

## Do Halophytes Really Require Salts for Their Growth and Development? An Experimental Approach

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### Abstract

Halophytes are salt-tolerant plants found exclusively in habitats with high levels of soil salinity. It is generally assumed that salt stress is the most important limiting factor for plant growth in natural saline environments, and that halophytes have developed specific adaptations to elevated salinity which make them unfitted to grow in the absence of salt, thus explaining their distribution in nature. To address experimentally this question, two halophytic species (*Inula crithmoides* L. and *Plantago crassifolia* Forssk.) and a maritime dune species (*Medicago marina* L.) were grown in the greenhouse for several weeks in different substrates: peat, vegetable garden soil, saline soil and sand from maritime dunes. Measurements of growth parameters-number of leaves, plant length, fresh and dry weights-showed that all three species grew much better on the salt-free and nutrient-rich substrates, peat and garden soil, than on saline soil and dune sand. These results indicate that salts are not compulsorily required for development of halophytic species, and suggest that limitation of water and nutrients, rather than soil salinity *per se*, are the most important restrictive factors for plant growth in saline habitats. The distribution of halophytes in nature is probably dependent on their limited ability to compete with glycophytes in non-saline areas, while remaining highly competitive under environmental conditions stressful for non-tolerant species.

**Keywords:** *Inula crithmoides*, *Medicago marina*, *Plantago crassifolia*, sand dunes, salt marsh, salt tolerance

### Introduction

Halophilic ('salt-loving') plants, or halophytes, are salt-tolerant plants specific for saline environments. Although they have been recognized since the time of Goethe (cf. Flowers *et al.*, 1986), halophytes were brought to scientific attention in the late 19<sup>th</sup> century, through the papers of Schimper (1891, 1898) and especially of Warming (1895, 1897, 1906, 1909). Despite the advances in our knowledge on halophytes biology during the last decades, their definition remains ambiguous and controversial (Grigore *et al.* 2010, and references therein). For example, halophytes have been defined as those plants able to grow and complete their life cycle in habitats with soil salinity higher than 200 mM NaCl (Flowers *et al.*, 1986; Flowers and Colmer, 2008). This operational definition, which excludes 99% of all angiosperm species, may be useful but, obviously, this concentration threshold is rather arbitrary; in fact, there is a continuous spectrum of salt tolerance among plant species, from typical glycophytes (salt-sensitive plants) to extreme halophytes. Another matter of discussion is the possible positive effect of salt on the growth and development of halophytes. Several definitions (Aronson and Le Floe, 1996; Chapman, 1936; Dansereau, 1957) suggest that

salts-especially NaCl-are compulsorily required during the life cycle of halophytes, due to their stimulating effect upon several biological processes in this type of plants. To emphasize this requirement, a sub-category of halophytes has been described: *euhalophytes*, sometimes called 'absolute halophytes', 'exclusive halophytes', or 'obligatory halophytes'; however, by extrapolation or by misinterpretation of the original definition, the term 'euhalophytes' is about to be used for all categories of salt tolerant plants.

In experiments performed under controlled conditions, many extreme dicotyledonous halophytes show, indeed, optimal growth in the presence of low or moderate salt concentrations; however, this stimulatory effect is not commonly detected in monocotyledonous halophytes, nor in other salt-tolerant dicots, which grow best in the absence of salt (Flowers *et al.*, 1977, 1986; Rozema, 1991; Vicente *et al.*, 2004). The degree of salt tolerance varies among halophytes and, for a given species, also at different developmental stages (Flowers *et al.*, 1986; Johnson *et al.*, 1992; Vicente *et al.*, 2004), but inhibition of plant growth is always observed at sufficiently high salinity levels, in all investigated taxa.

The deleterious effects of salt on plants are well known, including cellular dehydration and alteration of osmotic

balance, inhibition of many enzymatic activities and essential cellular processes (such as RNA processing or protein synthesis), interference with mineral nutrition, or generation of 'reactive oxygen species' (ROS), that is, of oxidative stress (Bartels and Sunkar, 2005; Dajic, 2006; Flowers et al., 1986; Flowers and Colmer, 2008; Forment et al., 2002; Läuchli and Grattan, 2007; Munns, 2002, 2005; Nawaz et al., 2010; Parida and Das, 2005; Serrano, 1996; Ungar, 1991). Plants activate different mechanisms to counteract the harmful consequences of exposure to high soil salinity, mechanisms which are based, for example, on the control of ion homeostasis and maintenance of cellular osmotic balance, the synthesis of 'protective' metabolites and proteins, or the activation of chemical and enzymatic antioxidant systems (Hussain et al., 2008; Flowers and Colmer, 2008; Vinocur and Altman, 2005; Zhu, 2001). There are many evidences that these mechanisms are used by all plants, sensitive as well as tolerant, to respond to salt stress, although with clear quantitative differences in the output: in most plants-which are glycophytes-these responses are not efficient enough to cope with even relatively mild salt stress conditions, whereas they lead to salt tolerance in halophytic species. Apart from these basic, conserved biochemical mechanisms of response to salt stress, some halophytes have developed specific ecophysiological or anatomical adaptations-salt glands, for example-which also contribute to their tolerance (Grigore et al., 2011a)

Regardless of the degree of salt tolerance and the mechanisms involved, typical halophytes are never found in the wild in non-saline environments, but are restricted to areas-such as salt marshes or saline arid lands-where the soil has a high content of salts, with an electric conductivity over 4 dS/m. Saline soils are generally poor in nutrients, and in some cases also arid or semiarid, but it is generally accepted that salt stress is the most important restrictive factor for plant growth in this type of habitats, and that halophytes-or, at least, 'obligatory' halophytes-are specifically adapted to these conditions and cannot survive in other habitats, less stressful for the vast majority of plant species, which are not salt-tolerant. In agreement with these ideas, the floristic composition of communities developed in saline habitats is rather poor, and generally less diverse than that of communities located in the adjacent non-saline areas (Ungar, 1991). Accordingly, also under controlled conditions, halophytes should be expected to grow better in the presence than in the absence of salt.

The aim of the present study was to challenge this simplistic explanation of the distribution of halophytic vegetation in nature, analysing the growth in the greenhouse, on different substrates, of three species from a littoral area in Valencia, two recognized and described as halophytes (*Plantago crassifolia* and *Inula crithmoides*) and the third one, *Medicago marina*, as a psammophyte, specific for sand dunes.

## Material and methods

### Species under study

Three species were selected for these experiments: *Inula crithmoides* L. (Asteraceae), *Plantago crassifolia* Forssk. (Plantaginaceae) and *Medicago marina* L. (Leguminosae).

*Inula crithmoides* is a chamaephyte up to 1 m high, with succulent leaves, distributed in the coastal areas of the South and East of Europe (Ball and Tutin, 1976); it is restricted to saline environments located at low altitudes, in the thermomediterranean belt in the Mediterranean, or in the coline belt in temperate climates. In SE Spain, *I. crithmoides* is common in littoral salt marshes, in plant communities of the vegetation class *Arthrocnemetea fruticosi* Br Bl. & R. Tx 1943. In the area of seed sampling, in the Natural Park 'La Albufera', near the city of Valencia (Spain), this species is accompanied by other halophytes, such as *Aster tripolium*, *Juncus maritimus*, *Limonium virgatum*, *Sarcocornia fruticosa* and *Arthrocnemum macrostachyum*.

*Plantago crassifolia* is a smaller chamaephyte, with fleshy leaves in rosettes and numerous stout scapes. The taxon has a Mediterranean distribution (Chater and Cartier, 1976) and is specific for borders of littoral salt marshes. Its ecological optimum is in the association *Schoeno-Plantagineteum crassifoliae* Br.-Bl. 1931 of the class *Juncetea maritima* Br.-Bl. Generally, the species is found at the transition from typical salt marsh to dune vegetation on sandy soils with lower salinity. In the area of study, *P. crassifolia* is very abundant, accompanied by *Schoenus nigricans*, *Scirpus holoschoenus* and *Juncus acutus*, all of them salt tolerant, but not extreme halophytes.

*Medicago marina* is a perennial, procumbent, white tomentose, of about 50 cm high. In Europe, it is distributed on maritime sands on the shores of the Mediterranean, the Black Sea and the Atlantic (Tutin, 1968). In the Natural Park of 'La Albufera', *M. marina* is present in the association *Medicago marinae-Ammophiletum arundinaceae* Br.-Bl 1931 (vegetation class *Ammophiletea* Br.-Bl. & R. Tx. 1943) specific for mobile dunes, which form a belt between the embryonic and fixed dunes (Costa and Mansanet, 1981)

### Plant material

Seeds were collected in autumn 2008 in 'La Albufera' Natural Park-those of *I. crithmoides* and *P. crassifolia* from a salt marsh, and those of *M. marina* from sand dunes-and stored at room temperature. Previous to germination tests, seeds were sterilised by 5 min treatment with 70% ethanol, followed by 5 min with 30% bleach containing 0.05% Triton X-100, and then thoroughly washed in distilled water. Seeds were germinated on Petri dishes in a germination chamber and young seedlings were transferred after one month to individual plastic pots of 9 cm diameter filled with different substrates, and placed in the greenhouse.

### Plant growth

Plants were cultivated in the greenhouse on the following substrates: commercial peat, garden soil, saline soil and sand from a littoral dune. The garden substrate was obtained from an experimental farm of the Polytechnic University of Valencia, and the saline soil and sand were sampled in the Natural Park from the same locations as the seeds. At the beginning of the experiment, pH and electrical conductivity (EC) of the four substrates were determined by standard methods. Soil pH was measured in soil suspensions in a soil-to-water ratio of 1:2.5 (w/v) in a Crison MicropH 2001 pH-meter. Electrical conductivity (EC) of saturated extract was measured in a Crison 522 conductivimeter. For each species, 20 plants were used per treatment. Irrigation was carried out with tap water twice a week and the number of leaves and plant length were measured weekly. After 12 weeks, all plants were harvested and individually weighed on a precision balance to obtain their fresh weight, and thereafter dried at 65°C until constant weight to measure their dry weight.

### Statistic analysis

Data were analysed using SPSS v. 16. Requirements of ANOVA were checked by normality plots and by testing the homogeneity of variance of residual means. Significance of differences among treatments was tested by applying one-way ANOVA, and post hoc comparison were carried out by the Tukey test.

### Results and discussion

Soil characteristics are synthesized in Tab. 1. As expected, the peat substrate showed an acidic pH and low EC, the soil sampled from the salt marsh is alkaline with a high EC, whereas both garden soil and dune sand had similar, slightly basic pH and low EC.

Tab. 1. pH and electrical conductivity (EC) in saturated extract (mean and standard deviation) of the four substrate types used for plant cultivation

Substrate	pH	EC (dS m <sup>-1</sup> )
Peat	5.33 ± 0.31	1.07 ± 0.26
Garden soil	7.95 ± 0.25	1.43 ± 0.35
Saline soil	8.51 ± 0.13	15.03 ± 4.01
Dune sand	7.8 ± 0.14	1.16 ± 0.01

The evolution of leaf number (Fig. 1) and plant length (Fig. 2) during ten weeks of growth clearly showed that all species grew better on peat, followed by garden substrate; development was slower and by far less efficient on the other two substrates used, saline soil and dune sand. Although growth patterns were similar for the three taxa analysed, quantitative differences were observed when comparing the effect of the substrate on the different species. Thus, after ten weeks, total leaf number in *I. crithmoides* was more than 10-fold higher on peat than on sand, while the variation between the same extreme values was about 5-fold in *P. crassifolia*, and only 3-fold in *M. marina* (Fig. 1). Similarly, maximum plant length was reached in peat and minimum in sand, with differences of more than 6-fold, and about 2.5-fold and 3.5-fold in the *Inula*, *Plantago*, and *Medicago* species, respectively (Fig. 2).

The experiment was stopped after 12 weeks of growth, when plants were harvested and their fresh weight (FW) and dry weight (DW) were determined (Tab. 2). Photos of one representative plant per treatment are shown in Fig. 3. The differences observed between treatments were in agreement with the two growth parameters previously analysed. In *I. crithmoides* and *P. crassifolia*, maximum mean fresh weight was registered in the plants grown on peat, followed by those on garden soil; lower values were measured on saline soil and, especially, on sand (Tab. 2). The dry weight of the plants, under all experimental conditions, was always a small fraction of the corresponding fresh weight, below ca. 10%-as expected, since *I. crithmoides* and *P. crassifolia* are both succulent plants-and the differences observed between different substrates were not so high. In fact, no significant differences were detected in the dry weight of plants grown on peat and garden soil, although it was significantly lower for those cultivated on saline soil or sand (Tab. 2). Therefore, maximal fresh weight, associated with optimal plant development on peat or garden soil must be due, at least in part, to a more efficient retention of water by the plants. On the other hand, *Medicago marina* plants are much smaller, and not succulent; in this species, the differences in fresh and dry weight between the four types of substrate were not so accentuated. Optimal growth was reached again on peat, followed by garden soil, but the differences were not significant. Plant growth was slower on the other two substrates, although slightly higher values were determined in plants

Tab. 2. Fresh weight (FW) and dry weight (DW) (means ± SE) of plants cultivated on different substrates. Within each column, values followed by a different lower case letter are significantly different from each other (Tukey test,  $p < 0.05$ )

Substrate	<i>Inula crithmoides</i>		<i>Plantago crassifolia</i>		<i>Medicago marina</i>	
	FW (g)	DW (g)	FW (g)	DW (g)	FW (g)	DW (g)
Peat	46.68 ± 7.57 <sup>a</sup>	2.14 ± 0.24 <sup>a</sup>	86.37 ± 6.68 <sup>a</sup>	1.49 ± 0.08 <sup>a</sup>	2.00 ± 0.30 <sup>a</sup>	0.47 ± 0.07 <sup>a</sup>
Garden soil	28.88 ± 2.01 <sup>b</sup>	2.38 ± 0.18 <sup>a</sup>	55.93 ± 5.71 <sup>b</sup>	1.52 ± 0.06 <sup>a</sup>	1.61 ± 0.63 <sup>a</sup>	0.37 ± 0.17 <sup>a</sup>
Saline soil	10.25 ± 0.97 <sup>c</sup>	0.83 ± 0.06 <sup>b</sup>	6.82 ± 0.89 <sup>c</sup>	0.63 ± 0.07 <sup>b</sup>	0.68 ± 0.14 <sup>b</sup>	0.06 ± 0.01 <sup>b</sup>
Sand	1.87 ± 0.22 <sup>d</sup>	0.15 ± 0.02 <sup>c</sup>	1.66 ± 0.72 <sup>d</sup>	0.18 ± 0.09 <sup>c</sup>	0.84 ± 0.32 <sup>b</sup>	0.21 ± 0.01 <sup>c</sup>

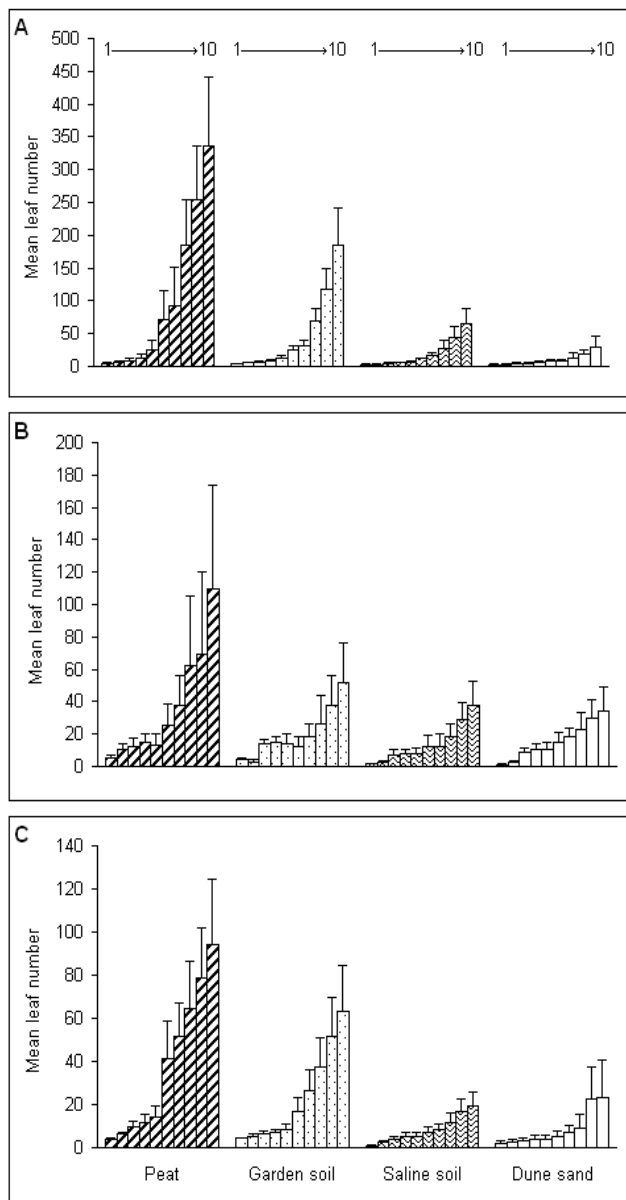


Fig. 1. Evolution of mean leaf number of *Inula crithmoides* (A), *Plantago crassifolia* (B) and *Medicago marina* (C) plants, grown for 10 weeks on the indicated types of substrate

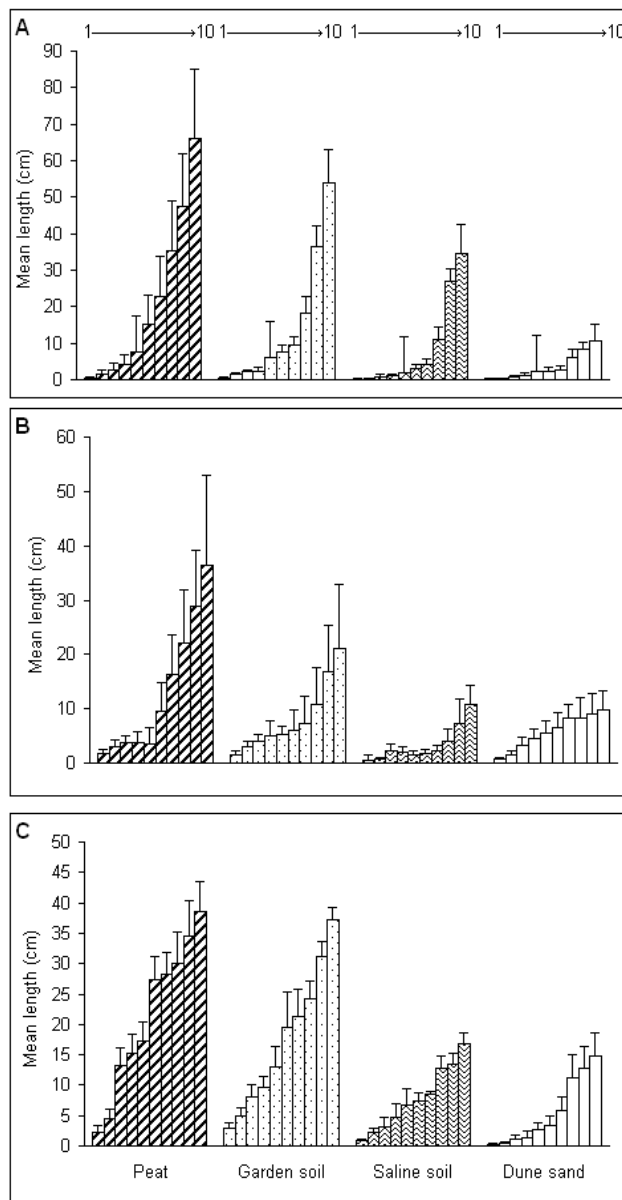


Fig. 2. Evolution of mean plant length (cm) of *Inula crithmoides* (A), *Plantago crassifolia* (B) and *Medicago marina* (C) plants, grown for 10 weeks on the indicated types of substrate

grown in sand than in saline soil, especially when referring to dry weight (Tab. 2).

These results, although preliminary and limited to only three species, strongly question the obligatory presence of salts for optimal development of halophytes. It is clear that these plants grow much better in non-saline substrates than in soil from their natural habitats-even though a further stimulation of growth by adding low or moderate concentrations of salt to the peat or garden substrates cannot be excluded. This is more striking for *I. crithmoides*, a typical succulent euhalophyte present in wet and strongly saline areas. *P. crassifolia*, is restricted to moderately saline patches within a salt marsh, but can also occur in sandy soils or making the transition from salt marsh to maritime dunes. Although *M. marina* is a psammophyte with a deep

root system, it shows a similar evolution of leaf number and plant length when cultivated on sand and saline soil.

As indicated in the Introduction, high soil salinity causes a strong reduction of plant growth, due to the two components of salt stress (Greenway and Munns, 1980): osmotically induced water stress and specific ion toxicity. The high concentration of sodium and chloride also results in nutrient imbalance, by reducing the uptake of  $K^+$ ,  $NO_3^-$  and  $PO_4^{3-}$ , and in the increased production of reactive oxygen species, which damage proteins and membrane structures. In addition, saline soils are physiological dry, due to the low water potential of the rooting medium. The implications for the ecology of halophytes are very important, since many adaptations occurring in halophytes are

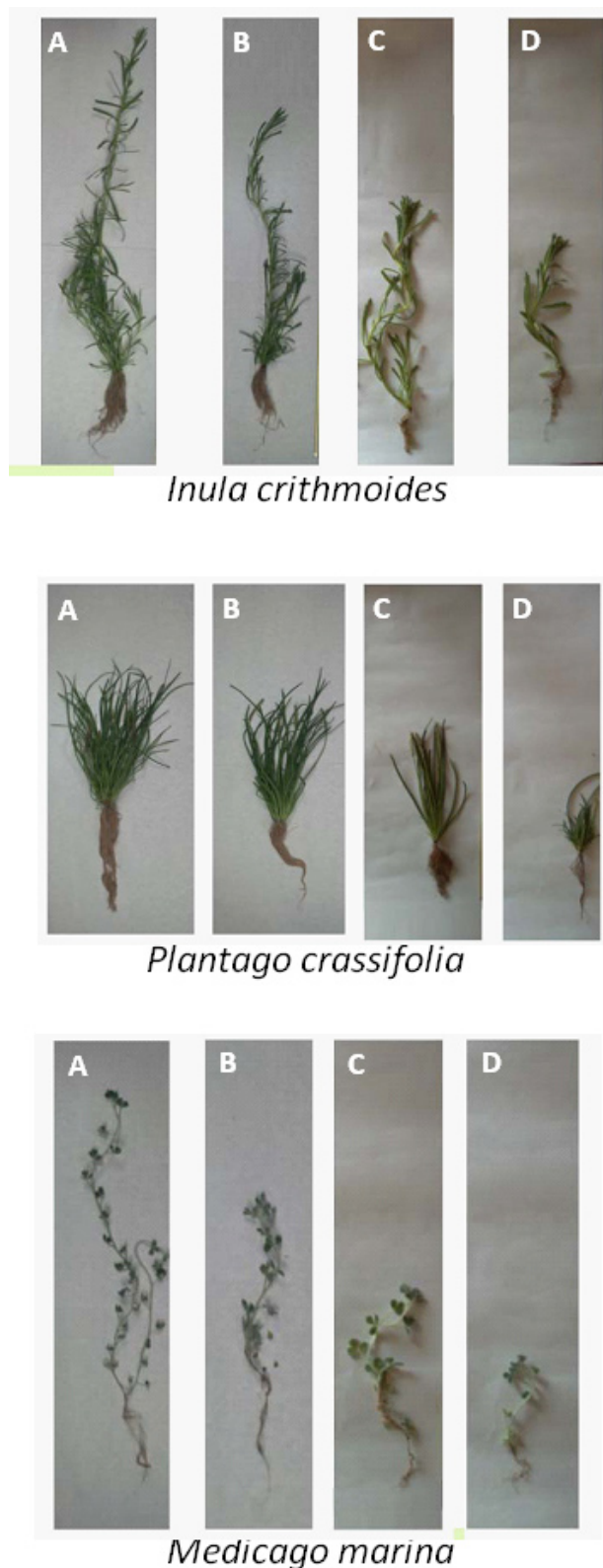


Fig. 3. Relative size of representative individual plants of *Inula crithmoides*, *Plantago crassifolia* and *Medicago marina* after 12 weeks of growth in different substrates: peat (A), garden soil (B), saline soil (C) and dune sand (D); note that plants of the different species are not shown at the same scale

of xerophytic nature (Grigore *et al.*, 2010, 2011b), as succulence in *I. crithmoides* and *P. crassifolia*.

However, data regarding the effect of salts on halophytes are not homogeneous. In many halophytic species, growth is inhibited by increasing salt concentrations; on the contrary, in several genera—such as *Salicornia*, *Suaeda*, or *Atriplex*—a stimulation of growth in the presence of salt has been observed, although no species has been shown to grow optimally at seawater or higher salt concentrations (Ungar, 1991; and references therein). For instance, dry mass production is stimulated by salinity in species that can be regarded as euhalophytes, such as *Aster tripolium* (Baumeister and Schmidt, 1962), *Salicornia brachystachya*, *S. patula* (Grouzis *et al.*, 1977), *S. europaea* (Ungar, 1978), *Spartina anglica* (Partridge and Wilson, 1987), *Suaeda monoica* (Storey and Wyn Jones, 1979), or *S. salsa* (Hekmat-Shoar, 1978). In other taxa, which could be considered less halophytic, a decrease in dry mass upon addition of salt has been observed, for example, in *Atriplex gmelini* (Matoh *et al.*, 1986), *A. hastata* (Black, 1956), *A. nummularia* (Uchiyama, 1987), *A. inflata* (Ashby and Beadle, 1957), *A. triangularis* (Drake and Ungar, 1989), *A. vesicaria* (Black, 1960), or *Spartina alterniflora* (Hopkins *et al.*, 1978).

Nevertheless, even in highly tolerant halophytes such as *Salicornia* species, increased biomass production has been shown to occur in the range from 170 to 340 mM NaCl (data summarized by Ungar, 1991), whereas hypersaline conditions in the field, ranging from 500 to 1000 mM total salts, were found to inhibit the biomass production of *S. europaea* (McGraw and Ungar, 1981). Similarly, growth of *Inula crithmoides* plants, submitted to increasing salt concentrations for a period of 87 days, was only affected by salinity exceeding 20 dS/m, and the accumulated biomass of plants irrigated with 40 dS/m saline water was nearly half of that of the control plants grown in the absence of salt (Zurayk and Baalbaki, 1996).

Most of the examples mentioned above refer to plants treated with salt under controlled artificial conditions. The question remains as to the relative importance of salt stress and other environmental conditions for the distribution of halophytes in nature. Our results suggest that the growth and development of plants present in saline habitats depend not so much on soil salinity *per se*, but rather on other factors such as the availability of water and nutrients. Moreover, it seems that halophytes, including the so-called 'obligatory' or 'exclusive' halophytes, can grow well also on non-saline substrates. Therefore, the absence of salt-tolerant species from natural habitats with low salinity cannot be simply explained by specific adaptations to high salt conditions that make them unfitted to grow in other types of habitats, but most likely because of competition with other plant species. Halophytes, to avoid the pressure of futile competition, might have been restricted during evolution to saline environments, where they would have

achieved several adaptations allowing them to persist and to eliminate subsequent possible competitors. However, these adaptations could not include a compulsory requirement of high salt for survival, due to the continue fluctuations in the salinity level in such ecosystems.

## Conclusions

This study clearly shows that the selected halophytic species do not require salt for their growth and development; in fact, they grow better on non-saline and nutrient-rich substrates. These results suggest that, in addition to soil salinity *per se*, limitation of water and nutrients are also very important restrictive factors for plant growth in natural saline habitats. Probably, halophytes are refugees outcompeted from non-saline areas by glycophytic species, while remaining highly competitive under suboptimal conditions, stressful for non-tolerant species, thus explaining the presence of halophytes exclusively on saline soils in the wild. Further research, extended to other species, would be required to confirm these ideas.

## Acknowledgements

This work was funded by the Spanish Ministry of Science and Innovation (project CGL2008-00438/BOS), with contribution from the European Regional Development Fund. M.-N.G. acknowledges the support provided by the Romanian POSDRU/89/1.5/S/49944 project 'Developing the innovation capacity and improving the impact of research through post-doctoral programmes', and by COST Action FA0901: 'Putting Halophytes to work-From Genes to Ecosystems' for his stay in Valencia in the frame of a Short Term Scientific Mission.

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