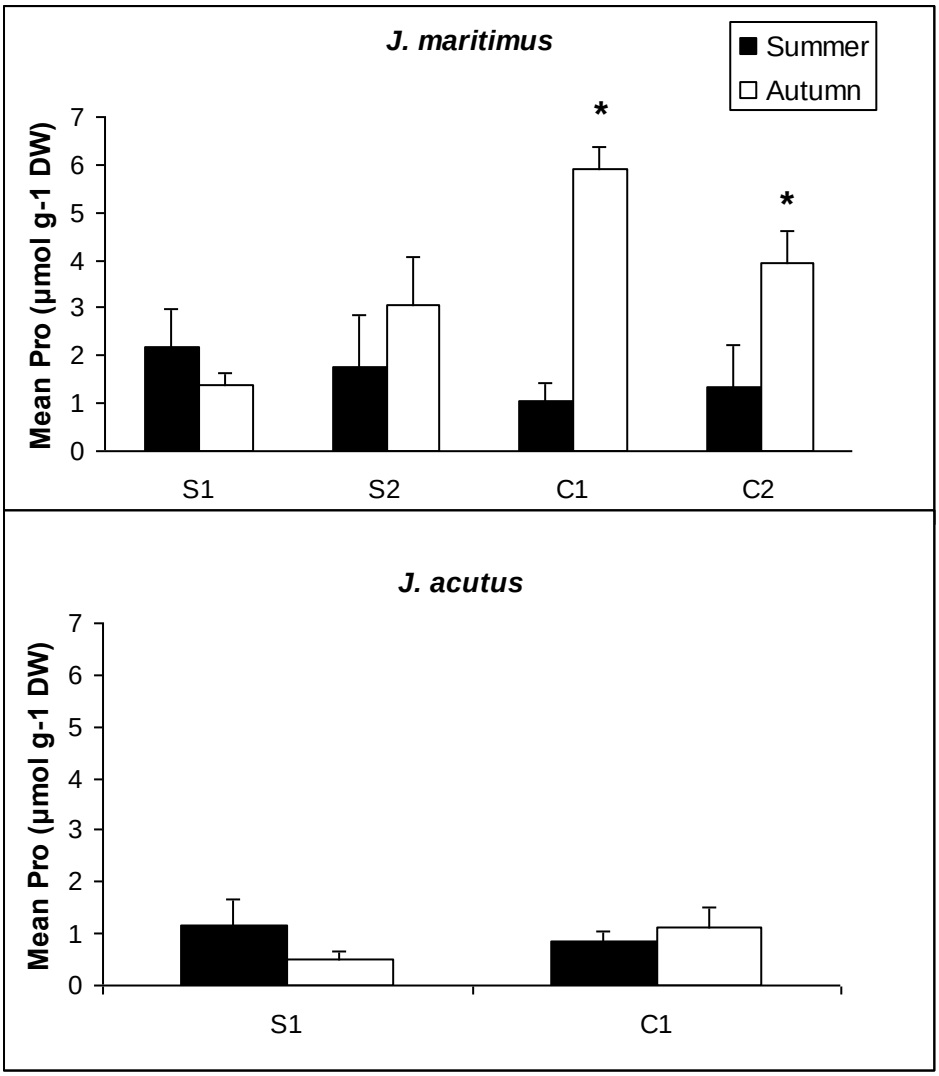

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623 Fig. 4.

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