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ECOPHYSIOLOGICAL STUDIES IN *JUNCUS ACUTUS* L. AND *J. MARITIMUS* LAM.

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Abstract: *Juncus acutus* and *J. maritimus* are two species taxonomically related, and with similar ecology. They both belong to subgenus *Juncus*, have a wide geographic distribution and are frequent in the Mediterranean region. This study has been carried out on plants from the Natural Park of Albufera, near Valencia, in eastern Spain. First, we tested seed germination capacity (in vitro) under salt stress conditions, from 0 to 0.5 M NaCl. In both species, the highest percentages of germination were found in the absence of salt, although seed viability was lower in *J. maritimus*. Germination was reduced by ca. 50% in the presence of 0.2 M NaCl, and completely inhibited at external salt concentration of 0.4 M or higher. Pre-incubation of the seeds in the presence of salt for one month did not negatively affect their germination capacity in water; in fact in *J. maritimus* seeds exposed to high salinity (0.4-0.5 M NaCl) germinated best. We also measured the levels of Na^+, K^+, Ca^{2+} and Mg^{2+} in leaves of plants harvested after three months of salt treatments. The analysis was performed by HPLC in a cation-exchange column, coupled to a conductivity detector. In both species, in parallel with increasing external NaCl concentrations, we detected the accumulation of Na^+ and a slight reduction of K^+ levels. In *J. acutus* the levels of Ca^{2+} and Mg^{2+} proportionally grow with increasing salinity, probably as a mechanism of defence induced by salt stress. In *J. maritimus* their variation is not significant, but is three-four times higher in absence of salt, which suggest the existence of a constitutive mechanisms of defence against high salinity. The results obtained agree with the ecology of the species. *J. maritimus* being more salt tolerant than *J. acutus.*

Key words: *Juncus acutus, Juncus maritimus*, halophytes, salt stress, seed germination, salt pre-treatment, recovery of germination, cation detection, HPLC, defence mechanism.

Introduction

The genus *Juncus*, distributed throughout the world in humid environments, includes more than 300 species (Mabberley, 1997), some of them halophytic. The two taxa studied, *Juncus maritimus* (sea rush) and *J. acutus* (spiny rush), are taxonomically and ecologically related. Both are grasslike, perennial, rhizomatous wetland plants. The sea rush is a perennial herb, up to 1 m high, with a thick and long rhizome and lax inflorescence, whereas the spiny rush is more vigorous, reaching 2 m, with a short rhizome, with a compact inflorescence and a long pungent bract.

They are grouped together within the subgenus *Juncus* and often grow in the same communities in salt marshes. *Juncus acutus* has a wider distribution, distributed in the Mediterranean Region, Western Europe, Canary islands and Madeira, W and N of North America, warm –temperate zones of South America, S of Africa, Australia and New Zealand, whereas *J. maritimus* is found in the Mediterranean region, western and central Europe, Northern Africa, Western Asia, and introduced to North America (Fernández-Carvajal, 1982). Both species are common in Spain, in coastal marsh communities, developed on permanently humid soil, rich in chlorides, especially NaCl and with alkaline carbonates. These Mediterranean salt meadows are included in the alliance *Juncion maritimae* Br.-Bl. 1931 of the class *Juncetea maritime* Br.-Bl. 1931. *Juncus acutus* is also frequent on dunes in zones of estuaries.

Although taxonomically the two species are well studied, not much is known about their tolerance to salt stress. According to their ecology, *Juncus maritimus* is more halophytic than *Juncus acutus*. In SE Spain it often grows on very saline soils, where the dominant vegetation is represented by succulent chamephytes of the class *Arthrocnemetea* Br.-Bl. & R. Tx. 1943 (Costa and Boira, 1981; Costa *et al.*, 1986). *Juncus acutus* grows in more variable habitats, and not always in saline environments. Both rush species have a fast germination rate which, combined with vegetative propagation, allow them to extend rapidly. The spiny rush, not readily eaten by grazing animals, eliminates in some conditions other vegetation and behaves as an invasive plant.

Germination represents the most salt-sensitive stage in the life cycle of halophytes. For most of these species, seed germination is maximal under non-saline conditions, and is strongly inhibited at salt concentrations much lower than those at which adult plants normally grow (Flowers *et al*., 1986; Ungar, 1995). Usually a reduction in the salinity of the surface layers is prerequisite for successful germination. Seed germination in saline environments usually occurs during spring or in the season with high precipitations, when soil salinity levels are reduced (Pujol *et al*., 2000). However, responses of germination to salt stress are very variable; extreme halophytes have the ability to germinate even at concentration in the range of the sea water (Keiffer and Ungar, 1997), whereas in other halophytes even medium salt concentration inhibits completely germination (Vicente *et al*., 2004).

Besides direct response of germination in NaCl external solutions, recovery of germination after a prolonged exposure to salt stress is especially meaningful. While glycophyte seeds lose their capacity of germination, seeds of halophytes generally maintain and often increase their rate of germination after salt pretreatments (Gulzar and Khan, 2001). Seed dormancy is recognized as a significant factor in the ecophysiology of salt-marsh species, which may be subjected to fluctuations in salinity and soil moisture throughout the year (Keiffer and Ungar, 1997). Still, the ability of seeds to remain dormant at extremely low water potentials and to recover germination capacity after inhibition under hypersaline conditions, indicates that they may be more salt tolerant than actively growing plants (Ungar, 1996; Pujol *et al*., 2000).

The most general effect of salt stress is inhibition of plant growth. In contrast to glycophytes, halophytes are able to complete their life cycle under high salinity conditions, but often they show optimal growth in the absence of salt. Salt stress includes two fundamental components: osmotic stress and ion toxicity. The osmotic component results from dehydration and loss of turgor induced by external solutes, and is not specific for NaCl (Serrano and Gaxiola, 1994). On the other hand, high intracellular concentrations of Na^+ and/or Cl^- are toxic, since they inhibit the activity of many enzymatic systems and some cellular processes, such as protein synthesis or mRNA processing (Serrano, 1996; Forment *et al*., 2002). Sodium also affects mineral nutrition, interfering with the uptake of essential cations, especially K^+ and Ca^{2+}, and promotes oxidative stress through generation of "reactive oxygen species" (ROS) (Serrrano and Gaxiola, 1994; Yeo, 1998; Zhu, 2001). Salt tolerance depends, to a great extent, on the cellular compartmentalization of toxic ions: tolerant plants have the ability to accumulate Na^+ and Cl^- in their vacuoles, so that the cytoplasm is maintained at lower ion concentrations, thus avoiding the inhibition of metabolic processes.

The present study reports the effects of salt stress on the germination of seeds of *Juncus acutus* and *J. maritimus*, as well as the recovery of germination after pre-treatment with different concentrations of NaCl. On the other hand, the accumulation in leaf tissue of different mono and divalent cations was tested in plants grown in the presence of salt.

Material and Methods

Plant material

Seeds of *Juncus acutus* and *J. maritimus* were obtained from Oficina Tecnica de la Devesa del Saler, collected in autumn of 2004 in the Natural Park of Albufera, Valencia, in the

East of Spain. The material was air dried thoroughly and hand-sieved. Previous to germination tests, seeds were sterilised with ethanol 70% and a mixture of 30% bleach and Triton X-100 and thoroughly washed in distilled water.

Mono and divalent cations were measured in adult plants, after three months exposure to salt stress conditions under greenhouse conditions.

Germination in the presence of salt

Four replicas of 25 seeds for each treatment were placed onto two layers of filter paper and cotton, in standard Petri dishes. The filter paper was moistened with distilled water for the controls, or with aqueous solutions of 100, 200, 300, 400 and 500 mM NaCl and the Petri dishes were sealed with parafilm. The filter paper was maintained wet by adding water or fresh salt solutions when necessary.

Germination was carried out in a germination chamber with a regime of 12 h light at 25ºC and 12 h darkness at 15ºC. The number of germinated seeds was counted every second day during 1 month from the start of the test.

Germination recovery

All seeds from the previous tests which did not germinate after one month at different salt concentrations, were placed in new Petri dishes with filter paper moistened with distilled water, and incubated under the same conditions for additional 30 days.

Determination of Na^+, K^+, Ca^{2+} and Mg^{2+} content

The levels of these cations were determined in leaves of adult plants, grown from seeds in the greenhouse. Once the plants reached an adequate size, they were subjected to different conditions of salt stress. Besides a control, plants were watered with increasing solutions of NaCl (from 100 to 500 mM NaCl). After three months, plants were harvested and leaf material (0,2 g fresh weight, from three independent plants per treatment) was frozen in liquid nitrogen, ground in a mortar until a fine powder was obtained, and then extracted with 100 mM perchloric acid by incubation at 95ºC for 10 min. Cell debris were removed by centrifugation at 14,000 g for 10 min. The supernatants were collected and processed as described by Vicente *et al*. (2004). Cation analysis was carried out in a Waters HPLC system, with a IC-Pak Cation M/D column coupled to a Waters 432 conductivity detector.

Statistic treatment

Data were analysed using SPSS, version 11. Germination data were arcsine transformed to ensure homogeneity of variance. Significance of differences among treatments was tested by applying one way ANOVA. A Tuckey test was applied to determine whether significant differences ($P<0.05$) occurred between pair-wise treatments.

Results

Seed germination in the presence of salt. Seeds of *Juncus acutus* were highly viable, reaching a germination frequency of 95% after two weeks in distilled water, with almost half of seeds germinating during the first week. Seeds of *J. maritimus* had lower viability; germination was also finalised after two weeks, but only 48% of seeds germinated.

Salt stress affected germination in both species (Fig. 1 and Fig. 2). At a concentration of 100 mM NaCl, a slightly lower percentage of seeds than in the control germinated, 84% in *J. acutus* and 40% in *J. maritimus*. Germination was clearly affected by the 200 mM NaCl treatment, where less than half of seeds germinated, as compared to control seeds germinating in water, 40% for *J. acutus* and 24% for *J. maritimus*, while only a small percentage of seeds germinated at 300 mM NaCl (5% and 4% respectively). Higher concentrations of sodium chloride, 400 or 500 mM, completely inhibited germination in both species.

One -way ANOVA showed that differences among treatments were significant.

Recovery of germination after salt pre-treatments

All seeds that did not germinate in the previous experiments, after 30 days of salt treatments, were transferred onto filter paper wetted with distilled water in fresh Petri dishes and incubated for 30 additional days. Germination percentages in both species were higher for seeds previously exposed to higher concentrations of NaCl. In *J. acutus*, more than 90% for the seeds coming from the 200, 300, 400 and 500 mM NaCl plates, and significantly lower (68.75%) for those from the 100 mM NaCl pre-treatment (Fig. 1). The differences registered in *J. maritimus* where more accentuated: germination in distilled water increased from 10.81% for the seeds pre-incubated in the presence of 100 mM NaCl, to 53.48% for those subjected to the 500 mM pre-treatment (Fig. 2).

Determination of Na^+, K^+, Ca^{2+} and Mg^{2+} contents

For cation measurements, leaf material was collected from plants treated for 90 days with different NaCl concentrations, from 100 to 500 mM, and from control plants grown in the absence of salt. The obtained values were expressed as µmol per gram fresh weight.

In *J. acutus* the mean values of Na^+ accumulated in the leaves increased about 20-fold from the control to the 500 mM NaCl treatment, whereas in *J. maritimus* the variation was not so high: the maximal concentration of Na^+, observed in the 300 mM salt treatment, was less than 10-fold bigger than that registered in the control. In this case, most leaves of the plants grown in the presence of 500 mM NaCl were senescent and there was not enough material to perform these measurements (Figs. 3 and 4)

Values of K^+ content varied very little with increasing salt concentrations, in both species. A slight tendency to a decrease in K^+ levels at higher NaCl concentrations was detected by regression analysis, but the differences were not significant according to one-way ANOVA. Mg^{2+} variation was also not significant for *J. maritimus*, whereas *J. acutus* plants grown in the presence of 500 mM NaCl accumulated double Mg^{2+} levels than the control plants (Figs. 3 and 4). Similarly, Ca^{2+} levels showed a significant increment with increasing external salinity in *J. acutus* (Fig. 3) but not in *J. maritimus* (Fig. 4). It is interesting to note that, in the absence of salt, the concentration of Mg^{2+} and Ca^{2+} is higher (3-4 fold) in *J. maritimus* than in *J. acutus*.

Discussion

The results presented here show that, as for most halophytic species, seeds of *Juncus acutus* and *J. maritimus* germinate best under non-saline conditions. Both tolerate high concentrations of salt as adult plants, but their germination is sensitive to salt stress, and is completely inhibited at NaCl concentrations of 400 mM or higher. The response of germination to saline stress was similar in both species, but germination percentages of *J. maritimus* were lower, since only half of its seeds germinated in control experiments, in the absence of salt. Considering its lower seed viability, *J. maritimus* showed a relatively higher salt tolerance during germination, as compared to *J. acutus*. Also, recovery of germination capacity after prolonged exposure to salt stress was again higher in *J. maritimus*. These data are in perfect agreement with the ecology of the two species, which often grow in same communities, but *J. maritimus* is frequent also in more saline environments. Our data suggest that germination in *Juncus maritimus* is optimal when the seeds are subjected to high soil salinity previous to the moment of germination.

At the sampling site both taxa grow in small endorheic basins, where salts accumulate after being washed out from the neighbouring zones. An important factor is that groundwater level is here high, and soils are clayey, and therefore they remain flooded for months after strong rainfalls, which occur in this typical Mediterranean climate mostly in autumn. In this season, salinity is probably considerably reduced as compared to the summer period, when soils are often completely dry. There is no data in SE Spain regarding the temperature and light conditions that could influence seed germination. However, with the exception of the hot summer, climate in this region is homogeneous along the year and therefore the main factor determining seed germination is very likely the variation of soil salinity (Pujol *et al*., 2000).

The two studied species often grow within the same communities (vegetation class *Juncetea maritimae*), but *J. maritimus* is also frequent in very saline environments (vegetation class *Artrhocnemetea*), where soil in summer is completely dry and covered by a crust of salt. Still the response of germination to salt stress is similar in the two species.

At high osmotic potentials of the soils in their environment, seeds of many halophytes enter in dormancy, recovering germination capacity when the stress condition is alleviated (Pujol *et al.*, 2000). In some cases, germination is stimulated by a previous treatment with salt (Ungar, 1978; Keiffer and Ungar, 1997), in other species germination percentages are similar with or without salt pre-treatments (Pujol *et al.*, 2000), and sometimes the salt decreases the germination capacity of the seeds (Gulzar and Khan, 2001). In *Juncus acutus*, seeds maintained their ability to germinate after one month exposure to salt stress, and germination was high in all treatments. In the case of *Juncus maritimus* we noted that seeds exposed to elevated salt stress germinate even better than the control, non-treated seeds, and that their germination capacity increased in parallel with the increase of NaCl concentrations in the salt pre-treatments. This data are in agreement with the ecology of these two species, as *J. maritimus* is a more obligate halophyte than *J. acutus*.

The salt tolerance of plants depends a great deal on their ability to compartmentalize toxic ions (mostly Na^+ and Cl^-) in the vacuoles, thus avoiding the inhibition by salt of key enzymatic activities and essential cellular processes in the cytosoplasm. In the two species studied, the increment of external NaCl concentration induced the accumulation of Na^+ in the aerial part of the plants. This is a fundamental response of halophytes to salt stress; the glycophytes, on the contrary, have the tendency to exclude these ions from the roots.

Regarding the other cations analysed (K^+, Mg^{2+}, and Ca^{2+}), the variations were small in both species. A slight tendency in the reduction of K^+ levels with increasing external NaCl concentrations was observed, in agreement with the competition between Na^+ and K^+ for the same cellular transporters. Ca^{2+} has a well-known protective role in conditions of salt stress (Rengel, 1992; Bressan *et al.* 1998), whereas inhibition by Na^+ of different enzymatic activities is due to displacement of the Mg^{2+} cofactor from the enzyme's active centre. Thus, the progressive increment of Ca^{2+} and Mg^{2+} with increasing external NaCl concentration in *J. acutus*, may be considered as a salt-inducible defence mechanism which confers some degree of salt tolerance to the plants. In *J. maritimus*, on the other hand, there is no accumulation of these cations in response to salt, but the levels of Ca^{2+} and Mg^{2+} in the leaves are already three to four fold higher than in *J. acutus* in the absence of salt; this fact could be explained as a constitutive mechanism of response to salt stress.

In conclusion, both species behave as halophytic plants, and many of their responses to salt stress are common, but *J. maritimus* appears to show more efficient defence mechanisms against soil salinity, fact that completely agrees with the ecology of these species in SE Spain.

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STUDII ECOFIZIOLOGICE LA *JUNCUS ACUTUS* L. ŞI *J. MARITIMUS* LAM.

(Rezumat)

Cele două specii studiate sunt apropiate din punct de vedere taxonomic şi ecologic. Ambele aparțin subgenului *Juncus*, au o distribuție amplă, şi sunt frecvente în regiunea mediteraneană. În Peninsula Iberică sunt prezente in toate zonele de litoral. Studiul s-a efectuat în Parcul Natural Albufera de Valencia, situat la 6 km de oraşul Valencia, în estul Spaniei. În primul rînd s-a testat capacitatea de germinare a semințelor în condiții de stres salin. Pentru aceasta semințele au fost germinate în condiții standard în camere de germinare în apă distilată şi în concentrații crescînde de sare de la 0,1 pînă la 0,5 M NaCl. S-a obervat că salinitatea afectează procesul de germinare în cazul ambelor specii. Procentajul maxim de germinare s-a înregistrat în ambele cazuri în tratamentele de control, în cel de 0,2 M doar jumătate din semințe au germinat, iar cele de 0, 4 si 0,5 M au inhibat complet germinarea. Semințele rămase negerminate au fost transferate în plăci Petri cu apa distilată pentru a verifica dacă îşi mențin capacitatea germinativă după o expunere indelungată la sare. În acest caz răspunsul a fost diferit, în cazul speciei *J. acutus* nu s-au detectat diferențe între pretratamente (ANOVA de un factor), toate semințele au germinat bine, în timp ce în *J. maritimus* s-a observat o tendință clară de creştere a procentajului de germinare în funcție de salinitate, rezultatul optim fiind după pretratamente în concentrații crescute de NaCl (0,4 şi 0,5 M). În paralel s-a determinat conținutul în cationi în extracte obținute din plante crescute în condiții de stres salin. Analizele s-au realizat cu o coloană de schimb ionic de HPLC legată de un detector de conductivitate. În ambele specii s-a observat o acumulare de Na^+ şi o reducere de K^+, pe măsură ce creşte salinitatea în mediu. Acest fenomen este cunoscut în plantele halofile şi se datorează concurenței celor doi cationi pentru transportatorii celulari. În *J. acutus* nivelele de Ca^{2+} şi Mg^{2+} cresc proporțional cu concentrația externă de sare, fapt corelat cu un mecanism de apărare indus de stresul salin. În *J. maritimus* variațiile acestor cationi nu sunt semnificative, dar nivelele în absență de sare sunt de 3-4 ori mai mari decît în *J. acutus*, ceea ce sugerează existența unui mecanism constitutiv de protecție contra stresului salin. Rezultatele obținute coincid cu ecologia celor două specii, *J. maritimus* fiind o specie mai tolerantă la salinitate decît *Juncus acutus*.

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