UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Mecanismos de Tolerancia a Estrés Salino e Hídrico en Plantas Endémicas, Raras o Amenazadas

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Resumen

Introducción

La sequía y la salinidad son los factores ambientales que más afectan a las plantas, aunque en general, las plantas mediterráneas están bien adaptadas a las condiciones adversas. Las previsiones estiman que debido al calentamiento global las condiciones ambientales se volverán más estresantes, especialmente en las zonas semi-áridas y áridas como muchas áreas de la Península Ibérica. Estas condiciones pueden afectar a la presencia de muchas especies silvestres, en especial, de las que ya de por si están amenazadas, son raras o endémicas.

Tanto la sequía como el estrés salino provocan la activación de una serie de mecanismos de defensa o respuesta de las plantas, que incluyen entre otros, el control del transporte iónico, la acumulación de solutos compatibles u osmolitos, y la activación de sistemas antioxidantes.

Para contribuir a la conservación y/o reintroducción de las especies de interés en hábitats prioritarios se ha realizado un estudio multidisciplinar abarcando los parámetros que pueden afectar sus poblaciones, como el clima, el suelo, y la vegetación acompañante, junto a estudios comparativos sobre las respuestas a la sequía y a la salinidad. Para entender mejor los mecanismos de tolerancia se han incluido en el estudio además de los taxones de interés conservacionista, especies relacionadas genéticamente con diferentes niveles de tolerancia.

El estudio presenta dos objetivos principales: i) establecer la tolerancia relativa al estrés hídrico y salino de las especies en base a su distribución en la naturaleza y en los análisis realizados en campo y, en función de la inhibición relativa de su crecimiento bajo condiciones provocadas de estrés; y, ii) evaluar los cambios bioquímicos inducidos por el estrés analizando diferentes mecanismos de respuesta (inhibición de la fotosíntesis, transporte iónico, acumulación de osmolitos, mecanismos antioxidantes).

Metodología

La mayoría de las especies estudiadas son halófitas, como las del género *Limonium*, o con comportamiento moderadamente halófito, como *Thalictrum maritimum* y *Bupleurum tenuissimum*, a excepción de una de las especies utilizada como material comparativo, *Bupleurum fruticosum*, una glicófita tolerante al déficit hídrico.

La metodología de trabajo incluye estudios efectuados en el campo, como censos poblacionales en algunas especies, análisis de las comunidades vegetales o de la morfología de las raíces en otras, completados con un análisis edafológico y climatológico.

Para analizar las respuestas en condiciones controladas de invernadero se han efectuado tratamientos de estrés salino con varias concentraciones de NaCl, dependiendo de las especies. El tratamiento de estrés hídrico se ha realizado en ausencia completa de riego.

La duración de los ensayos ha variado entre 21 y 30 días en función del grado de tolerancia de las especies.

Una vez finalizados los ensayos de invernadero se han analizado los parámetros de crecimiento, la degradación de los pigmentos fotosintéticos, el patrón de transporte iónico y la acumulación de osmolitos y compuestos antioxidantes, así como la activación de las enzimas antioxidantes.

Resultados y discusión

Los resultados obtenidos han permitido establecer el grado de tolerancia relativa al estrés hídrico y salino de las especies analizadas y sus relaciones con los factores de su entorno como el clima, vegetación y suelo. Por lo tanto, en base a esta información se podrán determinar las zonas óptimas para el desarrollo de las especies de interés.

Además, se han identificado los mecanismos relevantes para la tolerancia a estos dos tipos de estrés en cada especie analizada. Todas las plantas activan los mismos mecanismos de defensa en respuesta al estrés abiótico, pero su contribución difiere dependiendo del género e incluso de la especie. En general, se ha notado una intensificación del transporte de los iones hacia la parte aérea de las plantas y la activación de los sistemas antioxidantes enzimáticos, suficientes para mantener el equilibrio redox. Por lo tanto, la mayoría de las especies estudiadas no mostraron estrés oxidativo, salvo las dos especies del género *Bupleurum*.

Las especies del género *Limonium* mostraron la menor inhibición de crecimiento ante situaciones de estrés, sobre todo bajo condiciones de salinidad, y por lo tanto este género es claramente el más tolerante de los estudiados, basando su defensa principalmente en la activación del transporte de los iones tóxicos hacia la parte aérea y de los sistemas antioxidantes enzimáticos.

Thalictrum maritimum mostró ser más tolerante de lo esperado al estrés salino, pero menos resistente al estrés hídrico que le causó una mayor inhibición de crecimiento. Sus mecanismos de defensa parecen estar más relacionados con el transporte activo de iones a la parte aérea y el mantenimiento de la homeostasis del K^+ en las hojas, así como, la activación de sistemas antioxidantes enzimáticos.

Las especies del género *Bupleurum* mostraron una clara diferencia, siendo la especie de interés *B. tenuissimum* más tolerante a la salinidad mientras que la especie empleada como material comparativo, *B. fruticosum* mostró ser más tolerante a la sequía. Sus principales mecanismos de defensa están relacionados con la inmovilización de los iones tóxicos en las raíces, la acumulación de prolina y la activación de sistemas antioxidantes enzimáticos y no enzimáticos.

Conclusión

Los resultados obtenidos en el trabajo aportan información clave para la reintroducción y la conservación o mantenimiento de las poblaciones de las especies de interés, así como para la compresión de la reducción poblacional en los últimos años.

Por otra parte, los resultados también contribuyen a la mejor comprensión de los mecanismos de tolerancia en los géneros estudiados ante situaciones de estrés hídrico y salino, aportando información sobre las respuestas al estrés en especies que no han sido previamente analizadas.

Este trabajo ha dado lugar a siete manuscritos científicos (publicados, en revisión, o en preparación), que se consideran como subcapítulos de la sección de resultados de esta tesis y se enumeran a continuación:

1) González-Orenga, S.; Al Hassan, M.; Llinares, J.V.; Lisón, P.; López-Gresa, M.P.; Verdeguer, M; Vicente, O.; Boscaiu, M. Qualitative and Quantitative Differences in Osmolytes Accumulation and Antioxidant Activities in Response to Water Deficit in Four Mediterranean *Limonium* Species. *Plants* **2019**, *8*, 506; https://doi.org/10.3390/plants8110506

2) González-Orenga, S.; Llinares, J.V.; Al Hassan, M.; Fita, A.; Collado, F; Lisón, P.; Vicente, O.; Boscaiu, M. Physiological and Morphological Characterisation of *Limonium* Species in their Natural Habitats: Insights into their Abiotic Stress Responses. *Plant & Soil* **2020**, *449*, 267–284; https://doi.org/10.1007/s11104-020-04486-4

3) González-Orenga, S.; Ferrer-Gallego, P.P.; Laguna, E.; López-Gresa, M.P.; Donat-Torres, M.P.; Verdeguer, M.; Vicente, O.; Boscaiu, M. Insight on Salt Tolerance of Two Endemic *Limonium* from Spain. *Metabolites* **2019**, *9*, 294; https://doi.org/10.3390/metabo9120294

4) González-Orenga, S.; Donat-Torres, M.P.; Llinares, J.P.; Navarro, A.; Collado, F.; Ferrer-Gallego, P.P.; Laguna, E.; Vicente, O.; Boscaiu, M. Multidisciplinary Studies Supporting Conservation Programmes of Two Rare, Endangered *Limonium* Species from Spain. (Bajo revisión)

5) González-Orenga, S.; Grigore, M.N.; Vicente, O.; Boscaiu, M. Salt Tolerance Mechanisms and Potential Uses of *Limonium* Species. *Agronomy* **2021**, *11*, 413; https://doi.org/10.3390/agronomy11030413

6) González-Orenga, S.; Trif, C.; Donat-Torres, M.P.; Llinares, J.V.; Collado, F.; Ferrer-Gallego, P.P.; Laguna, E.; Boscaiu, M.; Vicente, O. Responses to Increased Salinity and Severe Drought in the Eastern Iberian Endemic Species *Thalictrum*

maritimum (Ranunculaceae), Threatened by Climate Change. *Plants* **2020**, 9, 1251; https://doi.org/10.3390/plants9101251

7) González-Orenga, S.; Leandro, M.E.D.A.; Tortajada, L.; Llorens, J.A.; Ferrer-Gallego, P.P.; Laguna, E.; Boscaiu, M.; Vicente, O. Comparative Study of Stress Responses in Two Species of *Bupleurum* (Apiaceae) in Support of Conservation Programs. (En preparación)

Otros manuscritos publicados durante el período de trabajo, más o menos relacionados con el tema, pero no incluidos en la tesis, se enumeran en el apéndice al final de este documento.

Resum

Introducció

La sequera i la salinitat són els factors ambientals que més afecten les plantes, encara que en general, les plantes mediterrànies estan ben adaptades a les condicions adverses. Les previsions estimen que a causa del calfament global les condicions ambientals es tornaran més estressants, especialment en les zones semi-àrides i àrides com moltes àrees de la Península Ibèrica. Aquestes condicions poden afectar la presència de moltes espècies silvestres, especialment, de les que ja de per si estan amenaçades, són rares o endèmiques.

Tant la sequera com l'estrès salí provoquen l'activació d'una sèrie de mecanismes de defensa o resposta de les plantes, que inclouen entre altres, el control del transport iònic, l'acumulació de soluts compatibles u osmolits, i l'activació de sistemes antioxidants.

Per a contribuir a la conservació i/o reintroducció de les espècies d'interès en hàbitats prioritaris s'ha realitzat un estudi multidisciplinari abastant els paràmetres que poden afectar les seues poblacions, com el clima, el sòl, i la vegetació acompanyant, al costat d'estudis comparatius sobre les respostes a la sequera i a la salinitat. Per a entendre millor els mecanismes de tolerància s'han inclòs en l'estudi a més dels tàxons d'interès conservacionista, espècies relacionades genèticament amb diferents nivells de tolerància.

L'estudi presenta dos objectius principals: i) establir la tolerància relativa a l'estrès hídric i salí de les espècies sobre la base de la seua distribució en la naturalesa i en les anàlisis realitzades en camp i, en funció de la inhibició relativa del seu creixement sota condicions provocades d'estrès; i, ii) avaluar els canvis bioquímics induïts per l'estrès analitzant diferents mecanismes de resposta (inhibició de la fotosíntesi, transport iònic, acumulació de osmolits, mecanismes antioxidants).

Metodologia

La majoria de les espècies estudiades són halòfites, com les del gènere *Limonium*, o amb comportament moderadament halòfit, com *Thalictrum maritimum* i *Bupleurum tenuissimum*, a excepció d'una de les espècies utilitzada com a material comparatiu, *Bupleurum fruticosum*, una glicòfita tolerant al dèficit hídric.

La metodologia de treball inclou estudis efectuats en el camp, com a censos poblacionals en algunes espècies, anàlisis de les comunitats vegetals o de la morfologia de les arrels en unes altres, completats amb una anàlisi edafològic i climatològic.

Per a analitzar les respostes en condicions controlades d'hivernacle s'han efectuat tractaments d'estrès salí amb diverses concentracions de NaCl, depenent de les espècies.

El tractament d'estrès hídric s'ha realitzat en absència completa de reg. La duració dels assajos ha variat entre 21 i 30 dies en funció del grau de tolerància de les espècies.

Una vegada finalitzats els assajos d'hivernacle s'han analitzat els paràmetres de creixement, la degradació dels pigments fotosintètics, el patró de transport iònic i l'acumulació de osmolits i compostos antioxidants, així com l'activació dels enzims antioxidants.

Resultats i discussió

Els resultats obtinguts han permès establir el grau de tolerància relativa a l'estrès hídric i salí de les espècies analitzades i les seues relacions amb els factors del seu entorn com el clima, vegetació i sòl. Per tant, sobre la base d'aquesta informació es podran determinar les zones òptimes per al desenvolupament de les espècies d'interès.

A més, s'han identificat els mecanismes rellevants per a la tolerància a aquests dos tipus d'estrès en cada espècie analitzada. Totes les plantes activen els mateixos mecanismes de defensa en resposta a l'estrès abiòtic, però la seua contribució difereix depenent del gènere i fins i tot de l'espècie. En general, s'ha notat una intensificació del transport dels ions cap a la part aèria de les plantes i l'activació dels sistemes antioxidants enzimàtics, suficients per a mantindré l'equilibri redox. Per tant, la majoria de les espècies estudiades no van mostrar estrès oxidatiu, excepte les dues espècies del gènere *Bupleurum*.

Les espècies del gènere *Limonium* van mostrar la menor inhibició de creixement davant situacions d'estrès, sobretot sota condicions de salinitat, i per tant aquest gènere és clarament el més tolerant dels estudiats, basant el seu defensa principalment en l'activació del transport dels ions tòxics cap a la part aèria i dels sistemes antioxidants enzimàtics.

Thalictrum maritimum va mostrar ser més tolerant de l'esperat a l'estrès salí, però menys resistent a l'estrès hídric que li va causar una major inhibició de creixement. Els seus mecanismes de defensa semblen estar més relacionats amb el transport actiu d'ions a la part aèria i el manteniment de l'homeòstasi del K^+ davant l'augment de sodi en les fulles, així com, l'activació de sistemes antioxidants enzimàtics.

Les espècies del gènere *Bupleurum* van mostrar una clara diferència, sent l'espècie d'interès *B. tenuissimum* més tolerant a la salinitat mentre que l'espècie emprada com a material comparatiu, *B. fruticosum* va mostrar ser més tolerant a la sequera. Els seus principals mecanismes de defensa estan relacionats amb la immobilització dels ions tòxics en les arrels, l'acumulació de prolina i l'activació de sistemes antioxidants enzimàtics i no enzimàtics.

Conclusió

Els resultats obtinguts en el treball aporten informació clau per a la reintroducció i la conservació o manteniment de les poblacions de les espècies d'interès, així com per a la compressió de la reducció poblacional en els últims anys.

D'altra banda, els resultats també contribueixen a la millor comprensió dels mecanismes de tolerància en els gèneres estudiats davant situacions d'estrès hídric i salí, aportant informació sobre les respostes a l'estrès en espècies que no han sigut prèviament analitzades.

Aquest treball ha donat lloc a set manuscrits científics (publicats, en revisió, o en preparació), que es consideren com a subcapítols de la secció de resultats d'aquesta tesi i s'enumeren a continuació:

1) González-Orenga, S.; Al Hassan, M.; Llinares, J.V.; Lisón, P.; López-Gresa, M.P.; Verdeguer, M; Vicente, O.; Boscaiu, M. Qualitative and Quantitative Differences in Osmolytes Accumulation and Antioxidant Activities in Response to Water Deficit in Four Mediterranean *Limonium* Species. *Plants* **2019**, *8*, 506; https://doi.org/10.3390/plants8110506

2) González-Orenga, S.; Llinares, J.V.; Al Hassan, M.; Fita, A.; Collado, F; Lisón, P.; Vicente, O.; Boscaiu, M. Physiological and Morphological Characterisation of *Limonium* Species in their Natural Habitats: Insights into their Abiotic Stress Responses. *Plant & Soil* **2020**, *449*, 267–284; https://doi.org/10.1007/s11104-020-04486-4

3) González-Orenga, S.; Ferrer-Gallego, P.P.; Laguna, E.; López-Gresa, M.P.; Donat-Torres, M.P.; Verdeguer, M.; Vicente, O.; Boscaiu, M. Insight on Salt Tolerance of Two Endemic *Limonium* from Spain. *Metabolites* **2019**, *9*, 294; https://doi.org/10.3390/metabo9120294

4) González-Orenga, S.; Donat-Torres, M.P.; Llinares, J.P.; Navarro, A.; Collado, F.; Ferrer-Gallego, P.P.; Laguna, E.; Vicente, O.; Boscaiu, M. Multidisciplinary Studies Supporting Conservation Programmes of Two Rare, Endangered *Limonium* Species from Spain. (En revisió)

5) González-Orenga, S.; Grigore, M.N.; Vicente, O.; Boscaiu, M. Salt Tolerance Mechanisms and Potential Uses of *Limonium* Species. *Agronomy* **2021**, *11*, 413; https://doi.org/10.3390/agronomy11030413

6) González-Orenga, S.; Trif, C.; Donat-Torres, M.P.; Llinares, J.V.; Collado, F.; Ferrer-Gallego, P.P.; Laguna, E.; Boscaiu, M.; Vicente, O. Responses to Increased

Salinity and Severe Drought in the Eastern Iberian Endemic Species *Thalictrum maritimum* (Ranunculaceae), Threatened by Climate Change. *Plants* **2020**, 9, 1251; https://doi.org/10.3390/plants9101251

7) González-Orenga, S.; Leandro, M.E.D.A.; Tortajada, L.; Llorens, J.A.; Ferrer-Gallego, P.P.; Laguna, E.; Boscaiu, M.; Vicente, O. Comparative Study of Stress Responses in Two Species of *Bupleurum* (Apiaceae) in Support of Conservation Programs. (En preparació)

Altres manuscrits publicats durant el període de treball, més o menys relacionats amb el tema, però no inclosos en la tesi, s'enumeren en l'apèndix al final d'aquest document.

Abstract

Introduction

Drought and salinity are the environmental factors that most affect plants, although in general Mediterranean plants are well adapted to adverse conditions. Predictions estimate that, due to global warming, environmental conditions will become more stressful, especially in semi-arid and arid areas, such as many areas of the Iberian Peninsula. These conditions may affect the presence of many wild species, especially those that are already threatened, rare or endemic.

Both drought and salt stress cause the activation of a series of defence or response mechanisms in plants, which include, among others, the control of ionic transport, the accumulation of compatible solutes or osmolytes, and the activation of antioxidant systems.

To contribute to the conservation and/or reintroduction of species of interest in priority habitats, a multidisciplinary study has been carried out covering parameters that may affect their populations, such as climate, soil and accompanying vegetation, together with comparative studies on responses to drought and salinity. To better understand tolerance mechanisms, genetically related species with different levels of tolerance have been included in the study, in addition to taxa of conservation interest.

The study has two main objectives: i) to establish the relative tolerance to water and salt stress of the species according to their distribution in nature and based on field analyses and, according to the relative inhibition of their growth under stress-induced conditions; and, ii) to evaluate stress-induced biochemical changes by analysing different mechanisms (inhibition of photosynthesis, ionic transport, osmolyte accumulation, antioxidant mechanisms).

Methodology

Most of the species studied are halophytes, such as those of the genus *Limonium*, or with moderate halophytic behaviour, such as *Thalictrum maritimum* and *Bupleurum tenuissimum*, with the exception of one of the species used as comparative material, *Bupleurum fruticosum*, a glycophyte tolerant to water deficit.

The work methodology includes field studies, such as population censuses in some species, analysis of plant communities or root morphology in others, completed with edaphological and climatological analyses.

To analyse the responses under controlled greenhouse conditions, salt stress treatments with different NaCl concentrations wereperformed, depending on the species. The water

stress treatment was carried out in the total absence of irrigation. The duration of the treatments varied between 21 and 30 days, depending on the degree of tolerance of the species.

Once the greenhouse tests were completed, were analysed growth parameters, photosynthetic pigment degradation, ion transport pattern and osmolyte accumulation, as well as the activation of antioxidant enzymes.

Results and discussion

The results obtained have made possible to establish the degree of relative tolerance to hydric and saline stress of the species analysed and their relations with environmental factors such as climate, vegetation and soil. Therefore, based on this information, the optimal areas for the development of the species of interest can be determined.

Furthermore, the relevant tolerance mechanisms to these two types of stress have been identified in each species analysed. All plants activate the same defence mechanisms in response to abiotic stress, but their contribution differs depending on the genus and even the species. In general, an intensification of the ionic transport towards the aerial part of the plants and the activation of the enzymatic antioxidant systems, sufficient to maintain the redox balance, has been noted. Therefore, most of the species studied did not show oxidative stress, except for the two species of the genus *Bupleurum.*

The results obtained have led to the establishment of the relative degree of tolerance to water and salt stress of the species analysed and their relationship with environmental factors such as climate, vegetation and soil. Therefore, based on this information, the optimal zones for the development of the species of interest can be determined.

In addition, the relevant tolerance mechanisms to these two types of stress have been identified for each species analysed. All plants activate the same defence mechanisms in response to abiotic stress, but their contribution differs according to genus and even species. In general, an enhancement of ionic transport to the aerial part of plants and the activation of enzymatic antioxidant systems, sufficient to maintain the redox balance, have been observed. Therefore, most of the species studied did not show oxidative stress, except for the two species of the genus *Bupleurum*.

The species of the genus *Limonium* showed the least inhibition of growth in stress situations, especially under salt conditions, and therefore this genus is clearly the most tolerant of those studied, basing its defence mainly on the activation of the transport of toxic ions towards the aerial part and of the enzymatic antioxidant systems.

Thalictrum maritimum showed to be more tolerant than expected to saline stress, but less resistant to water stress, which caused greater growth inhibition. Its defence mechanisms seem to be more related to the active ion transport to the aerial part and the maintenance of foliar K^+ homeostasis, as well as the activation of enzymatic antioxidant systems.

The species of the genus *Bupleurum* showed a clear difference, being the species of interest *B. tenuissimum* more tolerant to salinity while the species used as comparative material, *B. fruticosum,* showed to be more tolerant to drought. Their main defence mechanisms are related to the immobilization of toxic ions in the roots, accumulation of proline and the activation of enzymatic and non-enzymatic antioxidant systems.

Conclusion

The results obtained in this study provide key information for the reintroduction and conservation or maintenance of the species of interest, as well as for the understanding of the population reduction in recent years.

On the other hand, the results may also contribute to a better understanding of the mechanisms of water and stress tolerance in the studies genera, providing information on the responses to stress in species that have not been previously analysed.

This work has yielded seven scientific manuscripts (published, under review, or in preparation), that are considered as subchapters of the results section of this thesis and are listed below:

1) González-Orenga, S.; Al Hassan, M.; Llinares, J.V.; Lisón, P.; López-Gresa, M.P.; Verdeguer, M; Vicente, O.; Boscaiu, M. Qualitative and Quantitative Differences in Osmolytes Accumulation and Antioxidant Activities in Response to Water Deficit in Four Mediterranean *Limonium* Species. *Plants* **2019**, *8*, 506; https://doi.org/10.3390/plants8110506

2) González-Orenga, S.; Llinares, J.V.; Al Hassan, M.; Fita, A.; Collado, F; Lisón, P.; Vicente, O.; Boscaiu, M. Physiological and Morphological Characterisation of *Limonium* Species in their Natural Habitats: Insights into their Abiotic Stress Responses. *Plant & Soil* **2020**, *449*, 267–284; https://doi.org/10.1007/s11104-020-04486-4

3) González-Orenga, S.; Ferrer-Gallego, P.P.; Laguna, E.; López-Gresa, M.P.; Donat-Torres, M.P.; Verdeguer, M.; Vicente, O.; Boscaiu, M. Insight on Salt Tolerance of Two Endemic *Limonium* from Spain. *Metabolites* **2019**, *9*, 294; https://doi.org/10.3390/metabo9120294

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Other manuscripts published during the period of work, more or less related with its topic, but not included in the thesis, are listed in the appendix at the end of this document

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Capítulo 1:
Introducción

1.1 Cambio Climático y Estrés Abiótico: Estrés Hídrico y Salino

En general, las plantas mediterráneas están bien adaptadas a la sequía, ya que el clima mediterráneo se caracteriza por una drástica reducción de las precipitaciones estivales y una amplia variabilidad interanual (Lionello, 2010, 2012). Sin embargo, los pronósticos estiman que las condiciones ambientales se volverán más estresantes debido al calentamiento global, particularmente en esta región, y que las sequías serán más severas y frecuentes. Así, el cambio climático representa uno de los mayores retos ambientales del siglo XXI, acentuado por la deforestación y la degradación del hábitat. Por este motivo, en los últimos años ha aumentado el interés por el estudio de las respuestas y mecanismos de defensa de las plantas a los diferentes factores ambientales, ya que éstos están afectando a la conservación de muchas especies, así como a su producción y desarrollo.

Los factores de estrés que pueden afectar a las plantas son numerosos, tanto bióticos como abióticos (Azcón Bieto & Talón, 2008). El estrés abiótico se define como cualquier factor no vivo que disminuya la capacidad de la planta para prosperar a través de la fotosíntesis y convertir la energía recolectada en acumulación de biomasa (Grime, 1977). Los estreses abióticos pueden dividirse en físicos o químicos según el agente que los cause. Los factores físicos (fisicoquímicos) más importantes son el estrés por déficit o exceso de agua, las temperaturas extremas, la salinidad (en su componente osmótica) y la radiación. Por otro lado, los factores químicos que destacan son la contaminación atmosférica por metales pesados, la salinidad (en su componente iónico) y el déficit de sustancias minerales.

La sequía y la salinidad son los factores de estrés ambiental más importantes en la reducción del rendimiento de las especies vegetales en todo el mundo (Boyer, 1982; Mahajan & Tuteja, 2005).

La sequía afecta a más de la mitad del territorio de nuestro planeta, debido a la escasez de precipitaciones, y suele estar vinculada a la salinización secundaria del suelo debido al riego intensivo (Flowers, 2004). La sequía representa la condición de estrés abiótico más devastadora: la insuficiencia de lluvias provoca una reducción progresiva de la cantidad de agua disponible para las plantas en el suelo, afectando a su crecimiento y desarrollo y reduciendo la productividad de los cultivos, o incluso provocando la muerte prematura de las plantas y la pérdida total de las cosechas, si la sequía se prolonga en el tiempo (Osakabe et al., 2014).

La salinización del suelo es la acumulación de sales solubles en agua en el suelo a niveles que afectan a la producción agrícola, la salud ambiental y el bienestar económico (Rengasamy, 2006). La salinidad resultante de causas naturales se conoce como "salinidad primaria". Afecta a grandes extensiones de tierra en todo el mundo que nunca se utilizaron para actividades agrícolas, ya que los principales cultivos son sensibles al estrés salino. El término "salinidad secundaria" se acuñó para designar la salinización

del suelo debida a las actividades humanas, especialmente los sistemas de riego prolongados sin suficiente drenaje y el uso masivo e incontrolado de fertilizantes (Flowers, 2004; Parihar et al., 2015). La salinización del suelo ya está presente en más de 100 países del mundo, y aunque las regiones áridas y semiáridas son las más afectadas, ninguna zona está libre de ella (Rengasamy, 2006).

El estudio de la fisiología de las plantas sometidas a condiciones de estrés abiótico es de gran importancia. Según Tambussi (2004) las razones son: i) el conocimiento de los factores de estrés en las plantas puede ser crucial para el desarrollo de modelos mecanísticos de carácter predictivo como, por ejemplo, el estudio de los posibles efectos del cambio climático; y ii) desde una perspectiva ecofisiológica, el análisis de la interacción de las plantas con los factores ambientales es fundamental para entender la distribución de las especies en los diferentes ecosistemas.

Los cambios que se esperan en este siglo perjudicarán las áreas naturales de distribución de un gran número de especies vegetales. Por lo tanto, es necesario adoptar medidas de gestión adecuadas para reducir las alteraciones previstas con el fin de evitar la reducción o la extinción de estas especies y sus hábitats. Para ello es necesario conocer las respuestas de las especies de interés al estrés ambiental, así como el conocimiento de sus hábitats naturales. Por esta razón, además del interés académico de este tema, el estudio de las respuestas de las plantas al estrés abiótico se ha convertido en un área activa de investigación en biología vegetal.

1.2 Efectos de la Sequía y la Salinidad en Plantas

Las respuestas a uno u otro tipo de estrés varían mucho entre los distintos géneros de plantas y, a menudo, incluso entre especies congéneres. Las respuestas de las plantas al estrés hídrico y salino pueden estar correlacionadas y una condición puede llevar a la otra y viceversa (Hasegawa et al., 2000). Tanto el estrés salino como la ausencia de agua en el suelo provocan una disminución del potencial hídrico del medio. Ante este tipo de estrés, las plantas activan mecanismos para evitar la pérdida de agua. La primera respuesta de la planta para mantener el equilibrio hídrico es el cierre de los estomas y, a largo plazo, el aumento del tamaño de las raíces respecto a la parte aérea y la reducción de la superficie foliar.

Como ya se ha mencionado, la sequía induce un déficit hídrico en las plantas estresadas y esto afecta a los órganos de la planta en mayor o menor medida (Munns, 2002). Esta escasez de agua trae consigo una serie de efectos, desde la acumulación de especies reactivas de oxígeno (ROS), que producen estrés oxidativo (Van Breusegem & Dat, 2006), hasta un bajo potencial en la fotosíntesis (Mafakheri et al., 2010).

Casi todos los aspectos de la homeostasis de las plantas que se ven afectados por la sequía se ven alterados negativamente, lo que provoca una reducción del crecimiento

vegetativo, del rendimiento y, en última instancia, la muerte (Li et al., 2009). Sin embargo, las raíces de las plantas recurren a mecanismos eficaces que detectan el déficit hídrico. Este escenario puede producirse tanto por la falta de agua en el medio ambiente causada por las bajas precipitaciones como por el exceso de sal, dando lugar a una "sequía fisiológica" (Shabala et al., 2016). En ambos casos, las plantas son incapaces de absorber suficiente agua para un desarrollo y crecimiento normales, lo que activa las vías de transducción de señales relacionadas con el estrés (Schachtman y Goodger, 2008), seguido de un retraso en el crecimiento de los brotes y las hojas. Esta inhibición del crecimiento está relacionada con un cambio en los niveles de dióxido de carbono y oxígeno causado por el cierre parcial de los estomas.

La sequía, por lo tanto, está asociada a niveles altos de ROS que desencadenan el estrés oxidativo, que provoca cambios en la homeostasis redox celular y el metabolismo celular normal, y la activación de mecanismos antioxidantes (Kar, 2011; Sharma et al., 2012; Golldack et al., 2014).

El efecto de la salinidad en la fisiología de las plantas es doble: un efecto osmótico (como el estrés hídrico) y un efecto iónico. La acumulación de sales en el suelo en la zona radicular de la planta provoca una disminución del potencial hídrico y, por tanto, dificulta la absorción de agua y nutrientes. Como respuesta, las plantas activan diversos mecanismos para restablecer el equilibrio osmótico (Munns, 2002). En los suelos salinos, la concentración de sales implica la acumulación de ciertos iones en el suelo, que en altas concentraciones son tóxicos, como el sodio o el cloro. El estrés iónico, también denominado efecto específico de la salinidad (Greenway & Munns, 1980) suele seguir al estrés osmótico en las plantas afectadas por el estrés salino.

Las principales respuestas a nivel bioquímico generadas por la presencia de sal en el suelo son (i) transporte iónico entre células para lograr la homeostasis; (ii) síntesis de osmolitos y (iii) control del flujo de agua en los tejidos.

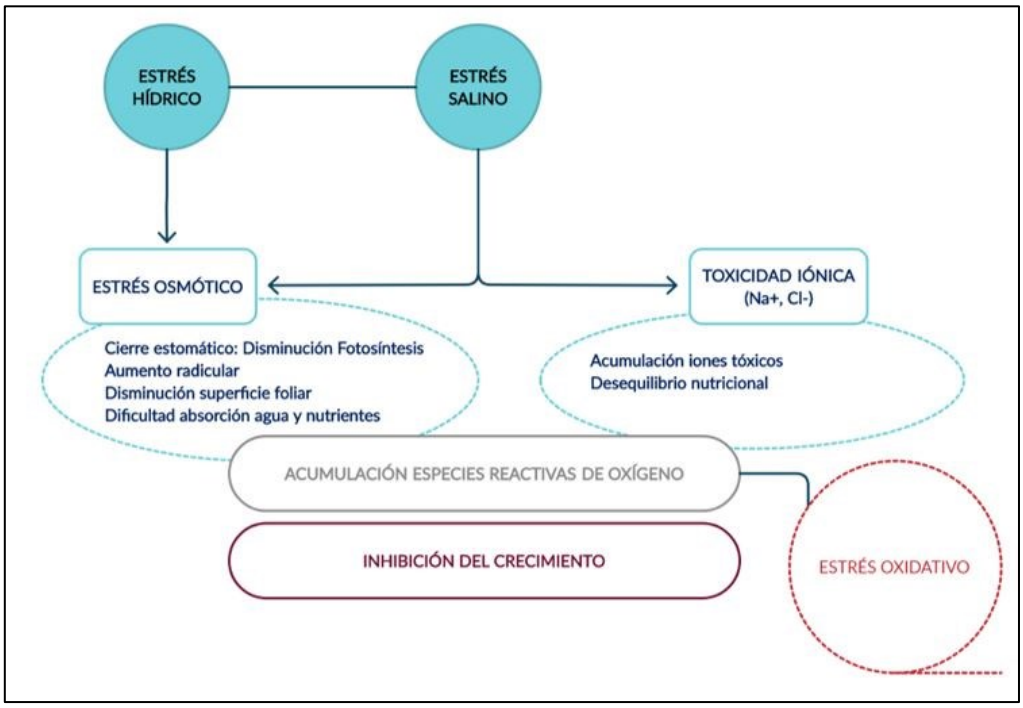

Figura 1. Efectos del estrés hídrico y salino que conducen a la inhibición del crecimiento.
Fuente: Propia

1.3 Halófitas, Plantas Tolerantes a la Sequía y la Importancia de sus Hábitats

Las halófitas son plantas que toleran en mayor o menor medida la salinidad del suelo. Existe una amplia gama de halófitas en función de su tolerancia, desde especies que crecen en los bordes de las marismas o lagunas, adaptadas a niveles de salinidad relativamente bajos, especies que alcanzan su desarrollo óptimo bajo niveles de salinidad moderados e incluso hay especies que toleran niveles de salinidad superiores a los del agua de mar. Sin embargo, una definición operativa generalmente aceptada es que las halófitas son plantas de ambientes salinos naturales que pueden completar su ciclo de vida con salinidades del suelo equivalentes al menos a 200 mM de NaCl (Flowers & Colmer, 2008). Las halófitas pueden sbrevivir en suelos salinos utilizando con gran eficacia mecanismos de respuesta conservados, y en algunas especies adaptaciones morfológicas y anatómicas más específicas (suculencia, glándulas salinas, etc.) que pueden ser de gran importancia para la halotolerancia en estos taxones (Flowers et al., 1977; Flowers & Colmer, 2008).

Las plantas halófilas son las que constituyen principalmente la vegetación de marismas, salinas, lagunas, zonas dunares y otros hábitats costeros de interés.

Sin embargo, la salinidad no es el único factor limitante para el desarrollo de las plantas en los hábitats objeto de estudio. Al encontrarse simultáneamente afectadas por otros factores de estrés, como ocurre en el clima mediterráneo, las plantas deben estar

adaptadas tanto a las fuertes lluvias de primavera/otoño como a la extrema sequía de verano.

En la Península Ibérica, así como en muchas otras regiones del mundo, los hábitats salinos sufren numerosas amenazas. En el pasado, los saladares se consideraban insalubres y, por lo tanto, se eliminaban si había asentamientos humanos cerca. Además, la expansión de la agricultura y la presión turística han contribuido a la reducción de la superficie ocupada por este tipo de ecosistema. A todo ello hay que añadir los efectos del cambio climático, especialmente en la zona mediterránea, que provoca un aumento de la temperatura y sequías cada vez más intensas y duraderas.

Debido a esta combinación de factores, los saladares son ecosistemas frágiles, muy amenazados y únicos. Estos hábitats se caracterizan por una elevada productividad primaria y albergan una gran variedad de flora y fauna. Los saladares costeros tienen un valor ecológico añadido, ya que representan importantes zonas de invernada para las aves acuáticas (Simas et al., 2001). Estos ecosistemas también son importantes desde el punto de vista económico, ya que pueden servir de criaderos para varias especies de peces y crustáceos (Dijkema et al., 1990; Reed, 1990). Los saladares constituyen los hábitats más abundantes, fértiles y accesibles del planeta, por lo que están muy amenazadas por las actividades humanas (contaminación industrial, urbanización, agricultura, etc.), que han deteriorado muchas de ellos en todo el planeta. En el litoral levantino español, y en particular en la Comunidad Valenciana, la situación de estos ecosistemas es especialmente crítica. Muchos ya habían sido destruidos en el pasado, debido a su transformación para uso agrícola o a su desecación por temor a la malaria. El hecho de que la franja costera de la Comunidad Valenciana soporte la práctica totalidad de la actividad agrícola, gran parte de la actividad industrial y grandes núcleos de población, junto con una enorme presión turística, ha sometido a estos ecosistemas a grandes impactos durante las últimas décadas del siglo pasado, provocando la degradación irreversible de muchos de ellos, ya que los hábitats costeros halófilos son muy frágiles y complejos.

La flora de los marjales y saladares litorales valencianos incluye taxones de gran interés, muchos endémicos o amenazados (Crespo & Lledó, 1998; Laguna & Atienza, 1998), lo que ha llevado a que gran parte de los que aún permanecen sin grandes alteraciones sean actualmente zonas protegidas. De hecho, todas las lagunas costeras han sido propuestas como LIC (Lugares de Interés Comunitario), integradas en la red Natura 2000 como ZEC (Zonas Especiales de Conservación) o ZEPA (Zonas de Especial Protección para las Aves). En particular, los hábitats 1150 (Lagunas costeras) y 1510 (Estepas salinas mediterráneas, Limonietalia), intrínsecamente relacionados con los saladares, son considerados prioritarios por la legislación europea (Directiva del Consejo, 1992). Además, numerosas microrreservas, algunas de ellas dedicadas a la protección de la vegetación halófila, se encuentran también en estos humedales (Laguna et al., 2003).

Además de las amenazas derivadas de la acción humana, los saladares costeros mediterráneos son ecosistemas especialmente sensibles a los efectos del cambio

climático, con un aumento de las temperaturas medias, un incremento de la frecuencia, intensidad y duración de los periodos de sequía, "olas de calor" y otros fenómenos extremos, así como cambios en los patrones climáticos estacionales (IPCC, 2014). Todo ello provocará probablemente un aumento de la salinidad del suelo, que puede afectar a la biodiversidad de la vegetación halófila, llegando a provocar la desaparición de algunas especies en zonas concretas, aunque, como se ha comprobado en otros estudios, algunas halófitas disponen de mecanismos que les permiten adaptarse rápidamente a un aumento del nivel de estrés salino, mediante la activación de respuestas de defensa específicas (Pardo-Domènech et al., 2015).

1.4 Área de Estudio: Parque Natural de l'Albufera

El Parque Natural de l'Albufera constituye uno de los humedales más representativos y valiosos de la Península Ibérica, de especial interés florístico y medioambiental. Ocupa una superficie de 21.120 hectáreas y se encuentra a tan solo 10 km de la ciudad de Valencia. Fue declarado Parque Natural en 1986 y desde 1989 está reconocido como "Humedal de importancia internacional". Además, la zona pertenece a los Humedales de Importancia Internacional de la Convención de Ramsar desde 1990, es parte integrante de la Red Natura 2000, al haber sido declarada como "Zona de Especial Protección para las Aves" (ZEPA) en 1990 y ha sido seleccionado como "Lugar de Importancia Comunitaria" (LIC) en 2006. En su territorio se encuentran también algunas "Microreservas de Flora" y "Reservas de Fauna".

Figura 2. Mapa hidromorfológico del Parque Natural de l'Albufera
Fuente: www.chj.es

Dentro del área de estudio, el proyecto se centra en la zona de Las Malladas de la Devesa de l'Albufera.

En la región valenciana, así como en otros territorios del SE de la Península Ibérica, los saladares litorales aparecen frecuentemente integrados en sistemas dunares, formando depresiones interdunares, denominadas localmente "malladas", donde predominan los suelos limosos. Este tipo de suelo hace que las malladas se encharquen con las lluvias y se formen costras salinas en verano con la evaporación del agua, estableciéndose gradientes de salinidad en el suelo, con las zonas más salinas localizadas en el centro de la mallada y las menos salinas en sus bordes. Generalmente se asume que la distribución de las diferentes especies de plantas en estas zonas salinas está determinada principalmente por su tolerancia relativa a la salinidad, de modo que las comunidades vegetales se instalan en las malladas como anillos concéntricos en función de la salinidad del suelo, aunque otros factores, como la competencia entre especies, pueden contribuir significativamente a la distribución de las plantas en los saladares (Barbour, 1978; Emery et al., 2001; Veldhuis et al., 2019).

Originalmente, las malladas de la Devesa de la Albufera eran una multitud de depresiones de distintos tamaños que se distribuían longitudinalmente por toda la restinga. Entre las décadas de 1960 y 1970, se llevó a cabo un proyecto de urbanización parcial en más del 70% de la Devesa, afectando negativamente a todos los hábitats existentes en esta zona. Sin embargo, los más afectados fueron los primeros cordones dunares, que se destruyeron por completo, y con su arena se rellenaron las malladas para crear grandes áreas urbanizadas e infraestructuras; esto provocó la falta de inundación natural de estas depresiones interdunares durante la estación húmeda, alterando su funcionamiento hidrológico. Además, muchas de las malladas fueron plantadas con especies alóctonas. Este conjunto de agresiones provocó la disminución o pérdida de las características específicas originales del hábitat, así como la reducción de las poblaciones de numerosas especies de interés y la desaparición completa de algunas especies vegetales; así, nueve especies se han extinguido localmente (ocho de ellas están incluidas en el Catálogo Valenciano de Especies Amenazadas, y cuatro en la Lista Roja de la UICN).

Afortunadamente, la situación ha cambiado en las últimas décadas, cuando se empezó a considerar el gran valor ecológico de estos hábitats. Desde principios de los años 80, el Servicio Devesa-Albufera del Ayuntamiento de Valencia ha realizado actuaciones y estudios encaminados a la regeneración de los ecosistemas de la Devesa de la Albufera. La actuación más destacada en relación con las malladas ha sido la regeneración geomorfológica de muchas de ellas. Actualmente se están llevando a cabo programas de conservación y recuperación de la vegetación halófila de las malladas.

La vegetación de las malladas incluye especies estructurales, que constituyen las comunidades típicas de saladar, como la asociación *Puccinellio festuciformis-Arthrocnemetum fruticosi* en las zonas más saladas, o los herbazales halohidrófilos (*Carici extensae-Juncetum maritimi; Schoenus–Plantaginetum crassifoliae*) en las zonas más externas

donde la salinidad es menor (Costa et al., 1986). Estas especies tienen un papel fundamental en las estructuras de estas comunidades vegetales, pero son bastante homogéneas entre los diferentes territorios y relativamente abundantes. Lo que confiere gran individualidad y un valor añadido a estos ecosistemas son las especies diferenciales, que aparecen con menor frecuencia, pero justamente son aquellas de las que depende la singularidad de cada mallada. En el territorio de referencia para este estudio, muchas de estas especies son endémicas (algunos endemismos valencianos exclusivos) o muy raras, amenazadas o incluso, como se ha mencionado anteriormente, han desaparecido del territorio del Parque Natural, aunque siguen presentes en otros saladares valencianos.

1.5 Importancia de los Estudios Comparativos de Laboratorio en Paralelo a los Estudios de Campo

Un enfoque útil para desentrañar los mecanismos que subyacen a la tolerancia de las plantas a la sal y al déficit hídrico - y otros estreses abióticos - es estudiar las respuestas al estrés de taxones relacionados taxonómicamente (implícitamente, también genéticamente).

Un enfoque útil para esclarecer los mecanismos que sustentan la tolerancia de las plantas al déficit de agua y a la salinidad - y a otros estreses abióticos- es estudiar las respuestas al estrés de taxones que están relacionados taxonómicamente (implícitamente, también genéticamente). Se han publicado varios estudios comparativos sobre respuestas al estrés hídrico y salino en taxones relacionados con diferentes niveles de tolerancia (por ejemplo, Al Hassan et al., 2016 a, b; Kozminska et al., 2018). Estos estudios han resultado esenciales para comprender los mecanismos de respuesta en las especies estudiadas, así como la función de ciertos osmolitos, que en algunos casos parecen ser específicos de cada especie o género, lo que contribuirá en gran medida a la comprensión de la tolerancia natural de las plantas tanto a la sequía como a la salinidad.

Para asegurar la efectividad de los programas de conservación y regeneración de las zonas de interés se requiere de un conocimiento profundo sobre los mecanismos de respuesta al estrés ambiental al que están sometidas en su hábitat natural. Mientras que se conocen los principales mecanismos bioquímicos y fisiológicos de tolerancia a la salinidad, la sequía u otras condiciones de estrés abiótico en muchas especies estructurales (Redondo-Gómez et al., 2009; Gil et al., 2011, 2014; Katschnig et al., 2013; Pardo-Domenech et al., 2016; Al Hassan et al., 2016a, b), prácticamente no hay información sobre las especies diferenciales, que a menudo son especies endémicas, raras y/o amenazadas.

Para complementar el estudio de los efectos y las respuestas de las especies bajo condiciones de estrés inducido y de este modo comprender el funcionamiento no solo

de las especies de interés de forma individual, sino también de su hábitat, se realizaron ensayos de germinación bajo condiciones de estrés y se realizaron estudios de campo.

Estos estudios de campo incluyeron el análisis bioquímico de algunas de las plantas muestreadas en su hábitat natural, además del estudio de sus hábitats naturales en cuanto a:

i) Análisis de la flora y la vegetación en las áreas de muestreo de las plantas y de las semillas utilizadas en el trabajo, así como, de las malladas del Parque Natural candidatas para la reintroducción de las especies estudiadas. Dentro de cada una de esas áreas se analizó de forma muy detallada la composición florística y estructura de las comunidades.

ii) Caracterización de las propiedades del suelo en las áreas de muestreo de las semillas utilizadas en el proyecto y en las malladas del Parque Natural de la Albufera candidatas para la reintroducción de estas especies.

iii) Análisis de los parámetros climáticos.

En general, se estudiaron todos los parámetros externos a las plantas que pudieron afectar de algún modo a su supervivencia, representado un enfoque clave para la elección de las mejores zonas para su reintroducción y/o aumento de las poblaciones de las especies consideradas diferenciales, endemismos y especies raras y/o amenazadas de alto valor ecológico.

Capítulo 2:
Objetivos

El objetivo general del trabajo se centra en abordar el estudio de los mecanismos de tolerancia a estrés ambiental, en concreto a la sequía y salinidad del suelo, en una selección de especies diferenciales de elevado interés ecológico y conservacionista presentes en saladares litorales de la Comunidad Valenciana, siendo taxones endémicos, muy raros y/o amenazados, incluso desaparecidos en la zona de estudio – el Parque Natural de La Albufera – con el fin de aportar información relevante para los programas de conservación y regeneración de las malladas del Parque. Los objetivos propuestos se adecúan al programa europeo de investigación "Horizonte 2020", en concreto al. Reto 5, '*Acción por el clima, medio ambiente, eficiencia de recursos y materias primas*', Apartado 5.2. '*Protección medioambiental y gestión sostenible de los recursos naturales (incluyendo agua, biodiversidad y ecosistemas)*', y también a la Estrategia Española de Ciencia y Tecnología y de Innovación 2013-2020, Reto 4.4.5 '*Acción sobre cambio climático y eficiencia en la utilización de recursos y materias primas*'.

Para abordar esta cuestión, analizamos las respuestas al estrés hídrico y salino en plantas relacionadas genéticamente con diferente potencial de tolerancia al estrés. La metodología se centró en: i) establecer la tolerancia a estos tipos de estrés en las especies de interés a través de su distribución en la naturaleza y el estudio de su entorno abiótico y biótico, ii) realizar tratamientos en condiciones de estrés controladas y iii) correlacionar los cambios inducidos por estos tipos de estrés en el nivel de "marcadores de estrés" bioquímicos asociados a vías de respuesta específicas (acumulación de osmolitos, transporte iónico, disminución de la actividad fotosintética, etc) con la tolerancia relativa de los taxones estudiados.

La investigación llevada a cabo a nivel bioquímico es una de las partes fundamentales de este trabajo, sin embargo, para completarlo, se realizaron estudios de campo para conocer el entorno en el que se desarrollan las especies de interés así como las zonas de interés para su reintroducción. Por tanto, los objetivos específicos del trabajo fueron:

1- Estudiar los patrones de crecimiento de las plantas en sus hábitats naturales.

2- Comprobación de la tolerancia relativa a condiciones de estrés abiótico (hídrico y salino) de las especies selecionadas, en base a ensayos de germinación *in vitro* y crecimiento de plantas en condiciones controladas de invernadero.

3- Determinación de los niveles de una serie de marcadores bioquímicos y enzimáticos característicos de rutas conservadas de respuesta a estrés abiótico, en plantas sometidas a tratamientos controlados de estrés o plantas silvestres.

4- Caracterización edafológica y climática de las zonas donde se han recolectado las semillas de las especies de saladar seleccionadas, y estudio detallado de la composición florística y fitosociológica de las poblaciones de origen.

5- Caracterización edafológica y selección de localizaciones concretas del Parque Natural para la reintroducción de las especies estudiadas en base a los resultados anteriores.

Capítulo 3:
Material Vegetal

Se han seleccionado especies con afinidades halófilas de tres géneros de gran interés por contar con especies endémicas, con distribución muy restringida, o desaparecidas recientemente del Parque Natural de l'Albufera, Valencia. Todas ellas están incluidas en los planes de gestión y programas de reintroducción y han sido seleccionadas de acuerdo con las directrices del Servicio Devesa-Albufera.

Las semillas empleadas para la obtención del material vegetal provienen de la Colección de Germoplasma de flora rara, endémica y/o amenazada perteneciente al Servicio de Vida Silvestre del Centro para la Investigación y Experimentación Forestal (CIEF) ubicado en Quart de Poblet (Valencia) o han sido recolectadas en el campo.

A continuación, se detallan las especies estudiadas agrupadas por género, mencionando sus principales características morfológicas, su distribución, hábitat, y nivel de protección, así como otros datos de interés.

3.1 Género *Limonium*

El género *Limonium*, perteneciente a la familia Plumbaginaceae, tiene una distribución cosmopolita, aunque la mayor diversidad se encuentra de la región del Mediterráneo a Asia Central. Incluye más de 400 especies, la mayoría halófitas, bien representadas en el Mediterráneo (Greuter et al., 1989) con numerosos endémismos en el área de estudio (Mateo & Crespo, 2014). Destaca su presencia en microhábitats que ocupan pequeñas áreas.

Limonium es el género con mayor número de especies reconocidas en España (Pignatti, 1972; Greuter et al., 1989; Lledó et al., 2005; Domina, 2011; Hassler, 2019), albergando nada menos que 107 especies (Erben, 1993). *Limonium* es también uno de los géneros con mayor relevancia de conservación en España, incluyendo 74 especies incluidas en la Lista Roja Española de Plantas Vasculares Amenazadas (Aedo et al., 2013), según los criterios de la UICN (2001). La Comunidad Valenciana destaca por tener 28 especies presentes en la actualidad, de las cuales 12 son endemismos exclusivos de esta región (Crespo & Lledó, 1998; Laguna, 1998; Aguilella et al., 2010; Mateo & Crespo, 2014).

Son plantas herbáceas perennes, raramente anuales, entre 10-70 (100) cm de altura a partir del rizoma, aunque también hay especies de arbustos leñosos de hasta 2 m. Las hojas son simples, enteras a lobuladas, de 1-30 cm de largo y 0,5-10 cm, la mayoría en una roseta basal densa. Las flores, agrupadas en panículas o corimbos, son pequeñas (4-10 mm de largo) con cinco lóbulos del cáliz y corola y cinco estambres. El color de la flor es rosa, violeta y púrpura en la mayoría de las especies, o blanco o amarillo en unas pocas. Muchas de las especies son apomícticas. El fruto es una pequeña cápsula que contiene una sola semilla, parcialmente rodeada por el cáliz persistente (Erben, 1993).

Suelen encontrarse en hábitats hostiles que otras plantas no pueden soportar como acantilados marítimos, suelos ricos en yeso, lagunas saladas, saladares y en general en suelos fuertemente salinos. Este hecho tiene relación con el elevado grado de halotolerancia del género ya que las especies de *Limonium* son plantas recreto-halófitas, que tienen la capacidad de excluir sales a través de las glándulas salinas (Leng et al., 2018), pero también son acumuladoras de sales en sus hojas como muchas otras halófitas dicotiledóneas (Wyn-Jones & Gorham, 2002; Flowers & Colmer, 2008).

En el presente trabajo se ha llevado a cabo el estudio de cuatro especies endémicas del género *Limonium* (*L. santapolense, L. girardianum, L. albuferae* y *L. dufourii*) con un alto valor de conservación. Como material comparativo se han incluido otras dos especies del mismo género con una distribución más amplia (*L. virgatum* y *L. narbonense*). Concretamente, se han estudiado por una parte *L. santapolense*, *L. girardianum*, *L. virgatum* y *L. narbonense* en su hábitat natural (Parque Natural de l'Albufera y Santa Pola (Alicante) así como, bajo condiciones controladas sometidas a estrés abiótico y, por otra parte, *L. albuferae* y *L. dufourii* bajo condiciones controladas de estrés abiótico junto a estudios de campo de su hábitat natural en la Parque Natural de l'Albufera (Valencia, España).

Todas las especies de *Limonium* seleccionadas son elementos importantes de los ecosistemas de los saladares, ya que su presencia y frecuencia en las comunidades vegetales aumenta la biodiversidad y el grado de diferenciación entre los hábitats locales de la zona de estudio. Las poblaciones de estas especies no han sido estudiadas en profundidad hasta el momento, y sus características morfológicas y bioquímicas han reflejado adaptaciones a nivel local, por lo que su análisis es importante para obtener un conocimiento más amplio de las especies, así como para predecir su posible respuesta en el escenario futuro del reto ante el cambio climático.

3.1.1 *Limonium santapolense* Erben

Las semillas fueron recolectadas del Clot de Galvany, un humedal localizado cerca de la ciudad de Elche en la provincia de Alicante (España) (39.12° N/0.20° E).

Es un endemismo local presente solamente en sustratos arenosos salobres del litoral, en una pequeña área con clima mediterráneo semiárido, en la provincia de Alicante, concretamente de Santa Pola a Crevillente, en los saladares del Hondo y de Aguamarga, Elche y Crevillente. En la Figura 3 se indica su distribución, de acuerdo con las citas del Banco de Datos de Biodiversidad (www.bdb.gva.es) y con el Sistema de Información sobre plantas (www.gbif.es).

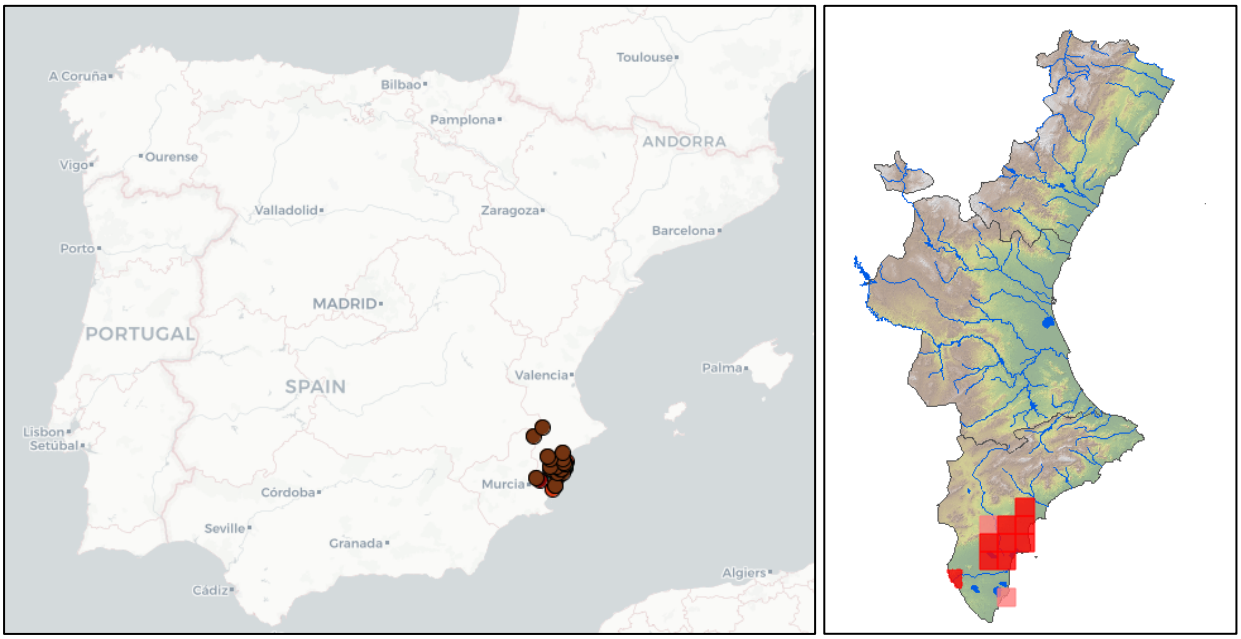

Figura 3. Distribución *Limonium santapolense.*
Fuente: www.gbif.es / www.bdb.gva.es

Está incluida en la lista roja española de flora vascular en la categoría de "vulnerable" de la UICN, por su riesgo de extinción (Moreno, 2008), así como en el anexo III de Especies Vigiladas del Catálogo Valenciano de Especies de Flora Amenazadas.

En cuanto a su descripción, es una planta perenne, glabra de hasta 70 cm de altura con hojas grandes elípticas u obovadas de ápice obtuso y mucronado, multinervias, persistentes en la floración. Pecíolo de longitud similar al limbo. Ramas estériles escasas. Con 3-4 espiguillas por centímetro. Brácteas internas de 3,8-4 mm de anchura, elípticas y con ancho margen hialino. Pétalos rojo-violáceos (Figura 4). Florece de mayo a julio (Erben, 1993).

Figura 4. Detalle flor, ramificaciones, hojas y planta entera de *Limonium santapolense*.

Fuente: Flora Silvestre

3.1.2 *Limonium girardianum* (Guss.) Fourr.

Las semillas fueron recolectadas del Parque Natural de l'Albufera, cerca de la ciudad de Valencia (España) (38.15° N/0.42° W).

Es un endemismo del Sur de Francia, Nordeste de la Península Ibérica y de las Islas Baleares, que se encuentra bien representado en toda la Comunidad Valenciana, principalmente en las provincias de Castellón y Valencia, aunque penetra en las áreas costeras de la provincia de Alicante, como se puede observar en la Figura 5, con áreas de distribución altamente fragmentadas. En cuanto a su hábitat, crece en zonas costeras con sustrato arenosos y en acantilados.

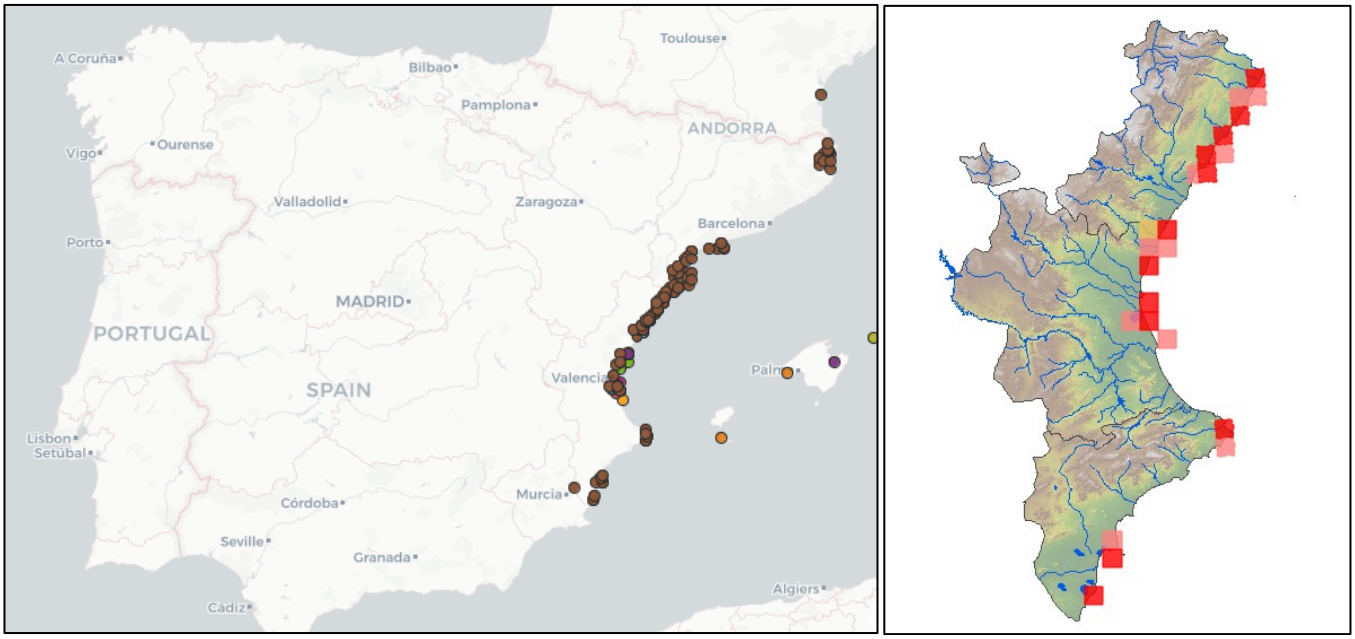

Figura 5. Distribución *Limonium girardianum*.
Fuente: www.gbif.es / www.bdb.gva.es

Esta especie también esta amenazada por la transformación de sus hábitats y esta protegida en Francia (Baumberger et al., 2012). Por otro parte, esta especie constituye un elemento vegetal de gran interés para la conservación de la flora de las malladas y microhábitats asociados.

Es una planta perenne, escaposa, ligeramente lignificada en la base, glabra o débilmente papilosa en el tercio inferior. Hojas de la roseta basal de 18-60 5-15 mm, oblanceoladas a espatuladas, agudas, con 1-3 (5) nervios y verdes durante la floración; pecíolo de 1-2 mm de anchura, de longitud mayor o subigual al limbo. Tallos de hasta 30 cm, robustos, poco ramificados, y en la base papilosos y con escamas de 4-6 mm de longitud. Inflorescencias poco desarrolladas, sin ramas estériles. Espigas de 6-15 mm de longitud. Espiguillas muy densamente dispuestas, 10-12 por centímetro, cada una con 2-4 (5) flores. Bráctea externa elíptica, grande, de 1.9-2.5 2.0-2.7 mm. Bráctea media hialina, de 1.7-2.4 1.1-2.0 mm. Bráctea interna de 3.8-4.8 3.5-4.7 mm, anchamente hialino-escariosa y con acumen de hasta 1 mm de longitud. Cáliz de 4.0-5.3 mm de longitud, con el tubo subigual al limbo. Pétalos cuneados, de 7.5-8.0 mm 2.5-2.9 mm, de color violáceo o, más raramente, rosado-blanquecino, emarginados en el ápice. Florece desde principios de junio hasta agosto (Erben, 1993).

Por lo general, es una planta de tamaño pequeño, con hojas pequeñas y estructuras subterráneas delgadas y poco profundas (Figura 6). Morfológicamente es muy similar a *L. virgatum*, que describiremos en el siguiente apartado, excepto por el mayor número de brotes y hojas, así como raíces más largas que *L. virgatum*.

Figura 6. Detalle flor, espigas, planta entera y hojas de *Limonium girardianum*.
Fuente: Flora Silvestre

3.1.3 *Limonium virgatum* (Willd.) Fourr.

Las semillas fueron recolectadas del Parque Natural de l'Albufera, cerca de la ciudad de Valencia (España) (38.15° N/0.42° W).

Esta especie tiene una distribución más amplia en la región mediterránea, concretamente en las costas occidentales. En la Comunidad Valenciana aparece de forma prácticamente continua en los acantilados y saladares situados de Vinaroz (Castellón) a La Punta del Mascarat en Altea (Alicante), desapareciendo hacia el sur y volviendo a estar presente en los saladares costeros de San Pedro del Pinatar (Murcia), desde donde se extiende hasta Portugal (Figura 7), hallándose también presente en el Norte de África y Oriente Medio. En cuanto a su hábitat, crece en playas arenosas y costas rocosas, siendo una especie de gran amplitud ecológica, ya que puede habitar desde acantilados marinos con salpicaduras directas de las olas hasta suelos húmedos salinos, siempre que sean zonas costeras.

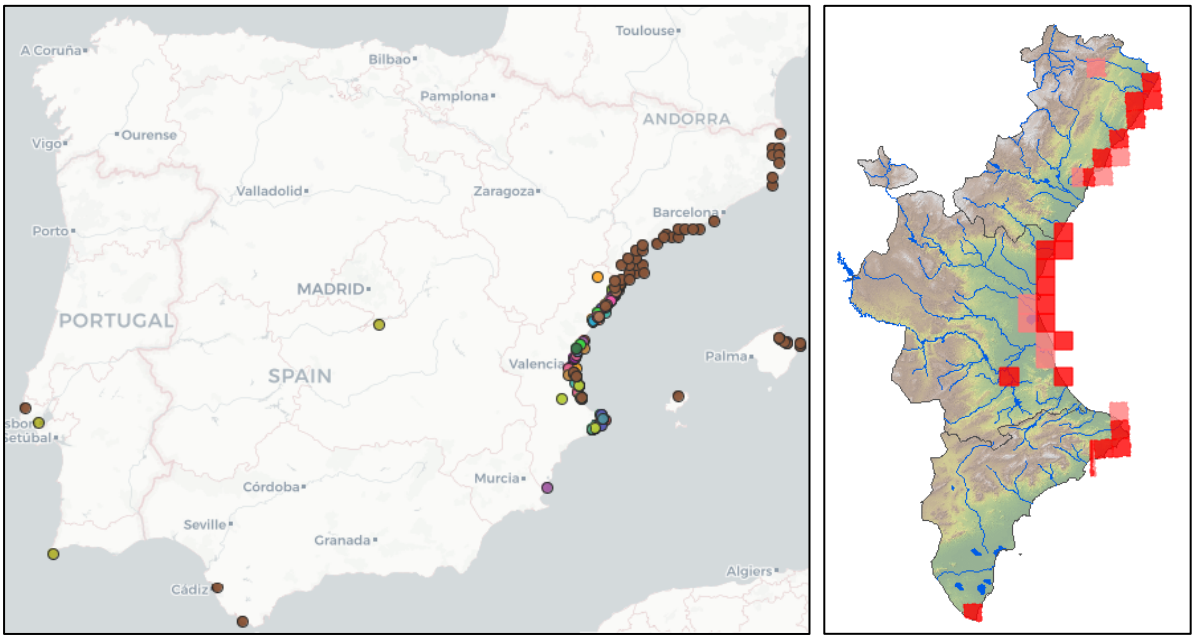

Figura 7. **Distribución** ***Limonium virgatum.***
Fuente: www.gbif.es / www.bdb.gva.es

En cuanto a su morfología, es una planta perenne, escaposa, glabra. Hojas de la roseta basal cuneadas a linear-espatuladas, obtusas, de 10-100 4-12 mm, uninervias, completamente secas durante la floración, con pecíolo de 2-4 mm de anchura y subigual o menor que el limbo. Tallos numerosos, de 1-8 (12) dm de altura, con escamas basales de 4-8 mm de longitud. Inflorescencia ramosa, consistente, la mitad inferior constituida por ramas estériles muy numerosas y gruesas, que forman entre ellas un ángulo agudo. Espigas de 1-7 cm de longitud, rectas o arqueadas en la base. Espiguillas dispuestas en número de (3) 4-5 por centímetro, cada una con (1) 2-4 (6) flores. Bráctea externa de 1.9-2.6 1.6-2.6 mm, triangular, aguda. Bráctea media hialina, de 1.8-2.6 1.6-2.1 mm. Bráctea interna de 5.0-7.0 2.9-3.9 mm, elíptica a obovada, con acumen de 0.7-1.3 mm de longitud y margen hialino. Cáliz de 6.0-7.0 mm de longitud, con limbo lacerado tras la floración y subigual al tubo, que frecuentemente aparece arqueado hacia la base. Pétalos de 9.5-10.3 3.5-3.8 mm, cuneados, emarginados y de color azul-violáceo. Florece desde julio hasta septiembre (Erben, 1993).

Por lo general, es una planta pequeña de menos de 10 gramos por planta, con hojas pequeñas y estructuras subterráneas delgadas y poco profundas, aunque con mayor número de brotes y de hojas que *L. girardianum*, así como raíces más profundas (Figura 8).

Figura 8. **Detalle flor, hojas y planta entera de *Limonium virgatum*.**
Fuente: Flora Silvestre

3.1.4 *Limonium narbonense* Mill.

Las semillas fueron recolectadas del Parque Natural de l'Albufera, cerca de la ciudad de Valencia (España) (38.15° N/0.42° W).

Está presente en los saladares de todo el Mediterráneo, en España también en la costa atlántica (Erben, 1993), bien representado en las tres provincias de la Comunidad Valenciana (Figura 9). Habita saladares costeros, donde se integra en diversas comunidades halófilas, principalmente en juncales y fenalares higrófilos. Además, este taxon tiene una particularidad, es halo-helófito, es decir, vive en matorrales de suelos salinos inundados gran parte del año.

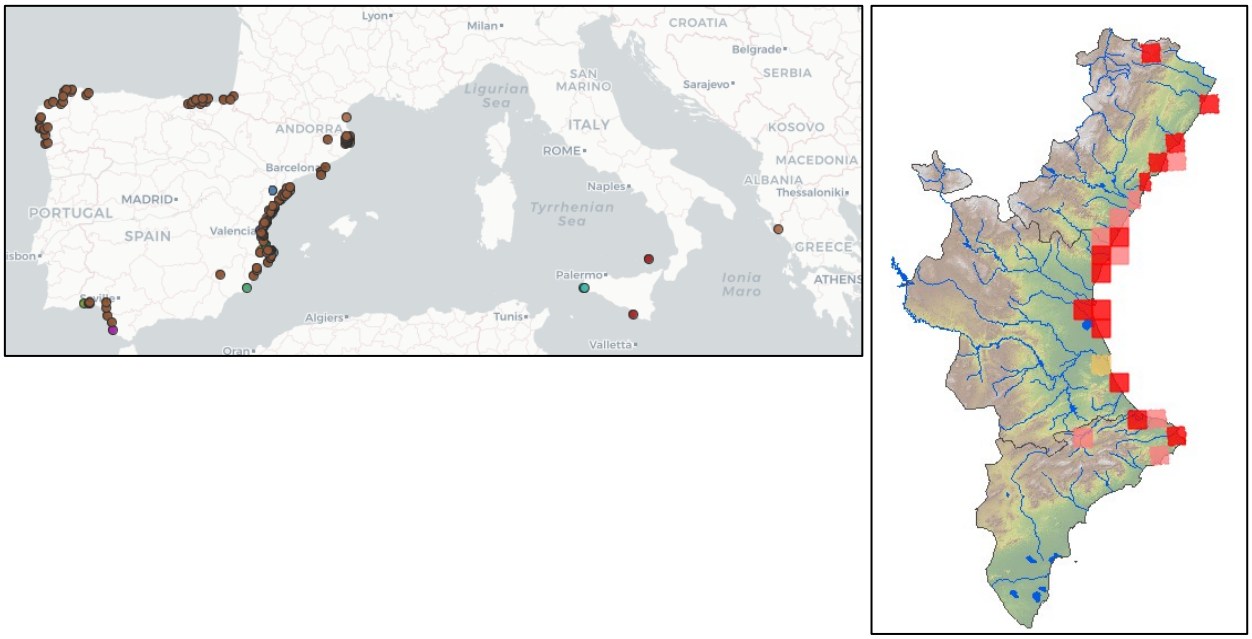

Figura 9. Distribución *Limonium narbonense*.
Fuente: www.gbif.es / www.bdb.gva.es

Planta perenne, escaposa, leñosa en la base, glabra. Hojas de tamaño y forma variables, generalmente de (5) 15-30 (45) 1-10 cm, lanceoladas, obtusas, con el nervio central prominente y muy visiblemente ramificado en toda su longitud, persistentes durante la floración; pecíolo de 3-5 mm de anchura, menor o igualando al limbo. Tallos de hasta 100 cm de altura, glabros y gruesos en la base, con 1-3 brácteas escuamiformes de hasta 4 cm de longitud. Inflorescencia ramosa y ancha, sin ramas estériles en la base o con 1-2 cortas. Espigas de 1-4 cm de longitud, con (3) 5-9 espiguillas por centímetro, portando (1) 2-4 flores cada una. Bráctea externa de 2.0-3.0 1.5-2.2 mm, con margen hialino. Bráctea media de 2.0-4.0 1.0-2.0 mm, hialina. Bráctea interna de 3.6-5.4 2.8-3.9 mm, con acumen de hasta 1 mm y margen hialino. Cáliz de 5.0-6.8 mm de longitud, con el limbo 2-3 veces menor que el tubo. Pétalos cuneados, de 12.0-14.0 2.0-2.5 mm, azul-violáceos. Florece tardíamente, desde agosto hasta noviembre, pero de forma vistosa y abundante (Erben, 1993).

Figura 10. Detalle flor, hojas y planta entera de *Limonium narbonense*.

3.1.5 *Limonium albuferae* P.P. Ferrer y col.

Las semillas fueron facilitadas por el CIEF (Centre per a l'investigació i l'experimentació forestal) de la Colección de Germoplasma de flora rara, endémica y/o amenazada provenientes de La Devesa (El Saler) con fecha de recolección 20/07/2016.

Limonium albuferae P.P. Ferrer y col. sólo se conoce de un pequeño paraje en una mallada del Parque Natural de la Albufera (Racó de l'Olla) (Figura 11). Esta mallada está situada entre las dunas de la Devesa y la reserva del Racó de l'Olla, representando un ecosistema de transición entre el mar y l'Albufera al ocupar una posición intermedia en términos topográficos y ecológicos. Esto implica una mayor dependencia del nivel freático de la mallada y una buena tolerancia a la salinidad de las especies que lo habitan.
La población ocupa suelos salinos y arenosos de la comunidad *Junco maritimi-Caricetum extensae* Géhu 1976 de *Juncetea maritimi*, y está asociada, por ejemplo, a *L. girardianum* (Guss.) Fourr., *L. narbonense* Mill., *L. virgatum* (Willd.) Fourr., *Juncus maritimus* Lam., *Bolboschoenus maritimus* (L.) Palla, y *Scirpioides holoschoenus* (L.) Soják (Ferrer-Gallego et al., 2016). Al igual que *L. narbonense*, *L. albuferae*, también es halo-helófito, viviendo gran parte del año en matorrales salinos inundados.

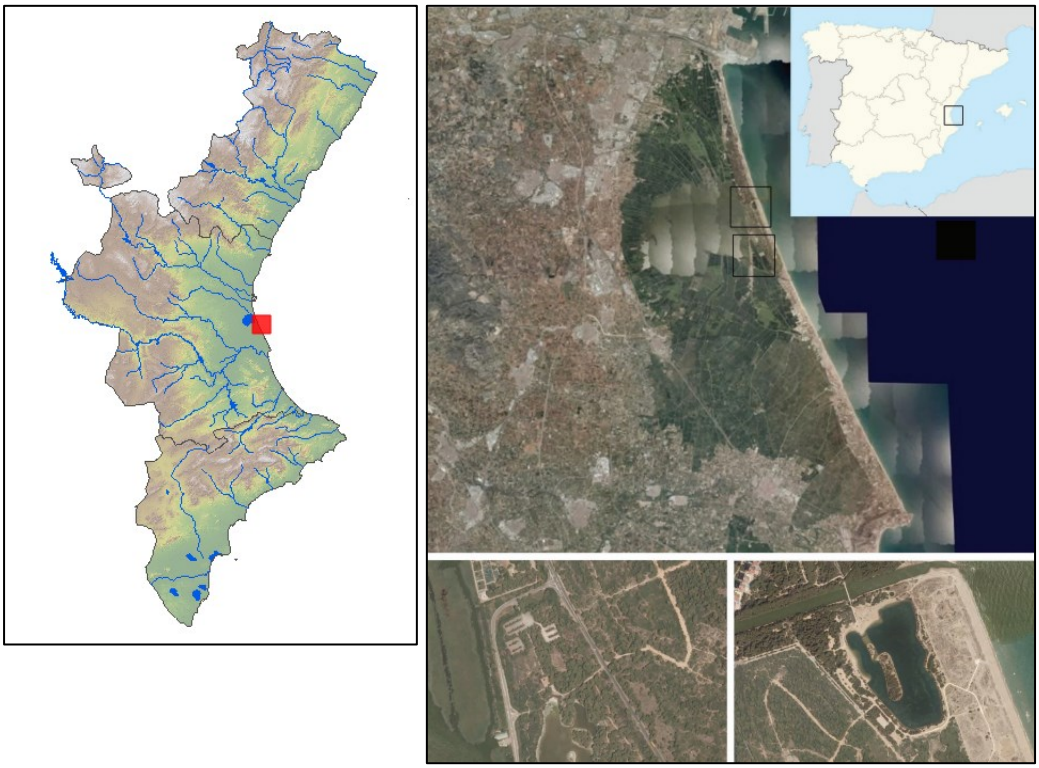

Figura 11. Distribución *Limonium albuferae*.
Fuente: www.bdb.gva.es / Ferrer-Gallego et al., 2016.

La primera descripción de la planta se realizó en el año 2016. A principios de 2020, se contabilizaron 255 plantas, con una superficie de unos 160 m². Por tanto, esta especie se incluirá en la categoría "En peligro de extinción" en la próxima edición del Catálogo Valenciano de Especies Vegetales Amenazadas, actualmente se encuentra "En peligro crítico de extinción".

Es una especie apomíctica triploide (2n = 26) con interacciones incompatibles polen-estigma (Ferrer-Gallego et al., 2016). Es una chamefita halófita perenne, (20) 45-55 cm, con 1 (2) escapos, glabra, cepa gruesa muy leñosa y fuertemente amacollada, multirrosulada, que varía desde rosetas aisladas (5-8 cm de ancho) hasta individuos densos en forma de cojín (hasta 45 cm de ancho) las superiores verde glaucas en la antesis, las inferiores marchitas y persistentes; limbo de ovado a espatulado, verde-glauco, de superficie áspera con numerosas papilas; nervio central marcado y visible hasta el tercio superior del limbo, con 2-4 nervios laterales menos marcados y mucho más finos, ápice agudo o subagudo, mucrón de hasta 2,5 mm, pecíolo hasta 7 mm de anchura. Escapo (15) 25-45 (50) cm, liso, erecto, recto, papiloso, grueso. Inflorescencia (4) 10-20 (25) cm de longitud, abierta o con espiguillas dispuestas de manera compacta, con silueta de tipo rómbica o lanceolada; con 0-2 (3) ramas estériles, cortas y de primer orden. Espigas (5) 8-18 (20) mm, compactas, rectas o muy poco arqueadas, erecto-patentes. Espiguillas 5-7 mm, con 6-7(9) espiguillas por centímetro y (2) 5-7 (8) flores por espiguilla. Bráctea externa 1,8-2 × 1,2-1,7 (1,8) mm, con ápice romo y subagudo, margen membranáceo. Bráctea media 1,8-2 (2,1) × 0,9-1,1 mm. Bráctea interna (3,5) 4-5 × (2,5) 3-

3,5 mm, obovada, con ápice romo a redondeado. Las brácteas acumulan a menudo fuertes eflorescencias salinas, que le dan un falso aspecto papiloso. Flores 3-4 mm de diámetro (Figura 12). Cáliz 4,3-4,6 mm, que sobrepasa 1-1,6 mm a la bráctea interna; nervios del cáliz sin alcanzar la base de los dientes. Pétalos 7,2-7,8 × 2,4-2,7 mm, emarginados, cuneiformes, violáceos. Semilla 1,5 × 0,5 mm. Florece durante los meses de julio y agosto. Fructifica durante los meses de julio a octubre (Ferrer-Gallego et al., 2016).

Figura 12. Detalle inflorescencias, espigas y hábitat natural *Limonium albuferae*.
Fuente: Ferrer-Gallego et al., 2016. (Foto de E. Laguna)

3.1.6 *Limonium dufourii* (Girard) Kuntze

Las semillas fueron facilitadas por el CIEF (Centre per a l'investigació i l'experimentació forestal) de la Colección de Germoplasma de flora rara, endémica y/o amenazada provenientes de La Marjal dels Moros (Sagunto) con fecha de recolección 20/11/2015.

Es un endemismo exclusivo de las costas de la Comunidad Valenciana entre Torreblanca y Cullera. Se extiende por 14 cuadrículas UTM de 10 km, por lo que se podría considerar amplia pero su presencia en cada una de ellas es escasa. En realidad, esta especie se agrupa en tres núcleos principales: zona litoral entre La Devesa (El Saler) y Cullera (fragmentado en dos áreas aisladas debidos a la urbanización), zona costera del Camp de Morvedre y arenales costeros en la zona de Torreblanca (Figura 13). En la actualidad, se conocen únicamente cinco poblaciones naturales restringidas a pequeñas zonas costeras en las provincias de Castellón (Torreblanca) y Valencia: Marjal dels Moros (con tres poblaciones), El Saler (Parque Natural de la Albufera) y Cullera (Laguna et al., 1994; Laguna, 1998; Aguilella et al., 2010), que albergan desde 232 individuos (en el año 2004) hasta 36.435 (en el año 2011), el último censo en el año 2019 indicaba 4.017 individuos

(https: //bdb.gva.es/bancodedatos/censos/). La mayoría de estas poblaciones tienen un número muy bajo de individuos, pero los análisis moleculares muestran que existe una variabilidad y diferenciación genética sustancial dentro y entre poblaciones (Palacios & González-Candelas, 1997; Palacios et al., 1999).

Crece en acantilados marinos y marismas sobre suelos arenosos (Laguna et al., 1994; Laguna, 1998; UICN, 2001; Aguilella et al., 2010;). Concretamente, en las comunidades de acantilado de *Crithmo-Staticetea Br.-Bl.* (por ejemplo, la población de Cullera) y marismas o suelos arenosos en *Juncetea maritimi Br.-Bl.* Está asociado *con Limonium girardianum* (Guss.) Fourr., *L. densissimum* (Pignatti) Pignatti, *L. virgatum* (Willd.) Fourr., *Juncus maritimus* Lam., *Bolboschoenus maritimus* (L.) Palla, *Scirpioides holoschoenus* (L.) Soják. En termotipos termomediterráneos y termotemperados, bajo ombrotipo seco-subhúmedo, según la clasificación de Rivas-Martínez (2007).

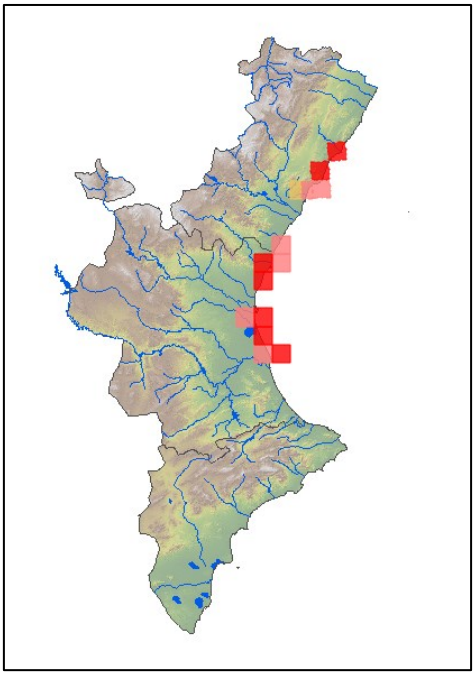

Figura 13. Distribución *Limonium dufourii.*
Fuente: www.bdb.gva.es

Todas las poblaciones de *L. dufourii* están incluidas en la red de Microreservas Vegetales o Parques Naturales (L'Albufera, Prat de Cabanes-Torreblanca) de la Comunidad Valenciana y también, adicionalmente, en la red Natura 2000 de espacios protegidos de la Unión Europea (como Lugar de Importancia Comunitaria, LIC). La especie se encuentra estrictamente protegida en la Comunidad Valenciana en la máxima categoría legal "En peligro de extinción", incluida en el Catálogo Valenciano de Especies Vegetales Amenazadas.

Es una especie triploide (2n=3x=27) con reproducción apomíctica obligada e interacciones polen-estigma incompatibles (Erben, 1993; Baker, 1996; Palacios & González-Candelas, 1997). Es una especie halófita perenne, hasta 50 cm, rosulada, hemicriptofita, densamente pelosa. Hojas 3-6 x 1 cm, en roseta basal,

obovadoespatuladas, obtusas, verdes durante la floración. Escapo florífero ramoso, con numerosas ramas estériles erguidas y divergentes en ángulo recto. Espigas 12-20 mm, gruesas; espiguillas 7-8 mm de longitud, densamente dispuestas, 10 o más por centímetro; brácteas densamente pelosas. Pétalos 8-9 mm, de color azul-violáceo (Figura 14). Florece de mayo a julio. Planta alógama, entomógama. Dispersión anemócora o mirmecocora. Presenta apomixis (Erben, 1993).

Figura 14. Detalle tallo, roseta basal, espiguillas e inflorescencias y flores de *Limonium dufourii*.
Fuente: Flora Silvestre

3.2 Género *Thalictrum*

El género *Thalictrum* pertenece al orden Ranunculales y a la familia Ranunculaceae. Ranunculaceae se divide en cuatro subfamilias según la estructura de flores y frutos y los datos citológicos, perteneciendo *Thalictrum* a la subfamilia *Thalictroideae* (Gregory, 1941).

Thalictrum está distribuido en muchas regiones, principalmente templadas y frías de ambos hemisferios. Es extremadamente polimorfo debido a la amplitud de su diversidad ecológico-geográfica. Consta de unas 110 especies de herbáceas perennes (Gubanov et al., 1976).

En el presente trabajo hemos estudiado el endemismo *Thalictrum maritimum* Dufour. En la actualidad sus poblaciones están amenazadas y en regresión, especialmente las

situadas en el Parque Natural de l'Albufera, por lo que esta especie tiene un alto interés de conservación. En la actualidad se realiza un seguimiento periódico de las poblaciones y se realizan recolecciones de semillas, que se almacenan a largo plazo en el Banco de Germoplasma de la Flora Silvestre Valenciana (CIEF). Además, se han desarrollado protocolos de propagación, y se han realizado traslocaciones y refuerzos poblacionales (Aguilella et al., 2010). Sin embargo, a pesar de los esfuerzos para su conservación, muchas de las poblaciones de *T. maritimum* están en declive y la especie ha desaparecido por completo de algunos lugares en los últimos años (Servicio de Vida Silvestre, datos no publicados y BDBCV). Por todo ello, la especie ha sido seleccionada para este estudio con la finalidad de optimizar las labores de conservación y/o reintroducción.

3.2.1 *Thalictrum maritimum* Dufour

Las plántulas de *T. maritimum*, fueron cedidas por el 'Centro de Conservación de Especies de Agua Dulce de la Comunidad Valenciana'.

T. maritimum fue identificado por Jean-Marie León Dufour en 1860 en saladares cerca de la ciudad de Valencia (Dufour, 1860). Es una especie endémica del este de la Península Ibérica, que crece solo en algunas zonas costeras (Aguilella et al., 2010; Ferrer-Gallego et al., 2015). En todo el mundo solo se puede encontrar en cuatro sitios, todos ubicados en la Comunidad Valenciana: en el Prat de Cabanes-Torreblanca (Castellón), donde se encuentra el mayor número de individuos, en la Marjal d'Almenara (Castellón), en la Marjal dels Moros (Valencia) y en el Parque Natural de l' Albufera, correspondiendo a su límite sur (Figura 15).

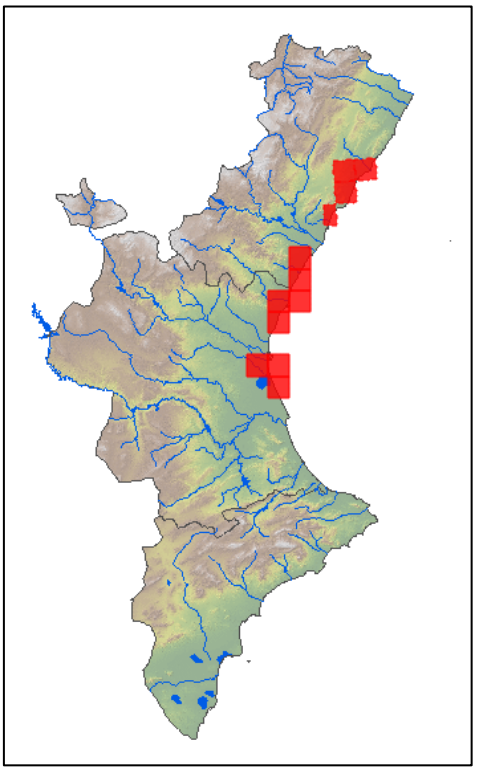

Figura 15. Distribución *Thalictrum maritimum*.
Fuente: www.bdb.gva.es

El número total de individuos adultos presentes en l'Albufera ha incrementado desde 2406 individuos en 2011 a 2516 en 2014 y 3333 en 2015, pero descendió a 2381 en 2017 (datos facilitados por Servicio de Vida Silvestre).

Es una planta propia de marjales litorales, formando parte de juncales y carrizales, más o menos salobres, en los bordes de depresiones temporalmente inundadas, de forma secundaria puede encontrarse en cañaverales y herbazales higrófilos en bordes de acequias.

La especie está clasificada como "Vulnerable" en el Catálogo Valenciano de Especies de Flora Amenazada, así como en el rango homónimo de la UICN en la Lista Roja Española de Flora Vascular.

En cuanto a su morfología (Figura 16), es un geófito perenne de tallo erecto simple de hasta 80 cm, glabro, glauco azulado. Las hojas están compuestas por pequeños foliolos, estrechos y lanceolados. La inflorescencia es suelta y las flores tienen pétalos amarillos y numerosos estambres que sobrepasan la corola. El fruto es un aquenio en forma de huso y con la cáscara dura. Florece de julio a octubre. La fructificación se produce en septiembre-octubre (Montserrat, 1986). Se desconoce el radio de dispersión de las semillas.

Figura 16. Detalle inflorescencias, hojas y tallo de *Thalictrum maritimum.*
Fuente: www.bdb.gva.es

3.3 Género *Bupleurum*

El género *Bupleurum* pertenece a la familia Apiaceae, que incluye alrededor de 450 géneros con 3540 especies (Judd et al., 2008; Mabberley, 2008). La mayoría de las especies de *Bupleurum* son hierbas perennes, de hasta 150 cm de altura con umbelas compuestas. Las flores son bisexuales, amarillentas o raramente violáceas con cinco estambres y los frutos se presentan principalmente como cremocarpos. Las hojas son simples, largas y delgadas. El género está representado por 180-190 especies.

En este estudio se analizó *B. tenuissimum* L. una especie de alto interés de conservación incluida en los programas de reintroducción. Como material comparativo se ha seleccionado *B. fruticosum* L., una especie mediterránea, relativamente frecuente en la vegetación arbustiva y forestal, adaptada a condiciones xéricas.

3.3.1 *Bupleurum tenuissimum* L.

Las semillas recolectadas en Font Amarga (Villanueva de Castellón) fueron facilitadas por el CIEF (Centre per a l'investigació i l'experimentació forestal) de la Colección de Germoplasma de flora rara, endémica y/o amenazada.

B. tenuissimum se distribuye en el oeste, centro y sur de Europa, el sur de Inglaterra y las zonas costeras del Mar del Norte hasta el sur de Suecia, el sureste de Asia y el Noreste de África. En la Península Ibérica y Baleares tiene una escasa presencia en marismas, límites de lagunas y cursos de agua, sobre suelos margosos, arcillosos o salobres. En la Comunidad Valenciana, aparece de forma bastante dispersa en diversos humedales litorales y con menos frecuencia en hábitats salobres de interior (Figura 17). Se puede encontrar entre el nivel del mar y los 800 m de altitud. *B. tenuissimum*, puede considerarse una halófita facultativa, ya que crece en suelos moderadamente salinos, pero también puede encontrarse en hábitats no salinos (Tyler et al., 2020) y sus semillas no germinan en presencia de sal (Al-Hawija et al., 2012).

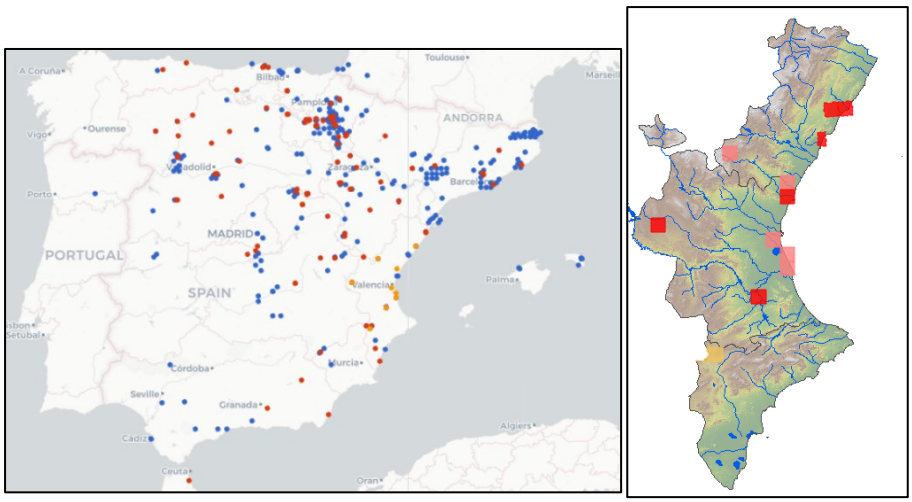

Figura 17. Distribución *Bupleurum tenuissimum*.
Fuente: www.gbif.es / www.bdb.gva.es

Es una especie extinta en l' Albufera recientemente y catalogada como "vulnerable" a nivel nacional, también está registrada como "Especie Protegida No Catalogada" en la Orden 6/2013, de 25 de marzo, del Ministerio de Infraestructuras, Territorio y Medio Ambiente de la Generalitat Valenciana (DOCV no. 6996 de 04.04.2013).

Es una hierba anual, erecta (5-70 cm) con tallos herbáceos, normalmente poco ramificados. Hojas (0,5-8 x 0,05-0,6 cm) subamplexicaules, lineares a linear-acuminadas, no atenuadas o gradualmente hacia la base, agudas o acuminadas, paralelinervias y sin nervio medio intramarginal grueso; hojas basales marchitas antes de la floración. Flores en umbelas terminales y laterales con 1-3 hasta 6 radios generalmente desiguales; inflorescencias laterales con pedúnculos muy cortos. Brácteas lineares más cortas o de longitud similar a los radios, persistentes en la fructificación. Flores con pétalos amarillos, verdosos o purpúreos, con o sin banda medio oscura. Frutos cortamente pedicelados (0,5-2 mm); mericarpos ovoideos o subglobosos, pailosos, con costillas aparentes y crenuladas. Florece normalmente entre julio y octubre, aunque puede ampliar este periodo en función de las condiciones ambientales (Neves, 2003) (Figura 18).

Figura 18. Detalle *Bupleurum tenuissimum.*
Fuente: *Herbario bajo Jarama, Atlas of the British and Iris Flora y Inventaire National du Patrimonie Naturel.*

3.3.2 *Bupleurum fruticosum* L.

Las semillas fueron facilitadas por el CIEF (Centre per a l'investigació i l'experimentació forestal) de la Colección de Germoplasma de flora rara, endémica y/o amenazada provenientes de La Sierra Calderona.

B. fruticosum crece por gran parte de la región mediterránea, en la Península se distribuye por los territorios costeros mediterráneos y atlánticos. Esta presente por gran parte de la región mediterránea (Figura 19).

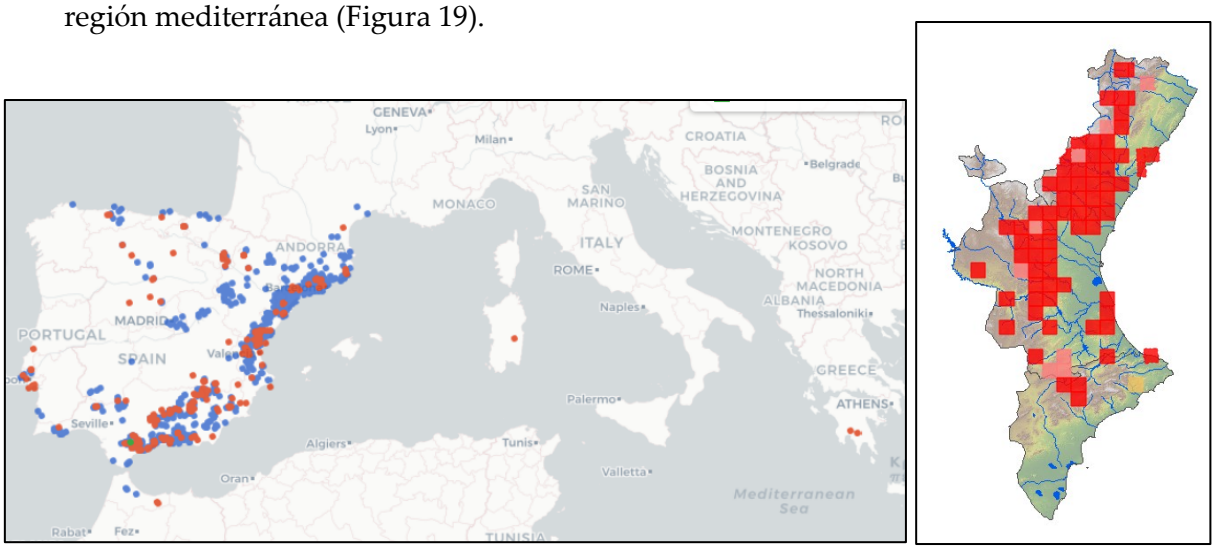

Figura 19. Distribución *Bupleurum fruticosum*.

Fuente: www.gbif.es / www.bdb.gva.es

En cuanto a su hábitat, se encuentra en bosques y matorrales mediterráneos, en todo tipo de suelos desde el nivel del mar hasta los 1200 m, generalmente acompañado de especies termofílicas como el madroño o la adelfa.

Es una especie sobre la que no pesan amenazas concretas.

Morfológicamente, es un arbusto con tallos leñosos que alcanza los 3 m de altura. Posee las hojas correosas, de color verde pálido algo azulado, lustrosas y de forma elíptica. Al carecer de peciolo se estrechan en su parte inferior y se une directamente al tallo. Las flores son pequeñas y de color amarillo-verdoso. Florece de junio hasta agosto (Neves, 2003) (Figura 20).

Figura 20. Detalle *Bupleurum fruticosum.*
Fuente: www.gva.bdb.es y www.floracatalana.es

Capítulo 4:
Resultados

Publicación I:
Subcapítulo 4.1

Qualitative and Quantitative Differences in Osmolytes Accumulation and Antioxidant Activities in Response to Water Deficit in Four Mediterranean *Limonium* Species

Referencia:
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Qualitative and Quantitative Differences in Osmolytes Accumulation and Antioxidant Activities in Response to Water Deficit in Four Mediterranean *Limonium* Species

Abstract

Limonium is a genus represented in the Iberian Peninsula by numerous halophytic species that are affected in nature by salinity, and often by prolonged drought episodes. Responses to water deficit have been studied in four Mediterranean *Limonium* species, previously investigated regarding salt tolerance mechanisms. The levels of biochemical markers, associated with specific responses –photosynthetic pigments, mono- and divalent ions, osmolytes, antioxidant compounds and enzymes– were determined in the control and water-stressed plants, and correlated with their relative degree of stress-induced growth inhibition. All the tested *Limonium* taxa are relatively resistant to drought based on both the constitutive presence of high leaf ion levels that contribute to osmotic adjustment, and the stress-induced accumulation of osmolytes and increased activity of antioxidant enzymes, albeit with different qualitative and quantitative induction patterns. *Limonium santapolense* activated the strongest responses and clearly differed from *L. virgatum*, *L. girardianum* and *L. narbonense,* as indicated by cluster and PCA analyses in agreement with its drier natural habitat and compared to that of the other plants. Somewhat surprisingly however, *L. santapolense* was the most affected species by water deficit in growth inhibition terms, which suggests the existence of additional mechanisms of defense operating in the field that cannot be mimicked in greenhouses.

Keywords: *Limonium santapolense*; *Limonium virgatum*; *Limonium girardianum*; *Limonium narbonense*; drought; water deficit; oxidative stress; ions; osmolytes; antioxidant enzymes.

4.1.1 Introduction

In general, Mediterranean plants are well adapted to drought, marked by drastically reduced in summer rainfall and wide inter-annual variability, both of which characterize the Mediterranean climate (Lionello, 2012). Nevertheless, forecasts estimate that environmental conditions will become more stressful from global warming, particularly in the Mediterranean Region, and that droughts will be severer and more frequent (Hoerling et al., 2015).

Drought induces water deficit in stressed plants, and this strongly impacts all plant organs to a greater or lesser extent (Munns, 2002). This shortage of water brings about a range of deleterious effects, from reactive oxygen species (ROS) levels building up and producing oxidative stress (Van Breusegem & Dat, 2006), to a low photosynthesis potential (Mafakheri et al., 2010). Almost every aspect of drought-affected plant homeostasis is negatively altered, which implies reduced vegetative growth, yield and eventually plant death (Li et al., 2009).

However, plant roots resort to effective mechanisms that sense low water potential. This scenario may emerge due to lack of water in the environment caused by low precipitation or excess salt ions are present in soil, which lead to 'physiological drought' (Shabala et al., 2016). In both events, plants are unable to take up enough water for normal development and growth, which activates stress-related signal transduction pathways (Schachtman & Goodger, 2008), which is immediately followed by stunted shoot and leaf growth. This inhibited growth is linked with a change in carbon dioxide and cellular oxygen levels prompted by partial stomata closure (Munns, 2002).

Nevertheless, plants possess several tolerance mechanisms to alleviate effects of drought-induced osmotic stress, including the synthesis of compatible solutes; e.g. proline, glycine betaine or soluble carbohydrates, etc. (Martínez et al., 2003; Miller et al., 2010; Fang & Xiong, 2015; Xi et al., 2018). Osmotic adjustment is also accomplished by inorganic ions accumulating. High K^+ and Na^+ levels in water-stressed plants have been described in the stress-tolerant *Atriplex halimus* (Martínez et al., 2003), whereas Na^+ accumulation has been found to act as an important drought tolerance strategy in a desert xerophyte (Xi et al., 2018).

Osmotic stress is linked with increased reactive oxygen species (ROS) production. Drought, thus, comes with raised ROS levels, which brings about changes in cellular redox homeostasis and normal cellular metabolism that trigger oxidative stress and the activation of antioxidant mechanisms (Kar, 2011; Sharma et al., 2012; Golldack et al., 2014). The commonest antioxidant metabolites include phenols, flavonoids, ascorbic, glutathione acid and carotenoids, whereas catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) (and other peroxidases), or redox regulatory enzymes like glutathione reductase (GR), are among the most relevant antioxidant enzymatic systems activated in plants to respond to deleterious oxidative stress effects (Sharma et al., 2012).

The genus *Limonium* L. of the Plumbaginaceae family comprises over 400 species, includes many endemics in the present study area and is well represented in the Mediterranean Region (Erben, 2013). The *Limonium* species have been well documented for responding to salt stress because this is an emblematic genus of halophytes that possesses salt excretory glands (Gagneul et al., 2007; Al Hassan et al., 2017). Moreover, the mechanisms of response and potential tolerance to drought of *Limonium* taxa, similarly to the majority of halophytes, have not drawn much interest and are still largely unknown. Nonetheless, salinity is not the only constraint for plants in salt marshes because, quite often, many other stressful factors occur simultaneously.

In this and former works, we examine the responses of four *Limonium* species to abiotic stress: *L. santapolense* Erben, *L. girardianum* (Guss.) Fourr., *L. virgatum* (Willd.) Fourr. and *L. narbonense* Mill. All four are found in littoral salt marshes in SE Spain. They are perennial, long day and C3, and their leaves differ in size and shape. The first two are endemics and have a high conservation value. *L. santapolense* is found on littoral sandy substrates in a small zone in the Province of Alicante. *L. girardianum* is endemic to E Spain, S France and the Balearic Isles, and grows on cliffs and sandy coasts. The other two species are broadly distributed and cover the Mediterranean Region, with *L. virgatum* found on rocky coasts and sandy beaches reaching North Africa and the Middle East, wheras *L. narbonense* is present in salt marshes throughout the Mediterranean, including Spain, as well as on the Atlantic coast (Erben, 2013).

The objective of this work is to analyze the responses of all four *Limonium* species to water stress generated artificially and under greenhouse conditions to plants grown from field-collected seeds. We hypothesize that the drought tolerance mechanisms of *L. santapolense* plants, whose origin lies in the aridest collection area, would be more efficient than those of the plants of the other three chosen species. In our extensive study, growth inhibition, induced by not watering plants for 1 month, correlated with: (a) degradation of photosynthetic pigments; (b) ionic homeostasis being maintained; (c) compatible solutes accumulating; e.g. proline, glycine betaine, and soluble carbohydrates; (d) leaf malondialdehyde (MDA, a reliable oxidative stress marker) and H_2O_2 contents, and α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging activity; (e) antioxidant compound (total phenolics and flavonoids) levels; (f) antioxidant enzymes activities (CAT, GR, SOD, and APX).

4.1.2 Material and Methods

Sampling Sites and Seed Sampling

Mature capsules of *L. santapolense* were collected from Clot de Galvany, a salt marsh located near the city of Elche in the Province of Alicante (39.12°N/0.20°E), and those of the other three species came from from the 'La Albufera' Natural Park near the city of Valencia (38°15N/0.42°W), Spain, in autumn 2016. Seeds were separated from capsules and stored at room temperature for 2 months.

Plant Growth, Drought Treatments and Sampling of Plant Material

Plants were obtained by directly sowing seeds on a mixture of commercial peat and vermiculite (3:1). Seedlings were watered twice weekly with Hoagland nutrient solution (Hoagland & Arnon, 1950). After 3 weeks, seedlings were transferred individually to 1 L pots and placed in plastic trays (5 pots per tray). One week later, stress treatments were initiated by entirely ceasing irrigation. The plants from the control treatment were watered every 5 days with 1 L water added to each tray. After 1 month of treatment, five stressed plants of each species and their control counterparts were harvested, together with a fraction of the corresponding substrates. All the experiments were conducted in a controlled environment chamber in a greenhouse under the following conditions: long-day photoperiod (16 h of light), temperature set at 20°C during the day and 17°C at night, and relative humidity between 50 and 80%, monitored by a Testo humidity data logger.

The leaves and roots of each harvested plant were collected and separately weighed. The following plant growth parameters were measured in the leaf fraction: fresh weight of leaves (FWL), leaf water content percentage (WCL) and leaf surface (LA). Five leaves from each plant were selected randomly and scanned to measure the leaf surface with the ImageJ software (Rasband, 1997-2012). A fraction of the fresh material was frozen in liquid N_2 and stored at -75°C. Most of the remaining material was dried for several days in an oven at 65°C until constant weight was achieved. The water content percentage in leaves was calculated as: WCL (%) = [(FWL-DWL)/FWL] × 100 (Gil et al., 2014). The root fraction of each harvested plant was thoroughly cleaned by brushing with a fine paintbrush. Then roots were briefly rinsed in Milli-Q water, quickly blotted on filter paper and dried at 65°C to calculate the water content percentage of roots (WCL), as for the leaf fraction. The total FW of roots could not be determined because it was not possible to recover the whole root system of each plant.

Substrate Analysis

Soil moisture was determined by the gravimetric method at the end of treatments: a fraction of each soil sample was weighed (SFW), dried in an oven at 105°C until reaching constant weight, and then weighed again (DSW). Soil water content (in %) was calculated as:

$$\text{Soil humidity: } WC\% = [(FSW - DSW) / FSW] \times 100 \quad (1)$$

Photosynthetic Pigments

Chlorophylls a and b (Chl a, Chl b) and total carotenoids (Caro) were determined as previously described (Lichtenthaler & Wellburn, 1983). Ten mL of ice-cold 80% (v/v) acetone was used to extract pigments from 0.05 g of fresh leaf material. After mixing overnight and centrifuging for 10 min at 12000 rpm, the supernatant was collected, and its absorbance was measured at 663, 646, and 470 nm. Chl a, Chl b, and Caro concentrations were calculated using described equations (Lichtenthaler & Wellburn, 1983) and their contents were expressed in mg g^{-1} DW.

Ion Content Measurements

Ion contents were determined in root and leaf aqueous extracts, essentially as described by Weimberg (1987), by heating samples (0.05 g of dried ground plant material in 15 mL of water) for 15 min at 99°C, followed by filtration through a 0.45 μm filter (Gelman Laboratory, PALL Corporation). Na^+ and K^+ were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA). Cl^- was measured using a chloride analyzer. Divalent cations (Ca^{2+} and Mg^{2+}) were determined in an atomic absorption spectrometer SpectrAA 220 (Varian, Inc., CA, USA).

Osmolyte Quantification

Proline (Pro) was extracted from 0.05 g of leaf fresh material with 2 mL of a 3% (w/v) sulfosalicylic acid solution to be quantified according to the acid-ninhydrin method (Bates et al., 1973). The extract, mixed with acid ninhydrin, was heated at 95°C for 1 h, cooled on ice and extracted with toluene. The absorbance of the organic phase was measured at 520 nm using toluene as a blank. Pro concentrations were expressed as μmol g^{-1} DW. Glycine betaine (GB) was determined in 1-mL aqueous extracts prepared from 0.05 g of the dry leaf material according to published procedures (Grieve & Grattan, 1983; Nawaz and Ashraf, 2010). The extract was supplemented with potassium iodide, kept on ice for 90 min and then extracted with 1, 2-dichlorethane (pre-cooled at -20°C). Finally, the absorbance of the sample was measured at 365 nm. GB content was expressed as μmol g^{-1} DW. To quantify total soluble sugars (TSS), 0.05 g of the dry ground leaf material was extracted with 3 ml of 80% (v/v) methanol and mixed on a rocker shaker for 24 h. The extract was centrifuged, concentrated sulfuric acid and 5% phenol were added to the supernatant, and absorbance was measured at 490 nm (Dubois et al., 1956). TSS contents were expressed as 'mg equivalent of glucose' (used as the standard) per g DW.

HPLC Analysis of Soluble Carbohydrates

Plant fresh material (0.05 g) was boiled in 2 mL of milliQ water for 10 minutes before being filtered using 0.22 μm filters. The soluble sugar fraction was analyzed using a Waters 1525 HPLC system coupled to a 2424 evaporative light scattering detector (ELSD), as previously described (Al Hassan et al., 2016b). The source parameters of ELSD were the following: gain 75, data rate one point per second, nebulizer heating 60%, drift tube 50°C, and gas pressure 2.8 Kg/cm^2. Samples of 20 μL were injected into a Prontosil 120-3-amino column (4.6 x 125 mm; 3 μm particle size) and were maintained at room temperature with a Waters 717 auto-sampler. An isocratic flux (1 mL/min) of 85% acetonitrile (J.T. Baker) was applied in each run for 25 minutes. Glucose, fructose, and sucrose standards were employed to identify peaks by co-injection. Sugars were quantified with peak integration using the Waters Empower software, and comparisons were made with the glucose, fructose, and sucrose standard calibration curves.

MDA, H_2O_2, DPPH, and Non-Enzymatic Antioxidants

MDA contents were determined essentially as described (Hodges et al., 1999), with modifications (Taulavuori et al., 2001). Methanol extracts (80% v/v) of the leaf material

were mixed with 0.5% thiobarbituric acid (TBA) in 20% TCA, and then incubated 15 min at 95°C. The reaction was stopped on ice, the absorbance of the sample was measured at 440, 600 and 532 nm, and the MDA concentration was determined using the described equations (Taulavuori et al., 2001).

The leaf hydrogen peroxide contents in both the control and treated plants were quantified as previously described (Loreto et al., 2002), with minor modifications. Dried leaf material (0.05 g) was extracted with a 0.1% (w/v) trichloroacetic acid (TCA) solution, followed by centrifuging the extract. The supernatant was thoroughly mixed with one volume of 10 mM potassium phosphate buffer (pH 7) and two volumes of 1 M potassium iodide. The absorbance of the sample was determined at 390 nm. Hydrogen peroxide concentrations were calculated against an H_2O_2 standard calibration curve and expressed as μmol g^{-1} DW.

The total antioxidant activity of extracts was evaluated by measuring the ability of samples to quench the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), a synthetic free radical product whose quenching by a scavenger substrate can be followed spectrophotometrically at 517 nm (Sagar et al., 2011). Plant dry material (0.05 g) was extracted in 2 mL of 90% methanol, sonicated for 10 min and centrifuged at 14000 rpm for 15 min. Then 50 μL of the soluble fraction were diluted with 2 mL of 96% ethanol. A fraction of the resulting solution was diluted 4 times with 96% ethanol containing 125 μM DPPH. The reaction mixture was incubated at 25°C for 10 min, and the absorbance of the sample was measured at 517 nm. A blank sample with no plant extract was included to check radical stability. The radical scavenging activity (S) of each extract was expressed as a percentage and calculated as:

$$S = 100 - [(A_x/A_0) \times 100] \quad (2)$$

where A_x is the absorbance of the DPPH solution in the presence of the extract, and A_0 is the absorbance of the blank.

Total phenolic compounds (TPC) and total flavonoid (TF) contents were determined in the same methanol extracts used for the TSS measurements. TPC were quantified by running a reaction with the Folin-Ciocalteu reagent (Blainski et al., 2013). Absorbance was measured at 765 nm, and the results were expressed as equivalents of gallic acid, used as the standard (mg·eq·GA g^{-1} DW). TF were measured following the method described by Zhishen et al. (1999), based on the nitration of catechol groups in aromatic rings and their reaction with $AlCl_3$ at an alkaline pH. Absorbance was measured at 510 nm, and the concentration of flavonoids was expressed in equivalents of the standard, catechin (mg eq C. g^{-1} DW).

Enzymatic Antioxidant Activities

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

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

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

Ascorbate peroxidase (APX) activity was determined following ascorbate oxidation in the presence of the plant extract by the decrease in absorbance at 290 nm. One APX unit was defined as the amount of enzyme to catalyze the consumption of 1 mmol of ascorbate per minute at 25°C (Nakano & Asada, 1981).

Glutathione reductase (GR) activity was quantified following the oxidation of NADPH, the cofactor in the reaction of oxidized glutathione (GSSG) reduction, by a reduction in absorbance at 340 nm. One GR unit was defined as the amount of enzyme to oxidize 1 mmol of NADPH per minute at 25°C (Connell & Mullet, 1986). The minor modifications introduced into the originally published assays of CAT, SOD, and GR are described in Gil *et al*. (Gil et al., 2014).

Statistics Analysis

Data were analyzed by program SPSS, v. 16 and SYSTAT v. XVI. Before the analysis of variance, the Shapiro-Wilk test was used to check for the validity of normality assumption and Levene's test for the homogeneity of variance. If the ANOVA requirements were accomplished, the significance of the differences among treatments was tested by a one-way ANOVA at the 95% confidence level and *post hoc* comparisons were made using the Tukey HSD test. A factorial ANOVA was performed for all parameters analyzed in the plants, considering two factors of variability, treatment and species, and their interaction. A dendrogram according to all the parameters recorded in the water-stressed plants was built by clustering the four species by the Nearest Neighbor Method, based on squared Euclidean distances. The parameters showing a significant variation between the treatments measured in all plants (control and water-stressed) were correlated using a PCA. All the means throughout the text are followed by SE.

4.1.3 Results

Substrate Moisture at the End of Treatments

After not irrigating the four analyzed species for 1 month, substrate humidity was significantly lower than that recorded in the control treatments, in which plants were watered twice weekly (Table 1). When comparing the four species, no significant differences were found in the moisture values of substrates (Table 1).

Table 1. Substrate humidity (%) of the control (C) and water-stressed (WS) *Limonium* plants grown in greenhouses. Asterisks indicate significant differences between treatments per species. Letters denote significant differences between species per treatment (capital letters for the control plants and lower-case letters for the plants subjected to 1-month water stress) at the 95% confidence level. Mean values are followed by SE (n=5).

Variable	Treatment	*L. santapolense*	*L. virgatum*	*L. girardianum*	*L. narbonense*
Humidity	C	45.42 ± 1.43*^{A}	45.42 ± 1.25*^{A}	45.39 ± 1.43*^{A}	43.67 ± 0.71*^{A}
(%)	WS	10.70 ± 1.25*^{a}	14.01 ± 1.43*^{a}	12.20 ± 1.10*^{a}	12.26 ± 0.34*^{a}

Effects of Drought on Plant Growth and Photosynthetic Pigments Levels

Water stress caused significantly inhibited vegetative plant growth in two of the selected species, *L. santapolense* and *L. narbonense*, as shown by the reduced fresh weight (FW) of aerial plant parts by about one third *versus* the corresponding controls (Figure 1, Table 2). Slight reduction (approx. 11%) was recorded in *L. girardianum*, whereas the mean FW of the water-stressed *L. virgatum* plants was slightly higher than in the control. However, the differences found for the two latter species were not significant (Table 2).

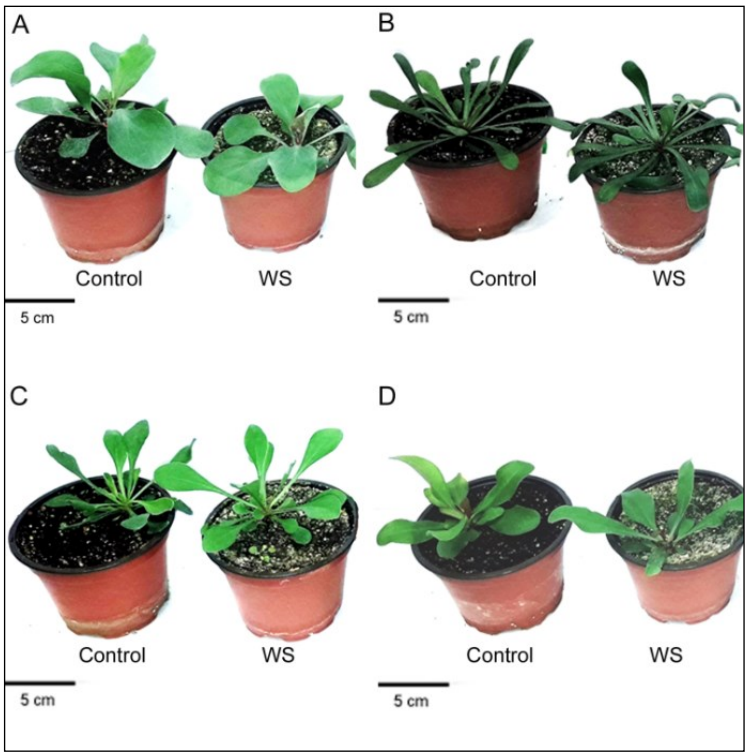

Figure 1. Effect of the 1-month water stress treatment (WS) in the four *Limonium* species under study: *L. santapolense* (A), *L. virgatum* (B), *L. girardianum* (C) and *L. narbonense* (D).

Regarding the reduced leaf area (LA), the effect of drought was statistically significant only in *L. santapolense*, the species with broader leaves, which lost about 40% of its foliar area compared to the non-stressed control. Despite complete withholding irrigation for 1 month, the dehydration of the *Limonium* plants was very low. Once again, the drought-induced reduction of the leaf water content (WCL) was significant only in *L. santapolense* and came to less than 2%. On the contrary, the root water content (WCR) reduction was significant only in *L. narbonense* (Table 2). According to these growth parameters, *L. santapolense* can be considered the species most affected by water stress under our experimental conditions.

Water stress induced only minor, non significant changes in the leaf contents of photosynthetic pigments (chlorophylls a and b, and total carotenoids) in the four *Limonium* species (Table 2). Pigment levels were similar in all four species, except for chlorophyll a in *L. virgatum* and *L. girardianum*, which showed a significant difference when comparing the control (untreated) plants of the two species.

Table 2. The mean leaf fresh weight (FWL), leaf area (LA), leaf water content (WCL), root water content (WCR) and photosynthetic pigments, chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Caro) levels of the control (C) and water-stressed (WS) *Limonium* plants. Asterisks indicate significant differences between treatments per species. Letters denote significant differences between species per treatment (capital letters for the control plants and lower-case letters for the plants subjected to 1-month water stress) at the 95% confidence level. Mean values are followed by SE (n=5).

Variable	Treatment	*L. santapolense*	*L. virgatum*	*L. girardianum*	*L. narbonense*
FWL (g)	C	5.02 ± 0.42*^{B}	2.33 ± 0.47^{A}	2.49 ± 0.43^{A}	2.05 ± 0.14*^{A}

LA (cm^2)	WS	3.44 ± 0.12*^{b}	2.54 ± 0.66^{b}	2.19 ± 0.27^{b}	1.47 ± 0.18*^{a}
	C	17.01 ± 1.24*^{C}	4.45 ± 0.20^{A}	6.06 ± 0.34^{AB}	8.60 ± 0.65^{B}
WCL (%)	WS	10.26 ± 0.74*^{b}	3.87 ± 0.25^{a}	6.27± 0.39^{a}	6.87 ± 0.63^{ab}
	C	84.55 ± 0.26*^{A}	87.50 ± 0.46^{C}	86.88 ± 0.37^{BC}	85.21 ± 0.97^{AB}
WCR (%)	WS	83.61 ± 0.41*^{a}	86.44 ± 0.88^{bc}	87.95 ± 0.40^{c}	84.26 ± 0.76^{ab}
	C	61.44 ± 5.50^{A}	74.85 ± 1.18^{B}	78.82 ± 1.47^{B}	80.36 ± 1.01*^{B}
Chl a (mg g^{-1} DW)	WS	52,74 ± 5.08^{a}	77.68 ± 1.24^{b}	73.46 ± 1.93^{b}	74.39 ± 0.36*^{b}
	C	2.37 ± 0.17^{AB}	1.74 ± 0.18^{A}	2.63 ± 0.13^{B}	1.97 ± 0.19^{AB}
	WS	2.27 ± 0.21^{ab}	2.18 ± 0.21^{ab}	2.75 ± 0.17^{b}	1.59 ± 0.13^{a}
Chl b (mg g^{-1} DW)	C	1.11 ± 0.01^{A}	1.04 ± 0.06^{A}	1.07 ± 0.10^{A}	0.99 ± 0.14^{A}
	WS	1.14 ± 0.25^{a}	1.22 ± 0.18^{a}	1.03 ± 0.06^{a}	0.76 ± 0.09^{a}
Caro (mg g^{-1} DW)	C	1.19 ± 0.07^{A}	1.36 ± 0.11^{A}	0.97 ± 0.08^{A}	1.30 ± 0.21^{A}
	WS	1.02 ± 0.11^{a}	1.12 ± 0.09^{a}	0.99 ± 0.04^{a}	0.93 ± 0.11^{a}

Ion Accumulation

No changes in the leaf contents of the mono- (Na^+, K^+ and Cl^-) and divalent (Ca^{2+} and Mg^{2+}) ions were induced under the water deficit conditions in any of the investigated *Limonium* species (Table 3). Some significant differences between the control and stressed plants were, however, observed in roots, but with no clear pattern of variation. For example, Na^+ and Cl^- generally increased in response to water stress, although the differences with the corresponding controls were not significant in all the *Limonium* taxa, while K^+ contents did not vary. The mean Ca^{2+} and Mg^{2+} levels drastically dropped in *L. santapolense* but rose in the other three species (Table 3.). The K^+/Na^+ ratio, which is considered relevant for maintaining ionic homeostasis, did not vary in the plant leaves, but significantly decreased in the roots of all the species, except *L. virgatum*, which showed a non-significant reduction. When comparing ion contents in the roots and leaves of the same plants, they were all higher in leaves in both the control and water-stressed plants: 2- to 4-fold, approximately, for the monovalent ions in all four *Limonium* species, and for Ca^{2+} and Mg^{2+} in *L. narbonense*. For the other three species, the divalent cation levels were about 6- to 15-fold higher in leaves than in roots (Table 3). The relatively high Mg^{2+} concentrations measured in the leaves of these species is worth mentioning.

Table 3. Mono- and divalent ions contents (μmol g^{-1} DW) and K^+/Na^+ ratios in the roots and leaves of the control (C) and water-stressed (WS) *Limonium* plants. Asterisks indicate significant differences between treatments per species. Letters denote significant differences between species per treatment (capital letters for the control plants and lower-case letters for the plants subjected to 1-month water stress) at the 95% confidence level. Mean values are followed by SE (n=5).

Ion	Treatment	*L. santapolense*	*L. virgatum*	*L. girardianum*	*L. narbonense*
Na^+ roots	C	137.22 ± 23.17*^{A}	127.99 ± 2.19^{A}	119.52 ± 1.68*^{A}	174.17 ± 16.09*^{A}
	WS	209.96 ± 42.13*^{ab}	116.44 ± 17.76^{a}	184.18 ± 11.05*^{a}	238.37 ± 22.70*^{b}
Na^+ leaves	C	450.01 ± 10.61^{A}	473.66 ± 27.32^{A}	534.93 ± 29.99^{A}	552.11 ± 67.78^{A}
	WS	499.65 ± 25.75^{b}	426.83 ± 7.46^{a}	513.47 ± 22.41^{b}	416.29 ± 18.90^{a}
K^+ roots	C	279.85 ± 35.35^{A}	353.33 ± 8.86^{AB}	278.78 ± 2.46^{A}	382.72 ± 32.19^{B}
	WS	242.79 ± 35.36^{a}	300.29 ± 42.01^{ab}	326.49 ± 22.23^{ab}	406.74 ± 36.69^{b}
K^+ leaves	C	833.33 ± 20.98^{A}	977.25 ± 43.11^{A}	974.47 ± 32.05^{A}	981.48 ± 97.45^{A}
	WS	839.27 ± 27.83^{ab}	897.79 ± 15.08^{ab}	1031.71 ± 75.86^{b}	823.25 ± 60.43^{a}
K^+/Na^+ roots	C	2.08 ± 0.18*^{A}	2.75 ± 0.01^{C}	2.33 ± 0.05*^{B}	2.20 ± 0.02*^{AB}
	WS	1.30 ± 0.16*^{a}	2.60 ± 0.07^{c}	1.77 ± 0.03*^{b}	1.72 ± 0.09*^{b}

K^+/Na^+ leaves	C	1.85 ± 0.04^{A}	2.08 ± 0.26^{A}	1.83 ± 0.04^{A}	1.80 ± 0.09^{A}
	WS	1.69 ± 0.06^{a}	2.11 ± 0.15^{b}	1.99 ± 0.06^{b}	1.96 ± 0.06^{b}
Cl^- roots	C	186.77 ± 18.28*^{A}	236.95 ± 5.35^{A}	203.10 ± 28.32*^{A}	355.43 ± 35.59^{B}
	WS	203.10 ± 51.70*^{a}	228.49 ± 17.34^{a}	299.01 ± 20.26*^{ab}	456.98 ± 41.80^{b}
Cl^- leaves	C	727.78 ± 51.33^{A}	787.02 ± 75.02^{A}	767.27 ± 32.36^{A}	971.50 ± 79.81^{A}
	WS	836.10 ± 47.45^{a}	892.80 ± 34.84^{a}	856.27 ± 17.34^{a}	999.35 ± 82.48^{a}
Ca^{2+} roots	C	13.29 ± 2.03*^{B}	4.48 ± 0.64*^{A}	6.18 ± 1.20*^{A}	14.23 ± 0.94*^{B}
	WS	6.18 ± 2.20*^{a}	10.35 ± 1.14*^{a}	9.69 ± 0.66*^{a}	24.28 ± 2.67*^{b}
Ca^{2+} leaves	C	84.88 ± 7.03^{A}	66.74 ± 10.43^{A}	61.93 ± 12.40^{A}	53.01 ± 12.13^{A}
	WS	97.12 ± 5.66^{bc}	68.46 ± 14.90^{ab}	117.57 ± 20.63^{b}	37.20 ± 1.70^{b}
Mg^{2+} roots	C	64.24 ± 3.36*^{C}	40.05 ± 0.36*^{A}	50.93 ± 3.01^{B}	76.33 ± 3.51^{D}
	WS	50.93 ± 5.51*^{a}	57.21 ± 6.28*^{a}	64.07 ± 5.07^{ab}	78.34 ± 4.52^{b}
Mg^{2+} leaves	C	456.63 ± 29.80^{A}	401.16 ± 40.36^{A}	486.11 ± 61.29^{A}	320.88 ± 34.63^{A}
	WS	538.36 ± 22.53^{c}	429.16 ± 49.70^{b}	555.07 ± 13.81^{c}	260.80 ± 38.60^{a}

Water Stress-Induced Osmolyte Accumulation

The levels of the commonest plant osmolytes –proline (Pro), glycine betaine (GB) and total soluble sugars (TSS)– were determined in the leaves of the investigated *Limonium* taxa when the water deficit treatments ended (Figure 2). With the exception of *L. narbonense*, Pro levels increased significantly compared to the untreated controls. However, this stress-induced increment was by far more pronounced in *L. santapolense*, where Pro reached 122 μmol g^{-1} DW, which represents a 6-fold increase over the control (Figure 2a).

The leaf GB contents in the control plants were similar to those of Pro (20 – 50 μmol g^{-1} DW), except for *L. virgatum*, in which a concentration of 76 μmol g^{-1} DW was determined. These values only slightly varied in general, and not significantly, in the plants subjected to the water deficit treatment (Figure 2b).

Limonium santapolense showed higher leaf TSS contents than the other three species in the control plants. For all four taxa, the water stress treatment induced only minimal changes in the TSS levels, as observed for GB (Figure 2c).

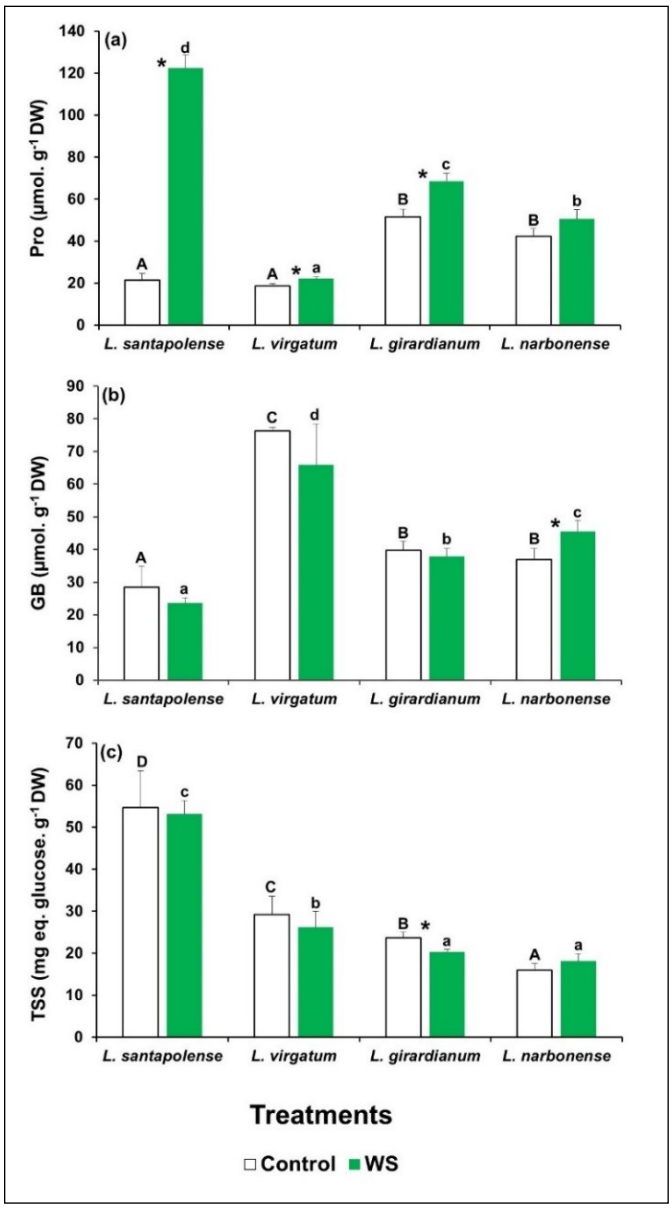

Figure 2. Osmolyte contents in the leaves of the four studied *Limonium* species. Proline (Pro) (A), glycine betaine (GB) (B), and total soluble sugar (TSS) levels (C), after 1 month of water stress treatment (WS) and in the control plants. The shown values are means with SE (n=5). Different letters above the bars indicate significant differences between species, for control (capital letters) and water-stressed (lowercase letters) plants. Asterisks denote significant differences between treatments for each species, according to Tukey's test (α = 0.05).

To check the possibility that the leaf levels of particular sugars could vary in response to the water deficit treatment, which cannot be detected by measuring TSS contents, soluble carbohydrates in aqueous extracts of plants were separated, identified and quantified by HPLC (Figure 3). Three major peaks in the chromatograms were observed, corresponding to glucose (Glu), fructose (Fru) and sucrose (Suc), but their concentrations showed entirely different patterns in the four analyzed *Limonium* species. Glu was detected only in *L. giradianum* and *L. narbonense*, but its concentration increased significantly in response to drought only in the former species by reaching 15.5 µmol g-1 DW (Figure 3a). In *L. santapolense* and *L. virgatum*, glucose levels were below the

detection limit of the evaporative light cattering detector (ELSD), both in control and stressed plants. Fru concentrations were very low (< 0.5 μmol g-1 DW) in the non-stressed *L. santapolense* plants and increased considerably to ca. 38 μmol g-1 DW, in response to water stress. The other three species had higher control Fru values (8 – 17 μmol g-1 DW), which either lowered or did not change significantly in the stressed plants (Figure 3b). Large differences in leaf Suc contents were also detected in the control plants of the four analyzed species, which went from extremely low values (< 0.2 μmol g-1 DW) in *L. giradianum* to 2.2 – 2.4 μmol g-1 DW in *L. narbonense* and *L. virgatum*, and to ~ 17 μmol g-1 DW in *L. santapolense*. In the latter species, water deficit stress induced a significant increase of 1.4-fold in the Suc concentration (Figure 3c), which was much lower than that observed for Fru in any case (Figure 3b).

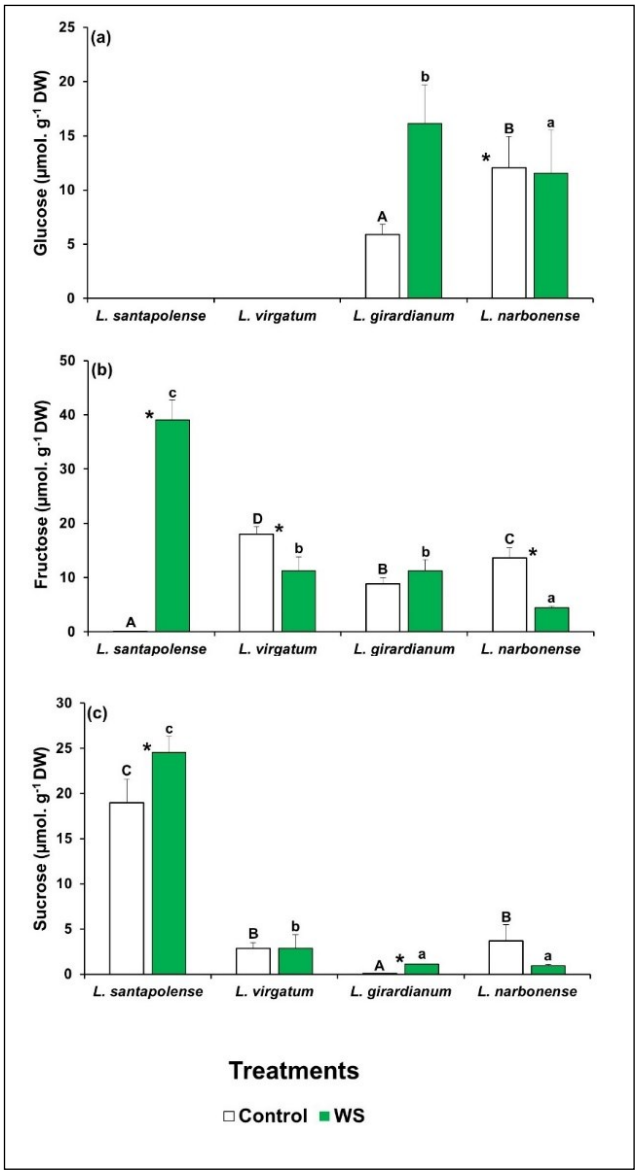

Figure 3. Soluble sugar contents in the leaves of the four studied *Limonium* species. Glucose (Glu) (A), fructose (Fru) (B) and sucrose (Suc) (C) levels after 1 month of water stress treatment (WS) and in the control plants. The shown values are means with SE (n=5). Different letters above the bars indicate significant differences between species, for control (capital letters) and water-stressed (lowercase letters)

plants. Asterisks denote significant differences between treatments for each species, according to Tukey's test (α = 0.05).

Oxidative Stress and Activation of Antioxidant Systems

Malondialdehyde (MDA) levels did not significantly differ in the control plants of the four *Limonium* species. After the water stress treatment, leaf MDA contents increased in all cases, albeit slightly, between 1.1- and 1.7-fold depending on the species. However, the differences with the non-stressed controls were statistically significant only in *L. narbonense* (Table 4). Hydrogen peroxide levels were similar in the control plants of all the *Limonium* taxa, and the water deficit-induced variations were also non-significant (Table 4). The overall antioxidant activity of plant extracts, as determined by the DPPH (α, α-diphenyl-β-picrylhydrazyl) free radical scavenging assay, did not change in the stressed plants compared to their corresponding controls. In this case, however, significant differences between taxa were observed, with *L. santapolense* showing the greatest antioxidant activity (Table 4).

Phenolic compounds, especially, the subclass of flavonoids, are well-established examples of antioxidant metabolites. Leaf levels of total phenolic compounds (TPC) and total flavonoids (TF) were determined in the control and water-stressed plants. Once again, TPC contents were higher in *L. santapolense* than in the other three species, but did not change as a result of the water deficit treatment in all cases. TF levels, however, were low and did not vary significantly either between species or between the control and stressed plants (Table 4). The leaf levels of anthocyanins, which can also be used to estimate the antioxidant activity of plant samples, were low in all the control plants and did not vary significantly in response to the water deficit treatment (data not shown).

Table 4. Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations, DPPH free radical scavenging activity, total phenolic compounds (TPC) and total flavonoids (TF) contents in the leaf extracts from the plants of the four selected *Limonium* species. Asterisks indicate significant differences between treatments per species. Different letters (capital for the non-stressed controls and lowercase for the plants subjected to 1 month of water deficit stress) indicate significant differences between species per treatment at the 95% confidence level. Mean values are followed by SE (n=5).

Variable	Treatment	*L. santapolense*	*L. virgatum*	*L. girardianum*	*L. narbonense*
MDA (nmol g^{-1} DW)	C	103.58± 16.92^{A}	83.93 ± 18.61^{A}	149.16± 16.74^{A}	80.09± 7.60*^{A}
	WS	152.59± 28.69^{a}	138.82± 21.224^{a}	162.53± 17.85^{a}	135.28± 14.73*^{a}
H_2O_2 (μmol g^{-1}DW)	C	17.93 ± 3.29^{A}	17.21 ± 2.26^{A}	20.86 ± 3.49^{A}	26.68 ± 3.24^{A}
	WS	25.25 ± 3.80^{b}	12.89 ± 1.28^{a}	18.59 ± 0.38^{ab}	21.94 ± 1.01^{b}
DPPH (%)	C	84.72 ± 6.10^{C}	76.94 ± 0.99^{BC}	15.28 ± 2.97^{A}	52.34 ± 11.27^{B}
	WS	82.60 ± 9.86^{b}	62.17 ± 6.90^{ab}	34.13 ± 7.97^{a}	41.81 ± 5.70^{ab}
TPC (mg eq. GA g^{-1} DW)	C	24.41 ± 4.18^{B}	11.50 ± 6.15^{A}	6.40 ± 1.31^{A}	12.57 ± 3.01^{A}
	WS	23.96 ± 1.81^{b}	6.15 ± 0.40^{a}	6.56 ± 1.18^{a}	9.25 ± 2.77^{a}
TF (mg eq. C g^{-1} DW)	C	1.96 ± 0.37^{AB}	1.26 ± 0.16^{A}	0.71 ± 0.13^{A}	2.95 ± 0.61^{B}
	WS	1.80 ± 0.28^{a}	1.00 ± 0.13^{a}	1.22 ± 0.18^{a}	1.90 ± 0.45^{a}

The specific activities of the four tested antioxidant enzymes (SOD, CAT, APX and GR) showed different patterns in the four species for both basal values in the controls and

quantitative changes in response to water deficit. In most cases however, the stress treatment led to greater antioxidant activities (Figure 4). SOD activity, for example, increased significantly in *L. santapolense* and *L. giradianum*, but did not change in *L. virgatum* and *L. narbonense*. The highest specific activity was measured in the stressed *L. santapolense* plants (Figure 4a). CAT activity was very low in the control *L. santapolense* and *L. virgatum* plants and slightly, but significantly, augmented in response to the drought treatment. The control values were much higher in *L. narbonense* and also grew under stress conditions, but did not vary in *L. giradianum* (Figure 4b). APX activity increased significantly in the four species. but reached much higher values in *L. santapolense* than in the other three taxa (Figure 4c). GR activity increased in response to the stress treatment in all the *Limonium* taxa, except for *L. giradianum*. Once again, the greatest activity was measured in *L. santapolense,* but the relative increment over the control values was lower than in *L. virgatum* and *L. giradianum* (Figure 4d).

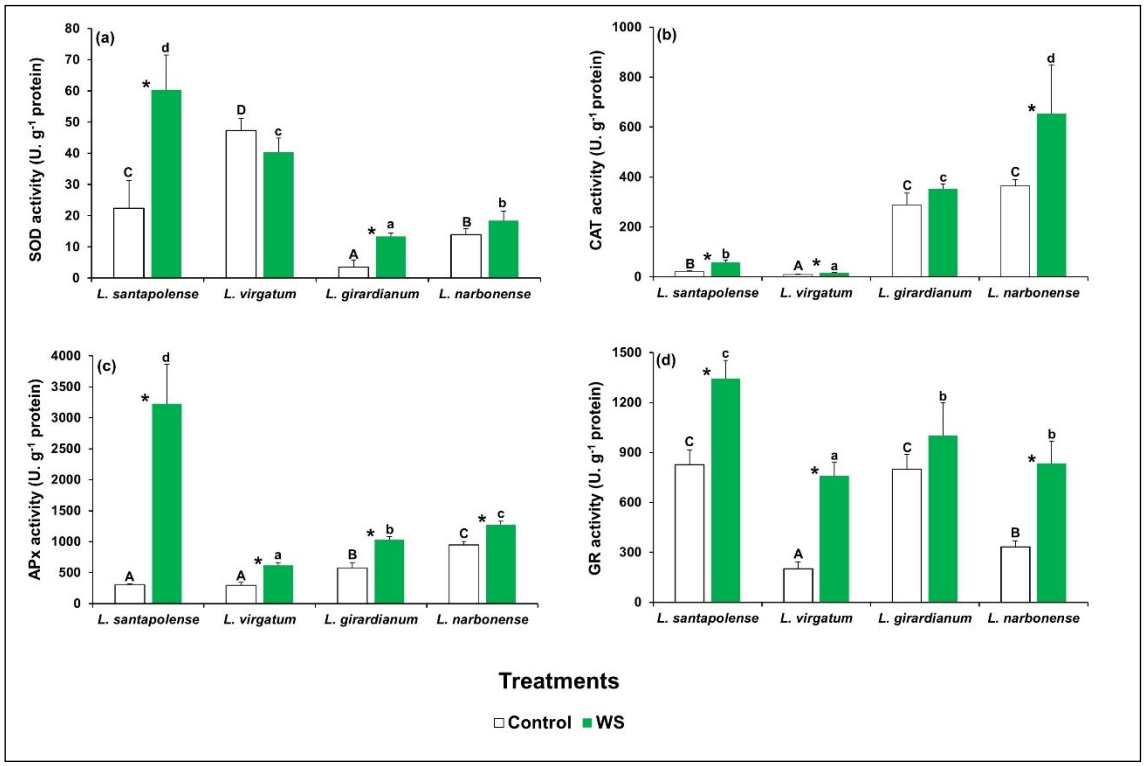

Figure 4. Activity of the antioxidant enzymes in the leaves of the four studied *Limonium* species after 1 month of water stress treatment (WS) and in the control plants. The graphs show the specific activities of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) ascorbate peroxidase (APX) and (D) glutathione reductase (GR) as mean values with SE (n=5). Different letters above the bars indicate significant differences between species, for control (capital letters) and water-stressed (lowercase letters) plants. Asterisks denote significant differences between treatments for each species, according to the Tukey test ($\alpha = 0.05$).

Statistical Analysis of Data. Factorial ANOVA, Clustering of Species and Principal Component Analysis

The results of a factorial ANOVA considering the effect of treatment, species and their interaction are shown in Table 5. Of the 31 parameters analyzed, 27 varied

significantly according to the species, but only 15 according to the treatment. The greatest variations between values measured in control and water-stressed plants were registered for osmolytes and antioxidant enzymes. Interestingly, the interactions between the two factors (Treatment x Species) were also significant for these parameters, indicating that the tested species do not show the same patterns of response to water stress.

Table 5. Factorial ANOVA (F values) considering the effect of Species (S), Treatment (T), and their interactions (S x T) on metabolic profiles of carbohydrates, organic acids and aminoacids in *L. albuferae* and *L. dufourii.* *, **, *** Significant at P = 0.05, 0.01 and 0.001 respectively; ns: not significant. FW: fresh weight; LA: leaf area; WC_l: water content of leaves; WC_r: water content of roots; Chl a: chlorophyll a; Chl b: chlorophyll b; Caro: carotenoids; Pro: proline, GB: glycine betaine; TSS: total soluble sugars; Glu: glucose, Fru: fructose: Suc: sucrose; MDA: malondialdehyde; TPC: total phenolic compounds; TF: total flavonoids; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase, GR glutathione reductase.

	Parameter	Treatment (T)	Species (S)	Interaction (T x S)
Growth	FW_l	2.88	10.83***	1.36
	LA	11.59**	39.65***	2.96*
	WC_l	1.23	14.81***	1.45
	WC_r	14.16***	30.38***	4.11*
Photosynthtetic pigments	Chl a	0.01	10.87***	2.05
	Chl b	0.1	1.91	0.66
	Caro	5.97*	1.77	1.06
Mono and divalent ions	Na^+_r	10.87**	6.06**	1.89
	Na^+_l	3.01	1.91	2.96*
	K^+_r	1.37	133.84***	6.09**
	K^+_l	1.31	3.33*	1.55
	Cl^-_r	8.68***	14.07***	2.90*
	Cl^-_l	5.45*	3.30*	1.33
	Ca^{2+}_r	0.05	7.39***	1.37
	Ca^{2+}_l	3.25	9.44****	2.80
	Mg^{2+}_r	3.29	19.11***	6.44**
	Mg^{2+}_l	2.08	14.55***	1.48
Compatible solutes	Pro	172.75***	80.14***	86.56***
	GB	1.68	22.21***	2.81
	TSS	0.27	37.72***	0.23
	Glu	5.51*	39.60***	6.09**
	Fru	25.67***	14.65***	79.13***
	Suc	5.35*	3.27*	1.15
Oxidative stress markers and antioxidants	MDA	9.26**	2.40	0.50
	H_2O_2	0.29	4.33*	2.29
	TPC	2.23	27.97***	0.73
	TF	1.05	10.06**	2.21
Antioxidants	SOD	10.84**	28.29***	10.04***
	CAT	12.63*	73.30***	5.39**
	APX	71.35***	21.84***	28.95***

	GR	42.53***	16.83***	1.47

The cluster analysis performed using all the variables measured in the water-stressed plants (including growth parameters) clearly distinguished *L. santapolense* individuals from the others as they were present on a separate branch of the dendrogram (Figure 5). Of the remaining three species, *L. narbonense* was the most distant, whereas the values recorded in *L. virgatum* and *L. girardianum* were entangled. A principal component analysis (PCA) was also performed, including the growth parameters, osmolytes, MDA and enzyme activities determined in the water stress and control treatments. Photosynthetic pigments, ions, H_2O_2 and antioxidant compounds were not included as they did not vary significantly under stress. Ten components had an eigenvalue above 1. The biplot of the two main principal components, which together explained 63% of total variability, is shown in Figure 6. The first component, which explains the highest variability of the data (41.79%) is related mostly to the treatment, whereas the second (20.18%) with the species. Water stress, reflected as reduced substrate moisture after not watering pots for 1 month, correlated positively with changes in water content in the plant roots and leaves, and negatively with proline and fructose (the osmolytes that strongly increased under stress), and with antioxidant enzymes (especially GR and APX), whose activity also increased as a response to water deficit. The PCA, like the cluster analysis, showed a clear separation of *L. santapolense* from the other taxa, which all appeared together in the negative sector of the X-axis. *L. santapolense* was the only species for which the control and stress treatment values were clearly distant. The latter correlated positively with changes in the Pro and Fru levels, and in the activities of SOD, APX and GR, which thus confirms the results of the above-described individual experiments (Figs. 2-4).

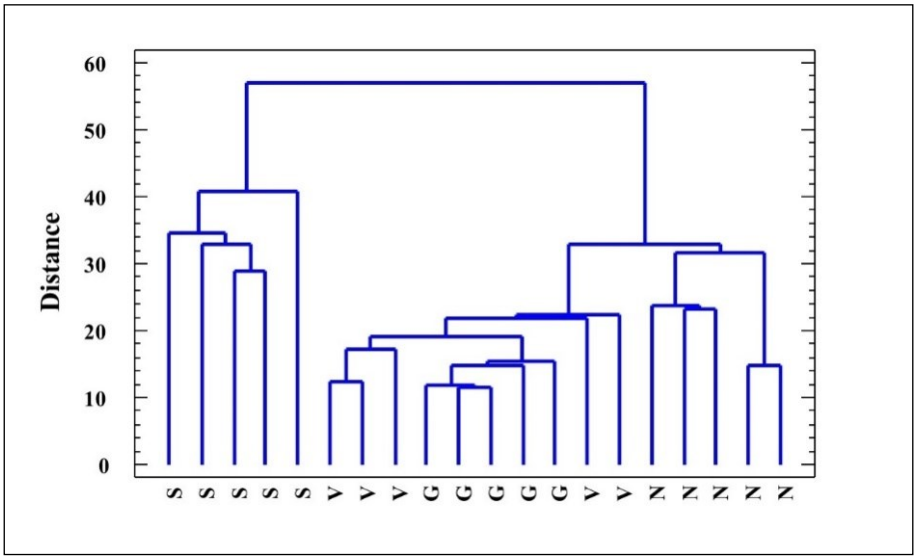

Figure 5. Clustering of the analyzed *Limonium* species: *L. santapolense* (S.), *L. virgatum* (V.), *L. girardianum* (G.), and *L. narbonense* (N.), by the Nearest Neighbor Method, based on squared Euclidean distances according to all the parameters registered in the water-stressed plant.

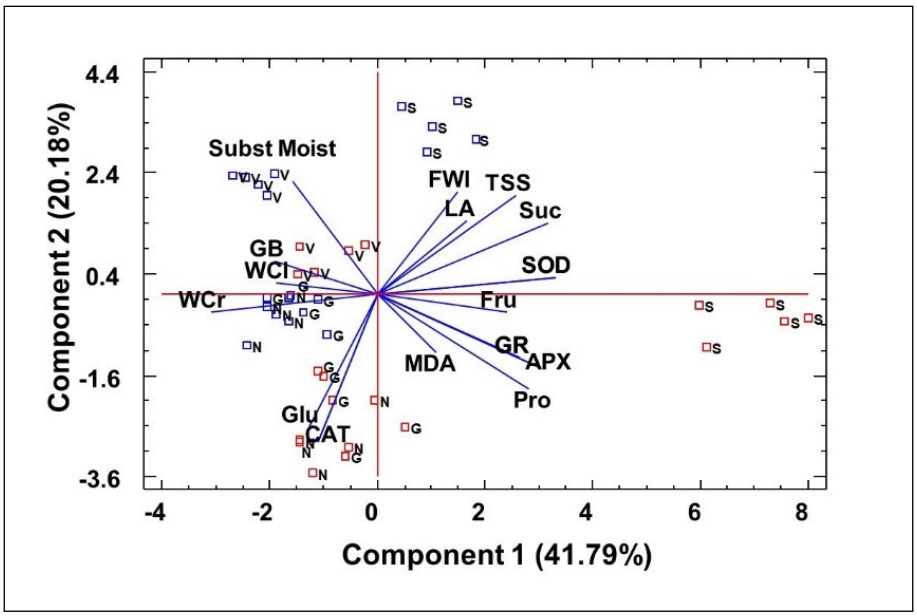

Figure 6. Principal Component Analysis (PCA). Changes in growth parameters, osmolytes levels and anti-oxidant enzyme activities in the plants grown under water stress conditions for 1 month (red squares) *versus* the corresponding control (blue squares), the non stressed plants of the investigated *Limonium* species: *L. santapolense* (S.), *L. virgatum* (V.), *L. girardianum* (G.), and *L. narbonense* (N.), in correlation to substrate moisture. Each square corresponds to an individual analyzed plant. FWL, leaf fresh weight; LA, leaf surface area; WCL, water content percentage in leaves; WCR, water content percentage in roots; Pro, proline; GB, glycine betaine; TSS, total soluble sugars; Glu, glucose; Fru, fructose; Suc, sucrose; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase, GR glutathione reductase.

4.1.4 Discussion

A considerable number of relevant physiological studies has been recently published on Mediterranean plant species that are adapted to severe stressful environments. Several have specifically centered on distinct *Limonium* species or have included some taxa of this genus along with other species (Galmés et al., 2005; Galmés et al., 2018). As regards the activation of specific stress responses at molecular and biochemical levels, the majority of former research works into *Limonium* have centered on responses to salt stress (Gagneul et al., 2007; Al Hassan et al., 2017). All this renders the data herein presented novel, and stresses the role played by antioxidant enzymes and specific osmolytes in mechanisms of defense against water deficit of *Limonium* plants.

Of all four studied species, *L. santapolense* was the most strongly affected in the greenhouse experiments by water deficit stress, as indicated by its severely inhibited growth (reduced mean leaf area and fresh weight vs. controls) under stress conditions. The only taxon to display a significant, albeit low, degree of leaf dehydration in the unwatered plants was *Limonium santapolense*. Our experiments revealed how the other three species quite well tolerated water stress. Of these, a significantly reduced fresh weight in the stressed plants was evidenced only for *L. narbonense*. In these two species,

the apparently diminished water deficit tolerance could be associated with the morphological characteristics of all four taxa, specifically with their leaf size because their leaves are larger and broader than those of *L. girardianum* and *L. virgatum*. This particular trait is generally thought to enhance plant sensitivity to dehydration, and there are reports that plants can respond to water deficit caused by the global warming by them diminishing their leaf areas (Guerin & Lowe, 2012).

Water stress had no effect on the levels of photosynthetic pigments in any studied *Limonium* taxon, including *L. santapolens*e. As inhibited photosynthesis and low chlorophyll contents are some frequent effects of drought and other abiotic stresses (Kumar et al., 2017; Marcek et al., 2019), these results also indicate that the studied species are quite resistant to water stress.

In the experiments performed herein, plant growth took place with low salt concentrations, those present in the nutrient solution and the peat substrate, and no major changes were expected in the plant ion contents in response to water stress. However, as inorganic ions can contribute to cellular osmotic adjustment with drought (Silva et al., 2010), the levels of the mono- and divalent cations, and those of Cl^-, were determined in the leaves and roots of the stressed and control plants. In roots, save *L. virgatum*, Cl^- and Na^+ contents significantly rose in the plants undergoing the water stress treatment *versus* the corresponding controls. This can be accounted for by the activation of ion transport in plants by counteracting, at least in part, the osmotic stress produced by not watering the plants. Increasing Na^+ concentrations are usually accompanied by loss of K^+ as Na^+ interferes with K^+ uptake by employing the same transport systems, and both cations compete for the same binding proteins (Munns, 2002). These changes in K^+ and Na^+ contents give rise to lower K^+/Na^+ ratios. Indeed, we recorded this reduction in the root K^+/Na^+ ratios in the *Limonium* plants (save *L. virgatum*), despite no significant drop in the K^+ concentration being detected. Ion leaf contents displayed a distinct pattern. Firstly, and most importantly, the levels of the three determined monovalent ions (K^+, Na^+ and Cl^-), and those of divalent cations Mg^{2+} and Ca^{2+}, were significantly higher in leaves than in roots. This finding clearly suggests the presence of active transport systems of these ions to aerial plant parts, which was also found under salt stress in *Limonium* (Al Hassan et al., 2017). High leaf concentrations of K^+ and Na^+ play an important role in osmotic adjustment under water stress also in quinoa, as it has been recently reported (Gámez et al., 2019). Secondly, significant differences were not found between the stressed and control plants in ion leaf contents for any ions or any species. This would indicate that ion transport activation is not induced by water deficit, but is likely a constitutive mechanism of response to stress that *Limonium* plants use to help contribute to the cellular osmotic balance in leaves.

As for the biochemical responses of all four *Limonium* taxa, *L. santapolense* behaved differently according to the cluster analysis, which showed a clear separation between this species and the remaining three. The most striking differences to appear among them indicate the activation of different antioxidant enzymes and the stress-induced accumulation of particular osmolytes. Water deficit led to the considerable accumulation of both fructose and proline in *L. santapolens*e leaves, at concentrations around 40 and 120 μmol g^{-1} DW, respectively, which are much higher than those recorded in the

controls. Sucrose levels also significantly increased, but by less than 1.5-fold, with lower absolute values (< 25 μmol g^{-1} DW). We conclude that osmotic adjustment to protect plants from dehydration in *L. santapolense* under water stress conditions is based on Fru and Pro accumulation, with Suc contributing less. The other *Limonium* taxa exhibited a much weaker water stress response for the mechanisms mediated by accumulating compatible solutes. In the three species, the leaf contents of all the tested putative osmolytes slightly increased, or not at all, as a response to stress treatment, except for glucose accumulation in *L. girardianum,* while the absolute concentrations of these compounds were too low to have a strong osmotic effect.

The lack of glucose accumulation at detectable levels in *L. santapolens*e and *L. virgatum*, both in unstressed plants and in those subjected to water deficit, should be mentioned. Similarly, very low, in most cases non-detectable, glucose leaf contents were determined in a previous study, in control and salt-stressed plants of the same taxa (Al Hassan et al., 2017). These data suggest that glucose does not play any relevant role in osmotic adjustment under stress in these species. The molecular basis of this behavior is not known, but could be possibly related to a rapid turnover of glucose in these taxa, used as an energy source and/or as a building block for the synthesis of polysaccharides required under stress conditions. For example, it has been recently reported that drought causes changes in the cell walls in leaves and stems of miscanthus, a biofuel crop, with an increase in their hemicellulose contents (Van Der Weijde et al., 2017). In any case, *Limonium* taxa are characterized by a large diversity in the type of compatible solutes that accumulate in response to abiotic strees treatments. The osmolytes herein identified have already been reported in other *Limonium* species, including plant material from natural habitats (Tabot & Adams, 2014; Al Hassan et al., 2017), but along with a considerable number of other compounds, like choline-O-sulfate, alanine, betaine or distinct polyalcohols (e.g., *myo*-inositol, pinitol or *chiro*-inositol) (Hanson et al., 1991; Rhodes & Hanson, 1991; Gagneul et al., 2007). The simultaneous synthesis of distinct osmolytes has been observed in *Limonium* (Gagneul et al., 2007) as in other Plumbaginaceae species (Murakeözy et al., 2003). The concomitant synthesis of distinct osmolytes is a helpful strategy adopted by stress tolerant taxa because it enables them to adapt better to the stressful environments they live in (Gil et al., 2011).

Drought, just like any other abiotic stress, increases levels of ROS, which, in excess, oxidize unsaturated fatty acids in cell membranes, amino acid residues in proteins, and DNA molecules, and thus provokes cellular damage (Halliwel, 2006). Hydrogen peroxide is the most relevant stable non radical among ROS that is produced in peroxisomes and chloroplasts, and is thought to be a good marker of the extent of oxidative stress (Sofo et al., 2015). According to our experiments, the leaf H_2O_2 levels showed no marked variation in the stressed plants, *versus* the non-stressed controls, in the studied four species. Malondialdehyde (MDA) is a lipid peroxidation product employed as a reliable oxidative stress marker in both animals and plants (Del Río et al.; 1996). The standard method followed to assess the ability of compounds to act as hydrogen donors or free radical scavengers is DPPH free radical scavenging by indicating specific biological samples' general antioxidant activity (Sagar et al., 2011). For all four *Limonium* taxa herein analyzed, neither the total free radical scavenging activity of the leaf extracts nor MDA contents significantly differed between the water-

stressed and control plants, except for a slight, but statistically significant, increase in MDA levels in the leaves of *L. narbonense*. These findings indicate that the water deficit treatment did not lead to a detectable degree of oxidative stress in the plants, which was likely owing to the activation of efficient antioxidant systems. However, such systems do not include antioxidant compounds likes flavonoids or phenolic compounds, in general, because their leaf concentrations did not vary in response to stress treatment. We found similar results in halophytes sampled in the wild, which did not show a seasonal variation of MDA, although environmental conditions drastically changed in summer (Gil et al., 2014). A review on ROS homeostasis in halophytes has demonstrated that they do not require high antioxidant activity levels because they do not generate ROS in excess thanks to their efficient mechanisms that avoid oxidative stress (Bose et al., 2014). It is believed that enzymatic antioxidant systems constitute the first line of defense against oxidative stress, while phenolic compounds (including flavonoids and other metabolites with antioxidant activity) are a secondary ROS scavenging system that is activated only under severe stress conditions, when antioxidant enzymes do not suffice (Fini et al., 2011). So, with the *Limonium* species chosen for this research, activation of antioxidant enzymes seemed sufficient to counteract the oxidative stress that the water deficit treatment generated, as formerly reported for other *Limonium* species under salt stress conditions (Souid et al., 2018).

SOD constitutes primary defense against ROS by catalyzing dismutation of superoxide radicals into O_2 and H_2O_2 (Alscher et al., 2002). SOD specific activity is enhanced when the superoxide substrate is present by the transcriptional activation of corresponding genes; i.e., by the *de novo* synthesis of the enzyme (Caverzan el al., 2016). After SOD, CAT acts by decomposing the produced H_2O_2 into O_2 and H_2O, induced by its substrate accumulating (Gunes et al., 2007). APX catalyzes the reduction of H_2O_2, coupled to ascorbate oxidation. GR contributes to recover and maintain the adequate cellular redox state by reducing GSSH to GSH, which it does by employing NADPH as a cofactor (Hameed et al., 2015).

In general, the specific activities of these antioxidant enzymes were enhanced in response to water stress in all four selected *Limonium* species, albeit quantitative differences were found in different taxa. *Limonium santapolense* displayed the strongest response as the four activities significantly increased in the stressed plants vs. the controls. The induced SOD, APX and GR levels were higher than those of the other three species, and only CAT displayed less activity, which was below that measured in *L. narbonense* or *L. girardianum*. Different patterns were observed for enzymatic antioxidant responses in the remaining taxa. SOD activity in *L. virgatum* did not vary in the water-stressed plants and remained quite high in the non-stressed controls. APX, CAT and GR activities significantly increased with stress, but the absolute activity values remained very low for APX and CAT. In *L. girardianum,* antioxidant defense was dependent on the constitutive presence of relatively strong GR and CAT activities, with water stress-induced SOD and APX contributing less. Finally, water stress in *L. narbonense* did not activate SOD, but instead induced marked increases in GR and CAT, and also in APX activities to a much lesser extent.

In summary, the four analyzed *Limonium* taxa are relatively resistant to drought, partly based on the presence of constitutive stress tolerance mechanisms, such as the active transport of mono an divalent cations to the leaves, contributing to osmotic balance, as well as on the water deficit-induced accumulation of specific osmolytes and the increased activity of antioxidant enzymes. These induced responses showed different qualitative and quantitative patterns, allowing a clear separation of *L. santapolense* from the other three taxa; this species activated the strongest drought responses, through the specific accumulation of high levels of Pro and Fru, as functional osmolytes, and the significant increase in the specific activities of the assayed antioxidant enzyme systems, SOD, APX, GR and, to a lesser extent, CAT.

4.1.5 Conclusions

The investigated *Limonium* species showed relatively good tolerance to water deficit stress under controlled experimental conditions, based on some constitutive mechanisms of defense, such as the active transport of mono- and divalent ions to aerial plant parts, which can help to maintain cellular osmotic balance and avoid drought-induced leaf dehydration, or the marked activity of some antioxidant enzymes detected in the non-stressed controls. In addition, water stress-induced responses contributed to drought tolerance, including the accumulation of specific osmolytes and the activation of enzymatic antioxidant systems. Interestingly, although the four species are closely related genetically, their induced responses to water stress differed qualitatively and quantitatively as regards the contribution of different osmolytes and enzyme activities to those tolerance mechanisms.

The behavior of *L. santapolense* under stress differed from that of the other three species, as indicated by the PCA and cluster analyses: *L. santapolense* was the species that showed the most strongly induced responses to the water stress treatment, which agrees with the fact that it naturally grows in a more arid environment than the habitats where the seeds of the other three taxa were collected. Surprisingly however, this apparently greater efficiency in the response to water deficit did not lead to higher tolerance in our experiments. On the contrary, the determination of several growth parameters demonstrated that *L. santapolense* was the most affected species by water stress. These results strongly suggest that other mechanisms of defense that are not activated in greenhouse experiments, most likely morphological adaptations of the plants, are responsible for this species' tolerance to drought in its natural habitat. Further studies in the field, which combine biochemical analyses of the plant material with morphological studies of plants, especially of their root system, are required to confirm this hypothesis.

Author Contributions

Conceptualization, O.V. and M.B.; methodology, S.G.O and M.A.H.; software, M.A.H; validation, M.P.L and M.P.L.G; formal analysis, O.V.; research, S.G.O, M.A.H, M.V., M.P.L., M.P.L.G and J.V.L.; resources, O.V. and M.B; data curation, M.V.; writing: original draft preparation, M.B.; writing: review and editing, O.V.; visualization, M.A.H.; supervision, O.V.; project administration, M.B.; funding acquisition, M.B.

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Publicación II:
Subcapítulo 4.2

Physiological and Morphological Characterisation of *Limonium* Species in their Natural Habitats: Insights into their Abiotic Stress Responses

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Physiological and Morphological Characterisation of *Limonium* Species in their Natural Habitats: Insights into their Abiotic Stress Responses

Abstract

Background and aims. Morphological and biochemical traits of four halophytes of the genus *Limonium* were analysed in plants sampled from salt marshes in SE Spain. This work aimed to explore the mechanism(s) behind the adaptation of these species to stressful habitats, with particular emphasis on responses to drought.
Methods. Plants of each species together with soil samples were collected in summer, which is the most stressful season in the Mediterranean. Soil parameters and plant morphological traits were determined, and the levels of several biochemical stress markers in plants were measured using spectrophotometric assays. A multivariate analysis was performed to correlate soil and plant data.
Results. Morphological characteristics regarding the underground system topology and several biochemical traits (higher foliar Ca^{2+}, sucrose and glucose, and lower proline, glycine-betaine and fructose) clearly separate *L. santapolense* individuals from plants of the other three species.

Conclusions. Drought tolerance of *L. santapolense* in the field is mostly dependent on morphological adaptations: when growing in an arid location, plants of this species develop long taproots that can extract water from the deep, moist layers of the soil.

Keywords: antioxidants, climate change, drought, endemics, osmolytes, salt marshes, soil analisis.

Abbreviations:
ETo: reference evapotranspiration
S: sand
Sl: silt
C: clay
OM: organic matter
ECsat: electric conductivity in the saturation extract
NS: number of apical shoots
NL: number of leaves
SFW: Shoot fresh weigh
RFW: Root fresh weight
LA: total leaf area
RL: total root length
LRL: lateral root length
PRL: taproot or principal root length
RSA: root surface area
D: average diameter of the roots
R/S: root to shoot ratio
SRL: specific root length

M: root magnitude
a: root altitude
Nd: numbers of root nodes
TI: topological index
Pro: proline
GB: glycine betaine
TSS: total soluble sugars
Fru: fructose
Suc: sucrose
Glu: glucose
MDA: malondialdehyde
DPPH: 2,2-diphenyl-1-picrylhydrazyl
TPC: total phenolic compounds
TF: total flavonoids
PCA: Principal component análisis

4.2.1 Introduction

Salt marshes, like many other coastal habitats, are considered as highly threatened ecosystems, intensively modified by anthropogenic actions (Barbier et al., 2011). In the Iberian Peninsula, as in many other regions of the world, these habitats have suffered numerous threats. Considered as insalubrious in the past, they were eliminated when located near human settlements; expansion of agriculture and touristic pressure also contributed to their reduction. In addition, effects of climate change represent another threat for salt marshes in the Mediterranean area. Not only rises of temperature and the risk of longer and more intense drought periods but also the sudden alteration of seasonal weather patterns may modify the existing conditions in these ecosystems (Thorne et al., 2012). The characteristic vegetation of the salt marshes is represented mainly by halophytic plants, which are tolerant to soil salinity in a greater or lesser degree. There is a wide range of halophytes, from plants present at the borders of salt marshes and adapted to only (relatively) low salinity levels, to plants that show optimal growth under moderate saline conditions and tolerate salt concentrations even higher than that of seawater. It is not possible to define a precise salinity threshold to separate halophytes from glycophytes, as plant species show a continuous range of sensitivity to salt stress (Grigore & Toma, 2017). Nevertheless, a generally accepted, operational definition is that halophytes are plants of natural saline environments, which are able to complete their life cycle at soil salinities equivalent to, at least, 200 mM NaCl (Flowers & Colmer, 2008). However, salinity is not the only limiting factor for plants in salt marshes, where they are simultaneously affected by other additional stressful conditions. For example, plants growing in such habitats under Mediterranean climatic conditions may switch from waterlogging, after heavy rains in spring, to extremely dry conditions in summer, when the soil surface is covered by a crust of salt due to intense evapotranspiration (Álvarez-Rogel et al., 2000). Responses to one or the other type of stress broadly vary among different genera of plants and often even between congener species.

A useful approach for unravelling the mechanisms underlying plant tolerance to salt – and other abiotic stresses – is to study the responses to stress of taxonomically (implicitly, also genetically) related taxa. A good candidate for this type of comparative studies is the genus *Limonium* L. of the Plumbaginaceae family. This genus includes more than 400 species, many of which are halophytes, and are well represented in the Mediterranean (Greuter et al., 1989) with numerous endemics in the area of study (Mateo & Crespo, 2014).

Four species of *Limonium* have been selected for our ongoing research on this genus: *L. santapolense* Erben, *L. girardianum* (Guss.) Fourr., *L. virgatum* (Willd.) Fourr. and *L. narbonense* Mill. The four species flower in summer, *L. santapolense* from May to July and the other three from July to September. Regarding their geographic distribution, *L. santapolense* is a local endemism, present only on littoral sandy substrates in a small area in the province of Alicante, whereas *L. girardianum* is endemic to S France, E Spain and Balearic Isles, growing on sandy coasts and cliffs. *L. virgatum* and *L. narbonense* have a

broader distribution throughout the Mediterranean region, the first on sandy beaches and rocky coasts, reaching the Middle East and North of Africa, and the second in salt marshes throughout the Mediterranean, in Spain also on the Atlantic coast (Erben, 1993). Besides the conservation value of the two endemic species, all four are important elements of the salt marsh ecosystems as their presence and frequency in plant communities increase the diversity and the degree of differentiation between the local habitats in the area of study. Populations of the four species growing in the wild in SE Spain have not been studied in depth so far, and their morphological and biochemical traits may reflect local adaptations. Thus, their analysis is important not only for obtaining a broader knowledge of the four species, but also to predict their possible future response to the challenge of climate change.

We have previously analysed the germination patterns of these species (Monllor et al., 2018), and their responses to salt stress (Al Hassan et al., 2017) and water stress (González-Orenga et al., 2019) under controlled greenhouse conditions. Tolerance to salinity was similar in the four species and was mainly based on the active transport and accumulation of ions in the leaves, with the concomitant synthesis of soluble sugars and proline as compatible solutes for osmotic adjustment (Al Hassan et al., 2017). On the contrary, water stress, induced by withholding irrigation of the plants, affected mostly *L. santapolense*, which appeared to be highly sensitive to dehydration – plants lost about one-third of their fresh weight after the water deficit treatment (González-Orenga et al., 2019). This finding was somewhat surprising since the natural habitat of this species is drier than those of the plants of the other three taxa analysed. Our working hypothesis to explain this apparent discrepancy is that *L. santapolense* plants possess some specific mechanisms that enable their survival in the field, under harsh natural conditions with very little water availability, but that cannot be mimic in the pot experiments in the artificial environment of the greenhouse. The analysis of plants sampled in the wild, in correlation with the climatic and edaphic conditions at the sampling sites, represents a useful complementary approach to study the stress response mechanisms of the four *Limonium* species, considering that the specific distribution of each taxon, within the same general habitat, may depend on local variations of soil characteristics.

With these ideas in mind, we undertook the present study on the mechanisms of stress tolerance in the selected *Limonium* species, with the following specific aims: (i) to analyse the climatic and soil conditions at the sampling sites of each species; (ii) to study the growth patterns of plants of the four species in the wild; (iii) to study stress response mechanisms based on the regulation of ion transport and osmolytes accumulation; and (iv) to determine the levels of oxidative stress affecting the plants, and the concentrations of representative non-enzymatic antioxidants.

4.2.2 Material and Methods

Sampling Sites and Material Sampling

Mature plants of *L. santapolense*, a rare endemic restricted to the province of Alicante (SE Spain), were collected from Clot de Galvany, a saltmarsh located near the city of Elche (39º 15′ N/0º 31′ W). Plants of the other three species were collected from 'La Albufera' Natural Park, near the city of Valencia (39° 20′ N/0º 19′ W). *Limonium* at the

sampling sites of the four species, four whole plants of each species and three soil samples were collected in July 2018.

Climatic Analysis

Climatic data were obtained from the nearest meteorological stations (Elche for 'Clot de Galvany' and Benifaió for El Saler), provided by the Agroclimatic Information System for Irrigation (SIAR), of the Spanish Ministry of Environment, Rural and Marine Affairs (MARM). The following bioclimatic indexes were calculated using available meteorological data of the last 16 years:
TI: Thermicity index, TI = 10 * (T + M + m)
CI: Continentality index, CI = T max – T min
OI: Ombrothermic index, OI = (P / 12) * 10 / ∑ Tm
Ppv: Summer precipitation in mm of the three consecutive warmest months in the year
Ttv: Value in tenths of degree resulting from the sum of the monthly average temperatures of the three consecutive warmest months in the year
ETo: Reference evapotranspiration, calculated according to Penman-Monteith equation (Allen et al., 1998)
GI: Giacobbe index, GI = ($P_{June} + P_{July} + P_{August}$) / Tª of the warmest month
where: T, yearly average temperature; m, average temperature of the minima of the coldest month of the year; M, average temperature of the maxima of the coldest month of the year; T max, average temperature of the warmest month; T min, average temperature of the coldest month; Tm, average temperature of each month; P, total yearly precipitation.
All indexes were calculated according to Rivas-Martínez & Rivas-Saenz (1996-2018), except for GI calculated according to Giacobbe (1938, 1959).
These specific indexes were chosen as they are the most suitable for local differentiations within the Mediterranean climate type (Ferriol et al., 2006).

Besides, meteorological data (mean, maximum and minimum temperatures, rainfall, air humidity and evapotranspiration) of the previous month to sampling were obtained from the same source.

Soil Analysis

Soil samples were taken at a depth of 0-15 cm. Once the samples had evenly lost moisture in open air at room temperature (approx. 25ºC), they were crushed with a roller to break aggregates and then passed through a 2-mm light sieve. Analyses were performed on fine soil (diameter < 2 mm). Soil texture was analysed by the hydrometer method (Bouyoucos, 1962), organic matter content (OM%) was determined as described by Walkey & Black (1934), and carbonates were measured with a Bernard Calcimeter.

The following parameters were determined in soil saturation extracts: pH, electric conductivity (EC), and concentrations of cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) and chlorides. A Crison pH-meter Basic 20 and a Crison Conductimeter Basic 30 were used to measure pH and EC, respectively. Sodium and potassium were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), chlorides were measured in a MKII Chloride Analyzer 92 6 (Sherwood, Inc., Cambridge, UK), and divalent cations of

calcium and magnesium were measured with an atomic absorption spectrometer SpectrA 220 (Varian, Inc., CA, USA). Cation exchange capacity (CEC) was determined following Rhoades (1982).

Plant Sampling in the Wild

Four plants were selected from distant areas of their natural location and then uprooted as in Fita et al. (2013), trying to recover intact roots systems. Roots were excavated digging a 30 cm depth-pit 50 cm away from de plant shoot without breaking any root that can go horizontally further than those 50 cm and then removing carefully the soil, as if it was in a pot. If roots grow deeper, the same procedure was repeated until reaching the end of the root. The number of shoots per plant (NS), number of leaves (NL), shoot fresh weight (SFW), and root fresh weight (RFW) were recorded. The roots were scanned (Epson LA 1600+, Epson America Inc. Long Beach, CA, USA,), and the pictures were analysed with the WinRhizo Pro software (WinRhizo Pro 2003b, Reagent Instruments Inc. Quebec Canada) to obtain the total root length (RL, cm), the lateral root length (LRL, cm), the primary root length (PRL, cm), the root surface area (RSA, cm^2) and the average diameter of the roots (D, mm).

To better assess the root architecture, the root topological parameters defined by Fitter (1987) were evaluated. Root magnitude (M) was evaluated as the number of external links of a root, root altitude (a), as the maximum external path length of the root, numbers of root nodes (Nd) were counted, and the topological index (TI) was calculated as the ratio of log altitude over log magnitude (Magalhães & Seifert, 2015). Other composite parameters were calculated, such as root to shoot ratio (R/S) as RFW/SFW, and the specific root length (SRL, cm/g) as RL/RFW.

A fraction of the plant material was stored at -20°C, and the remaining material was dried for several days in an oven at 65ºC until constant weight. Water content percentage in roots and leaves was calculated according to Gil et al. (2014).

Ion Concentration Measurements

Ion concentrations were determined in dry roots and leaves, after being eluted in aqueous extracts according to Weimberg (1987), by heating the samples (0.05 g of dried, ground plant material in 15 mL of water) for 15 min at 95°C, followed by filtration through a 0.45 μm filter (Gelman Laboratory, PALL Corporation).

Ion (Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}) concentrations in plant extracts were measured using the same instruments as for their determination in soil samples.

Osmolyte Quantification

Proline (Pro) was extracted with 2 mL of 3 % (w/v) sulfosalicylic acid, from 0.05 g of dry leaf material, and was quantified in toluene according to the acid-ninhydrin method of Bates et al. (1973). The extract, mixed with acid ninhydrin, was heated at 95ºC for one h, cooled on ice and extracted with toluene. The absorbance of the organic phase was

measured at 520 nm, using toluene as a blank. Pro concentrations were expressed as μmol g^{-1} DW.

Glycine betaine (GB) was extracted from 0.05 g dry leaf material with 1 mL water, according to Grieve & Grattan (1983) with the modifications proposed by Nawaz & Ashraf (2010). The extract was supplemented with potassium iodide, kept on ice for 90 min and then extracted with 1, 2-dichloroethane (pre-cooled at -20ºC); finally, the absorbance of the sample was measured at 365 nm. GB concentration was expressed as μmol g^{-1} DW.

Total soluble sugars (TSS), were measured in 0.05 g dry plant material extracted with 2 mL of 80% (v/v) methanol, following the method described by Dubois et al. (1956). The sample was mixed on a rocker shaker for 24 h; the extract was then centrifuged, concentrated sulfuric acid and 5% phenol was added to the supernatant, and the absorbance was measured at 490 nm. TSS concentrations were expressed as 'mg equivalent of glucose' (used as the standard) per g DW.

HPLC Analysis of Soluble Carbohydrates

Plant dry material (0.05 g) was boiled in 2 mL Milli-Q water for 10 minutes and then filtered through 0.22 μm nylon filters. The soluble sugar fraction was analysed using a Waters 1525 high performance liquid chromatography (HPLC) coupled to a 2424 evaporative light scattering detector (ELSD), according to Al Hassan et al. (2016). 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/cm2. The 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% acetonitrile (J.T. Baker) was applied for 25 min in each run. Standards of glucose, fructose, and sucrose were employed to identify peaks by co-injection. Sugars were quantified with peak integration using the Waters Empower software and comparison with glucose, fructose, and sucrose standard calibration curves.

Malondialdehyde and Total Antioxidant Activity

Malondialdehyde (MDA, a reliable oxidative stress marker) concentrations were determined in the same 80% methanol leaf extracts used to quantify TSS, according to the method of Hodges et al. (1999). The extracts were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA (or with 20% TCA without TBA for the controls), and then incubated at 95°C for 15 min. After the reaction was stopped by placing the tubes on ice for a few minutes, absorbance was measured at 600 and 532 nm, and the concentration of MDA was determined using the equation described by Hodges et al. (1999).

The antioxidant activity was evaluated according to Falchi et al. (2006), by measuring the ability of the samples to quench the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), a synthetic and stable free radical product, whose quenching by a scavenger substrate could be followed spectrophotometrically at 517 nm. Leaf dry material (0.05 g) was

extracted using 2 mL of 90% methanol by sonication during 10 min. The sample was centrifuged at 14000 rpm for 15 min, and the supernatant was collected. Then, 50 μL of this methanol-soluble phenolic fraction was diluted with 2 mL of 96% ethanol, 0.5 mL of the resulting solution was added to 1.5 mL 96% ethanol and 0.5 mL of an ethanolic solution containing 0.5 mM DPPH. To check the radical stability, a blank sample was prepared without the plant extract. Mixtures were then incubated at 25°C for 10 min, and the absorbance was measured at 517 nm.

The radical scavenging activity (S) of each extract was expressed in percentage and calculated as $S = 100 - [(A_x/A_0) \times 100]$; A_x is the optical density of the DPPH solution in the presence of the extract, and A_0 in its absence.

Non-Enzymatic Antioxidants

Total phenolic compounds (TPC) and total flavonoid (TF) concentrations were determined in 80% methanol extracts, as for TSS. TPC were measured by its reaction with the Folin-Ciocalteu reagent and sodium bicarbonate, according to Blainski et al. (2013). Absorbance measurements were taken at 765 nm, and TPC concentrations were expressed as equivalents of gallic acid (mg eq. GA g^{-1} DW). TF was determined according to Zhishen et al. (1999); the extracts were mixed with sodium nitrite, and then aluminium chloride was added under alkaline conditions before absorbance was measured at 510 nm. TF concentrations were expressed as equivalents of catechin (mg eq. C. g^{-1} DW).

Statistical Analysis

Statistical analyses were performed 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 the Levene test for the homogeneity of variance. The significance of the effects of stress was evaluated by one way ANOVA. Tukey's HSD test was applied to identify the homogeneous groups when significant differences were found between the studied species. Correlations between soil and biochemical plant parameters were performed by the Pearson product-moment coefficient. Parameters that showed significant correlations were used for a principal component analysis (PCA). A cluster analysis was applied to discriminate the four species based on their growth and biochemical responses to water stress, using Squared Euclidean distances for the proximity procedure. All means throughout the text include the standard error (SE). A dendrogram based on the nearest neighbour method, using squared Euclidian distances between biochemical parameters and ion concentrations, was also performed with Stagraphics Centurion XVI.

4.2.3 Results

Climatic Analysis

Individuals of three of the four *Limonium* species under study were collected from salt marshes in El Saler, near the city of Valencia, and those of the fourth, *L. santapolense*, from a more southern location (Clot de Galvany) near Elche, in the province of Alicante. Both areas have a similar climate with the highest temperatures in summer, coinciding

with a drastic reduction of rainfall, which is characteristic of the Mediterranean climate. However, the amount of precipitation differs in the two areas; the average annual rainfall is much higher in El Saler than in the Clot area. The mean rainfall calculated for the last 18 years is 240.02 mm in Clot and 441.66 mm in El Saler, although ETo is similar in the two zones. The two areas are located near the beach, and therefore have similar Continentality index, and both are classified within the Thermomediterranean thermotype, characterised by warm temperatures (a yearly mean of 16-18°C) and mild winters, and have very similar thermicity index (TI) (Table 1). Based on their ombrothermic index calculated according to Rivas-Martínez & Rivas-Saenz (1996-2019), Clot is classified as arid, and El Saler as semi-arid.

Table 1. Values of the climate variables in the collection sites of *Limonium santapolense* (Clot) and *L. giradianum*, *L. narbonense* and *L. virgatum* (El Saler).

Bioclimatic Indexes	Clot	El Saler
TI	406.1	389.3
CI	15.2	15.1
OI	0.9	1.6
Ppv	18.8	38.2
Ttv	74.1	73.9
ETo	97.1	98.9
GI	0.6	1.4

Data were obtained from the nearest meteorological stations (Elche for 'Clot de Galvany' and Benifaió for El Saler) and calculated for the period 1999–2018)

TI, Thermicity index; *CI*, Continentality index; *OI*, Ombrothermic index; *Ppv*, Summer precipitation in mm of the three consecutive warmest months in the year; *Tt*, Value in tenths of degree resulting from the sum of the monthly average temperatures of the three consecutive warmest months in the year; *ETo*, Evapotranspiration; *IG*, Giacobbe index.

Meteorological data for the four weeks previous to sampling (from 15th of June to 15th of July, 2018) in the two areas, summarised in Table 2, indicate that they differ mainly in the amount of rainfall in the last two weeks of June, which were extremely dry in Clot, but more than 100 mm were registered in El Saler. As the evapotranspiration was similar in both locations, the water deficit was obviously much more intense at Clot, the sampling area of *L. santapolense*. During the first two weeks of July, the meteorological conditions were similar in the two areas, with a pronounced water deficit, as it is characteristic for the Mediterranean climate in summer.

Table 2. Meteorological data in the period previous to the sampling (from 15th of June to 15th of July, 2018) in the collection sites of *Limonium santapolense* (Clot) and *L. girardianum*, *L. narbonense* and *L. virgatum* (El Saler).

Meteorological Data	Clot		El Saler	
	June	July	June	July
Mean T (ºC)	23.6	26.8	22.7	26.1
T max (ºC)	32.4	37.4	32.6	37.1
T min (ºC)	12.4	19.6	12.7	27.8
Mean H (%)	61.9	64.8	65.4	69.7
H max (%)	96.4	95.2	95.2	97.8
H min (%)	23.5	17.2	25.0	17.6
Pp (mm)	9.6	0.0	136.9	5.8
Eto (mm)	163.1	187.8	163.0	184.6

T: temperature, *H*: atmospheric humidity; *Pp*: Precipitation; *Eto*: Evapotranspiration. Data were obtained from the nearest meteorological stations (Elche for 'Clot de Galvany' and Benifaió for El Saler).

Soil Analysis

The textural classes of the soils were determined according to their corresponding percentages of sand, silt and clay, based on the USDA classification (Soil Survey Division Staff, 1993). As shown in Table3, all soils in El Saler area contain a high percentage of sand, between 88% and 94%, belonging therefore to the 'sandy' textural class. The texture is sandy loam at the collection site of *L. santapolense* (Clot de Galvany), as the percentage of sand in the soil is lower (55%), and the percentage of silt is higher (35%), whereas differences in % of clay are small in respect to the other areas.

All analysed soils had a basic pH, with values ranging from 8.25 to 9.05, being slightly higher in the growth area of *L. girardianum* and lower for that of *L. santapolense*. Organic matter content commonly ranges between ≤ 0.5 and 2.0%, for surface soils from arid regions (Bresler et al., 1982). Very low and similar values have been determined in the soils of Clot and El Saler, except in the growing area of *L. narbonense*, where the soil is not so poor in organic matter, although it is still below 2% (Table 3).

Statistically significant differences have been found in the carbonate content of the soils from Clot, where *L. santapolense* grows, and those of El Saler, the area where the remaining species were sampled. According to the scale of Yáñez (1989), the soils of El Saler area should be classified as soils with a high carbonate content, whereas that of Clot as soil with a very high carbonate content.

The results obtained for the cation exchange capacity (CEC) indicate similar values for *L. santapolense*, *L. girardianum* and *L. virgatum*, but a higher mean value for soil samples from the area of *L. narbonense*.

Sodium and chloride concentrations in the saturation extract of all tested soils were much higher than those of the other ions measured, K^+, Ca^{2+} and Mg^{2+}. Significantly higher levels of sodium were found in the sampling areas of *L. virgatum* and *L.*

narbonense, as compared to that of *L. santapolense*, with intermediate values measured for the *L. girardianum* location, whereas chlorides contents were similar in all soil samples. Magnesium was the next chemical in concentration, with higher values in the areas of the species sampled from El Saler, significantly higher than in Clot, especially in the collection zone of *L. narbonense*. Higher values in El Saler than in Clot were also registered for potassium. Finally, calcium values were significantly lower in the area of *L. girardianum* than in the sampling areas of the other three species.

Table 3. Soil variables at the collection sites of the four analysed *Limonium* species. Values shown are means ± SD (n = 3); different lower-case letters in each row indicate statistically significant differences between the different locations, according to Tukey test ($\alpha = 0.05$).

Soil variable	*L. santapolense* Clot de Galvany	*L. virgatum* El Saler	*L. girardianum* El Saler	*L. narbonense* El Saler
Texture	Sandy loam (55 S, 35 Sl, 10 C)	Sandy (94 S, 2 Sl, 4C)	Loamy-sandy (88 S, 6 Sl, 8 C)	Sandy (92 S, 2 Sl, 6 C)
pH	8.2 ± 0.0 a	8.7 ± 0.1 b	9.1 ± 0.0 c	8.5 ± 0.1 d
OM (%)	0.5 ± 0.0 a	0.4 ± 0.0 a	0.3 ± 0.0 a	1.7 ± 0.2 b
$CaCO_3$ (%)	42.3 ± 3.6 b	26.3 ± 0.3 a	24.3 ± 0.8 a	22.0 ± 0.9 a
EC_{sat} (dS/m)	31.4± 2.7 a	53.1 ± 1.0 b	33.9 ± 4.4 a	50.3 ± 9.3 b
CEC (cmol+/kg)	2.9 ± 0.9 b	1.4 ± 0.06 ab	0.9 ± 0.2 a	8.8 ± 0.7c
Na^+_{sat} (meq/L)t	183.7± 17.0 a	321.2 ± 1.0 b	239.1 ± 33.3 ab	291.5 ± 54.1 b
Cl^-_{sat} (meq/L)	352.6 ± 39.6 a	339.9 ± 22.8 a	327.2 ± 45.3 a	361.7 ± 116.8 a
K^+_{sat} (meq/L)	3.6 ± 0.3 a	10.0 ± 1.2 b	10.2 ± 1.8 b	10.4 ± 2.4 b
Ca^{2+}_{sat} (meq/L)	17.1 ± 2.4 b	14.9 ± 4.3 b	6.0 ± 0.8 a	15.7 ± 1.7 b
Mg^{2+}_{sat} (meq/L)	16.6 ± 1.5 a	23.6 ±0.1 b	30.7 ± 0.3 c	37.9 ± 4.3 d

S, % sand; *Sl*, % silt; *C*, % clay; *OM*, organic matter; *ECsat*: electric conductivity in saturation extract.

Morphology of Plants Growing in the Wild

Limonium virgatum and *L. girardianum* were small (less than 10 g per plant), with small leaves and thin shallow underground structures. Plants of these two species were similar, except for the higher number of shoots and leaves and longer roots of *L. virgatum*, and the higher specific length of *L. girardianum*. According to the topological measurements, both had a herringbone development in which the primary root predominates among others and penetrates deeply in the soil without extensive branching (Magalhães & Seifert, 2015), (Table 4). *L. santapolense* and *L. narbonense* had both an average biomass above 75 g but showed very different morphology (Table 4, Fig. 1). *L. santapolense* had an average of five shoots per plant and showed a larger leaf

area in comparison with the other three selected species. It also showed a long underground system (averaging 187 cm) that deeply penetrate in the soil, reaching the proximity of the soil water table, as we observed in the field.

Table 4. Means and standard deviations of shoot and root traits evaluated from wild plants of the four *Limonium* species (n = 4).

Traits	*L. santapolense*	*L. virgatum*	*L. girardianum*	*L. narbonense*
NS	5.0 ± 0.7 b	11.5 ±1.8 c	1.0 ± 0.0 a	2.8 ± 0.7 ab
NL	43.8 ± 7.5 bc	63.3 ± 8.3 c	23.8 ± 3.7 ab	15.2 ± 8.6 a
SFW (g)	70.0 ± 21.4 a	6.6 ± 0.8 a	2.0 ± 0.30 a	47.3 ± 22.4 a
RFW (g)	5.2 ± 0.7 a	1.0 ± 0.2 a	0.2 ± 0.1 a	50.2 ± 18.1 b
LA (cm^2)	552.3 ± 150.3 b	33.6 ± 5.6 a	23.9 ± 4.7 a	98.2 ±47.4 a
RL (cm)	187.4 ± 49.2 a	120.4 ± 43.2 a	57.4 ± 14.6 a	83.7 ±28.4 a
LRL (cm)	152.1 ± 55.7 a	113.7 ± 42.7 a	54.7 ± 13.7 a	45.0 ± 21.9 a
PRL (cm)	35.3 ±10.6 b	6.5 ± 1.1 a	2.6 ± 1.2 a	38.7 ± 10.5 b
RSA (cm^2)	85.0 ±14.3 ab	35.1 ± 10.9 a	15.6 ± 4.1 a	162.5 ± 49.1 b
D (mm)	1.6 ± 0.3 a	1.0 ± 0.0 a	0.9 ± 0.1 a	6.9 ± 1.2 b
R/S	0.1 ±0.0 a	0.1 ±0.0 a	0.1 ± 0.0 a	2.2 ± 1.4 a
SRL (cm/g)	37.9 ± 9.6 a	113.5 ± 19.5 ab	273.9 ± 68.3 b	2.0 ± 0.5 a
M	27.7 ± 11.6 ab	56.7 ± 8.7 c	40.7 ± 10.4 bc	5.5 ± 1.2 a
A	9.7 ± 2.9 ab	22.2 ± 4.8 c	20.7 ± 5.7 bc	5.2 ± 0.6 a
Nd	21.0 ± 8.1 ab	53.0 ± 9.8 c	34.7 ± 9.5 bc	4.5 ± 0.9 a
TI	0.7 ± 0.1 a	0.8 ± 0.0 a	0.8 ± 0.0 a	1.0 ± 0.1 b

NS, number of apical shoots; *NL*, number of leaves, *SFW*, shoot fresh weight (g); *RFW*, root fresh weight (g); *LA*, total leaf area (cm^2); *RL*, total root length (cm); *LRL*, lateral root length (cm); *PRL*, taproot or principal root length (cm); *RSA*, root surface area (cm^2); *D*, average diameter of the roots (mm); *R/S*, root to shoot ratio; *SRL*, specific root length (cm/g); *M*, root magnitude; a, root altitude; *Nd*, numbers of root nodes; *TI*, topological index. Numbers of the same row followed by different letter differ significantly in the ANOVA test at $P < 0.05$

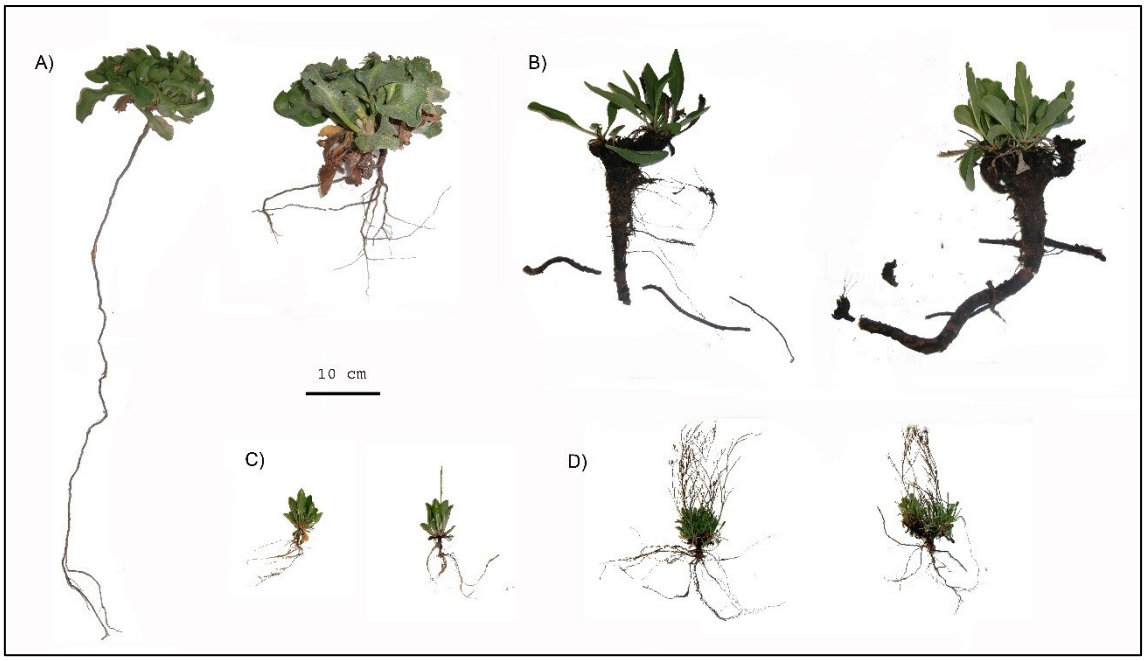

Figure 1. Examples of the four *Limonium* species sampled in the wild: *L. santapolense* (a), *L. narbonense* (b), *L. girardianum* (c) and *L. virgatum* (d).

However, it must be noticed that differences were found between *L. santapolense* plants growing close to the water level and those growing far from it. The first ones had a short taproot with nodes evenly distributed over it (Fig. 1a, right), whereas the plants growing distantly from the water develop a very long taproot (more than 1 m), which only ramifies when the root reaches deep moist areas of the soil (Fig. 1a, left). Regarding the topological indexes, it was a moderate herringbone root even though, as we have pointed out above, it can grow very deep as a single root (Fig. 1a). In terms of underground systems, *L. narbonense* was very different from the rest due to its rhizomatous root system. It has a rhizomatous structure of 2-3 cm in diameter, which penetrates the soil up to 40 cm and then starts to branch. This structure was covered by fine roots of 1-2 mm in diameter and up to 10 cm in length. The topological index of *L. narbonense* corroborates its herringbone structure (Fig. 1b).

Ion Accumulation

Levels of ions and their interspecific variation were generally higher in leaves than in roots, and these differences were, in most cases, statistically significant (Fig. 2). Mean Na^+ concentrations were higher in *L. girardianum* and *L. narbonense* than in *L. santapolense* and *L. virgatum*, ranging in leaves from 631 μmol g^{-1} DW in *L. santapolense* to 1993 μmol g^{-1} DW in *L. narbonense*, but the differences were significant only in roots, due to the wide individual variation within each species in the leaves (Fig. 2a). Cl^- concentrations in roots were significantly lower than in leaves in all four species and did not vary between species, whereas in leaves significantly higher Cl^- levels of 467 μmol g^{-1} DW were found in *L. narbonense*, as compared to around 200 μmol g^{-1} DW in leaves of the other three taxa (Fig. 2b). Also, root K^+ concentrations did not differ in the four species, whereas K^+ concentrations in leaves were significantly higher in *L. santapolense* and *L. virgatum* than in *L. girardianum*, whereas *L. narbonense* showed intermediate values (Fig. 2c). The most considerable differences between root and leaf Ca^{2+} concentrations were observed in *L.*

santapolense, which showed very low levels of this cation in the roots but stood out by its high levels of foliar Ca^{2+} concentrations, up to 319 μmol g^{-1} DW, much higher than those measured in the other three species (Fig. 2d).

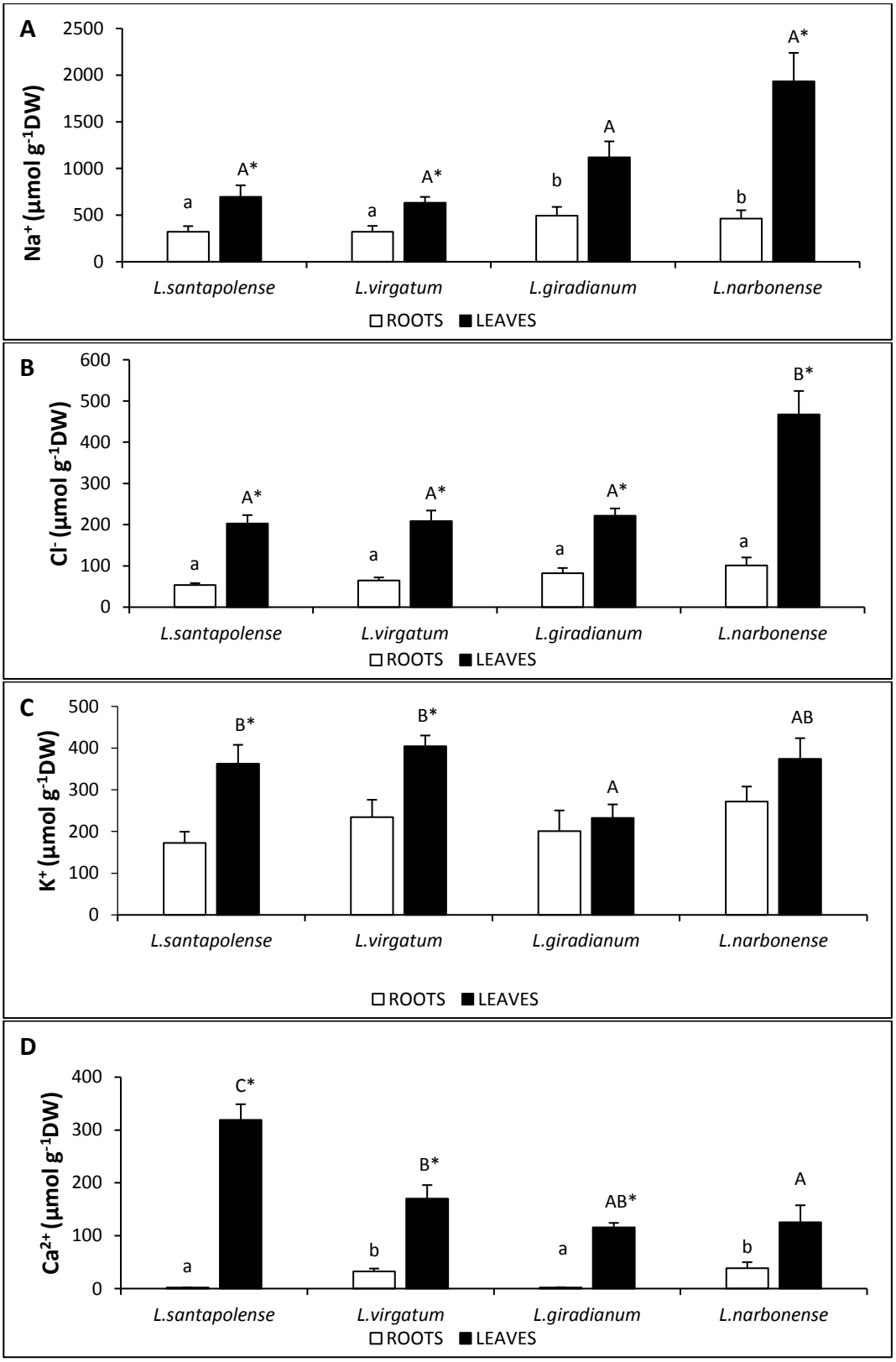

Figure 2. Ions levels in roots and leaves of the four *Limonium* species sampled in the field. Na^+ (a); Cl^- (b); K^+ (c); Ca^{2+} (d). Bars represent means ± SD (n = 4). Asterisks indicate

significant differences between roots and leaves for each species, and letters significant differences between species (lower-case letters for roots and capital letters for leaves) at $P < 0.05$.

Osmolytes

The most common osmolytes in plants, proline (Pro), glycine betaine (GB) and total soluble sugars (TSS) were quantified in plant leaves of the four *Limonium* species (Table 5). Levels of Pro in *L. santapolense* and *L. virgatum* were lower – below 20 μmol g^{-1} DW – than those measured in *L. girardianum* and *L. narbonense* – ca. 50 and 75 μmol g^{-1} DW, respectively. Regarding leaf GB concentrations, *L. santapolense* showed the lowest value, about 14 μmol g^{-1} DW, whereas similar concentrations, ca. 40 μmol g^{-1} DW, were measured in the other three species. TSS ranged from the lowest value (around 40 mg eq. glucose g^{-1} DW) in *L. virgatum* to the highest (~ 70 mg eq. glucose g^{-1} DW) in *L. santapolense* (Table 5).

In addition to the spectrophotometric determination of TSS, individual sugars in the leaf water-soluble fraction were separated, identified and quantified by HPLC. Three peaks were detected in the chromatograms, corresponding to fructose (Fru), sucrose (Suc), and glucose (Glu). Fru concentrations were very low in *L. santapolense* (25.5 μmol g^{-1} DW) in comparison with the other taxa (656 μmol g^{-1} DW in *L. girandianum*, for example), whereas the reverse pattern was observed in the case of Suc, which showed very low levels in all species (bellow 1 in *L. girardianum* and *L. narbonense*) except in *L. santapolense* (75.5 μmol g^{-1} DW). Finally, Glu levels were also low in all species, somewhat higher in *L. santapolense* (13.5 μmol g^{-1} DW) and not detectable in *L. girardianum* (Table 5).

Oxidative Stress and Antioxidant Compounds

Malondialdehyde (MDA) is a product of peroxidation of unsaturated fatty acids, used as a reliable marker of free radical damage to cell membranes in plants and animals (del Rio, 2005; Suzuki and Mittler 2006). The DPPH-free radical scavenging assay is a useful method for quantifying the ability of compounds in an extract to act as free radical scavengers or hydrogen donors, indicating the overall antioxidant capacity of the sample (Sagar et al., 2011). Both biochemical markers were determined in plants of the four *Limonium* taxa. No significant differences were found between *L. santapolense*, *L. girardianum* and *L. narbonense,* and somewhat lower values were measured in *L. virgatum* (Table 5), indicating that the degree of oxidative stress affecting the plants in the field and the total antioxidant activity of the leaf extracts were roughly the same in all cases.

In response to oxidative stress, plants activate enzymatic and non-enzymatic antioxidant mechanisms. Synthesis of phenolic compounds, including flavonoids, many of them possessing strong antioxidant activities, is one of the most frequent and most efficient strategies used by plants to reduce oxidative stress. Total phenolic compounds (TPC) were measured in leaves of plants of the four *Limonium* species and ranged from 23.9 mg eq. GA g^{-1} DW in *L. virgatum* to 39.2 in *L. girardianum* with no significant interspecific differences observed. Flavonoid concentrations were significantly lower in *L. girardianum* (4.0 mg eq. C g^{-1} DW) and *L. narbonense* (2.9) than in *L. santapolense* (5.9) and

L. virgatum (6.9) but these differences are probably irrelevant in terms of antioxidant capacity since absolute TF values were very low in all cases (Table 5).

Table 5. Biochemical parameters quantified in the leaves of plants sampled in the wild of four *Limonium* species. Mean followed by SE, $n = 4$.

Biochemical Parameters	*L. santapolense*	*L. virgatum*	*L. girardianum*	*L. narbonense*
Pro (μmol g^{-1} DW)	19.3 ± 1.8 a	14.7 ± 0.4 a	47.7 ± 2.2 b	75.6 ± 25.5 c
GB (μmol g^{-1} DW)	14.2 ± 1.3 a	42.6 ± 6.8 b	40.3 ± 3.2 b	42.0 ± 3.2 b
TSS (mg eq. G g^{-1} DW)	70.2 ± 5.2 c	38.7 ± 2.7 a	67.0 ± 0.7 c	54.7 ± 4.8 b
Fru (μmol g^{-1} DW)	27.6 ± 2.1 a	632.0 ± 22.1 c	656.5 ± 244.2 d	291.0 ± 91.6 b
Suc (μmol g^{-1} DW)	73.5 ± 14.6 b	9.0 ± 1.4 a	0.7 ± 0.1 a	0.3 ± 0.0 a
Glu (μmol g^{-1} DW)	13.6 ± 0.2 c	8.2 ± 2.2 b	0.0 a	6.5 ± 0.4 b
MDA (nmol g^{-1} DW)	171.4 ± 5.3 b	97.4 ± 8.7 a	210.3 ± 25.4 b	191.6 ± 35.5 b
DPPH (%)	84.2 ± 0.3 b	67.6 ± 7.3 a	82.8 ± 1.1 b	74.5 ± 4.0 ab
TPC (mg eq. GA g^{-1} DW)	37.1 ± 3.7 a	23.9 ± 6.2 a	39.2 ± 4.8 a	24.9 ± 5.2 a
TF (mg eq. C g^{-1} DW)	5.9 ± 0.3 b	6.9 ± 0.5 b	4.0 ± 0.4 a	2.9 ± 0.6 a

Pro, proline; *GB*, glycine betaine; *TSS*, total soluble sugars; *Fru*, fructose; *Suc*, sucrose; *Glu*, glucose; *MDA*, malondialdehyde; *DPPH*, 2,2-diphenyl-1-picrylhydrazyl; *TPC*, total phenolic compounds; *TF*, total flavonoids

PCA and Cluster Analysis

A Pearson Moment Correlation was performed with all analysed parameters and those plant and soil variables that showed a significant correlation were further subjected to a Principal Component Analysis. (PCA) (Table 6 and Fig.3). Climatic data could not be included since three of the species analysed, *L. virgatum*, *L. girardianum* and *L. narbonense* were sampled from the same area, so plants grew in the same climatic conditions. The PCA extracted four components with an eigenvalue higher than one out of the total 25 parameters considered. Together, the four components account for 88.6% of the total variability. The first component, explaining 45.2% of the variability, was mostly related to soil parameters (positively with EC, K^+, Mg^{2+} and Na^+ levels in the soil, and negatively with the percentage of $CaCO_3$), and also correlated with osmolytes, positively with glycine betaine (GB) and negatively with sucrose (Suc). The second component, which explained an additional 23.8% of the total variability, was positively related to the levels of Ca in soil and with glucose (Glu), and negatively related to the concentrations of the oxidative stress marker (MDA), total antioxidant activity (DPPH), total phenolics (TPC) and total soluble sugars (TSS) in the plants (Table 6).

Table 6. Weights of the main four principal components extracted by the PCA in the four *Limonium* species.

Component	Component 1	Component 2	Component 3	Component 4
Eigenvalue	11.30	5.96	3.75	1.13
Variance (%)	45.20	23.86	15.00	4.53
pH	0.16	-0.29	-0.18	-0.09
EC	0.23	0.24	-0.00	0.11
$CaCO_3$	-0.28	0.07	-0.06	0.14
OM	0.09	0.17	0.38	-0.17

Na^+s	0.27	0.14	-0.06	0.084
K^+s	0.28	-0.07	0.01	-0.11
$Mg^{2+}s$	0.25	0.16	0.12	-0.02
$Ca^{2+}s$	-0.08	0.37	0.10	0.18
Cl^-s	0.19	0.27	-0.08	0.19
Na^+l	0.08	0.05	0.45	0.08
Cl^-l	0.13	0.13	0.29	-0.07
$Ca^{2+}l$	-0.19	0.07	-0.14	0.33
$Ca^{2+}r$	0.21	0.25	-0.01	0.18
MDA	-0.03	-0.24	0.36	0.11
GB	0.25	-0.08	0.01	0.16
TSS	-0.18	-0.21	0.22	-0.05
TPC	-0.11	-0.26	0.12	0.44
Pro	0.10	-0.10	0.41	0.05
DPPH	-0.14	-0.22	0.192	0.22
Fru	0.20	-0.23	-0.16	-0.07
Glu	-0.18	0.30	-0.00	0.17
Suc	-0.27	0.09	-0.02	0.20
LA	-0.24	0.12	0.062	-0.29
RL	-0.19	0.14	0.01	-0.29
SFW	-0.21	0.15	0.12	-0.35

EC, soil electric conductivity; *$CaCO_3$*, calcium carbonate in soil; *OM*, soil organic matter; *Na^+s*, sodium in soil; *K^+s* potassium in soil; *$Mg^{2+}s$*, magnesium in soil; *$Ca^{2+}s$*, calcium in soil; *Cl^-s* chloride in soil; *Na^+l*, sodium in leaves; *Cl^-l*, chloride in leaves; *$Ca^{2+}l$*, calcium in leaves; *$Ca^{2+}r$*, calcium in roots; *MDA*, malondialdehyde; *GB* glycine betaine; *TSS*, total soluble sugars; *TPC*, total phenolic compounds; *Pro*, proline; *DPPH*, 2,2-diphenyl-1-picrylhydrazyl; *Fru*, fructose; *Glu*, glucose; *Suc*, sucrose; *LA*, total leaf area; *RL*, total root length; *SFW*, shoot fresh weight.

The loading plot indicates that the concentrations of Na^+ and Cl^- in the soil correlated positively with those in the leaves of plants, and with the osmolytes Pro, GB and Fru. Growth parameters [fresh weight of shoots (SFW), leaf area (LA) and length of the roots (RL)] are grouped together (Fig.3). The projection of the four individuals of the four species in the PCA score plot shows a clear separation along the first component of *L. santapolense*, due to the particularity of its habitat with higher soil level of $CaCO_3$ (Fig.3); this taxon is also separated from the other species based on its high levels of foliar sucrose (Suc) and low values of fructose (Fru). *L. narbonense* and *L. virgatum* were situated on the opposite side, as they grow in soils with higher concentrations of Na^+, K^+ and Mg^{2+}, and therefore higher EC. *Limonium girardianum* was separated along the second component due to its higher values of MDA.

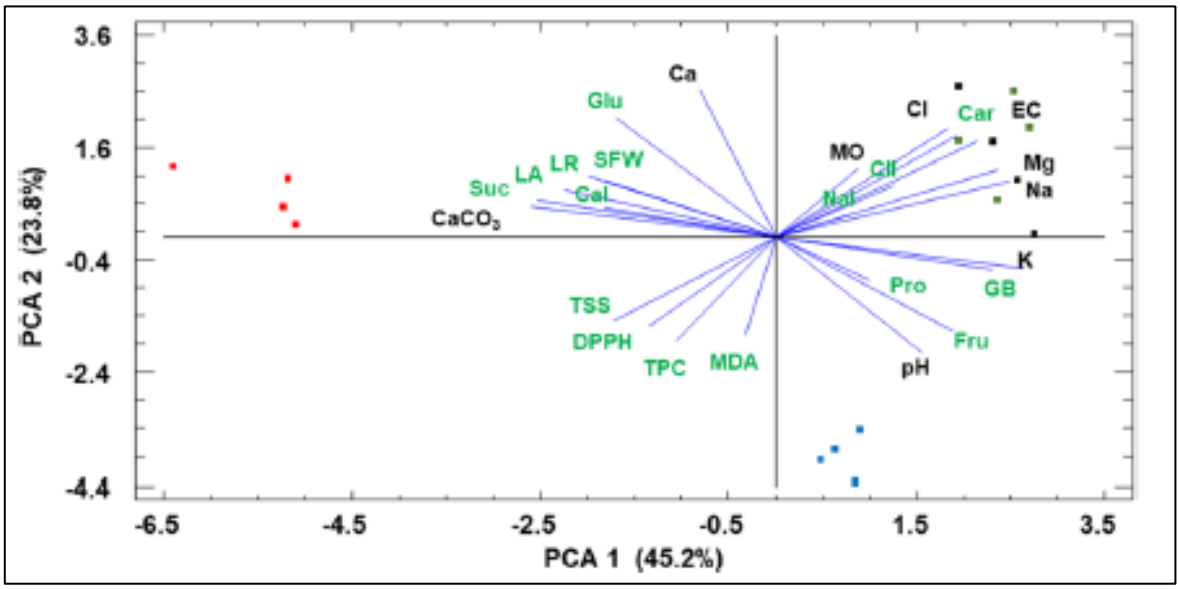

Figure 3. Diagram showing the relationships among the plants' traits (3 morphological, 13 biochemical) with nine soil parameters measured in their collection sites and among the 16 individuals from the four species of *Limonium*: *L. santapolense* (red), *L. narbonense* (black), *L. girardianum* (blue) and *L. virgatum* (green) based on the two first principal components of a principal components analysis (PCA). Soil parameters in black and plant parameters in green. Abbreviations: *EC*, soil electric conductivity; *CaCO3*, calcium carbonate in soil; *OM*, soil organic matter; *Na+s*, sodium in soil; *K+s* potassium in soil; *Mg2+s*, magnesium in soil; *Ca2+s*, calcium in soil; *Cl-s* chloride in soil; *Na+l*, sodium in leaves; *Cl-l*, chloride in leaves; *Ca2+l*, calcium in leaves; *Ca2+r*, calcium in roots; *MDA*, malondialdehyde; *GB*, glycine betaine; *TSS*, total soluble sugars; *TPC*, total phenolic compounds; *Pro*, proline; *DPPH*, 2,2-diphenyl-1-picrylhydrazyl; *Fru*, fructose; *Glu*, glucose; *Suc*, sucrose; *LA*, total leaf area; *RL*, total root length; *SFW*, shoot fresh weight

A cluster analysis based on the nearest neighbour method, including only morphological traits, biochemical parameters and ion concentrations of the plants, also separated the four species and supported the results of the PCA (Fig. 4). The more distant species was again *L. santapolense*, and the most related, based on the analysed characteristics, were *L. narbonense* and *L. girardianum.*

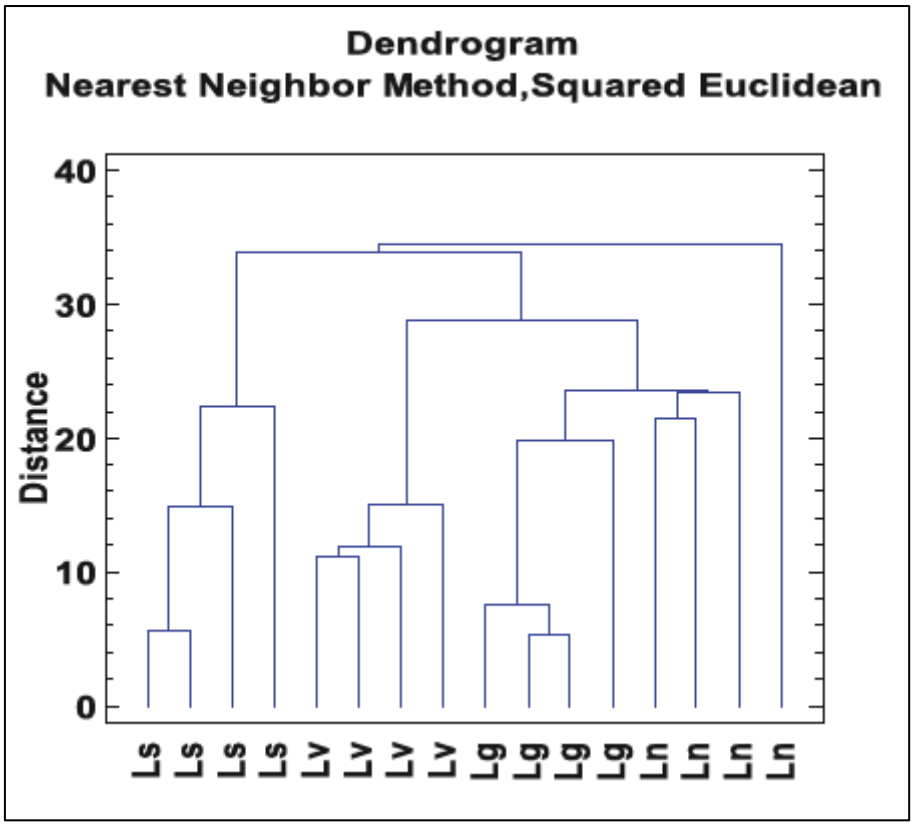

Figure 4. Cluster analysis based on the morphological and biochemical traits measured in plants of *L. santapolense* (Ls), *L. narbonense* (Ln), *L. girardianum* (Lg) and *L. virgatum* (Lv).

4.2.4 Discussion

In previous studies performed in our laboratory, it was found that once the bottleneck of germination was overcome, the four studied *Limonium* species tolerated high salinity levels, up to 800 mM NaCl in the irrigation solution (Al Hassan et al., 2017), but they differed in their responses to water deficit. Plants of three taxa, *L. virgatum*, *L. girardianum* and *L. narbonense*, grown in pots did not show a drastic reduction of growth after one month of lack of irrigation, whereas those of *L. santapolense* lost one-third of its fresh weight (González-Orenga et al., 2019).

Climate analysis of the sampling zones of *L. santapolense* at Clot de Galvany and the other three species at El Saler revealed differences between the two areas in the amount of rainfall, which lead to different climate types: arid for the first and semi-arid for the second. Although dry summers represent a characteristic trait of the Mediterranean climate, during the month previous to sampling the water deficit was much more pronounced in the area of *L. santapolense* at Clot than in El Saler, where in the last two weeks of June the rainfall was over 130 mm. Soil analyses did not reveal big differences between the sampling sites, except the texture of the soil, which was sandy-loam for the *L. santapolense*'s collection area and sandy in the other three sites. This difference is related to the geology of the two zones. The Clot area belongs to the undifferentiated quaternary and combines a series of more recent formations such as colluvia, alluvium, brackish deposits and debris in general (IGME, 1973) that increased the levels of

carbonates, whereas the salt marshes in El Saler area, also of quaternary origin, were formed behind the dunar belt, with deposits of grey sandy silts (IGME, 1974). This difference in texture can be significant after rain periods, as the soil in Clot has a slightly better ability to retain moisture than the sandy soils in El Saler. Although the substrate is sandy in El Saler, with very low water-holding capacity, this area is located in the immediate proximity of the sea, which makes the climate more humid and intensifies the cryptoprecipitation. Therefore, despite the differences in the texture of the soil, Clot de Galvany represents a drier habitat than El Saler. In fact, soil humidity registered by a WET 2 sensor simultaneously with plant' uprooting did not show apparent differences between the areas of the four species and varied in surface (10 cm depth) from 5 to 15%. The cation exchange capacity (CEC) is an essential trait of soils, influencing their stability and nutrient availability (Hazelton & Murphy, 2007). CEC depends on the organic matter and clay proportion in soils. The more elevated CEC in the area of *L. narbonense* is explained by its higher content in organic matter. However, although soil from Clot had a higher proportion of clay, due to its very low OM %, CEC is only slightly higher in this area than in those of *L. girardianum* and *L. virgatum*.

When studies conducted in the greenhouse (Gonzalez-Orenga et al., 2019) indicated that *L. santapolense* is the species most sensitive to water deficit, it was clear that plants growing in their natural environments possess some specific mechanisms of defense against drought, which are not effective under artificial conditions in potted plants. The root system of plants has an essential function in their adaptation to drought, as roots serve as the interphase between plants and soil and play a key role in plant nutrition and development. Root functional traits are achieving more considerable attention in recent studies. As they are directly sensing the physicochemical parameters of the soil, roots are essential in the adaptation of plants to different environments (Franco et al., 2011; Fry et al., 2018). According to databases such as PLANTATT, there are several main categories of roots, such as tap-rooted, rhizomatous, stoloniferous or fibrous (Hill et al., 2004. Three of the *Limonium* species analysed in the present work (*L. santapolense*, *L. girardianum* and *L. virgatum*) have a simple morphology consisting of a central primary underground system with few lateral roots and a low surface area to volume ratio. This type of roots are poor foragers for resources in shallow soils and are not optimal for microbial symbiosis (Fry et al., 2018, and references therein), but can reach deep soil layers (Alvarez-Flores et al., 2018). Some tap-rooted species in arid and semi-arid areas have the ability to produce a hydraulic lift (an upwards transport of water from the more profound, moister layers of soil to the shallow, drier zone), and act as 'nurse plants', beneficial for other plant species by redistributing water from deeper soil layers (Prieto et al., 2011). The fourth species under study, *L. narbonense*, presented a rhizomatous root as described in other *Limonium* species (Eber & Veenhuis, 1991; Antonelli-Ushirobira et al., 2015). Such structures are adapted for storing high amounts of carbohydrates and nitrogenous reserves (Suzuki & Stuefer, 1999; Schmidt & Gaudin, 2017).

Although the main morphological types are stable within species, underground system development shows high plasticity concerning different types of abiotic stresses. Substantial variations in their length, branching and other morphologic and structural aspects may appear even within the same species when environmental conditions are different (Franco et al., 2011, and references therein). In the case of *L. santapolense*,

collected from the arid area at Clot de Galvany, a substantial variation in root length was noticed. Plants growing in more humid soils near the waterlogged depressed part of the salt marsh had short taproot with nodes evenly distributed over it, whereas the plants growing distantly from the water develop a very long taproot and the main root is branched only in deeper, more humid layers of the soil. In the case of the species sampled from El Saler, roots did not show such a strong morphological variation. The development of long roots able to explore deeper moist layers of the soil may explain the fact that *L. santapolense* grows in arid areas, although under controlled conditions was the species most affected by water stress. When growing in a standard pot of 9 cm diameter, plants of this species were affected by one month of imposed water stress while in the wild they tolerate much longer periods of drought, sometimes extending to more than three months without precipitations.

In addition to the root type, many ecophysiological traits may have an adaptive value, enabling plants to inhabit stressful natural environments. *Limonium* species are well-known recretohalophyes, plants that have the ability to exclude salts through salt glands (Leng et al., 2018), but they also accumulate salts in their leaves like many other dicotyledonous halophytes (Wyn-Jones & Gorham, 2002; Flowers & Colmer, 2008). By sequestration of toxic ions in their vacuoles, these plants achieve a cheap osmoticum (Flowers & Yeo, 1986) and also require little K^+ for cytosolic metabolism (Zia et al., 2008; Hameed et al., 2015). When examining Na^+ in roots and leaves, its level was higher in leaves, as it was already reported in these species (Al Hasan et al., 2017); on the contrary, differences between species were not significant. Cl^- followed a similar pattern, accumulating considerably larger concentrations in leaves than in roots. For both ions, the most significant difference between roots and leaves was detected in *L. narbonense*, sampled in a strongly saline area. The active transport and sequestration in leaf vacuoles of toxic ions was reported in other *Limonium* species (Hameed et al., 2015) and may also represent a mechanism of avoidance of toxic ions at the underground level, where osmoregulation is achieved by the accumulation of free osmotic solutes (Alarcon et al., 1999). In addition to Na^+ and Cl^- transport to the aerial part of the plants, Ca^{2+} accumulation in the leaves can also contribute to salt tolerance mechanisms in the analysed *Limonium* taxa, especially in *L. santapolense*, the species showing highest leaf concentrations of this cation, as the essential role of Ca^{2+} in alleviating the deleterious effects of salinity is well established (Hasegawa et al., 2000; Hadi & Karimi, 2012).

Limonium santapolense also showed a striking difference with the other three selected species, regarding the major osmolytes synthesised for osmotic balance under the stressful conditions of their natural habitats. Plants of this species showed much lower leaf concentrations of Fru and also, in general, significantly lower concentrations of Pro and GB than those of *L. virgatum*, *L. girardianum* and *L. narbonense*. Osmotic adjustment in *L. santapolense* seems to be achieved by the accumulation of Suc – and, to a lesser extent, Glu – which is present at much higher levels than in the other three taxa. In contrast, under experimental conditions in the greenhouse, when plants of this species were strongly affected by drought, levels of Pro, Fru and Suc were much higher than those measured in the other species (Gonzalez-Orenga et al., 2019). These data, together with published reports on other *Limonium* species (e.g., Hanson et al., 1991; Liu & Grieve, 2009), support the notion that osmolyte biosynthesis is extremely variable in this genus,

in contrast to other genera that use a single compound as the primary functional osmolyte, for example, sorbitol in *Plantago* (Flowers & Colmer, 2008).

In the present work, measurements in the four selected *Limonium* species of leaf concentrations of MDA – which is routinely used to assess the oxidative damage induced in plants by different stress treatments (e.g. Demiral & Türkan 2004; Aghaleh et al. 2009) – did not reveal, in general, significant interspecific differences. Similarly, the total antioxidant activity of leaf extracts – determined by the DPPH-free radical scavenging assay – or the total phenolic compounds concentrations – as relevant non-enzymatic antioxidants – varied little between the four taxa, non-significantly in most cases. This indicated that *L. santapolense* plants were affected in the field roughly by the same degree of oxidative stress than plants of the other three taxa and that the antioxidant responses were also similar in all cases, despite the different aridity of the corresponding habitats.

The analysis of the responses to environmental stress of the four studied *Limonium* species using field-collected material complements previous work in which plants of the same species were subjected to salt stress (Al Hassan et al., 2017) or water stress (Gonzalez-Orenga et al., 2019)) treatments under controlled greenhouse conditions. Although the general physiological and biochemical responses to stress may be qualitatively similar, comparisons between the two types of experiments should be taken with caution. First, in the greenhouse plants are likely affected by much higher levels of salt or water stress, as their root systems are constrained in the pots to closed and limited environments of relatively homogeneous salinity and moisture. On the contrary, plants in the wild may develop longer roots, with different morphology better adapted to a more heterogeneous environment, where salinity and soil moisture largely varies in different locations and in time, as we show here for *L. santapolense*. Second, the developmental stage of the plants was different, young plants grown from seeds used in the greenhouse experiments *versus* adult plants of unknown age (the four species are perennial) grown in the field. Moreover, the salt and water stress treatments applied in the greenhouse cannot mimic the conditions in nature – specifically, in this case, in Mediterranean salt marshes in summer – where plants are simultaneously affected by different types and varying degrees of environmental stress, including drought, soil salinity, elevated temperatures, and UV irradiation.

4.2.5 Conclusions

The analysis of the biochemical responses to environmental stress of four *Limonium* species in littoral salt marshes in SE Spain, suggested that their mechanisms of stress tolerance are mostly based on the active transport of different ions (Na^+, Cl^-, K^+ and Ca^{2+}) to the leaves, where they contribute to osmotic adjustment, together with the synthesis and accumulation of specific compatible solutes. Our data clearly separated L. santapolense from the other three taxa, *L. virgatum*, *L. girardianum* and *L. narbonense*, as it contains higher leaf Ca^{2+} concentrations and uses different compounds as functional osmolytes, namely sucrose and, to a lesser extent, glucose, which are present at very low levels in the other species. Specific morphological features of the L. santapolense root system – development of a very long taproot to reach deeper, more humid layers of the soil – explains the adaptation of this species to a drier environment than the habitats of the other three selected congeners and the apparent contradiction that *L. santapolense* was

found to be the taxon most sensitive to water deficit in the greenhouse. Root morphology is a trait that should be more often considered in the studies on responses of plants to drought and salinity, as root growth is an essential functional mechanism of adaptation of plants to their natural environments.

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4.2.6 References

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Publicación III:
Subcapítulo 4.3

Insights on Salt Tolerance of Two Endemic *Limonium* Species from Spain

Referencia:
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Insights on Salt Tolerance of Two Endemic *Limonium* Species from Spain

Abstract

We have analysed the salt tolerance of two endemic halophytes of the genus *Limonium*, with high conservation value. In the present study, seed germination and growth parameters as well as different biomarkers – photosynthetic pigments, mono and divalent ion contents – associated to salt stress have been evaluated in response to high levels of NaCl. The study was completed with an untargeted metabolomics analysis of the primary compounds including carbohydrates, phosphoric and organic acids, and amino acids, identified by using a gas chromatography and mass spectrometry platform. *Limonium albuferae* proved to be more salt-tolerant than *L. dufourii*, both at the germination stage and during vegetative growth. The degradation of photosynthetic pigments and the increase of Na^+/K^+ ratio under salt stress were more accentuated in the less tolerant second species. The metabolomics analysis unravelled several differences between the two species. The higher salt tolerance of *L. albuferae* may rely on its specific accumulation of fructose and glucose under high salinity conditions, the first considered as a major osmolyte in this genus. In addition, *L. albuferae* showed steady levels of citric and malic acids, whereas the glutamate family pathway was strongly activated under stress in both species, leading to the accumulation of proline (Pro) and γ-aminobutyric acid (GABA).

Keywords: *Limonium albuferae*; *Limonium dufourii*; growth parameters; photosynthetic pigments; ionic homeostasis; metabolic profiles; carbohydrates, organic acids, aminoacids; multivariate analysis.

4.3.1 Introduction

Salt-affected habitats house plant species with particular adaptations to withstand the deleterious effects of high salt concentrations in the soil, which may include the synthesis of specific metabolites (Flowers & Colmer, 2008; Kumar et al., 2017). Only halophytes, or salt-tolerant species, belonging to different plant families, are well adapted to survive in saline ecosystems; a good example is the *Plumbaginaceae* family, which consists mainly of perennial shrubs, subshrubs, and herbs, most of them growing in arid and saline habitats (Kubitzi, 1993; Koutroumpa et al., 2018); some of these species, mainly belonging to the genera *Limonium* Mill., *Limoniastrum* Fabr. and *Plumbago* L., are used as ornamental and medicinal plants.

The Mediterranean basin is one of the regions in the world with the greatest plant diversity (Davis et al., 1994), and is particularly rich in saline habitats. Within this region, the Spanish territory of the Iberian Peninsula and the Balearic Islands contain several major hotspots of plant diversity (Médail & Quézel, 1997; Médail & Quézel, 1999); besides, this area holds an extensive list of endemic and threatened species (Moreno, 2008; Aedo et al., 2013). *Limonium* is the genus with the highest number of recognised species in Spain (Pignatti, 1972; Greuter et al., 1989; Lledó et al., 2005; Domina, 2019; Hassler, 2019), hosting not less than 107 species (Erben, 1993). *Limonium* is also one of the genera with more conservationist relevance in Spain, including 74 species listed in the Spanish Red List of Threatened Vascular Plants (Aedo et al., 2013), according to IUCN Criteria (2001).

One of the Spanish richest territories in *Limonium* species is the Valencian Community, in which at least 26 endemic species are present, many of them listed as threatened (Crespo & Lledó, 1998; Laguna, 1998; Moreno, 2008; *Decreto 70/200*, 2009; Aguilella et al., 2010; Mateo & Crespo, 2014). The area called El Saler or Devesa de l'Albufera, near the city of Valencia, is one of the best known botanical sites in Spain, as well as the classical site for two of the most relevant Valencian *Limonium* endemics, *L. dufourii* (Girard) Kuntze, and *L. albuferae* P.P. Ferrer, R.A. Rosello & E. Laguna (Crespo & Laguna, 1993; Laguna et al., 1994; Crespo & Lledó, 1998; Laguna, 1998; *Decreto 70/200*, 2009; Aguilella et al., 2010; *Orden 6/2013*,2013; Mateo & Crespo, 2014; Ferrer-Gallego et al,. 2016). It is located in the l'Albufera Natural Park, the most renowned protected area of the Valencian Community. So far, little research has been conducted on these two endemic species and none specifically on their metabolite profiles.

Limonium dufourii is a triploid species (2n = 27) with obligate apomictic reproduction and incompatible pollen-stigma interactions (Erben, 1993; Baker, 1996; Palacios & González-Candelas, 1997). It grows on sea-cliffs and salt marshes on sandy soils (Laguna et al.,1994; Laguna, 1998; IUCN, 2001; Aguilella et al., 2010;). Although this species was more widely distributed in the past along the Valencian coasts and salt marshes, its current distribution is restricted to four populations, hosting from 232 (in the year 2004) to 36,435 (in the year 2011) individuals (https://bdb.gva.es/bancodedatos/censos/). However, molecular analyses show that there is substantial genetic variability and differentiation within and between populations (Palacios & González-Candelas, 1997; Palacios et al., 1999).

Limonium albuferae is a triploid (2n = 26), recently described apomictic species with incompatible pollen-stigma interactions (Ferrer-Gallego et al., 2016). It is a perennial halophyte, multi-rosulate chamaephyte, varying from isolated rosettes (5-8 cm in width) to dense cushion-shape individuals (up to 45 cm in width) bearing tall flowering stems up to 80 cm (personal observations). *Limonium albuferae* has been found so far only in two small sites, both of them located in Devesa de l'Albufera. Despite the significant interest of these two threatened, endemic *Limonium* species for biodiversity conservation, there are no reports regarding their germination, salt tolerance and metabolite profiles. Seed germination and seedling establishment often represent the bottleneck of survival in saline environments (Rubio-Casal et al., 2001; Donohue et al., 2010). Germination of salt marsh halophytes occurs when soil salinity is alleviated during the rainy season (Waisel, 1972; Ungar, 1991; Khan, 2003; Khan, 2006), which is generally autumn in the Mediterranean façade of the Iberian Peninsula (Martín-Vide & López-Bustins, 2006; Del Río et al., 2011).

Plant growth under stressful conditions depends on the efficiency of the mechanisms of stress tolerance of each particular species. A distinctive trait of many halophytes is their ability to absorb toxic ions from saline soils, transport them to the aerial part of the plant and sequester them into vacuoles of the foliar tissue, thus ensuring a low cytosolic concentration; this response is especially efficient in highly salt-tolerant dicotyledonous halophytes (Grieve & Poss, 2005). In parallel to mineral ion compartmentalisation in vacuoles, plants accumulate in the cytoplasm non-toxic, compatible solutes necessary for osmotic balance. Such compounds, known as osmolytes, are not specific for halophytes, but are also synthesised by glycophytes under different abiotic stress conditions that cause depletion of cellular water, such as drought, high salinity in the soil or extreme temperatures (Kumari et al., 2015; Slama et al., 2015). A wide range of metabolites are involved in responses to salinity, including mono-, di-, oligo-, and polysaccharides, sugar alcohols, amino acids, quaternary ammonium compounds, betaines, and tertiary sulphonium compounds (Kumari et al., 2015). Sugars are direct products of photosynthesis that play diverse essential functions in plant cells, so that the assessment of their specific roles as compatible solutes is not so simple. An increase in their concentration is not necessarily a primary response to stress but could be due to activation of other cellular processes (Gil et al., 2013). The major roles played by soluble carbohydrates in stress mitigation involve osmoprotection, carbon storage, and scavenging of 'reactive oxygen species' (ROS) (Kumari et al., 2015). Amino acids are the constituents of proteins, but they have also regulatory and signalling functions. Many amino acids have a well-established role in stress tolerance, such as the flagship compatible solute Pro. Recent microarray studies and amino acids profiles in stressed *Arabidopsis thaliana* plants indicated that an increase in their contents under stress is a general trend; however, only some abundant amino acids such as Pro, Arg, Asn, Gln, and GABA are involved in stress tolerance mechanisms as compatible osmolytes, precursors of secondary metabolites, or storage forms of organic nitrogen**.** On the contrary, low abundant amino acids accumulate under stressful conditions mostly due to protein degradation (Hildebrandt, 2018).

This work aimed at a better understanding of the mechanisms of salt tolerance in halophytes of the genus *Limonium*. Apart from increasing our basic knowledge on these mechanisms, this study is relevant also for conservation purposes, as the information obtained could be useful for the conservation and reintroduction programmes of these two endangered species. The specific objectives were to check the ability of seeds of the two species to germinate under saline conditions and to maintain their germination capacity after a period of exposure to salt, as well as to compare their limits of salt tolerance during vegetative growth. To address the latter question, growth parameters, chlorophyll degradation and the pattern of variation of different mono and divalent ions were determined in plants of the two species subjected in the greenhouse to salinity levels similar and beyond those existents in their natural environments. As the two species showed different degrees of tolerance to salinity, we hypothesised that this would be reflected in qualitative and quantitative differences in their metabolic profiles. Therefore, the work was completed with an untargeted metabolomics study of primary metabolites (sugars and polyols, phosphoric and organic acids, and amino acids) in control and stressed plants. The analysis of salt-induced changes in the metabolite profiles can provide relevant information on the possible mechanisms of salt tolerance in the investigated *Limonium* species.

4.3.2 Material and Methods

Seed Germination

Seeds of *L. albuferae* and *L. dufourii* were provided by Centro para la Investigación y Experimentación Forestal (Centre for Forest Research and Experimentation, CIEF). Before germination, seeds were disinfected with a 10% hypochlorite solution and then thoroughly washed with distilled water. For the germination assays, four replicas of 20 seeds were placed in standard Petri dishes (90 mm in diameter) with a sterile cotton layer covered by two layers of filter paper, which had been wetted with 20 mL of distilled water (for the control treatments) or NaCl solutions of increasing concentration (150, 300, 450 and 600 mM). The plates were incubated in a germination chamber (Equitec, EGCHS HR), with a photoperiod of 16 hours of light at 30°C, and 8 hours of darkness at 20°C. The number of germinated seeds was counted every two days, considering a seed as germinated when the radicle emerged and reached 2-3 mm. After 30 days, all seeds that did not germinate were rinsed several times in distilled water and transferred to new Petri dishes for the 'recovery of germination' assays in distilled water. The number of germinated seeds was recorded for another 30 days. The percentages of germination were calculated as means of the four replicates for each species and treatment. The germination velocity was estimated by calculating the 'Mean Germination Time' (MGT), as described by Ellis and Roberts (Ellis & Roberts, 1981):

MGT= Σ D n/Σn, where D is 'days from the beginning of the germination test', and n is the number of seeds newly germinated on day D.

Plant Growth and Salt Treatments

Seeds without any prior sterilisation were sown on a mixture of commercial peat and vermiculite (3:1) and watered occasionally with Hoagland nutrient solution (Hoagland, 1950). After three weeks, seedlings were transferred to individual 1 L pots placed in plastic trays, with five pots per tray. One week later, salt treatments were started, by watering the plants with water (for the control treatments) or with aqueous salt solutions containing NaCl at 200, 400, 600 and 800 mM final concentrations (for the salt stress treatments). In all cases, 1 L of water or the corresponding salt solution was added to each tray every five days. Five biological replicas (five individual plants) were used per species and per treatment. All experiments were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 h of light), temperature of 23°C during the day and 17°C at night, and 50-80% relative humidity.
After one month of treatment, the aerial parts and the roots of the plants were harvested and weighed separately. The following plant growth parameters were measured: fresh weight of leaves (FWL) and roots (FWR), water content percentage of leaves (WCL) and roots (WCR), and leaf number (LN). Water content percentage in leaves was calculated as: WC% = [(FW-DW)/FW] × 100. For an easier comparison of the two species, which differ in size, fresh weights of leaves and roots were expressed in percentages of the corresponding mean values of the control, non-stressed plants, considered as 100%.

Photosynthetic Pigments

Chlorophylls a and b (Chl a, and Chl b) and total carotenoids (Caro) contents, were determined as described by Lichtenthaler and Wellburn (Lichtenthaler & Wellburn, 1983). Pigments were extracted from 0.05 gr of fresh leaf material in 10 mL of ice-cold acetone 80%. After mixing overnight and centrifuging for 10 min at 12,000 rpm, the supernatant was collected, and its absorbance was measured at 663, 646, and 470 nm. Pigment concentrations were calculated using the equations formulated by Lichtenthaler and Wellburn (Lichtenthaler & Wellburn, 1983) , and their contents were expressed in mg g^{-1} DW.

Ion Content Measurements

Ion contents were determined in roots and leaves, after being eluted in aqueous extracts according to the protocol of Weimberg (Weimberg, 1987), by heating the samples (0.05 g of dried, ground plant material in 15 mL of water) for 15 min at 95°C in a water bath, followed by filtration through a 0.45 μm filter (Gelman Laboratory, PALL Corporation). Cations Na^+ and K^+ and bioavailable Ca^{2+} were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), Cl^- was measured using a chloride analyser Corning 926.

Primary Metabolite Extraction and Metabolite Profiling Analysis

Primary metabolite analysis was performed by the Metabolomics Platform of the "Institute for Plant Molecular and Cell Biology" (Polytechnic University of Valencia, Spain) as previously described (Roessner et al., 2000) with some modifications, following the platform's standard procedures. Each sample (100 mg of leaf material) was homogenised in liquid N_2 and extracted with 1.4 mL 100% methanol and 60 µL internal standard (0.2 mg ribitol in 1 mL of water). The mixture was extracted for 15 min at 70°C; subsequently, the extract was centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a glass vial, and 750 µl of chloroform and 1.5 ml of water were added. The mixture was vortexed for 15 s and centrifuged for 15 min at 14,000 rpm. Finally, 150 µl aliquots of the methanol/water upper phase were dried *in vacuo* for 6-16 h.
For derivatisation, dry residues were redissolved in 40 µL of 20 mg/ml methoxyamine hydrochloride in pyridine and incubated for 90 min at 37°C, followed by addition of 70 µL MSTFA (N-methyl-N-[trimethylsilyl]trifluoroacetamide) and 6 µL of a retention time standard mixture (3.7% [w/v] mix of fatty acid methyl esters ranging from 8 to 24°C) and further incubation for 30 min at 37°C. Sample volumes of 2 µL were injected in split and splitless mode, to increase metabolite detection range, in a 6890 N gas chromatograph (Agilent Technologies Inc. Santa Clara, CA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI). Gas chromatography was performed on a BPX35 (30 m × 0.32 mm × 0.25 µm) column (SGE Analytical Science Pty Ltd., Australia) with helium as carrier gas, at a constant flow of 2 ml/min. The liner was set at 230°C. The oven programme was 85°C for 2 min, followed by an 8°C/min ramp up to 360°C. Mass spectra were collected at 6.25 spectra s^{-1} in the m/z range 35–900 and ionisation energy of 70 eV. Chromatograms and mass spectra were evaluated using the CHROMATOF programme (LECO, St. Joseph, MI).

Statistical Analysis

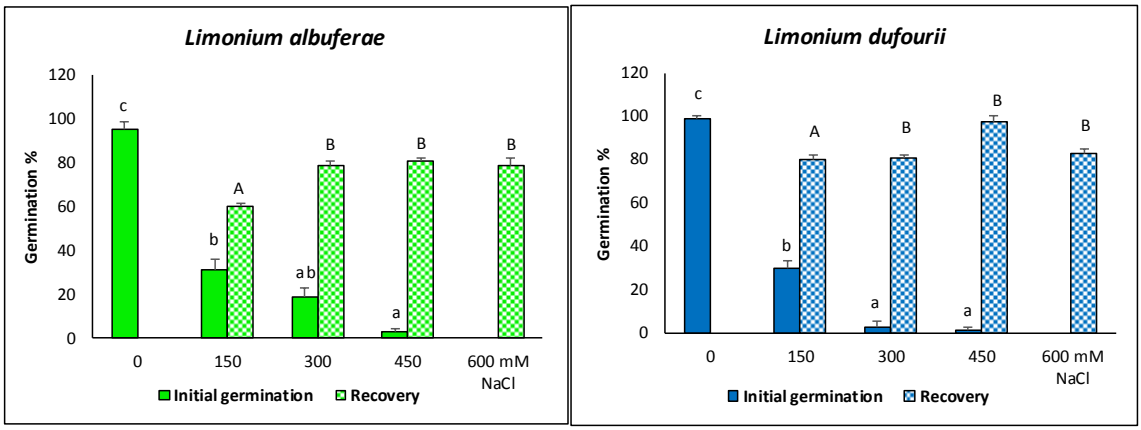
Percentages of germination were arcsine transformed prior to the statistical analysis. Analysis of variance was carried out separately on data from each species. When the ANOVA null hypothesis was rejected, differences between treatments were evaluated by the Tukey's test.
A multifactorial ANOVA was applied to assess the interaction between species, treatments and organs (leaves or roots). Data were analysed using the software Statgraphics Centurion v.16 (Statpoint Technologies, 132 Warrenton, VA, USA).
For the untargeted NMR-metabolomics analysis, the 1H-NMR spectra were automatically reduced by AMIX (v. 3.7, Bruker Biospin) to ASCII files. Partial least square (PLS), and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were performed with the SIMCA-P software (v. 13.0.3, Umetrics, Umeå, Sweden) using the Pareto scaling method. For the targeted GC-MS-metabolomics analysis, the area of each primary metabolite relative to the ribitol area was used as X variable of Principal Component Analysis (PCA) using unit variance (UV) scaling method.

4.3.3 Results

Seed Germination

Final germination percentages after 30 days of incubation were very high in the controls (seeds germinated in water) for the two species, 95% in *L. albuferae* and 98% in *L. dufourii.* Salinity inhibited seed germination in both species, reducing the germination percentages to ca. 30% in the presence of 150 mM NaCl. A significant difference between the two species was found at 300 mM NaCl, with *L. albuferae* showing a higher percentage of germinated seeds (18.5%) than *L. dufourii* (2.5%). Only a few seeds of the two species germinated in the presence of 450 mM NaCl, whereas 600 mM NaCl completely inhibited germination (Figure 1). Most seeds that did not germinate after one month in the presence of salt in the initial germination assays, recovered their germination capacity when they were washed with water and transferred to new Petri dishes. Germination percentages after one additional month of incubation in water were elevated, ranging from 60 to 80% in *L. albuferae* (Figure 1a) and from 80 to ca. 95% in *L. dufourii* (Figure 1b). The lower values corresponded to seeds that had been pre-incubated with 150 mM NaCl, whereas the higher germination percentages were measured for those recovered from the 300, 450 and 600 mM NaCl plates, without statistically significant differences between these pre-treatments (Figure 1).

As expected, germination velocity decreased with increasing salinity, as shown by the parallel increase in the mean germination time (MGT, Table 1); the seeds of *L. dufourii* generally germinated faster than those of *L. albuferae*, but the pattern of concentration-dependent variations was more irregular. Germination in water in the 'recovery' assays was generally faster than in the initial germination experiments, with small differences in the calculated MGT values, irrespective of the salt concentration used in the seed pre-treatments (Table 1).



(a) (b)

Figure 1. Final germination percentages in the two *Limonium* species after 30 days exposure to the NaCl concentrations indicated in the graphs: (a) *L. albuferae,* (b*) L. dufourii*. Bars represent mean ± SEvalues (n = 4). Same letters indicate homogeneous groups between treatments for each species ($p < 0.05$). Lower-case letters were used

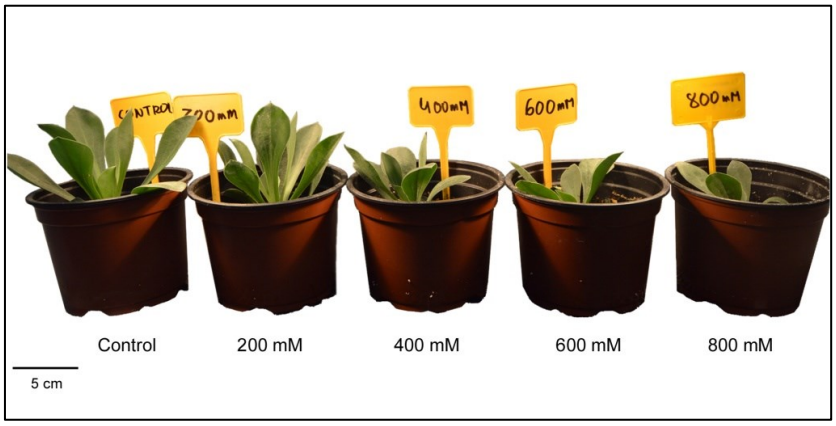
for initial germination in the presence of salt and capital letters for the recovery of germination.

Table 1. The seed mean germination time (MGT) (in days, in the initial germination and recovery of germination assays). Mean ± SE values are shown (n = 4). Same letters indicate homogeneous groups between treatments for each species according to the Tukey test ($p < 0.05$).

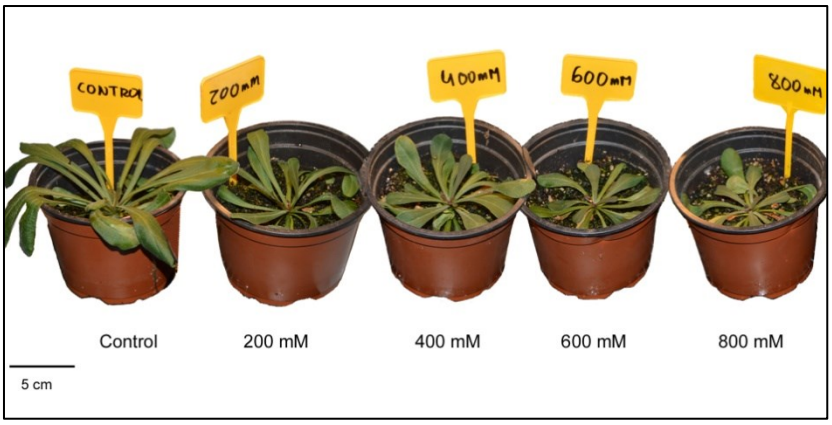
NaCl (mM)	*Limonium albuferae*		*Limonium dufourii*	
	Initial germination	Recovery	Initial germination	Recovery
0	5.78 ± 0.33 a	-	5.32 ± 0.38 a	-
150	10.86 ± 0.78 b	4.41 ± 0.15 ab	11.99 ± 1.16 bc	5.97 ± 0.45 b
300	16.67 ± 0.59 c	4.41 ± 0.20 ab	6.50 ± 0.71ab	4.88 ± 042 ab
450	20.00 ± 1.41 c	4.51 ± 0.17 b	13.00 c	3.89 ± 0. 28 a
600	-	3.73 ± 0.16 a	-	3.97 ± 0.68 ab

Growth Performance under Salt Stress Conditions

Salt stress inhibited growth in both *Limonium* species (Figure 2), affecting mostly the aerial part of the plants, as indicated by a significant reduction in the number of leaves and the leaf fresh weight, in relation to the corresponding controls (Table 2). Comparing the two species, *L. dufourii* showed a more accentuated reduction of these growth parameters in parallel with increasing external salinity. For example, leaf fresh weight (FW) of *L. albuferae* plants treated with 200 mM NaCl did not differ significantly from the non-stressed controls, whereas the same concentration caused a reduction of about 50% in *L. dufourii*.



(a)



(b)

Figure 2. Effects of 30 days salt treatments on growth of young of plants of *L. albuferae* (a) and *L. dufourii* (b). Treatments started one month after sowing the seeds.

The first species tolerated better all salt concentrations, and a drastic inhibitory effect was noticed only in the presence of 600 mM NaCl, when plants lost half of their aerial FW (Table 2). The reduction of leaf FW was partially due to loss of water, as the salt treatments induced a slight (but significant) dehydration of the plants; in this case, however, very small differences were observed between the two species (Table 2). Interestingly, root FW increased significantly, especially in *L. dufourii*, in the presence of moderate (200 mM) or relatively high (400 mM) NaCl concentrations, to decrease again at even higher salinities (600-800 mM NaCl), but never below the values measured in the control plants (Table 2). Moreover, no salt-induced dehydration was detected in roots, as their water content did not suffer any significant reduction in either species.

Regarding photosynthetic pigments, salt stress induced a significant, concentration-dependent reduction in the levels of chlorophylls a (Chl a) and b (Chl b), and carotenoids (Caro) in *L. dufourii*. On the contrary, in *L. albuferae* Chl a contents remained practically unchanged at all NaCl concentrations tested, as the small variations with respect to the controls were not statistically significant; Chl b and Caro contents did show a reduction when comparing control and salt-stressed plants, but without significant differences between the values obtained at different salinities (Table 2). These findings are in agreement with the responses to salt of the two species in terms of growth, indicating a relative higher tolerance to salinity in *L. albuferae*.

Table 2. Growth responses and photosynthetic pigments in the two *Limonium* species after 30 days of treatment with the indicated NaCl concentrations. Mean ± SE values are shown (n = 5). Same letters within each column indicate homogeneous groups between treatments for each species according to the Tukey test ($p < 0.05$). *Leaf FW and root FW were expressed as percentages of the average weights in control plants, taken as 100%, with absolutes values of 6.08 g and 1.49 g (for *L. albuferae*) and 7.15 g and 1.22 g (for *L. dufourii*), respectively.

Parameters	**NaCl (mM)**	***L. albuferae***	***L. dufourii***
Leaf number	0	13.4 ± 0.24 c	24 ± 2.83 d
	200	12.4 ± 0.81 c	20.4 ± 1.14 c
	400	12 ± 0.77 c	18.6 ± 2.19 bc
	600	9.8 ± 0.58 b	16.2 ± 3.56 b
	800	7.4 ± 0.24 a	12.4 ± 2.19 a
Leaf FW*	0	100 ± 0.60 c	100 ± 0.82 c
(% of control)	200	106.27 ± 7.91 c	46.94 ± 3.28 b
	400	83.26 ± 6.42 b	37.72 ± 1.50 ab
	600	49.93 ± 2.55 a	31.65 ± 7.59 a
	800	36.86 ± 1.76 a	29.84 ± 3.40 a
Roots FW*	0	100 ± 0.18 a	100 ± 0.38 a
(% of control)	200	204.11 ± 36.73 b	240.50 ± 29.61 c
	400	187.98 ± 18.56 b	214.38 ± 17.13 bc
	600	141.50 ± 34.02 ab	159.88 ± 35.61 ab
	800	108.72 ± 13.96 a	142.12 ± 14.15 a
WC % in	0	85.22 ± 0.54 e	87 ± 0.30 c
leaves	200	82.46 ± 0.10 d	84.77± 0.32 c
	400	79.45 ± 0.54 c	79.50 ± 0.92 b
	600	75.82 ± 0.5 b	75.54 ± 2.55 a
	800	72.45 ± 0.55 a	76.04 ± 0.65 ab
WC% in roots	0	77.73 ± 0.64 a	83.75 ± 4.37 a
	200	79.14 ± 0.70 a	78.04 ± 9.73 a
	400	78.42 ± 0.57 a	79.60 ± 5.83 a
	600	77.96 ± 0.63 a	72.81 ± 5.67 a
	800	76.80 ± 0.36 a	75.47 ± 6.36 a
Chl a	0	4.99 ± 0.69 a	3.61 ± 0.62 b
(mg g^{-1} DW)	200	3.48 ± 0.13 a	2.00 ± 0.30 a
	400	2.35 ± 0.44 a	1.96 ± 0.34 a
	600	2.47 ± 0.32 a	1.64 ± 0.26 a
	800	3.22 ± 0.76 a	1.64 ± 0.31 a
Chl b	0	3.56 ± 0.97 b	2.06 ± 0.29 c
(mg g^{-1} DW)	200	1.50 ± 0.34 a	1.42 ± 0.25 b
	400	0.81 ± 0.21 a	0.95 ± 0.13 ab
	600	0.76 ± 0.16 a	0.66 ± 0.13 a
	800	1.00 ± 0.26 a	0.65 ± 0.06 a
Caro	0	1.76 ± 0.52 b	1.04 ± 0.12 c
(mg g^{-1} DW)	200	0.95 ± 0.30 a	0.88 ± 0.07 bc
	400	0.57 ± 0.09 a	0.71 ± 0.06 ab
	600	0.63 ± 0.15 a	0.69 ± 0.08 ab
	800	0.91 ± 0.23 a	0.56 0.04 a

Ion Accumulation

Plant Na^+ and Cl^- contents increased in parallel to increasing external salinity in the two *Limonium* species, both in roots and leaves. In general, at all external NaCl concentrations tested, the levels of these two ions were higher in leaves than in roots, and in *L. dufourii* than in *L. albuferae*, except for root Cl^- contents, which were similar in both species. Also, in most cases, Na^+ accumulated to higher levels than Cl^-, for each particular species, organ and salt treatment (Table 3). Regarding K^+ levels, they were also higher in leaves than in roots in the two species, and did not vary significantly in response to salt stress in roots of *L. albuferae*; the pattern of salt-induced changes in K^+ contents was different in *L. dufourii* roots, where they first decreased at 200 and 400 mM NaCl, to increase again at higher salinities, reaching control values in the presence of 800 mM NaCl. When leaf K^+ contents are considered, however, the two *Limonium* species responded in the same way to salt stress, with a significant reduction with respect to the controls, but no differences between the different salt treatments applied (Table 3). Finally, bioavailable Ca^{2+} concentrations were also measured in the samples, showing salt-induced patterns of variation qualitatively similar to those of Na^+ and Cl^-: higher Ca^{2+} contents in leaves than in roots, and a concentration-dependent increase in the levels of this cation in response to increasing salinity, in roots and leaves of both species (Table 3). It is important to highlight the relatively high concentrations of all measured ions in the leaves of control, non-stressed plants, generally much higher than the corresponding values in roots (Table 3).

Table 3. Ion contents in the two *Limonium* species after 30 days of treatments with the indicated NaCl concentrations. Mean ± SE values are shown (n = 5). Same letters within each column indicate homogeneous groups between treatments for each ion and species according to the Tukey test ($p < 0.05$).

Parameters	NaCl (mM)	Species	
		L. albuferae	*L. dufourii*
Na^+ in roots ($\mu mol\ g^{-1}$ DW)	0	150.26 ± 19.54 a	325.60 ± 71.19 a
	200	861.19 ± 71.59 b	1104.48 ± 108.85 b
	400	1081.68 ± 87.38 b	1693.58 ± 119.04 c
	600	1169.40 ± 97.91 b	1744.62 ± 119.59 c
	800	2186.31 ± 272.50 c	2575.18 ± 63.85 d
Na^+ in leaves ($\mu mol\ g^{-1}$ DW)	0	499.48 ± 31.68 a	275.66 ± 68.10 a
	200	2170.93 ± 121.15 b	1990.42 ± 426.87 b
	400	2644.25 ± 70.25 c	2989.64 ± 160.04 c
	600	2845.45 ± 308.40 c	3042.36 ± 199.10 c
	800	3033.51 ± 115.87 c	4271.14 ± 135.61 d
Cl^- in roots ($\mu mol\ g^{-1}$ DW)	0	110.208 ± 19.83 a	123.51 ± 28.55 a
	200	766.74 ± 75.35 b	646.08 ± 85.31 b
	400	1025.84 ± 83.21 c	892.34 ± 55.12 c
	600	1383.95 ± 72.81 d	1120.20 ± 83.55 d
	800	1575.37 ± 55.61 d	1473.89 ± 111.90 e
	0	437.95 ± 32.76 a	671.27 ± 31.86 a

Cl^- in leaves (μmol g^{-1} DW)	200	1120.56 ± 67.86 b	1530.25 ± 74.81 b
	400	1451.80 ± 35.61 c	1723.05 ± 32.51 b
	600	1508.57 ± 137.70 c	1746.18 ± 110.38 b
	800	1689.12 ± 65.28 c	2472.19 ± 75.76 c
K^+ in roots (μmol g^{-1} DW)	0	180.38 ± 31.32 a	149.00 ± 18.31 b
	200	222.38 ± 26.56 a	93.85 ± 24.03 ab
	400	172.02 ± 26.04 a	64.62 ± 12.72 a
	600	177.11 ± 11.67 a	78.94 ± 17.08 a
	800	170.94 ± 16.49 a	146.49 ± 9.15 b
K^+ in leaves (μmol g^{-1} DW)	0	607.93 ± 31.41 b	774.63 ± 78.57 b
	200	410.06 ± 25.32 a	490.56 ± 30.28 a
	400	458.93 ± 19.28 a	494.16 ± 38.64 a
	600	436.83 ± 35.94 a	533.84 ± 81.54 a
	800	414.70 ± 6.06 a	444.43 ± 32.07 a
Ca^{2+} in roots (μmol g^{-1} DW)	0	30.16 ± 2.14 a	43.00 ± 9.44 a
	200	106.50 ± 9.37 b	84.50 ± 8.96 b
	400	121.91 ± 13.30 bc	130.32 ± 5.31 c
	600	145.20 ± 14.16 c	129.29 ± 3.73 c
	800	191.83 ± 5.00 d	176.31 ± 4.11 d
Ca^{2+} in leaves (μmol g^{-1} DW)	0	249.57 ± 13.90 a	210.18 ± 14.96 a
	200	305.30 ± 15.32 ab	322.30 ± 24.60 b
	400	360.52 ± 18.77 b	394.36 ± 23.20 b
	600	302.40 ± 30.14 ab	341.84 ± 10.12 b
	800	353.95 ± 19.94 b	349.03 ± 38.56 b

Statistical Analysis of the Differences in Growth Parameters, Photosynthetic Pigments and Ion Accumulation between the Two Species

A factorial ANOVA was performed, considering three sources of variation: Species (A), Treatment (B), and Organ (C). The effects of the three factors and their interactions are summarised in Table 4. This analysis revealed that the most distinctive trait for assessing the effect of salinity in the two species was FW, which varied significantly according to the three parameters mentioned above: the salt concentration applied, the organ (roots or leaves), and also on a genetic basis, that is, according to species. Therefore, we consider FW as a reliable morphological marker of salt tolerance in the selected *Limonium* species.

Table 4. Factorial ANOVA (F values) considering the effect of Species (A), Treatment (B), Organ (C), and their interactions (A x B; A x C; B x C; A x B x C) on growth parameters (leaf number, FW, WC%), photosynthetic pigments (Chl a, Chl b, Caro) and ions (Na^+, K^+, Cl^-,Ca^{2+}) in *L. albuferae* and *L. dufourii*. *, **, *** significant at P = 0.05, 0.01 and 0.001 respectively; ns: not significant

Parameter	A (Species)	B (Treatment)	C (Organ)	A x B Interaction	A x C Interaction	B x C Interaction	A x B x C Interaction
Leaf (number)	0.000***	0.498	-	0.004**	-	-	-
FW	0.000***	0.007**	0.000***	0.002**	0.000***	0.081^{ns}	0.032*
WC%	0.001**	0.631^{ns}	0.407^{ns}	0.471^{ns}	0.382^{ns}	0.586^{ns}	0.466^{ns}
Chl a	0.062^{ns}	0.030*	-	0.533^{ns}	-	-	-

Chl b	0.000***	0.180^{ns}	-	0.350^{ns}	-	-	-
Caro	0.002**	0.098^{ns}	-	0.006**	-	-	-
Na^+	0.000***	0.000***	0.000***	0.010**	0.000***	0.481^{ns}	0.039*
K^+	0.000***	0.899^{ns}	0.242^{ns}	0.241^{ns}	0.000***	0.000***	0.465^{ns}
Cl^-	0.000***	0.000***	0.000***	0.039*	0.018*	0.000***	0.026*
Ca^{2+}	0.000***	0.862^{ns}	0.000***	0.088^{ns}	0.010*	0.326^{ns}	0.259^{ns}

The interactions between the effects of 'species' and 'treatment', and between 'organ' (roots or leaves) and 'treatment', are shown in Figure 3. Both species exhibited different responses as salinity increased. *L. albuferae* was more tolerant than *L. dufourii* to NaCl concentrations between 200 and 400 mM (Figure 3a), showing higher FW values (Figure 3a). However, at salt concentrations of 600 or 800 mM NaCl both species were equally sensitive (Figure 3a). Comparing the FW of leaves and roots (Figure 3b), a different pattern could be observed. Root t FW slightly increased at low salinity (between 0 and 200 mM NaCl) in response to the salt treatment, whereas a progressive reduction of FW with increasing salinity was observed in leaves. The pattern of WC% variations supported the above results: up to 600 mM external NaCl, a stronger dehydration was observed for *L. dufourii* as compared to *L. albuferae*, which showed a more progressive reduction of WC (Figure 3c). A concentration-dependent loss of water was detected in plant leaves in response to increasing external salinity, whereas the pattern of salt-dependent WC reduction was more irregular in roots (Figure 3d).

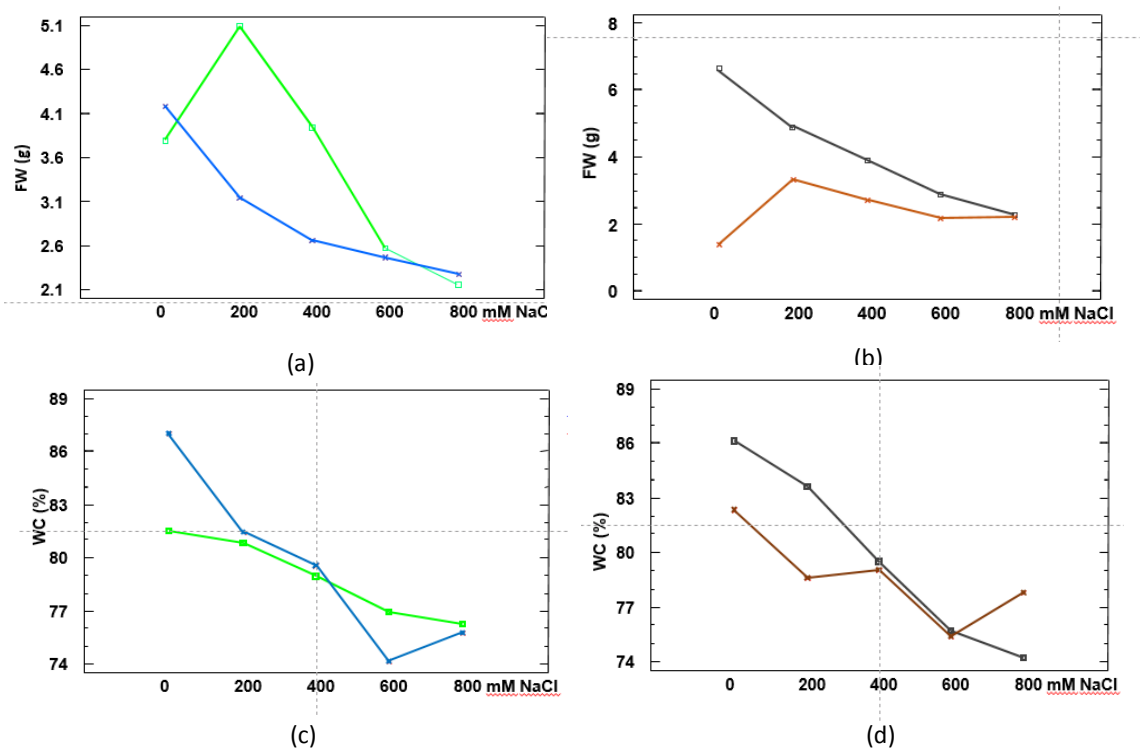


Figure 3. Three-way interaction plots (Species x Treatment x Organ) for fresh weight (FW) (a, b) and for water content (WC) (c, d) in plants *of L. albuferae* (green), *L. dufourii* (blue), leaves (black) and roots (brown). In a and b, interactions Species x Treatment are shown (not differentiating 'organs', leaves and roots, values); in b and d, interactions Organ x Treatment are shown (not differentiating 'species' values).

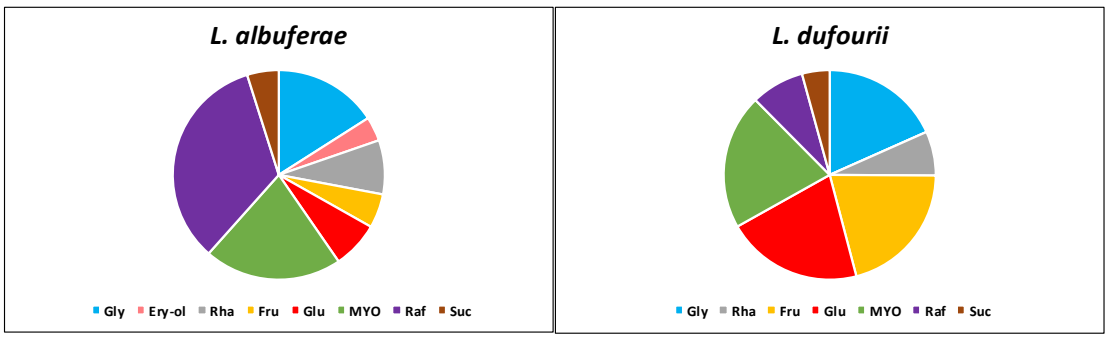
Regarding the photosynthetic pigments, the effect of species was significant for Chl b and Caro, but not for Chl a, whereas the effect of the treatment was significant for Chl a, and the interaction species x treatment only for carotenoids (Table 4).
Ion contents were strongly influenced by the factor 'species' and, with the exception of K^+, also by the organ, roots or leaves. As expected, the effect of the salt treatment was highly significant for the accumulation of Na^+ and Cl^-, as were many of the interactions between different factors (Table 4).

Table 4. Factorial ANOVA (F values) considering the effect of Species (A), Treatment (B), Organ (C), and their interactions (A x B; A x C; B x C; A x B x C) on growth parameters (leaf number, FW, WC%), photosynthetic pigments (Chl a, Chl b, Caro) and ions (Na^+, K^+, Cl^-,Ca^{2+}) in *L. albuferae* and *L. dufourii*. *, **, *** significant at P = 0.05, 0.01 and 0.001 respectively; ns: not significant

Parameter	A (Species)	B (Treatment)	C (Organ)	A x B Interaction	A x C Interaction	B x C Interaction	A x B x C Interaction
Leaf (number)	0.000***	0.498	-	0.004**	-	-	-
FW	0.000***	0.007**	0.000***	0.002**	0.000***	0.081^{ns}	0.032*
WC%	0.001**	0.631^{ns}	0.407^{ns}	0.471^{ns}	0.382^{ns}	0.586^{ns}	0.466^{ns}
Chl a	0.062^{ns}	0.030*	-	0.533^{ns}	-	-	-
Chl b	0.000***	0.180^{ns}	-	0.350^{ns}	-	-	-
Caro	0.002**	0.098^{ns}	-	0.006**	-	-	-
Na^+	0.000***	0.000***	0.000***	0.010**	0.000***	0.481^{ns}	0.039*
K^+	0.000***	0.899^{ns}	0.242^{ns}	0.241^{ns}	0.000***	0.000***	0.465^{ns}
Cl^-	0.000***	0.000***	0.000***	0.039*	0.018*	0.000***	0.026*
Ca^{2+}	0.000***	0.862^{ns}	0.000***	0.088^{ns}	0.010*	0.326^{ns}	0.259^{ns}

Differences in the Metabolite Profiles of the Two Species in the Absence of Stress

The metabolite profiles were analysed by Gas Chromatography-Mass Spectrometry in control plants, not subjected to the salt stress treatments. Three categories of primary metabolites were detected: carbohydrates, organic acids (together with phosphoric acid) and amino acids (Figure 4). Seven carbohydrates were found in the two species (glycerol, rhamnose, fructose, glucose, *myo*-inositol, raffinose and sucrose), and one (erythritol) was detected only in *L. albuferae*. The relative contents of most carbohydrates were roughly similar in the two *Limonium* species, except for raffinose, which was much more abundant in *L. albuferae* (Figure 4a), and for fructose and glucose, present at higher relative levels in *L. dufourii* (Figure 4b).



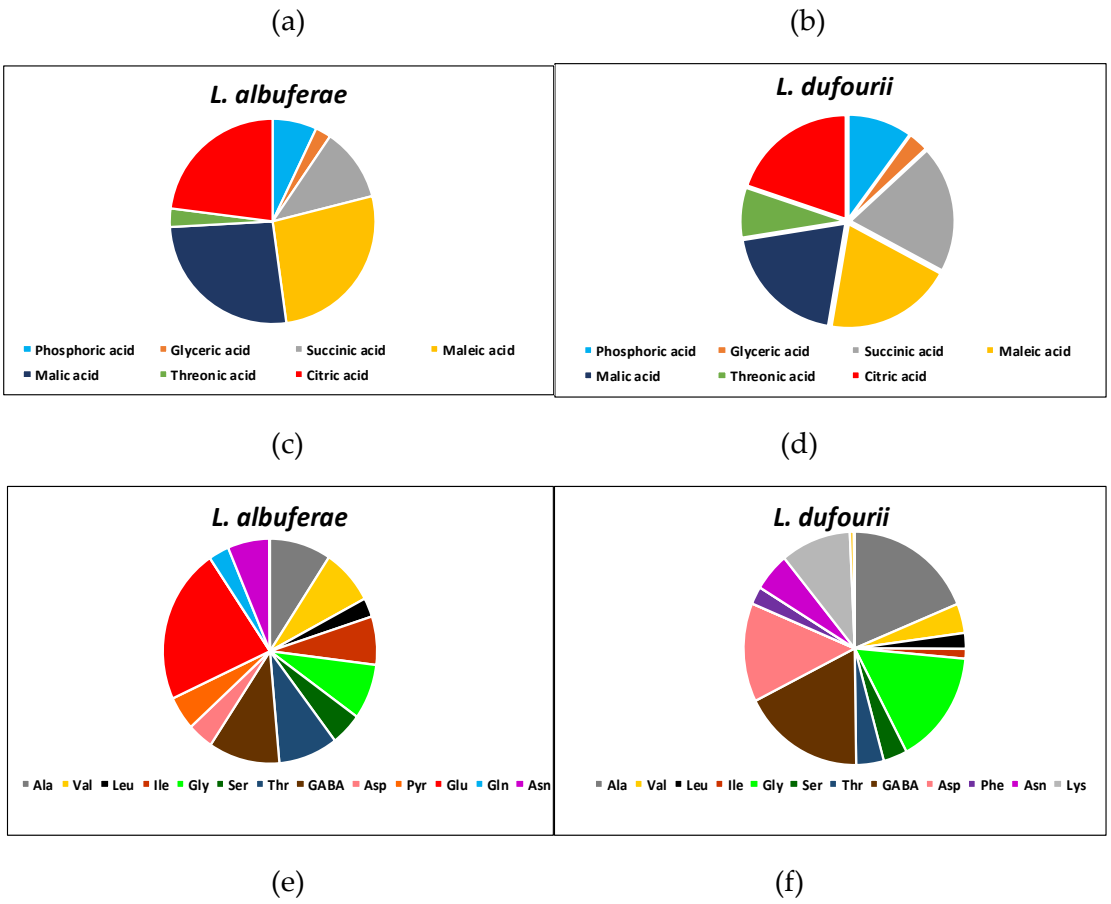


Figure 4. Relative contents of leaf carbohydrates (a, b), phosphoric and organic acids (c, d) and aminoacids (e, f) detected in absence of stress in plants from control treatments (n = 5) of the two *Limonium* species.

Phosphoric acid and six organic acids (glyceric, succinic, maleic, malic, threonic, and citric) were detected in the two species, without marked differences observed in their relative contents in both of them (Figures 4c, d).

Of the 17 amino acids detected, 12 were found in the two species: Ala, Val, Leu, Ile, Gly, Ser, Thr, GABA, Asp, Asn, , Trp and Pro. Pyro-Glu, Glu and Gln were detected only in *L. albuferae* (Figure 4e) and Lys and Phe only in *L. dufourii* (Figure 4f). Significant quantitative differences between the two species were observed regarding the relative contents of most amino acids. The most abundant amino acid in *L. albuferae*, showing substantial differences with all the others, was Glu – not detected in *L. dufourii* – whereas very low levels of Trp and Pro were measured in this species (Figure 4e). In *L. dufourii* there was no single predominant amino acid, as four of them (Ala, Gly, GABA and Asp) showed similarly high relative contents and, in all four cases, significantly higher than in *L. albuferae*. Very low Trp and Pro relative contents were also measured in *L. dufourii* (Figure 4f).

Changes in Metabolites Relative Contents in Response to Salt Stress

The salt treatments induced changes in the relative contents of many of the metabolites mentioned above, with quantitative and qualitative differences often observed between the two *Limonium* species. A summary of all results is presented in Supplementary Table S1. Concerning sugar and polyol relative contents, in both *Limonium* species erythritol, rhamnose and sucrose increased with increasing salt concentrations, glycerol showed oscillations, generally not significant, and myo-inositol levels decreased in response to salt stress; glucose and fructose relative levels were higher in non-stressed L. dufourii plants, but showed significant increases over control values only in *L. albuferae*.

The relative levels of phosphoric acid, and the organic acids glyceric and threonic increased in salt-stressed plants of the two species. In the case of threonic acid, when comparing the two species, the most statistically significant increase was observed in *L. dufourii* plants treated with 400 mM NaCl. No significant changes in the foliar contents of maleic, malic and citric acids were detected in *L. albuferae* in response to increasing external salinity, whereas a general decrease was observed in *L. dufourii* regarding the relative contents of these compounds. Succinic acid showed an irregular oscillation pattern in both species.

A general trend of increasing amino acid relative contents under salt stress was detected for most amino acids in both species, but especially in *L. albuferae*: Ala, GABA, Gly, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, and Val. For example, in *L. albuferae*, Phe and Trp increased from undetected levels in the control plants to maximum values in the presence of 800 mM NaCl; in *L. dufourii*, the same amino acids showed a peak at 400 mM NaCl, decreasing at higher salinities. The same accumulation pattern – i.e., maximum contents at the highest salt concentrations tested (600-800 mM NaCl) in *L. albuferae* and a peak at 400 mM in *L. dufourii* – was observed for other amino acids, such as Asn, Asp, Gly, Ser, Thr, or Val. In fact, a statistically increase was observed for these aminoacids along with Ile, Leu, Phe and Pro in *L. albuferae* plants upon maximum salt stress (600-800 mM NaCl), when comparing both species. Besides, Glu, Gln and pyro-Glu were only found in *L. albuferae*, but their levels did not vary significantly, or the changes were not clearly correlated with the external salt concentration.

Statistical Analysis of the Untargeted Metabolomics Results

A two-way ANOVA was performed to analyse the effects of the treatment, species and their interactions, for each of the identified metabolites (Table 5). Concerning the carbohydrates group (sugars and polyols), the effect of the 'species' factor was significant for fructose, erythritol, rhamnose and sucrose, and that of 'treatment', for all detected carbohydrates except glycerol. The highest interactions of the two factors were observed for raffinose and erythritol, which varied in different directions (increasing/decreasing with salinity) in the two species.

The two-way ANOVA also revealed a significant effect of 'species' for two organic acids, citric and threonic, and that of 'treatment' for glyceric and threonic acids (Table 5). Regarding the amino acids, the 'species' effect was highly significant for Glu, Gln and

pyro-Glu – as expected since these amino acids were only detected in *L. albuferae* – but also for Ile, Lys, Thr and Val. The effect of the treatment was significant for all detected amino acids except GABA, Glu and Gly (Table 5).

Table 5. Factorial ANOVA (F values) considering the effect of Species (S), Treatment (T), and their interactions (S x T) on metabolic profiles of leaf carbohydrates, phosphoric and organic acids and aminoacids in *L. albuferae* and *L. dufourii.* *, **, *** Significant at P = 0.05, 0.01 and 0.001 respectively; ns: not significant

	Parameter	S	T	S x T
Carbohydrates	Erythritol	0.0099**	0.0000***	0.0419**
	Fructose	0.0000***	0.0408**	0.0660*
	Glucose	0.6060^{ns}	0.0082**	0.2290^{ns}
	Glycerol	0.1200^{ns}	0.4000^{ns}	0.2700^{ns}
	Myoinositol	0.5321^{ns}	0.0109*	0.8501^{ns}
	Raffinose	0.2707^{ns}	0.0000***	0.0097**
	Rhamnose	0.0191*	0.0000***	0.6961^{ns}
	Sucrose	0.0240*	0.0000***	0.0650^{ns}
Inorganic Acids	Phosphoric Acid	0.3124^{ns}	0.4720^{ns}	0.2133^{ns}
Organic Acids	Citric Acid	0.0044**	0.0907^{ns}	0.0303*
	Glyceric Acid	0.3516^{ns}	0.0000***	0.0210*
	Maleic Acid	0.2020^{ns}	0.0840^{ns}	0.3300^{ns}
	Malic Acid	0.0737^{ns}	0.0533^{ns}	0.0201*
	Succinic Acid	0.6010^{ns}	0.5586^{ns}	0.0064**
	Threonic Acid	0.0018**	0.0000***	0.0754^{ns}
Amino acids	Alanine	0.6222^{ns}	0.0034**	0.2083^{ns}
	Asparagine	0.9672^{ns}	0.0322*	0.0053**
	Aspartic Acid	0.1198^{ns}	0.0064**	0.0039**
	GABA	0.2916^{ns}	0.4754^{ns}	0.1937^{ns}
	Glutamic acid	0.0000***	0.5378^{ns}	0.5780^{ns}
	Glutamine	0.0000***	0.0000***	0.0000***
	Glycine	0.7987^{ns}	0.0669^{ns}	0.0084**
	Isoleucine	0.0115*	0.0009***	0.0343*
	Leucine	0.4357^{ns}	0.0024**	0.0102*
	Lysine	0.0335*	0.0001***	0.0975^{ns}
	Phenylalanine	0.9134^{ns}	0.0000***	0.0000***
	Proline	0.2250^{ns}	0.0000***	0.0353*
	Pyroglutamic acid	0.0000***	0.0003**	0.0003**
	Serine	0.6344^{ns}	0.0006***	0.1424^{ns}
	Threonine	0.0002**	0.0000***	0.0564^{ns}
	Tryptophan	0.1810^{ns}	0.0170*	0.0353*
	Valine	0.0422*	0.0000***	0.0106*

A comparison of all samples included in the metabolomics analysis was performed through multivariate data analysis (MVDA). Specifically, a partial least square (PLS) analysis was applied, defining as the X variable the area of the characteristic ion mass spectra, and as the step-wise Y variables the species (*L. albuferae* and *L. dufourii*) and the treatments (control and salt stress). The score plot of PLS analysis according to model

terms clearly separated the effect of treatment in the OX axis (explaining 29.5% of the total variability) and that of species in the OY axis (11% of variability). When analysing the distribution of plants from different salt treatments (Figure 5), control and 200 (200 mM NaCl) of the two species were grouped together with 400 of *L. albuferae* in the negative part of PLS component 1, whereas 400 in *L. dufourii* falls together with higher concentrations (600 and 800) of *L. albuferae* and were placed in the positive side. The second component of PLS clearly separated the metabolite relative contents of both species, locating *L. dufourii* (LD) and *L. albuferae* (LA) in the positive and in the negative part, respectively. This analysis indicated that the salt stress provoked important changes in the metabolic profiles of both species, being different in each one.

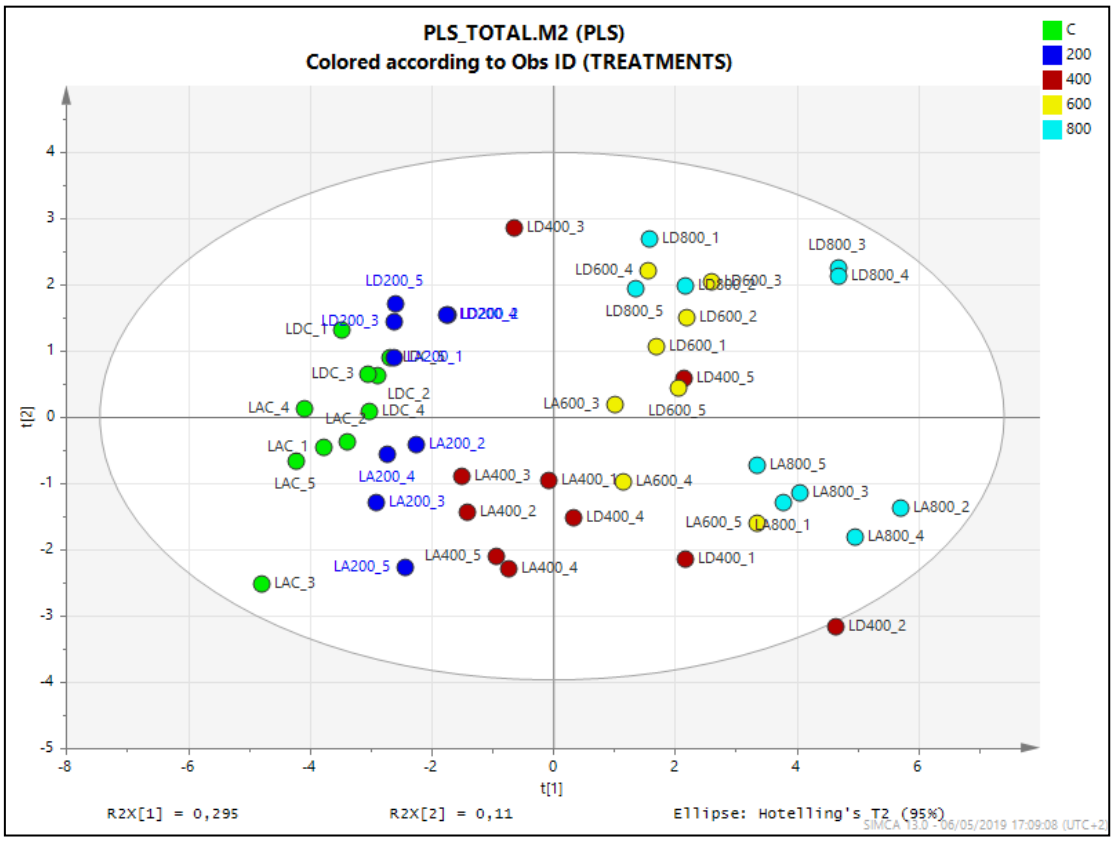


Figure 5. Score plot of partial least square analysis (PLS) based on the characteristic ion of the mass spectra from the primary metabolites measured in the m/z range 35–900, of the *L. albuferae* (LA) and *L. dufourii* (LD) control (C) plants and after salt stress (200, 400, 600 and 800 mM NaCl).

The loading scatter plot of PLS analysis (Figure 6) showed that Pro, rhamnose, erythritol, threonic acid and sucrose were the metabolites that mostly increase in conditions of salinity whereas Thr, citric, malic and maleic acids are those most related to the species. Out of them, Thr was the only metabolite that showed a peak at lower salt concentration in *L. albuferae* (at 200 mM NaCl) than in *L. dufourii* (400 mM NaCl), and the remaining ones showed also a different pattern of variation in the two species.

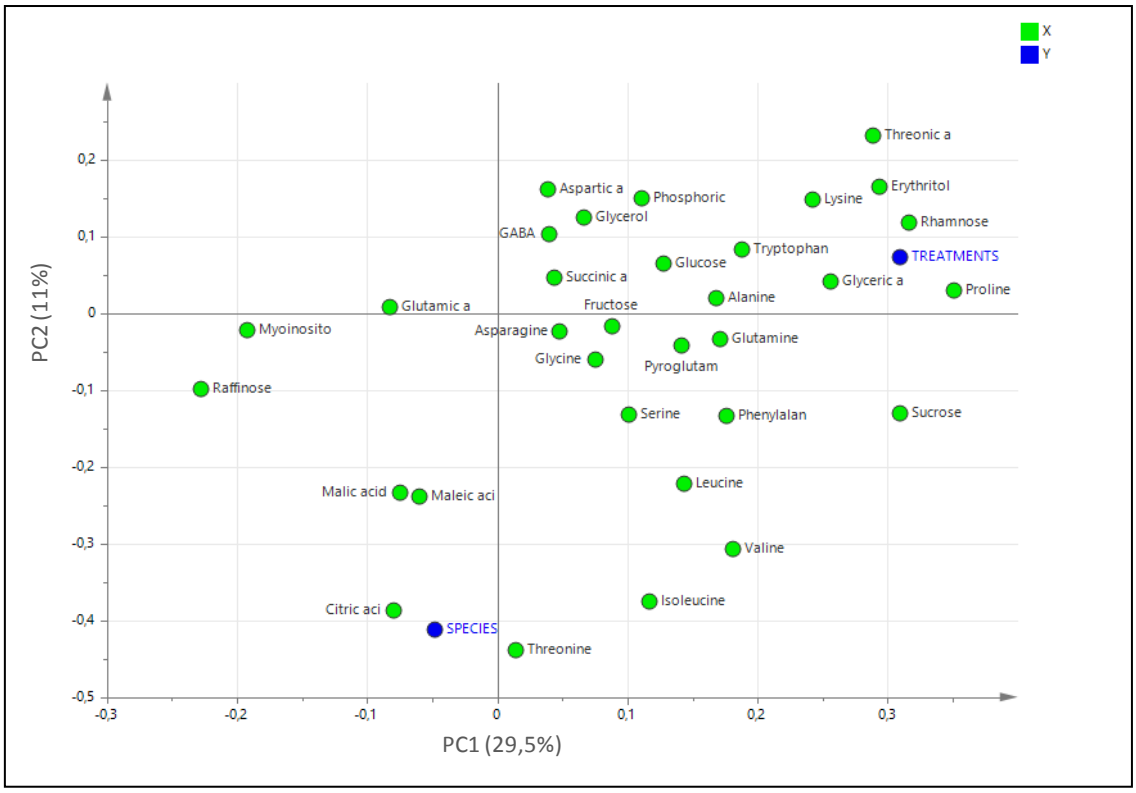


Figure 6. Loading scatter plot analysis of PLS analysis of the showing the metabolites involved in salt stress responses in the metabolomics study in the two *Limonium* species.

4.3.4 Discussion

Relative Salt Tolerance of *Limonium albuferae* and *L. dufourii*

As highlighted in the introduction, the two species under study present a special interest, as they are unique endemics from salt marshes in the area of Valencia. Such ecosystems are highly dynamic, characterised by large variations in the salinity of the soil, at the temporal and spatial scales (Gil et al., 2014). Therefore, reintroduction or reinforcement of populations should be based on a deep knowledge not only of the soil characteristics and their seasonal variations, but also on the tolerance limits of the species of interest. Increased temperature due to global warming leads to increased evapotranspiration, which can generate hypersaline conditions; even acting over a short time period, this may inflict a greater stress of plants living in salt marshes, eventually causing, in the worst-case scenario, the dieback of those less tolerant (Touchette et al., 2019) The two species of *Limonium* studied in the present work share the same area in l'Albufera Natural Park, the site of origin of their seeds, but they differ in their tolerance to salt stress. *Limonium albuferae* proved to be more salt tolerant than *L. dufourii*, as a higher percentage of its seeds germinated at 300 mM NaCl, its growth was not affected in the presence of 200 mM NaCl, and higher salt concentrations (400 and 600 mM) inhibited vegetative growth to a lesser extent than in *L. dufourii*. Seed germination and seedling establishment are critical phases in the life cycle of halophytes (Houle et al., 2001) and a reduction of the salinity in the superficial soil layers, to allow germination, is a prerequisite for their survival in salt marshes. The concentration of 200 mM NaCl drastically affects or, in combination with high temperatures, is even the salinity limit

allowing germination for several Iberian *Limonium* endemics (Giménez et al., 2013; Delgado-Fernández et al., 2015; Delgado-Fernández et al., 2016). On the contrary, in other species of this genus seeds still maintain their germination ability at higher salt concentrations (Zia & Khan, 2004; Redondo-Gómez et al., 2008; Al Hassan et al., 2017), as in other halophytes (Vicente et al., 2004; Boscaiu et al., 2011; Zhang et al., 2012; Manzoor et al., 2017). However, a distinctive trait of halophtyes is the recovery of germination capacity even after long periods of exposure to high salt concentrations, a phenomenon that does not occur in glycophytes (Gul et al., 2013). The cessation of dormancy triggered by the alleviation of salt stress is significant in salt marshes, which are subjected to seasonal fluctuations of salinity (Keiffer & Ungar, 1997; Pujol et al., 2000). Although *L. dufourii* showed lower germination rates under saline conditions, after the salt pre-treatments its seeds had an excellent recovery capacity, reaching germination percentages in water as high as in the control and up to ~ 95%, whereas the percentage of recovery ranged from 60 to 80% in *L. albuferae*. The ecological significance is that seeds of both species maintain their viability under high soil salinity conditions and at this stage, and their responses to salt stress are similar to those of other *Limonium* species.

During vegetative growth, once the critical phase of seedling establishment is overcome, the ability to tolerate different types of stresses, including salinity, increases in parallel to the age of plants (Vicente et al., 2004). For this reason, an optimal method to assess the limits of salt tolerance of one species, but also to compare stress tolerance of genetically related taxa, is the quantification of the salt-induced inhibition of growth of the plants (Al Hassan et al., 2016 a; Al Hassan et al., 2016 b). Growth reduction under salt stress is a general trait in glycophytes, but also in most halophytes. However, in many dicotyledonous halophytes, especially in those more salt-tolerant, moderate concentrations of NaCl stimulate growth (Flowers et al., 1986). Stimulation of growth under moderate salinity conditions has also been reported in some *Limonium* species, such as *L. delicatulum* (Souid et al., 2016), *L. girardianum*, or *L. virgatum* (Al Hassan et al., 2017). Of the two species studied here, only *L. albuferae* experienced an increase of FW in the presence of 200 mM NaCl, whereas plants of *L. dufourii* subjected to the same treatment suffered a reduction of growth of more than 50%. In fact, all analysed growth parameters clearly indicated that *L. dufourii* is more sensitive to salt during vegetative growth than *L. albuferae*; this finding should be considered in the management programmes of these two endangered species. Plants of both species appear to be tolerant to soil salinities much higher than those normally present in the salt marshes that represent their natural habitats, considering that they survived for one month in the greenhouse in the presence of NaCl concentrations as high as 600 – 800 mM. This explains how these plants can withstand in the field episodes of hypersalinity, occurring generally in summer due to increased temperatures and evapotranspiration, as it has been reported for the area of study (Gil et al., 2011; Gil et al., 2013; Boscaiu et al., 2013).

Main Salt Tolerance Mechanisms in the Investigated Species

The strategy of using inorganic ions as cheap osmotica to maintain growth under saline conditions, is a relevant mechanism of salt tolerance in many halophytes, including *Limonium* species (Alarcón et al., 1999; Morales et al., 2001; Carter et al., 2005; Al Hassan et al., 2016c; Al Hassan et al., 2017). In our experiments, ion contents were generally higher in leaves than in roots, at all external salinities tested, in agreement with those previous data. Accumulation of Na^+ in plants is generally associated with a decrease of K^+ levels, as it has been found in the present work in the leaves of the two *Limonium* species. The drop in K^+ levels is mainly due to the competition of Na^+ for the same binding sites in proteins, including the physiological K^+ membrane transport proteins. Moreover, increased Na^+ concentrations induce depolarisation of the plasma membrane, causing the loss of cellular K^+ by activation of outward rectifying K^+ channels (Greenway & Munns, 1980). In the selected *Limonium* species, K^+ levels were constant in roots but progressively decreased in leaves, in parallel with increasing external salinity. The reduction in K^+ was more pronounced in *L. dufourii*, which also showed a relatively higher accumulation of Na^+ in salt-stressed plants, in comparison to the controls. For this reason, the increase in the leaf Na^+/K^+ ratio in plants grown in the presence of 800 mM NaCl (the highest salinity tested) with respect to the controls, was much higher in *L. dufourii* (~ 27-fold) than in *L. albuferae* (~ 2.5-fold). Maintaining a balanced cytosolic Na^+/K^+ ratio is regarded as an essential mechanism for salt tolerance (Assaha et al., 2017), and it seems logical to assume that the lower Na^+/K^+ ratios in *L. albuferae* contribute to its better tolerance to high salinity as compared to *L. dufourii.*

The observed increase in Ca^{2+} contents in response to increasing external salinity, both in roots and in leaves of the two *Limonium* species is probably also involved in the salt tolerance mechanisms of these species, as calcium plays a key regulatory and signaling role in plant growth and development under salt stress (Hepler, 2005); for instance, extracellular Ca^{2+} is beneficial for maintaining Na^+ and K^+ homeostasis via the SOS pathway (Mahajan et al., 2008). Moreover, an increase in Ca^{2+} concentration stimulated the development and the salt-secretion rates of salt glands in the leaves of *L. bicolor*; salt secretion is an effective strategy used by recretohalophytes (as the two species studied here) to adapt to highly saline soils (Ding et al., 2010).

It is interesting to mention the relatively high concentrations of all measured ions in leaves of control, non-stressed plants, generally much higher than the corresponding values in roots. These data support the existence of constitutive mechanisms of tolerance based in the active transport of inorganic ions to the leaves to contribute to osmotic adjustment, even under low soil salinity conditions.

The primary metabolites that were detected in the untargeted metabolomics analysis belong to the groups of carbohydrates (sugars and polyols), organic acids, together with phosphoric acid, and amino acids. Carbohydrate profiles were qualitatively similar in the two analysed species, in the absence of stress. Several of the identified compounds did not vary significantly, or did not show a clear correlation of the variation patterns with the intensity of the salt treatments, whereas the leaf relative contents of erythritol, sucrose and rhamnose clearly increased in parallel to increasing external salinities in the

two species. Fructose, which has been reported as a major osmolyte in different *Limonium* species (Gagneul et al., 2007; Liu & Grieve, 2009; Al Hassan et al., 2017), showed a salt-induced increment only in *L. albuferae*, and a similar pattern was observed for glucose. Therefore, the relatively higher salt tolerance of *L. albuferae* may be partly due to the specific accumulation of fructose and glucose under high salinity conditions in this species, but not in *L. dufourii.*

Among the organic acids identified in the leaf extracts, the most abundant in the two species were malic, citric and maleic. Their concentrations did not vary significantly when *L. albuferae* plants were subjected to the salt treatments, but they decreased significantly in *L. dufourii*. Citric and malic acids also decreased in salt-treated *L. latifolium* plants (Gagneul et al., 2007). On the contrary, citric acid levels increased upon salt stress in the halophyte *Leymus chinensis*, and improved growth when applied exogenously. Higher constitutive levels of citric and malic acids were detected in the salt-tolerant *Thelungiella halophilla* in comparison to *Arabidospis thaliana* (Sanchez et al., 2008). These and other data support the positive role of citric and malic acids in the mechanisms of salt tolerance and, accordingly, it can be suggested that *L. albuferae* respond to salt stress better than *L. dufourii*, in part, by maintaining steady levels of these two organic acids.

Amino acids are synthesised by various distinct metabolic pathways, some strongly influenced by environmental conditions. For example, the glutamate family pathway is strongly activated under stress, leading to the accumulation of Pro and GABA (Planchet et al., 2015). Pro is the commonest osmolyte in plants, directly participating in cellular osmotic adjustment under stress conditions, but also playing additional roles as 'osmoprotectant' – low-molecular-weight chaperon and ROS scavenger (Szabados & Savouré, 2010). Together with fructose, Pro has been reported as the main osmolyte in four species of *Limonium* from the area under study (Al Hassan et al., 2017), but it seems to play only a minor osmoregulatory role in *L. latifolium* (Gagneul et al., 2007). Besides its function in maintaining carbon-nitrogen balance, GABA is involved in responses to different types of abiotic stress in plants. Increased concentrations of GABA were detected in conditions of hypoxia, drought, salinity and low or high temperatures, and its role as effective osmolyte and in ROS scavenging is well-known (Cheng et al., 2018).

The metabolite profile of *L. albuferae* and *L. dufourii* samples showed, as a general trend, a salt-induced increase in the levels of most amino acids, especially in the former species, suggesting a positive role of these compounds in the mechanisms of salt tolerance of both *Limonium* species. GABA and Pro contents gradually increased in the two species in parallel with the external salt concentration, reaching maximum values in the presence of 800 mM NaCl. A similar pattern, with peaks at 800 mM or 600 mM NaCl, was observed for many other amino acids in *L. albuferae*, whereas in *L. dufourii* maximum concentrations were measured in the 400 mM NaCl treatment, decreasing at higher salinities. This pattern of variation correlates with the relative tolerance of the two species: it appears that, at high salinities, the more salt-sensitive *L. dufourii* cannot use as efficiently as *L. albuferae* this mechanism of defence based on the accumulation of specific amino acids.

Relevance of the Obtained Results for Conservation Strategies of the Two Endemic *Limonium* Species

As stated in the Introduction, *L. albuferae* and *L. dufourii* are highly threatened local endemics, represented by a few populations with a rather low number of individuals in salt marshes located near Valencia. Both species require management programmes for ensuring their persistence, mostly due to habitat loss but also to other factors, including for example pressure of invasive plants, which should be taken into consideration when establishing new populations in the frame of reintroduction strategies. The data presented here indicate that soil salinity, *per se*, is not a restrictive factor as both species are halophytic, although with different degrees of salt tolerance. The more tolerant *L. albuferae* could be reintroduced in the most saline areas where there will be less competition with aggressive invasive species, such as those of the genus *Spartina***,** which are increasing in the area (personal observations**).** On the other hand, new sites for *L. dufourii* should be considered only in areas with moderate salinity, as its salt tolerance is lower than in other species of the genus (Al Hassan et al., 2017)**.** Given the uncertainty of climate change effects, conservation efforts of the two species – but especially of the less tolerant *L. dufourii* – should include continuous monitoring of soil salinity and moisture in the areas of location or reintroduction, as well as the presence and evolution of invasive plants.

4.3.5 Conclusions

The germination study indicated that both species behave as halophytes, their germination being enhanced after one month exposure to salt concentrations. *Limonium albuferae* proved to be more salt tolerant than *L. dufourii*, with higher germination percentage at 300 mM NaCl, but higher salt concentrations completely inhibited germination in both species. The same pattern was observed during growth, as *L. dufourii* was more affected by salt treatments than *L. albuferae*, which had an optimal growth when watered with 200 mM NaCl. Other parameters, such as photosynthetic pigments and the Na^+/K^+ ratio also indicated that *L. albuferae* can better tolerate saline conditions. The metabolomics analysis revealed only a few differences between the two species in absence of stress, in plants from the control treatment, but the pattern of accumulation of several compounds under stress was different. Fructose and glucose, the first reported as a major osmolyte in the genus, were accumulated only in *L. albuferae*, which also showed steady levels of citric and malic acids. Proline and γ-aminobutyric acid (GABA), both with osmoregulatory functions, increased in both species.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/9/12/294/s1, Table S1: Metabolic profiles of the two *Limonium* species after 30 days of treatment with the indicated NaCl concentrations.

Author Contributions

Conceptualization, M.B. and O.V.; methodology, S.G.O, M.P.L.G. and M.P.D.T.; software, M.V.; validation, E.L., P.P.F.G., O.V. and M.B.; formal analysis, M.B.;

investigation, S.G.O. and M.P.L:G; resources, M.B.; data curation, M.P.D.T.; writing—original draft preparation, P.P.F.G, E.L. and M.B.; writing—review and editing, O.V.; visualization, M.P.D.T. and P.P.L.G.; supervision, O.V.; project administration, M.B.; funding acquisition, M.B.

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Publicación IV:
Subcapítulo 4.4

Multidisciplinary Studies Supporting Conservation Programmes of Two Rare, Endangered *Limonium* Species from Spain

Este artículo ha sido enviado a la revista; *Plant and Soil,* y está actualmente bajo revisión.

Referencia:
González-Orenga, S.; Donat-Torres, M.P.; Llinares, J.P.; Navarro, A.; Collado, F.; Ferrer-Gallego, P.P.; Laguna, E.; Vicente, O.; Boscaiu, M. Multidisciplinary Studies Supporting Conservation Programmes of Two Rare, Endangered *Limonium* Species from Spain. (Bajo revisión)

Multidisciplinary Studies Supporting Conservation Programmes of Two Rare, Endangered *Limonium* Species from Spain

Abstract

Background and aims. Two local threatened endemics from Valencian salt marshes were analysed from a multidisciplinary perspective combining field studies with experiments performed under greenhouse-controlled conditions. The work aimed to investigate the habitat of the two species but also to explore their limits of tolerance to severe drought and salinity and the mechanisms behind their stress responses.

Methods. The number of individuals in several populations, climatic conditions, soil characteristics and accompanying vegetation in the natural habitats were analysed in the field study. Plants obtained by seed germination were grown in the greenhouse and subjected to one month of water and salt stress treatments. Growth and biochemical parameters were analysed after the treatments were finalised.

Results. No correlation between climatic parameters and number of individuals censed of the two *Limonium* species could be established. Although *L. dufourii* was found in more saline soils in the natural habitats, under controlled greenhouse conditions, this species was more severely affected by salt treatment than *L. albuferae*, which is more susceptible to water stress. A common biochemical response was the increase of proline under all stress treatments, but mostly in water-stressed plants. Oxidative stress markers, MDA and H_2O_2, did not indicate significant differences between the treatments. The differences in the two species' responses to the two kinds of stress were correlated with the activation of the antioxidant enzymes, more pronounced in conditions of salt stress in *L. albuferae* and of water stress in *L. dufourii*.

Conclusions. Although *L. albuferae* is found in sites with lower salinity in the natural habitats, the greenhouse experiment indicated that it tolerates higher concentrations of salt than *L. dufouri*, which is more resistant to drought. The two species efficiently mitigate the oxidative stress by activation of antioxidant enzymes. The results obtained may be useful in conservation management of the two species: whereas salinity is not problematic, as the two species tolerated under controlled conditions salinities far beyond those in their natural environments, water scarcity may be a problem for *L. albuferae*, which proved to be more susceptible to water deficit.

Keywords: Salt marshes, Salinity, Water stress, Endemics, Osmolyes, Antioxidants

4.4.1 Introduction

Coastal salt marshes represent ecosystems of great biodiversity and great ecological value (Gedan et al., 2010; Barbier et al., 2011; Gardner et al., 2015; Mitsch et al., 2015; Sutton-Grier & Sandifer, 2019; Wolanski et al., 2009). In the region of Valencia (E Spain), they often appear as depressions integrated into dune systems, with the most saline areas located in the centre and the least saline at the edges of the salt marsh. The distribution of the different plant species in these saline areas is mainly determined by their relative tolerance to salinity, so that the plant communities are installed in concentric rings, depending on the salinity of the soil – although other factors, such as competition between species, can contribute significantly to the distribution of plants in the salt marsh. The complex of salt marshes developed in the Albufera Natural Park territory, located a few kilometres south of the city of Valencia, is of special floristic and environmental interest (Ballester et al., 2003; Soria, 2006). It shelters the unique populations of endemic *Limonium albuferae*, only found in this area (Ferrer-Gallego et al., 2016), and *Limonium dufourii*, which is present also in a few other salt marshes outside the Natural Park (Crespo & Laguna, 1993; Laguna, 1998; Navarro et al., 2006; Aguilella et al., 2010). Initially, the area was covered by a multitude of depressions of different sizes that were distributed longitudinally throughout the shoreline. The area suffered a strong environmental impact during the 1960s and 1970s, when an urbanisation project was started, negatively affecting all existing habitats in this area (Benavent et al., 2004, 2014; Soria, 2006). The most affected were the littoral dunes, which were utterly destroyed, and their sand was used to fill the salt marshes, for extending the urbanised areas and infrastructures. These actions prevented natural flooding of these depressions during the wet season, altering their hydrological functioning and destroying their vegetation. Furthermore, in many salt marshes, non-native species were planted. This combination of aggressions caused an alteration or loss of the original specific characteristics of the habitat, as well as the reduction of the populations of numerous species of interest, and even the complete disappearance of some of them. In fact, nine species have become locally extinct (eight are included in the Valencian Catalogue of Endangered Flora Species, and four on the IUCN Red List). Fortunately, the situation has changed in recent decades, after the great ecological value of these habitats began to be recognised. Since the early 1980s, several actions were undertaken with the aim of regenerating the ecosystems of this area (Sanjaume & Pardo-Pascual, 2005, 2011; Vizcaíno, 2009; Zuccarini et al., 2016), starting with the geomorphological regeneration of many salt marshes, followed by recovery programmes of their native vegetation that are still in progress (Soria, 2006).

It is evident that effective programmes for the conservation and regeneration of salt marshes require an in-depth knowledge of the response mechanisms of salt marsh species to the environmental stress conditions to which they are subjected in their natural habitat. In the case of many halophytes with large areas of distribution, the main biochemical and physiological mechanisms of tolerance to drought, salinity or other abiotic stresses are known. However, similar studies have not been conducted, and

practically nothing is known about these mechanisms, in endemic, rare and/or threatened species.

The genus *Limonium* Mill. (Plumbaginaceae) is outstanding in the region of Valencia; of the 28 species present, 20 are Iberian endemics and 12 grow exclusively in this region (Crespo & Lledó, 1998; Laguna, 1998; Anonymous, 2009, 2013; Aguilella et al., 2010; Mateo & Crespo, 2014). One of the most threatened endemic species of *Limonium* of the Valencian territory is *L. dufourii* (Girard) Kuntze (Aguilella et al., 2010). Historically, this species was more widely distributed along the coast and salt marshes of Valencia and Castellón provinces (Crespo & Laguna, 1993; Crespo & Lledó, 1998). Today, it is represented only by five natural populations restricted to small coastal areas in the provinces of Castellón (Torreblanca) and Valencia: Marjal dels Moros (with three populations), El Saler (Albufera Natural Park) and Cullera (Aguilella et al., 2010; Laguna et al., 1994; Laguna, 1998). Most of these populations have a very low number of individuals, and molecular analyses show that substantial genetic variability and differentiation exist within and between populations (Palacios & González-Candelas, 1997; Palacios et al., 1999). *Limonium dufourii* is a triploid species ($2n = 3x = 27$), with obligate apomictic reproduction and an incompatible pollen-stigma combination (Baker, 1966; Erben, 1993; Palacios et al., 1999). It is a perennial halophyte, a rosulate, hemicryptophyte, densely hairy species, with racemose inflorescences (Erben, 1993; Crespo & Lledó, 1998). The flowering time is from June to September, and the fruiting time is from July to October. This species grows on sea-cliffs in *Crithmo-Staticetea* Br.-Bl. in Br.-Bl., Roussine & Nègre 1952 communities (e.g., the Cullera population) and salt marshes or sandy soils in *Juncetea maritimi* Br.-Bl. in Br.-Bl., Roussine & Nègre 1952 communities. It is associated with *Limonium girardianum* (Guss.) Fourr., *L. densissimum* (Pignatti) Pignatti, *L. virgatum* (Willd.) Fourr., *Juncus maritimus* Lam., *Bolboschoenus maritimus* (L.) Palla, *Scirpioides holoschoenus* (L.) Soják., in thermomediterranean and thermotemperate termotyps, under dry-subhumide ombrotype, according to the classification of Rivas-Martínez (2007). All the populations of *L. dufourii* are included in the Plant Micro-reserve network or Natural Parks (L'Albufera, Prat de Cabanes-Torreblanca) of the Valencian Community and also, additionally, in the European Union's Natura 2000 network of protected sites (as Site of Community Importance, SCI). The species is strictly protected in the Valencian region at the highest legal category "In danger of extinction", included in the Valencian Catalogue of Threatened Plant Species (Anonymous, 2009, 2013; Aguilella et al., 2010).

Limonium albuferae P.P. Ferrer *et al.* is known only from a small site in the Albufera Natural Park (Racó de l'Olla). The population inhabits the saline and sandy soils in *Juncetea maritimi* communities (*Junco maritimi-Caricetum extensae* Géhu 1976), and is associated to, for example, *L. girardianum, L. narbonense* Mill., *L. virgatum, Juncus maritimus, Bolboschoenus maritimus, and Scirpioides holoschoenus* (Ferrer-Gallego et al., 2016). At the beginning of 2020, 255 plants were counted, covering an area of about 160 m^2. Therefore, this species will be included in the category "In danger of extinction" in the next edition of the Valencian Catalog of Threatened Plant Species.

In a previous study on the two species (*L. dufourii* and *L. albuferae*), based on metabolites profiling and the analysis of ion transport and accumulation, *L. albuferae* was found to be more salt-tolerant than *L. dufourii*, mostly due to its ability to accumulate fructose as a specific osmolyte (González-Orenga et al., 2019a). However, there is no information on the responses of the two species to drought, which can also affect their natural populations, especially in the changing climatic conditions of the global warming scenario. The present work analyses responses to water stress and salinity not previously investigated in the two species, emphasising their antioxidant mechanisms. The study has been completed with the detailed analysis of the soil characteristics and plant communities at the location sites of the two species in the Albufera Natural Park.

4.4.2 Material and Methods

Area of Study

The field study was conducted in several salt marshes from the Albufera Natural Park, located in the "Devesa de l'Albufera" (Valencia province, Spain). The area belongs to Wetlands of International Importance of the Ramsar Convention since 1990, and in 1991 it was declared as Special Protection Area under the EU Directive on the Conservation of Wild Birds (79/409/EEC). It contains as well habitats and refuges of species included in the EU Habitats Directive (92/43/EEC), and it is also classified within the Special Protection Areas in the Mediterranean, according to the Geneva Protocol (Soria, 2006). The populations of the two species of *Limonium* (*L. dufourii* and *L. albuferae*) are located in small salt marshes, locally named 'malladas', which are inter-dune depressions, often inundated during the winter period.

Climatic Analysis

To establish a correlation with the evolution of the number of individuals in the censed populations, climate data were retrieved from SIAR, the Agroclimatic Information System for Irrigation (SIAR, 2020) of the Spanish Ministry of Agriculture, Fisheries and Food. Data on the mean, maximum and minimum temperatures, rainfall and reference evapotranspiration (ETo) were collected for the past 19 years, on a monthly basis, from the agroclimatological station Benifaio (Valencia province), located at 11 km from the area of study. For correlating the variation in the number of individual censed with climatic conditions the trimestrial variation of mean rainfall, evapotranspiration and temperatures (mean, minimal and maximal) were calculated.

Population Censuses

Since *L. dufourii* and *L. albuferae* have no genetic exchange between individuals of the same species, and their seed dispersal is carried out at a very short distance – mainly by ants or floating during the flooding of the saline basins – each group of plants colonising a small salt marsh was considered as one monitoring unit. In each monitoring unit, censuses were made following the methodology of the Spanish Atlas and Red Data Book of Vascular Plants (Iriondo et al., 2003, 2009), adapted for the monitoring of

endangered Valencian plant species by Navarro et al. (2010). Following this method, each census was carried out by counting all individuals – summing adults and young plants – and georeferencing them individually or by the centroid of small groups. A Garmin GPS was used, taking UTM coordinates (ETRS 89, zone 30). Censuses were made from late July to late August, coinciding with the blooming period, where both species of *Limonium* are easier to be detected.

For *L. dufourii*, each monitoring unit was established after a detailed tracking of each site for which there are references to the plant's old presence or where it was recently detected. Thus, five natural population monitoring units were established, referred to as Devesa A (monitored since 2004), B (since 2005), C (since 2005) and D (since 2006); a new E unit has been established in 2020, as a result of the tracking made for the present work. Additionally, Devesa 1 and 2 units were established for two new artificial populations, planted in winter 2013-2014. Although the species vanished in monitoring units A, C and D in 2008-2009, their sites have been revisited every year, corroborating the absence of the species.

In the case of *L. albuferae*, the descriptive article of the species (Ferrer-Gallego et al., 2016) considered its presence in two sites in Devesa de l'Albufera, but today the species only exists in one place, known as Racó de l' Olla.

Vegetation Analysis

Vegetation inventories were carried out in the areas where the populations of the two species are located in the territory of study. As for the population censuses, the coordinates were obtained with the Garmin GPS in UTM coordinates (ETRS 89, zone 30); they have not necessarily been taken in the centre of the inventory to avoid trampling, which is aggravated when the soil is waterlogged. The phytosociological method was used to note the proportions in which the species appear (Braun Blanquet, 1932). Nomenclature of the taxa follows EuroMed (2020) and the syntaxonomic nomenclature, according to Rivas-Martínez et al. (2001, 2002a, 2002b). Three measurements of soil electrical conductivity (EC, dS m^{-1}) were performed with a WET sensor (Delta Devices, Cambridge, England) at 10 cm depth in each inventory. The inventories were carried out mainly from mid-June to mid-November 2019, which is an ideal time to cause the least possible disturbance to the nesting fauna (Ballester et al., 2003).

Soil Characteristics

Soil sampling was performed in July 2019. Samples were taken at 0-10 cm and at 10-20 cm depth in the vicinity of specimens of the two species, from one salt marsh where the present unique population of *L. albuferae* is located, and from three salt marshes for *L. dufourii*. From each salt marsh, three soil samples were taken (n = 3). The samples were air-dried at room temperature and then were crushed with a roller to break aggregates and passed through a 2-mm sieve. Analyses were performed on fine soil (diameter < 2 mm). Soil texture was analysed by the hydrometer method (Bouyoucos, 1962). Organic

matter was determined by the Walkley & Black method (1934) and carbonates by the technique of Bernard calcimeter (Loeppert & Suarez, 1996). The following parameters were analysed in a saturation extract: pH, electrical conductivity (EC), Cl^-, Na^+, K^+, Ca^{2+}, and Mg^{2+}. A Crison pH-meter Basic 20 and a Crison conductivity-meter Basic 30 (Crison, Barcelona, Spain) were used to measure pH and EC, respectively. Sodium and potassium were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), chlorides were measured in a MKII Chloride Analyzer 92 6 (Sherwood, Inc., Cambridge, UK). Divalent cations (Ca^{2+} and Mg^{2+}) were measured with an atomic absorption spectrometer SpectrA 220 (Varian, Inc., CA, USA).

Plant Growth under Greenhouse Conditions

Seeds of *L. albuferae* and *L. dufourii* provided by the Centre for Forest Research and Experimentation of the Valencian Region (CIEF, Valencia), were sown on a mixture of commercial peat and vermiculite (3:1) and watered with Hoagland nutrient solution (Hoagland, 1950). After three weeks, plantlets were transferred to individual 1 L pots placed in plastic trays, with five pots per tray, and watered one further week with Hoagland solution. One week later, when plants had achieved a sufficient size, stress treatments were started. Plants subjected to the salt treatments were watered with aqueous solutions of 200, 400, 600, and 800 mM NaCl, those for the controls with distilled water, and those for the water stress treatment were not irrigated at all. Watering was performed by adding 1 L of the corresponding salt solution or water to each tray every five days. Five replicas (individual plants) were used per species and per treatment. All experiments were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 h of light), temperature of 23 °C during the day and 17 °C at night, and 50–80% relative humidity.

Moisture and EC in the pots were measured with the WET sensor (Delta Devices, Cambridge, England) at the beginning and during the treatments, as long as it was permitted by the device's limitations. At the end of the treatments, pot substrates were collected and moisture and EC were determined in the laboratory. Moisture was determined by the gravimetric method. The samples were dried in an oven at 105 °C until they reached constant weight and then weighed again to calculate the water content as $WC\% = [(FW-DW)/FW] \times 100$, where FW and DW are the fresh and dry weights of the substrate samples.

For EC measurements, samples were collected from each pot, air-dried and then passed through a 2 mm sieve. A soil: water suspension (1: 5) was prepared in deionised water and mixed for one hour at 600 rpm, and 21 °C before being filtered. Electric conductivity was measured with a Crison 522 conductivity-meter and expressed in dS m^{-1}.

After one month of treatment, the aerial parts and the roots of the plants were harvested and weighed separately, and several growth parameters were measured: Fresh weight of leaves (FWL) and roots (FWR), water content percentage of leaves (WCL) and roots (WCR), and leaf number (LN). Water content percentage in leaves was calculated as indicated above for the soil samples, except that the plant material was dried at 65 °C.

Photosynthetic Pigments

Chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids (Caro) were quantified according to the method reported by Lichtenthaler & Wellburn (1983), from 0.1g of fresh leaves ground in 3 mL of ice-cold 80 % acetone, mixed by vortexing and then centrifuged. The absorbance of the supernatant was measured at 663, 646 and 470 nm, and the concentration of each group of compounds was calculated according to equations previously described (Lichtenthaler & Wellburn, 1983). Pigment concentrations were expressed in mg g^{-1} DW.

Osmolytes

Proline (Pro) content was quantified using fresh leaf material, according to the ninhydrin-acetic acid method of Bates et al. (1973). Pro was extracted in 3% aqueous sulfosalicylic acid, the extract was mixed with acid ninhydrin solution, incubated for one h at 95°C, cooled on ice and then extracted with two volumes of toluene. The absorbance of the supernatant was read at 520 nm, using toluene as a blank. Pro concentration was expressed as μmol g^{-1} DW.

Total soluble sugars (TSS) were measured according to a previously published procedure (Dubois, 1956). Fresh leaf material was ground in liquid N_2 and extracted with 80% (v/v) methanol. After mixing in a rocker shaker for 24 h., the samples were centrifuged at 12,000 rpm for 10 min; supernatants were collected, appropriately diluted with water and supplemented with concentrated sulfuric acid and 5% phenol. After 20 min incubation at room temperature, the absorbance was measured at 490 nm. TSS concentrations were expressed as equivalents of glucose, used as the standard (mg eq. glucose g^{-1} DW).

Oxidative Stress Markers and Non-Enzymatic Antioxidants

Leaf hydrogen peroxide contents in both, control and salt-treated plants, were quantified as previously described (Loreto & Velikova, 2002). Fresh leaf material (0.05 g) was extracted with a 0.1% (w/v) trichloroacetic acid (TCA) solution, followed by centrifugation of the extract. The supernatant was thoroughly mixed with one volume of 10 mM potassium phosphate buffer (pH 7.0) and two volumes of 1 M potassium iodide. The absorbance of the sample was determined at 390 nm. Hydrogen peroxide concentrations were calculated against an H_2O_2 standard calibration curve and expressed as μmol g^{-1} DW.

Malondialdehyde (MDA), total phenolic compounds (TPC), and total flavonoids (TF) were quantified in the same methanol extracts of fresh leaf material used for TSS measurements. MDA was determined according to the method of Hodges et al. (1999), with some modifications (Taulavuori et al., 2001). The extracts were mixed with 0.5 % thiobarbituric acid (TBA) prepared in 20% TCA and then incubated at 95 °C for 20 min. After subtracting the non-specific absorbance at 440 and 600 nm, the MDA contents were calculated using the equation included in Taulavuori et al. (2001), based on the extinction

coefficient at 532 nm of the MDA-TBA adduct (155 mM^{-1} cm^{-1}). Control samples (extracts mixed with 20% TCA without TBA) were processed in parallel. The concentration of MDA was finally expressed as nmol g^{-1} DW.

TPC were quantified, according to Blainski et al. (2013), by reaction with the Folin–Ciocalteu reagent. The methanol extracts were mixed with sodium bicarbonate and the reagent, incubated at room temperature in the dark for 90 min and the absorbance was recorded at 765 nm. Gallic acid (GA) was used as standard, and the measured TPC concentrations were expressed as GA equivalents (mg eq. GA g^{-1} DW).

Total 'antioxidant flavonoids' (TF) were determined by a previously described method (Zhishen et al., 1999), based on the nitration of aromatic rings containing a catechol group, by incubation with $NaNO_2$, followed by reaction with $AlCl_3$ at alkaline pH. After the reaction, the absorbance of the samples was determined at 510 nm, and TF contents were expressed as equivalents of the catechin standard (mg eq. C g^{-1} DW).

Antioxidant Enzymatic Activity

Antioxidant enzyme activities were determined, at room temperature (25 °C), in crude protein extracts prepared from fresh plant material as described by Gil et al. (2014). Samples were ground in the presence of liquid N_2 and then mixed with extraction buffer [20 mM Hepes, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.2% (w/v) polyvinylpyrrolidone, 0.2 % (w/v) polyvinylpolypyrrolidone and 5 % (v/v) glycerol]. A 1/10 volume of 'high salt buffer' (225 mM Hepes, pH 7.5, 1.5 M KCl and 22.5 mM $MgCl_2$) was added to each sample, and the homogenates were centrifuged for 20 min at 20,000 *rpm* and 4 °C. Supernatants were collected, concentrated in U-Tube TM concentrators (Novagen, Madison, WI, USA), and centrifuged to remove precipitated material. The final samples (referred to as 'protein extracts') were divided into aliquots, flash-frozen in liquid N_2 and stored at -75 ºC until used for enzyme assays. Protein concentration in the extracts was determined by the Bradford's (1976) method, using the Bio-Rad commercial reagent and bovine serum albumin (BSA) as the standard.

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

Catalase (CAT) activity was determined, according to Aebi (1984), following the decrease in absorbance at 240 nm due to the consumption of H_2O_2 added to the protein extracts. One CAT unit was defined as the amount of enzyme that will decompose one mmol of H_2O_2 per minute at 25 °C.

Ascorbate peroxidase (APX) activity was determined as described by Nakano & Asada (1981), by measuring the decrease in absorbance at 290 nm, which accompanies ascorbate oxidation as the reaction progresses. One APX unit was defined as the amount of enzyme required to consume one mmol of ascorbate per minute, at 25 °C.

Glutathione reductase (GR) activity was determined according to Connell & Mullet (1986), following the oxidation of NADPH [the cofactor in the GR-catalysed reduction of oxidised glutathione (GSSG)] by the decrease in absorbance at 340 nm. One GR unit was defined as the amount of enzyme that will oxidise one mmol of NADPH per minute at 25 °C.

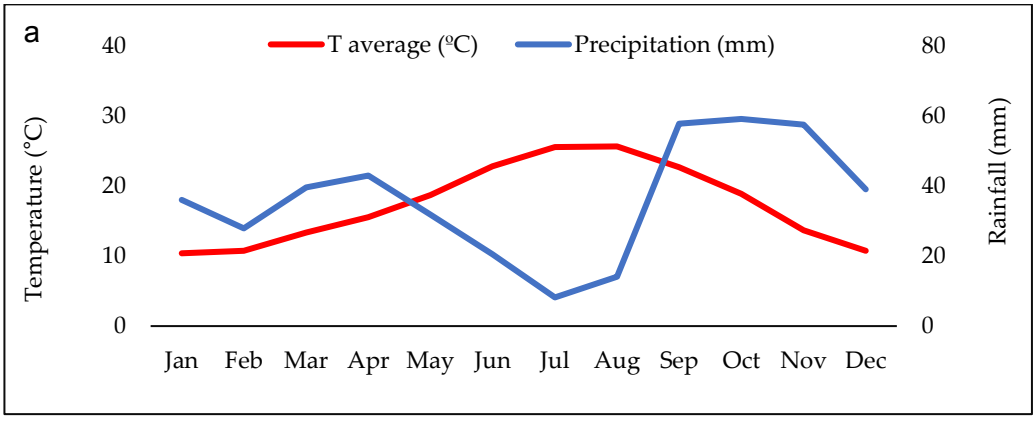
Statistical Analysis

Data were analysed using the programme Statgraphics Centurion XVII (Statgraphics Technologies, The Plains, VA, USA). All mean values throughout the text are based on five biological replicates. Significant differences between treatments were tested by one-way analysis of variance (ANOVA) at the 95% confidence level, and post hoc comparisons were made using the Tukey's HSD test at $p < 0.05$. A two-way analysis of variance (ANOVA) was performed for all traits analysed to check the interaction between the species and the treatments. A principal component analysis (PCA) was used to check the similarity in the responses to water and salt stress between the two species.

4.4.3 Results

Climatic Analysis

As it can be observed in the climatic diagram calculated for the period 2001-2019 (Figure 1a), there is a strong water deficit in summer in the area of study, which belongs to the thermo-Mediterranean climate belt, specific for coastal and low-altitude zones, according to the Worldwide Bioclimatic Classification System (1996-2020). The evapotranspiration surpasses the rainfall amount in all the years analysed (Figure 1 b).



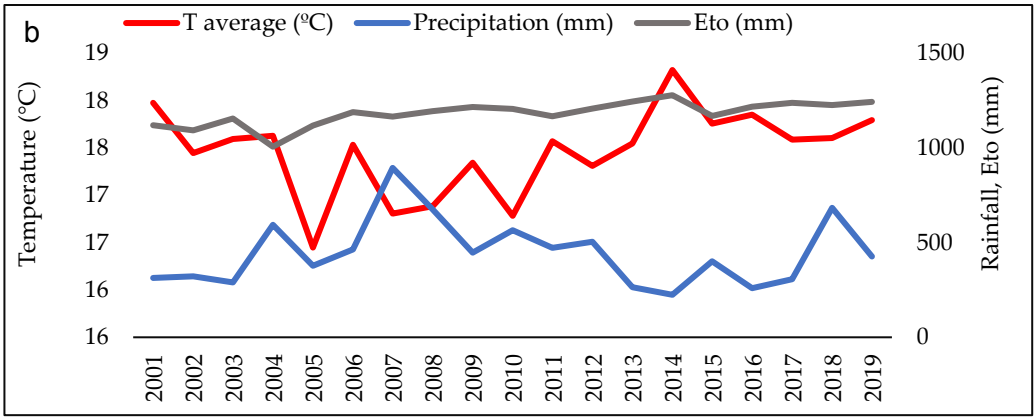


Figure 1. Climatic characteristics in the area of study. Climatic diagram calculated for the period 2001-2019 (a); Evolution of mean temperature, rainfall and evapotranspiration in the study area (b). Data from the meteorological station of Beinfaio (Valencia), located near the site of the field study, obtained from SIAR (2020).

Evapotranspiration did not vary much during the last two decades, although a slight increase can be noticed in the last few years. On the contrary, both mean temperatures and evapotranspiration curves showed a substantial variation from one year to another.

Trimestrial variation of the main climatic parameters (mean, maximal and minimal temperatures, rainfall and evapotranspiration) is summarised in Table 1. A strong variation of the trimestrial rainfall was detected, with minimal values in the third trimester and maximal in the fourth, coinciding with the general Mediterranean climate pattern, characterised by dry summers and rainfall mainly in autumn.

Table 1. Trimestrial variation of climatic conditions from 2005 to present. Rainfall and evapotranspiration values represent the sum of each trimester, whereas temperatures are means of the same period.

		Rainfall				Evapotranspiration (mm)				Mean Temperature (°C)				Mean Maximum Temperature (°C)				Mean Minimum Temperature (°C)			
Year	No of plants	R1	R2	R 3	R 4	Eto 1	Eto 2	Eto 3	Eto 4	Mean T 1	Mean T2	Mean T 3	Mean T 4	T max 1	T max 2	T max 3	T max 4	T min 1	T min 2	T min 3	T min 4
2005	13	77.2	119.6	73.0	107.6	173.8	411.7	393.0	140.1	9.2	19.2	24.0	13.4	24.7	32.1	36.3	25.0	-4.5	7.8	12.5	2.3
2006	15	158.8	83.8	32.0	189.8	196.7	376.3	440.8	175.7	11.0	19.2	24.7	15.2	23.3	31.4	34.3	27.1	-1.6	9.1	14.9	5.3
2007	9	218.6	107.4	208.4	360.0	207.0	408.2	413.7	142.0	11.9	19.0	23.6	12.7	24.9	32.8	35.8	24.9	1.8	8.1	13.2	0.5
2008	12	84.6	191.0	76.4	322.4	208.1	411.0	428.1	147.0	11.9	18.7	24.3	12.7	24.6	32.1	36.9	24.2	-0.3	8.3	14.3	1.3
2009	34	146.0	11.8	127.0	161.6	201.2	430.1	399.3	187.8	10.7	19.0	24.1	15.5	22.8	31.3	37.2	28.1	-0.2	8.4	14.6	3.1
2010	37	197.2	131.8	114.0	122.0	185.4	408.4	442.8	172.0	10.9	18.3	24.9	13.1	23.1	32.0	37.6	26.1	-0.4	7.2	15.0	0.4
2011	44	142.2	95.4	53.0	179.8	184.9	398.9	438.2	150.7	10.9	19.5	24.9	14.9	24.2	33.4	37.7	26.5	-1.8	9.1	15.4	4.7
2012	50	127.0	40.0	150.7	186.1	194.0	451.1	414.4	159.1	9.8	20.0	24.8	14.6	25.2	32.9	35.9	27.8	-1.0	8.1	14.8	2.0
2013	45	124.5	82.5	20.3	36.5	250.7	401.6	409.6	183.5	12.7	17.9	24.7	14.9	24.3	31.4	35.5	28.5	1.3	6.6	14.9	2.1
2014	40	68.4	13.8	43.0	99.2	250.6	424.8	430.8	172.0	13.1	19.8	25.1	15.4	25.1	32.9	37.8	27.5	1.9	9.7	16.7	3.8
2015	241	85.2	59.3	130.7	126.1	206.9	420.7	402.8	138.7	11.6	19.8	24.9	14.7	26.0	34.8	35.8	27.0	-0.7	9.1	16.0	4.0
2016	263	22.5	49.1	73.3	114.7	247.6	411.9	418.3	140.7	13.2	18.9	24.5	14.8	25.1	31.1	36.7	25.7	-0.9	6.3	15.3	5.2
2017	133	177.5	52.3	41.1	36.4	202.5	440.6	415.1	180.6	12.0	19.8	24.4	14.2	25.0	32.4	36.3	26.2	1.5	9.0	13.9	2.7
2018	374	94.2	147.3	70.5	372.0	227.5	423.8	433.1	141.3	11.8	19.0	25.4	14.3	25.9	30.2	35.9	26.3	0.2	8.7	15.7	2.8
2019	94	21.5	149.1	112.0	144.4	201.0	401.7	429.3	211.9	11.5	18.5	25.3	16.0	25.5	32.8	37.9	29.5	0.5	7.5	16.2	4.8

Population Censuses

Results of censuses for the two species are shown in Table 2. During the period of population monitoring (2004-2020) three of the four previously known populations of *L. dufourii* disappeared: A (in 2007-2008), C (in 2008-2009) and D (in 2007-2008).

Table 2. Censuses of the *L. albuferae* (LA) and *L. dufourii* (LD) populations, performed in the Albufera Natural Park. MU = monitoring units, - not censed.

X	731022	731395	731469	732091	730987	731592	731386	730217	730543
Y	4357733	4358286	4358185	4356743	4357713	4357785	4358355	4362094	4361434
Species	LA	LD	LD	LD	LD	LD	LD	LD	LD
MU	Racó de l'Olla 1	Devesa B	Devesa E	Devesa 2	Racó de l'Olla	Devesa A	Devesa C	Devesa D	Devesa 1
2004						10			
2005		13				10	28		
2006		15				7	31	21	
2007		9				3	98	11	
2008		12				0	31	0	
2009		34				0	0	0	
2010		37				0	0	0	
2011		44				-	0	0	
2012		50				0	0	-	
2013		45				0	0	-	
2014		40		328		0	0	0	170
2015		241		648		-	0	0	175
2016		263		1822		0	0	0	89
2017	238	133		120	38	0	0	0	0
2018	243	374		809	38	-	0	-	11
2019	39	94		27	0	-	0	-	3
2020	255	77	17	3	0	0	0	0	17

The extinction of populations A, C and D may be closely related to the high rainfall amount recorded in 2007 and 2008 (see Table 1 and Figure 1b). The plants in these monitoring units lived in sunken or slightly elevated sites, which remained flooded or with very wet soils for long periods during the year. A similar pattern has been observed in another natural protected site, known as Marjal dels Moros (approx. 30 km north from Devesa de l'Albufera), where the populations of *L. dufourii* have decreased drastically when the saline basins have been artificially flooded to favour the migratory bird in danger of extinction *Marmaronetta angustirostris* (Marbled teal) (EL. AN & PPFG. pers. obs.)

A similar situation could occur for *L. dufourii* plants in the Devesa B monitoring unit, situated at a slightly higher place than the surrounding saline basins, unlike the previous ones. After several years with a low number (50 or less) of registered individuals, this monitoring unit experienced a notable increase between 2014 and 2015, from 40 to 241 specimens. This increase occurred after the succession of two very dry years, 2013 (only

263.8 mm) and 2014 (224.40 mm). Population levels remained high in subsequent years, with a minimum of 133 individuals (in 2017) and a maximum of 374 (in 2018), but after the intense rainfall recorded in 2018 (684.02 mm), the population showed a sharp decline again, with only 94 specimens registered in 2019.

The monitoring unit 'B' is the only one with enough number of censuses between 2005 and 2020 to allow an analysis of correlations with climatic factors. However, after performing the correlation analysis between the Devesa B population censuses and the climatic variables, using both the raw data and the censuses' logarithms, no clear relationship has been found between the number of specimens and the variables studied (Table 3). Data for *L. albuferae* are still insufficient to establish population trends and relate them to climatic variables since detailed censuses have only been carried out since 2017.

Table 3. Pearson coefficients (r) and their probability (p) found by correlating the number of *L. dufourii* individuals from the Devesa B population, censed from 2005 to 2019 and the trimestrial climatic variables: Rainfall (R), evapotranspiration (Eto), mean temperature (T), and mean of maximal temperatures (Tmax), as indicated in Table 1.

Climatic parameters	r	p
R1	-0.398298	0.1414
R2	0.0025	0.9931
R3	-0.0707	0.8023
R4	0.1397	0.6195
Eto1	0.3862	0.1551
Eto2	0.2412	0.3864
Eto3	0.0118	0.9668
Eto 4	0.3295	0.2304
T 1	0.3384	0.2173
T2	0.1032	0.7143
T 3	0.4598	0.0846
T 4	0.1895	0.4988
T max 1	0.600172	0.018*
T max 2	-0.2032	0.4676
T max 3	-0.1104	0.6952
T max 4	-0.0108	0.9695
T min 1	0.0811	0.7738

Vegetation Analysis

Vegetation inventories of plants communities were performed in 22 locations, corresponding to different salt marshes in the Albufera Natural Park. Each inventory was accompanied by the collection of soil data obtained with a portable sensor. Table 4 summarises the habitat characteristics of each site (the extension and coverage of the plant community), soil moisture and electrical conductivity, the list of species present in the community and their coverage on the Braun-Blanquet´s scale (Braun-Blanquet, 1932).

Table 4. Phytosociological inventories with *Limonium albuferae* and *L. dufourii* from the Albufera.

Relevé nº	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Area (m^2)	7	5	10	10	7	6	10	15	15	9	15	10	8	20	9	15	12	4	5	7	1.5	15
Coverage (%)	60	50	70	80	70	70	70	95	100	40	40	70	80	50	85	40	90	100	90	70	90	80
Conductivity EC dSm^{-1}	43.0	32.0	17.2	30.1	22.4	31.3	11.1	36.0	9.4	11.6	2.5	14.5	11.1	4.8	27.1	4.9	11.2	6.1	19.2	2.4	2.0	27.6
Species																						
Vegetation class																						
Salicornietea fruticosae																						
Limbarda crithmoides	1	2	1	1	1	1	3	2	3		+	2	3									
Limonium albuferae																**1**	**+**	**3**	**+**	**1**	**1**	**+**
Limonium dufourii	**3**	**2**	**+**	**+**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**+**	**+**							
Limonium girardianum										1	1			2								
Limonium narbonense															2	3	4	+	4	1	4	3
Limonium virgatum							+		2	2	1				1							
Sarcocornia fruticosa															2		1		2			4
Thero-Suaedetea																						
Salicornia ramosissima					1	4				1	1	+										
Suaeda spicata	3	3	2	4	3	1		+	+			3			3	1			1			1
Juncetea maritimi																						
Tripolium pannonicus									+								+					
Dorycnium gracile					+	+				+	+											
Elytrigia elongata										2	2	1								+		+
Juncus acutus							+							2	3	+	1	+	+	1	+	
Juncus maritimus							1	2	2			3	3	+	1							
Linum maritimum	1	+	1	1	+	1																
Plantago crassifolia							2		1					1		+	+	+				
Samolus valerandi					+	+							1									
Spartina patens							1		1					2			1	4				
Sporobolus pungens	+																					
Artemisietea vulgaris																						
Agrostis stolonifera							1		1							+	1	+		1		+
Ditrichia viscosa					+		2	+	3		1	1	2	2						2		+
Piptatherum miliaceum										1	+									1		
Molinio-Arrhenatheretea																						
Schoenus nigricans	1	1	+	1	1	1																

Scirpoides holoschoenus														+									
Sonchus maritimus	1	1	+	1	1	1								+									
Phragmito-Magnocaricetea																							
Phragmites australis subsp. *chrysanthus*									+		2			1	1		+	1	1	1			
Phragmites australis subsp. *australis*	1	2	3	2	2	1	1	4	2	+		+	1	2									
Polygono-Poetea annuae																							
Plantago coronopus										2	1							1		+			
Quercetea ilicis																							
Phyllirea angustifolia																				1	1	+	
Pistacia lentiscus							+			2	2		+	+				1		1	2	+	
Smilax aspera										1	1												
Rubia peregrina										+													
Asparagus acutifolius										+										+	+	+	
Stellarietea mediae																							
Lagurus ovatus															1	1	1	1					
Erigeron bonariensis										+													

Two species had a higher presence in some of the inventories, equal to 4 in the Braun-Blanquet scale: *Sarcocornia fruticosa*, a structural shrubby species of the Mediterranean salt marshes, but also *Spartina patens*, an invasive with an increasing presence in recent years in the area of study. The soil electrical conductivity measured by the WET sensor near the plants of the two studied species in all inventories was higher in case of *L. dufourii,* but it is necessary to take into consideration that *L. albuferae* was found in one unique location, thus this finding does not demonstrate a better salt tolerance of *L. dufourii.*

The assignment of phytosociological associations is difficult and not included in the objective of this study. Therefore, the different inventories were grouped according to their corresponding vegetation classes, which are summarised in Table 5, according to Rivas-Martínez et al. (2001, 2002a, 2002b).

Soil Characteristics

Soil samples, collected in summer of 2019 from the unique location of *L. albuferae* and three saltmarshes with *L. dufourii*, all located in the Albufera Natural Park, were analysed. From each location, three soil samples were taken in the vicinity of the plants at two depths, 0 – 10 cm and 10- 20 cm. Their physical and chemical properties are summarised in Table 6.

Table 6. Soil characteristics in the salt marshes with *Limonium albuferae* and *L. dufourii* in the Albufera Natural Park. Values represent means followed by SD (n = 3). CEC, cationic exchange capacity, EC, Electric conductivity in saturated paste. Same letters indicate homogenous groups according to the Tukey test ($p \leq 0.05$).

	0-10 cm depth		10-20 cm depth	
Parameter	*L. albuferae*	*L. dufourii*	*L. albuferae*	*L. dufourii*
Sand (%)	99.50 ± 0.07^{b}	92.69 ± 1.79^{a}	94.90 ± 0.61^{b}	91.11 ± 0.63^{a}
Silt (%)	0.35 ± 0.05^{a}	5.11 ± 1.25^{b}	3.57 ± 0.43^{ab}	6.23 ± 0.22^{b}
Clay (%)	0.15 ± 0.02^{a}	2.19 ± 0.53^{b}	1.53 ± 0.18^{a}	2.67 ± 0.19^{b}
Bulk density (g cm^{-3})	1.10 ± 0.07^{a}	1.30 ± 0.05^{a}	1.17 ± 0.09^{a}	1.31 ± 0.06^{a}
Porosity (%)	58.46 ± 2.73^{b}	51.00 ± 2.14^{aA}	55.80 ± 3.45^{a}	50.57 ± 3.73^{a}
Carbonates (%)	23.21 ± 0.64^{a}	22.07 ± 1.63^{a}	24.56 ± 1.56^{a}	26.05 ± 4.09^{a}
Organic Matter (%)	1.66 ± 0.38^{a}	0.97 ± 0.37^{a}	0.71 ± 0.08^{a}	0.61 ± 0.25^{a}
pH	7.86 ± 0.21^{a}	7.42 ± 0.18^{a}	7.87 ± 0.03^{a}	7.79 ± 0.14^{a}
EC (dS m^{-1})	12.75 ± 4.93^{a}	37.91 ± 6.24^{b}	10.33 ± 2.38^{a}	15.73 ± 1.98^{a}
Na^{+} (meq L^{-1})	97.87 ± 28.63^{a}	295.52 ± 51.05^{b}	74.02 ± 22.53^{a}	107.63 ± 12.95^{a}
K^{+} (meq L^{-1})	2.68 ± 0.61^{a}	7.26 ± 2.13^{b}	2.32 ± 0.36^{a}	2.78 ± 0.15^{a}
Cl^{-} (meq L^{-1})	54.85 ± 13.02^{a}	252.64 ± 49.15^{b}	44.51 ± 9.28^{a}	76.49 ± 15.89^{a}
Ca^{2+} (meq L^{-1})	11.04 ± 1.78^{a}	18.62 ± 1.86^{b}	11.24 ± 0.72^{a}	12.10 ± 0.76^{a}
Mg^{2+} (meq L^{-1})	8.80 ± 2.75^{a}	15.62 ± 5.37^{a}	7.24 ± 1.14^{a}	12.97 ± 5.78^{a}

All soils had a sandy texture. The percentage of sand represented the primary component, with low amounts of silt and clays. The soil pH is neutral, and the salinity in the superficial layer (0-10 cm) was higher than at 10-20 cm depth – which is the area explored by the roots of the plants –, especially in the areas with *L. dufourii*. When comparing the two species, higher EC values were obtained at the two depths for *L. dufourii*, confirming the measurement performed by the WET sensor in the natural habitats. The most abundant ion in the soil was Na^{+}, found at a higher concentration than that of Cl^{-}. The soil samples are also characterised by a high percentage of carbonates and divalent cations, Ca^{2+} and Mg^{2+}.

Plant Growth under Greenhouse Conditions

Substrate EC was measured with the WET Sensor at the beginning and after one week of treatment, but further measurements were not possible due to the high EC reached in salt treatments of 400-800 mM NaCl, which were beyond the capacity of the device. Therefore, the final EC was measured in an extract 1:5. EC in the pots gradually increased in parallel to the concentration applied, reaching values over 10-fold higher than in the control in those watered with 800 mM NaCl, for the two species (Figure 2a). Substrate moisture decreased drastically in the WS treatment already after one week, and even more after 17 days, in the two species; no further measurements were possible with the WET sensor. Thus, the final moisture determination was carried out using the gravimetric method. The results indicated a similar reduction in soil moisture in the two species at the end of the WS treatments (down to around 3%), whereas only a slight decrease was found in the presence of 400, 600 and 800 mM NaCl, with respect to the control and 200 mM NaCl treatments (Figure 2b).

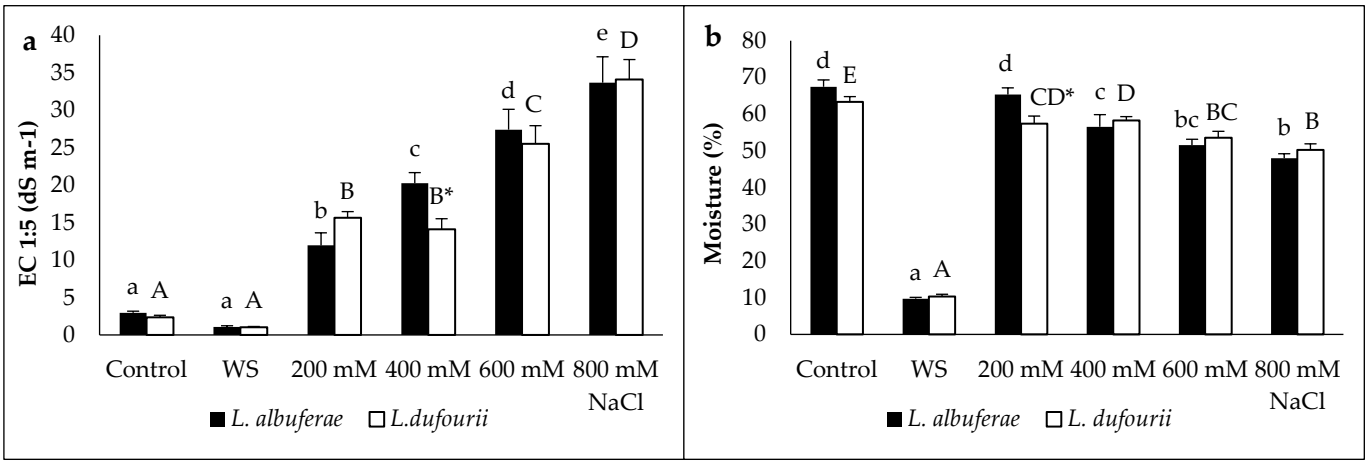


Figure 2. Substrate electrical conductivity in a soil: water suspension (1:5) (a), and moisture (b), measured in pots with *L. albuferae* or *L. dufourii* at the end of 30 d of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$).

Analysis of the Growth Parameters

Stress treatments had a strong effect on all analysed growth parameters, and also on the photosynthetic pigments contents, whereas the effect of species was significant only for the leaf fresh weight (LFW) and leaf water content (LWC), as well as for Chl a content. The interaction of the two factors was also significant only for leaf traits: number of leaves (Lno), leaf area (LA), mean fresh weight (LFW) and mean water content (LWC) (Table 7). Chlorophyll a showed a predominantly uncontrolled variation, as shown by the higher SS percentage of the residual.

Table 7. Two-way analysis of variance (ANOVA) of treatment, species and their interactions, for the measured growth parameters. Numbers represent percentages of the sum of squares (SS) at the 5% confidence level. RFW, root fresh weight; RWC, root water content; LA, leaf surface area; Lno, increment in the number of leaves; LFW, leaf fresh weight; LWC, leaf water content percentage; chlorophyll a, Chl a; chlorophyll b, Chl b; carotenoids, Caro.

Parameter	Treatment	Species	Interaction	Residual
RFW	57.48***	0.43	1.35	40.73
RWC	87.19***	0.12	1.51	11.17
Lno	56.79***	1.14	13.21**	28.81
LA	34.10***	1.31	46.12***	18.54
LFW	72.24***	2.84**	11.68***	13.22
LWC	88.98***	1.91***	5.21***	3.88
Chl a	37.45***	6.04**	5.54	50.96

Chl b	48.85***	2.52	5.50	43.11
Caro	45.13**	2.53	9.34	42.98

Figure 3 shows the variation of leaf traits according to the various applied treatments. Leaf area decreased mostly in *L. dufourii*, whereas *L. albuferae* showed only a smaller reduction under water stress (Figure 3a). The leaf number strongly decreased under the highest salt concentration, but the formation of new leaves was also reduced under water stress and salt treatments in the two species (Figure 3b). Leaf fresh weight suffered a reduction under water stress in both species, and under all salt concentrations in *L. dufourii*, but only under the higher salt concentrations in *L. albuferae* (Figure 3c). Leaf water content showed a similar variation in the two species being affected only by the water stress (Figure 3d).

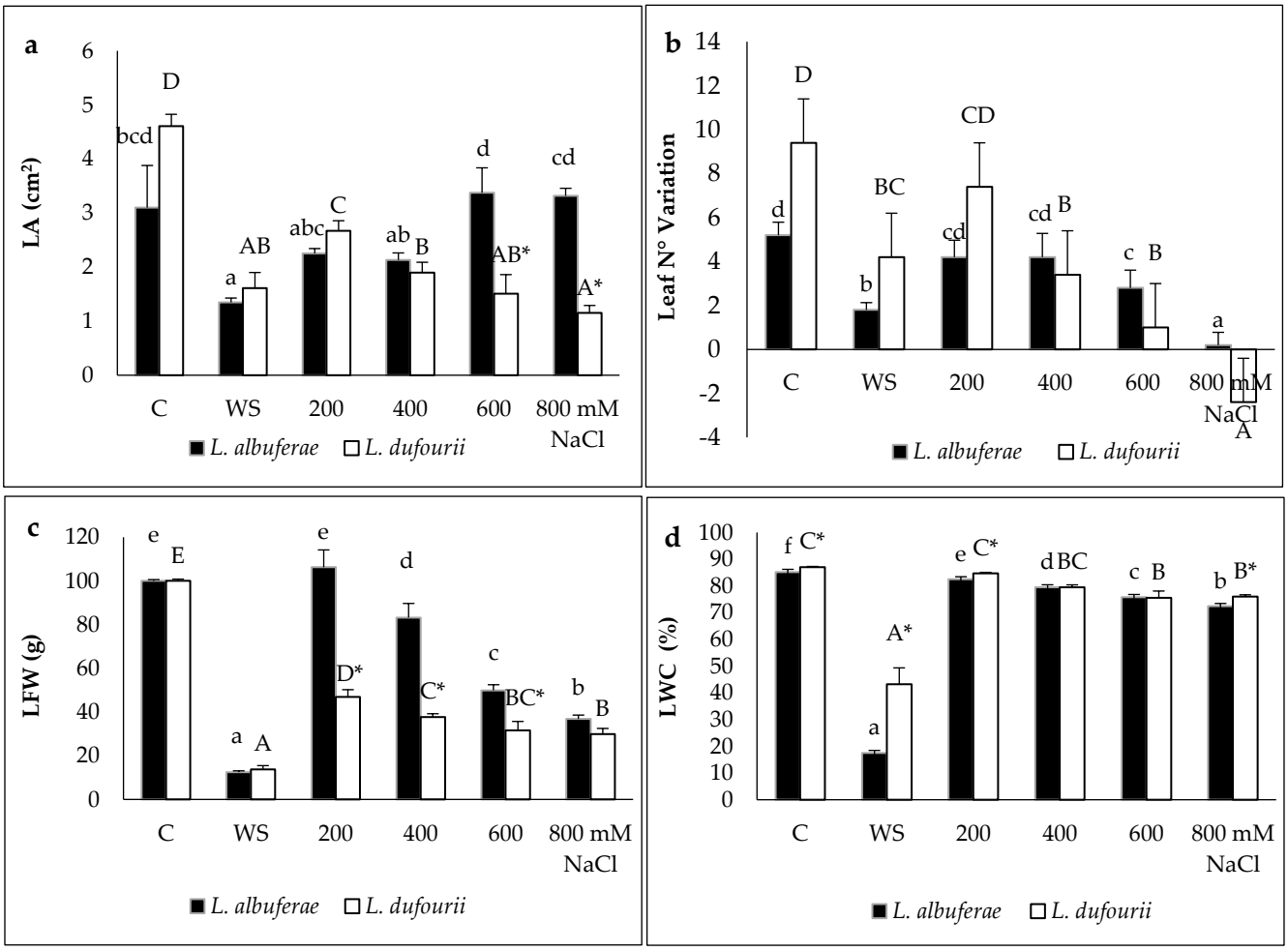


Figure 3. Growth parameters in the two *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Leaf area (a), variation in the number of leaves (b), leaf fresh weight (c) and leaf water content (d). Mean ± SE values are shown (n = 5). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p<0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$)

Osmolytes, Oxidative Stress Markers and Antioxidants Systems

Several biochemical parameters, such as osmolytes (proline and total soluble sugars), oxidative stress markers (MDA and H_2O_2), non-enzymatic antioxidants (total phenolic compounds and flavonoids) and the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase) were determined in leaves of plants sampled at the end of the water stress and salt treatments.

The two-way ANOVA showed that, except MDA, all other analysed biochemical traits were significantly influenced by the treatments and, with the exception of MDA and total phenolic compounds (TPC), also by the species. The interaction between the two factors was highly significant for total soluble sugars contents (TSS) and the antioxidant enzymes activities, but not significant for Pro and TPC (Table 8). The most significant contribution to variation of MDA, TPC and TF) is accounted for by the residual source of variation.

Table 8. Two-way analysis of variance (ANOVA) of treatment, species and their interactions for the biochemical parameters considered. Numbers represent percentages of the sum of squares at the 5% confidence level, Pro, proline; TSS, total soluble sugars; MDA, malondialdehyde, H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; GR glutathione reductase.

Parameter	Treatment	Species	Interaction	Residual
Pro	61.32***	21.35***	1.07	16.25
TSS	36.55***	6.97**	20.53***	36.03
MDA	6.09	5.72	18.28*	69.91
H_2O_2	57.95***	4.71**	9.22*	28.10
TPC	33.80***	0.004	8.48	57.71
TF	17.85*	10.86**	17.06*	54.21
SOD	39.90***	7.60***	24.90***	27.51
CAT	14.85***	50.01***	13.19***	21.95
GR	64.90***	4.27**	8.59**	22.30

As indicated above, the variation of proline (Pro) leaf contents followed a similar pattern in the two species, increasing in parallel to the external concentration of NaCl, and especially under water stress. The relative increase over control values in plants subjected to water deficit was 29-fold for *L. albuferae* and 2.7-fold for *L. dufourii*; the corresponding values in the presence of the highest salt concentration applied, 800 mM NaCl, were 17-fold and 1.7-fold, respectively. However, it should be noted that Pro concentrations differed in the two species, being significantly higher in *L. dufourii* than in *L. albuferae* in the control plants and under all tested stress conditions (Figure 4a).

Under water stress, total soluble sugars (TSS) significantly increased in *L. dufourii* and decreased in *L. albuferae*, although the levels in non-stressed, control plants were more

than four-fold higher in the latter species. Watering the plants with 800 mM NaCl induced a significant increase of TSS contents in both species. When comparing the two species, apart from the controls, significant differences in TSS levels were found under water deficit and moderate (200 mM) salinity conditions, but not in the presence of 400 mM or higher NaCl concentrations (Figure 4b).

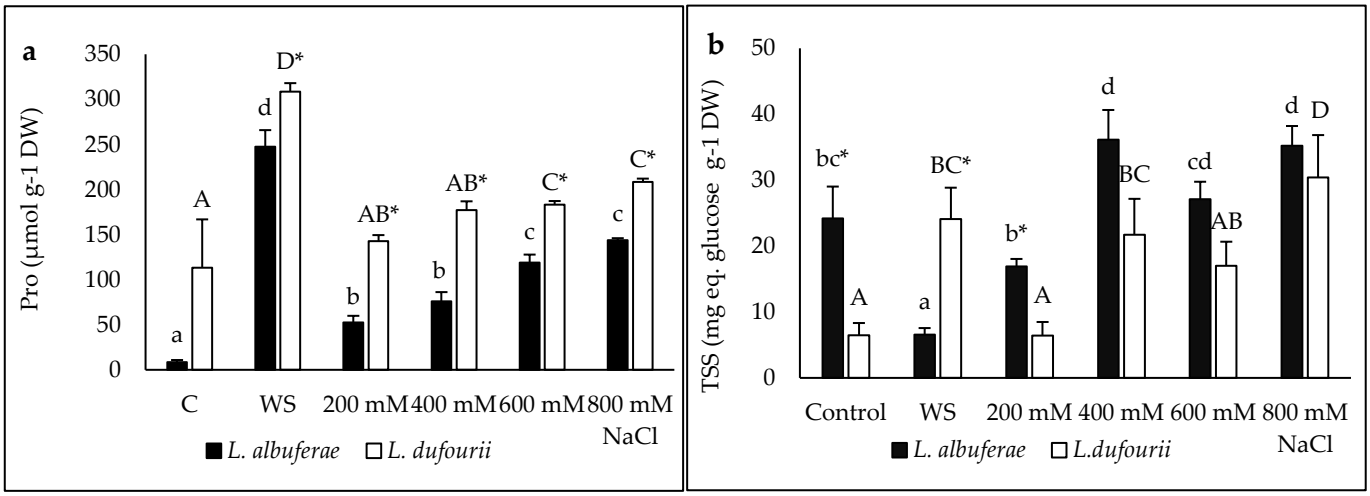


Figure 4. Proline (a) and total soluble sugars (b) concentrations in leaves of the two *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Mean ± SE values are shown (n = 5). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p<0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$).

Malondialdehyde (MDA) contents did not vary in *L. albuferae* under any of the applied stress treatments and showed a significant (albeit small) increment only in water-stressed *L. dufourii* plants (Figure 5a), whereas hydrogen peroxide decreased with respect to the corresponding controls in both species (Figure 5b). Total phenolic compounds (TPC) showed a similar pattern of variation in response to stress in the two species, with small, in most cases non-significant changes as compared to the controls (Figure 5c), whereas total flavonoids (TF) contents increased significantly only in plants of *L. albuferae* treated with 400 mM or higher NaCl concentrations (Figure 5d).

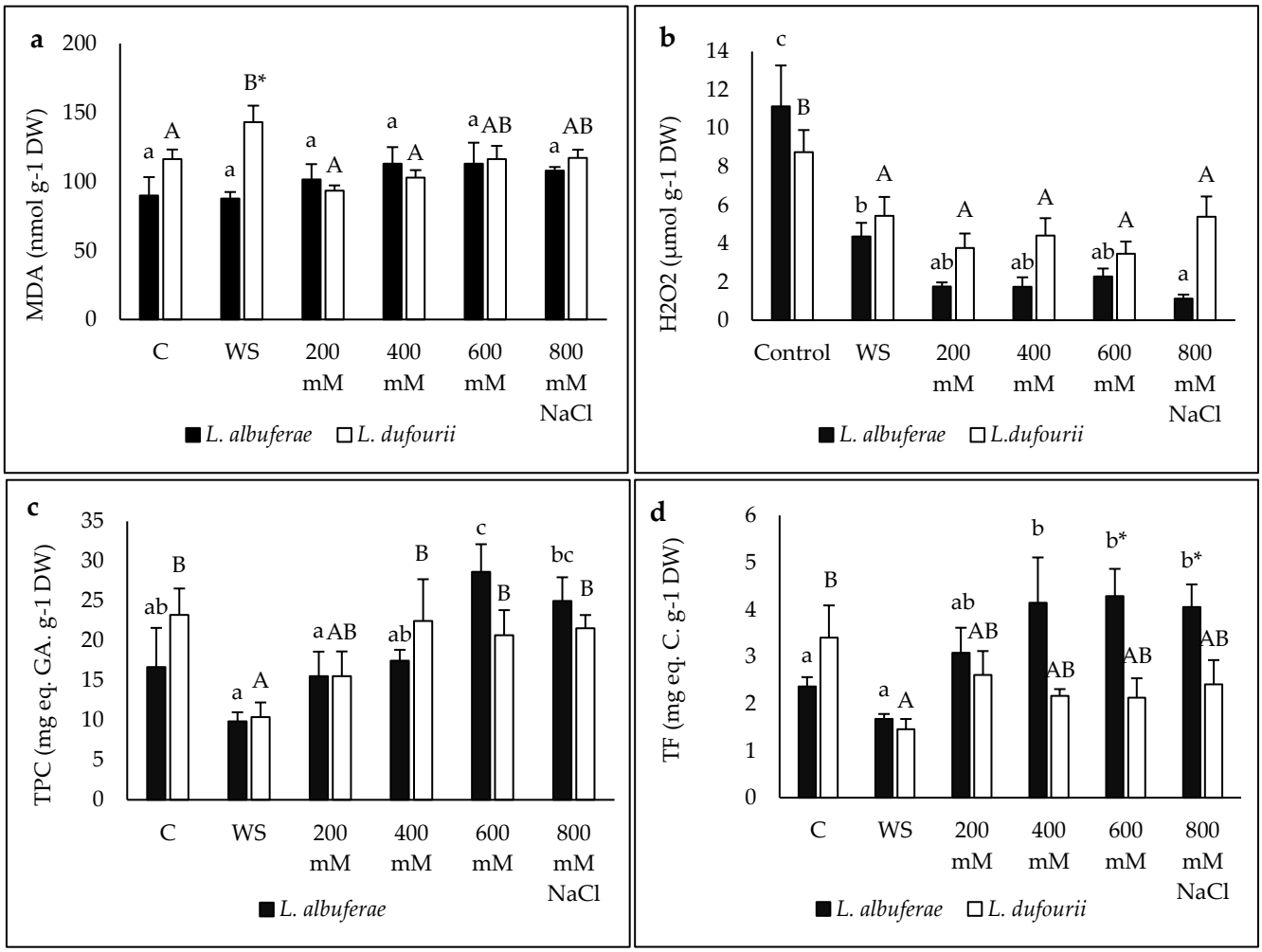


Figure 5. Malondialdehyde (a), hydrogen peroxide (b), total phenolic compounds (c), and total flavonoids (d) concentrations in leaves of the two *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Mean ± SE values are shown (n = 5). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$)

Activity of Antioxidant Enzymes

The specific activities of the three tested antioxidant enzymes (SOD, CAT, and GR) showed different qualitative and quantitative patterns of variation in the two species, in response to the applied stress treatment (Figure 6). In *L. albuferae*, compared to the basal levels in non-stressed plants, the activity of the three tested enzymes increased significantly at very high salinities (600-800 mM NaCl) but not at lower NaCl concentrations or under water deficit conditions (Figure 6 a, b, c). In *L. dufourii*, SOD increased significantly only in the presence of 800 mM NaCl and in water-stressed plants (Figure 6a), and GR also at the highest salt concentration tested, but not under water deficit stress (Figure 6c). In contrast, CAT activity did not show significant changes in

any of the treatments (Figure 6b). Comparing the two species, significant differences were found, higher activation of SOD and CAT under salt tress in *L. albuferae*, whereas, on the contrary, higher activation of SOD and GR under water stress in *L. dufourii*.

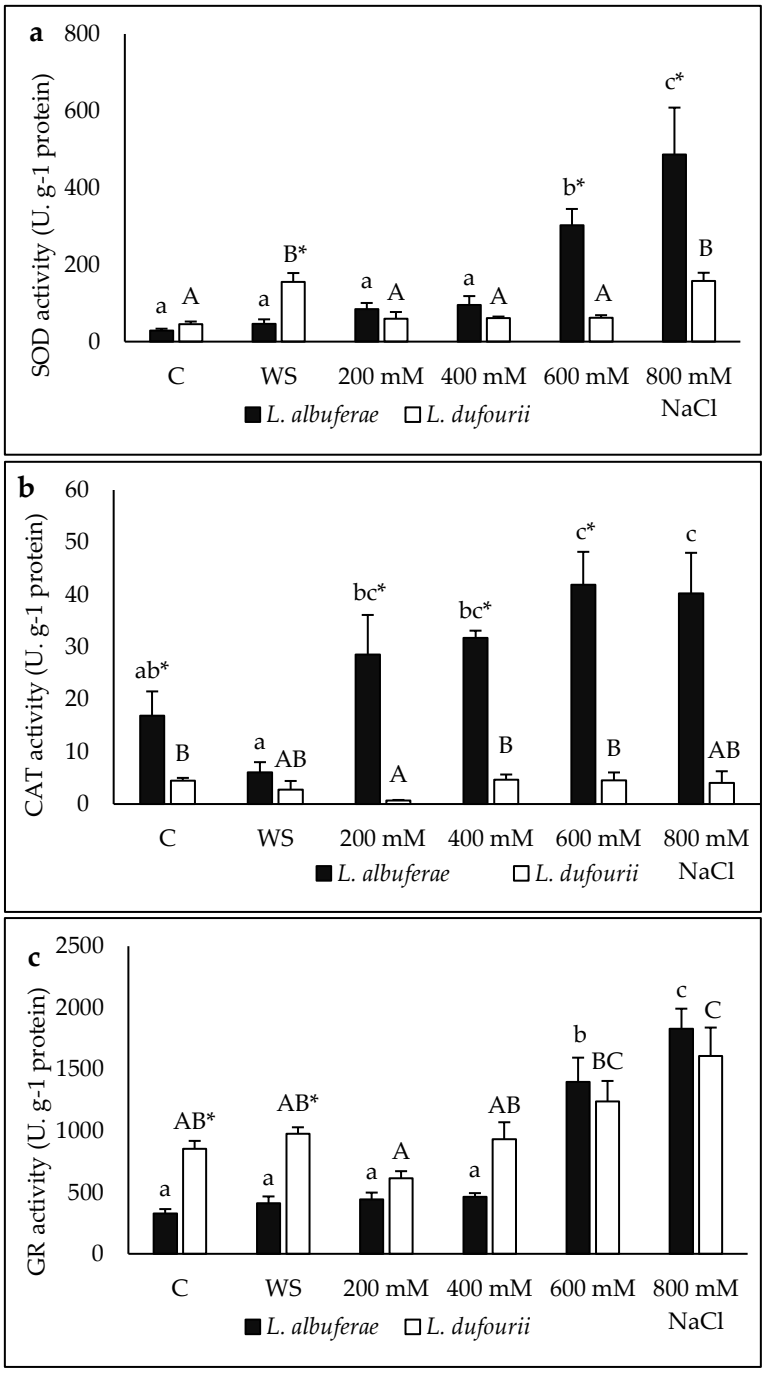


Figure 6. Activity of the antioxidant enzymes, superoxide dismutase (SOD) (a), catalase (CAT) (b) and glutathione reductase (GR) (c), in the leaves of the two studied *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Mean ± SE values are shown (n = 5). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$).

Principal Component Analysis

A principal component analysis (PCA) was also performed, including all growth parameters, osmolytes, oxidative stress markers, antioxidants and enzyme activities determined in control and stressed plants. Five components had an Eigenvalue above 1. The biplot of the two main principal components, which together explained 67.96% of total variability, is shown in Figure 7. The first component (X-axis), explaining 39.57% of variability, is related to the moisture of the substrate and, therefore, mostly to the water stress effect. The second component (Y-axis), explaining an additional 28.12% of variability, is related to the EC of the substrate and, as such, mostly to the salt treatments. Changes in substrate moisture correlated positively with changes in all growth parameters – especially the water content of root and leaves and leaf fresh weight – and photosynthetic pigments concentrations, which agrees with the inhibition of growth and the decrease in pigments contents observed under water stress (Figure 7a). On the other hand, a strong *negative* correlation was detected between substrate water content and Pro, reflecting the large increase in Pro levels induced by water deficit (Figure 7a). Regarding changes in the substrate EC, strong positive correlations were found with the antioxidant systems, especially with the activity of the SOD, CAT and GR enzymes, which increased with the salt treatments, at least at high salinity (Figure 7a). The PCA also showed a clear separation of control, water stress and salt treatments but not of the two species, which responded in a similar manner to each applied stress treatment (Figure 7b).

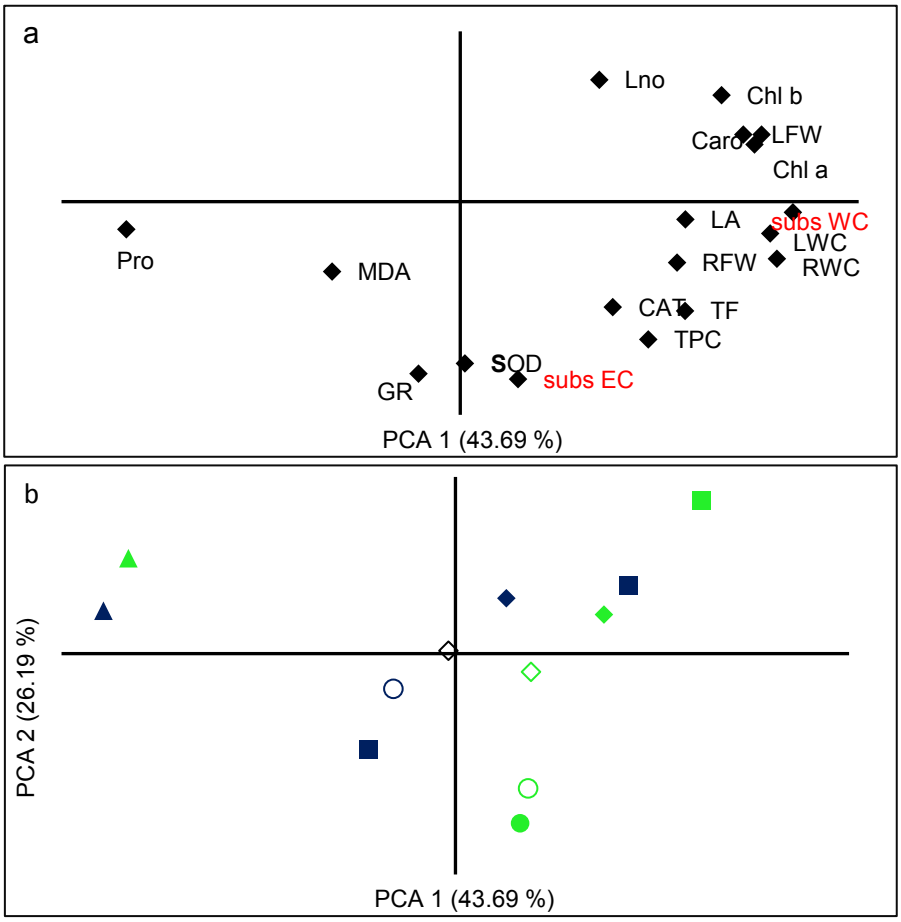


Figure 7. Principal Component Analysis (PCA). Loading plot of the principal component analysis performed with the analysed traits (a): growth parameters, osmolytes, oxidative stress markers and non-enzymatic antioxidants levels, and antioxidant enzyme activities, in correlation to substrate moisture and EC. Scatter plot of the PCA scores (b): *L. albuferae* (green) and *L. dufourii* (blue) plants, grown under water stress (triangles), and 200 (full rhombs), 400 (empty rhombs), 600 (full circles), and 800 (open circles) mM NaCl, versus the corresponding non-stressed controls (squares). Each symbol corresponds to the mean of the analysed plants per species and treatment (n = 5). Abbreviations: RFW, root fresh weight; RWC, root water content; LA, leaf surface area; Lno, increment in the number of leaves; LFW, leaf fresh weight; LWC, leaf water content; Chl a, chlorophyll a; Chl b, chlorophyll b; Caro, carotenoids; Pro, proline; TSS, total soluble sugars; MDA, malondialdehyde, H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase.

4.4.4 Discussion

As stated in the introduction, the two studied *Limonium* species are extremely interesting from the conservationist perspective. Both are endemic, with a small area of distribution in Eastern Spain and highly threatened due to the scarcity of their populations (a single one was known for *L. albuferae*), and the large fluctuations in the number of their individuals. One of the *L. dufourii* populations, surveyed in successive censuses, was analysed in an attempt to correlate these fluctuations in population size with climatic factors; however, no statistically significant correlation was found. Yet, the highest number of individuals were registered in drier years, when flooding of the salt marshes did not occur or was very brief; conversely, the population declined after a year with intense rainfall. It should be taken into account that in this area of Eastern Spain, the highest concentration of precipitation occurs in autumn and, therefore, its effects on the censuses of *Limonium* species are detected when they are carried out in the summer of the following year. The possible effects of climatic conditions on the number of individuals of *L. albuferae* could not be assessed, as the species has been described only recently (Ferrer-Gallego et al. 2016). In 2019, an apparent decrease in the population of *L. albuferae* was observed; only 39 specimens were initially counted, which could be related to the intense colonisation of this site by the invasive species *Spartina patens* (Aiton) Muhl. (see González-Orenga et al., 2019a). However, after manual elimination of the invasive plants, 255 individuals of *L. albuferae* were censed in 2020. Furthermore, this site (Racó de l'Olla) is the closest to the Albufera lake of all monitoring units of both species. This aspect is relevant because, starting with winter 2016-2017, the water level of Albufera lake has been artificially modified to favour rice cultivation in the fields surrounding a large part of the lake's shores. The effect of this artificial regulation on the water table of the closest salt marshes is still unknown.

The soil salinities in the salt marshes where the two species were found, were moderate, and in the case of *L. albuferae* significantly lower than those registered for the less salt-tolerant *L. dufourii*. Moreover, the salinity of the natural habitat of *L. albuferae* was well below the limit of tolerance of the species established in the greenhouse experiments. However, due to the extreme scarcity of this species, represented by a single population, the soil analyses were performed from only one area and are not conclusive for its ecological characterisation. Definitely, the peculiar rarity of *L. albuferae* is not related to edaphic conditions but most likely to evolutionary factors. The species probably

appeared recently by hybridisation, introgression or apomixis, which are very active in this genus, with many new and evolving species in the Iberian Peninsula (Cortinhas et al., 2015). It is worth mentioning that, during the preparation of this manuscript, a new population of *L. albuferae* has been discovered in an area strictly protected for bird nesting and for a long time not visited in floristic campaigns. Plants of *Triglochin barrelieri* Loisel., a Cyperaceae reported as extinct in the Natural Park of Albufera fifty years ago (Mansanet, 1979), were also found in this site.

Regarding the analysed soil parameters, Na^+ and Cl^- contents in samples from 0-10 cm depth were 3 and 4.5-fold higher, respectively, in the areas of *L. dufourii* than in that of *L. albuferae* at the same depth. These differences explain the higher mean EC also detected with the WET sensor in the areas where *L. dufourii* was present. The same pattern was found for K^+, Ca^{2+} and Mg^{2+} soil concentrations, although the differences between the areas of the two species were not as marked as for Na^+ and Cl^-. Other soil properties relevant to plants' life, such as texture or pH, were similar in all soil samples.

This multidisciplinary study included a phytosociological analysis; however, ascribing the inventories to associations was not possible, for several reasons. The specimens of the two species are often located in areas that have been extensively altered and have suffered a geomorphological restoration. The vegetation dynamics is very rapid, subjected to flooding and, therefore, to changes in salinity. This triggers the advance and retreat in a short time of different species, as is the case of the two species of *Phragmites* present in the area. The rainfall regime has been very variable in recent years, also greatly altering the plant communities. Moreover, some of the specimens were located in contact areas between different types of vegetation, and there are no previously recognised associations in the area for these *Limonium* species. Nevertheless, the performance of phytosociological inventories indicated the presence of similar species, typical halophytes of the vegetation classes *Salicornietea fruticosae*, *Thero-Suaedetea* and *Juncetea maritimae*, in the areas of the two *Limonium* species. The study also revealed the abundant presence in some inventories of the invasive species *Spartina patens*, which was recently reported as a major threat for native halophytes in this area (Martínez-Fort & Donat-Torres, 2020). *Spartina patens* appears to pose a severe risk also to the two endemic *Limonium* species, as the salt marshes where *L. dufourii* disappeared (Devesa A, C, and D) are completely invaded by this species. In the remaining sites, the large yearly variation in the number of individuals is related with genetic factors of *L. dufourii*, which shows very different flowering patterns, occasionally behaving as annual or monocarpic perennial (plants die after the first reproductive stage), or flowering every year. Also, it should be noticed the high frequency of *Dittrichia viscosa* (L.) Greuter, regarded as a native invasive species, extremely competitive at low and moderate salinities in salt marshes of the region (Al Hassan et al., 2016).

Salt marsh ecosystems are highly dynamic, characterised by large variations in the salinity of the soil at the temporal and spatial scales, as reported in previous studies performed on the territory of the Albufera Natural Park (Boscaiu et al., 2013; Gil et al., 2014; González-Orenga et al., 2020). Therefore, reintroduction or reinforcement

programmes for endemic and/or rare salt marsh species should also consider information on their limits of tolerance to stressful environmental factors. In Mediterranean salt marshes, a general increase in average temperatures and short-term 'heatwaves', due to climate change, will lead to increased evapotranspiration. Consequently, drought and soil salinity will also intensify, inflicting greater stress on plants and potentially causing the dieback of those less tolerant (Touchette et al., 2019).

The analysis of growth inhibition in response to the applied stress treatments indicated that the two species were mostly affected by water stress. In both, the strongest reduction in the most relevant growth parameters, leaf fresh weight and water content, was found in plants that were subjected to one month of water deficit, especially those of *L. albuferae*. This indicated that *L. albuferae* is much more susceptible to drought than other *Limonium* species growing in the study area, which were the subject of previous work (González-Orenga et al., 2019b). On the contrary, the salt-induced changes in growth parameters suggested that *L. dufourii* is more sensitive to high soil salinity than *L. albuferae*. Growth reduction under salt stress is a general trait in glycophytes but also in many halophytes (Flowers et al., 1986; Flowers & Colmer, 2008). Only in some dicotyledonous halophytes, especially in those more salt-tolerant, low and moderate concentrations of NaCl stimulate growth, as we have observed in *L. albuferae*, in which foliar fresh weight was slightly higher in the presence of 200 mM NaCl than in control plants. Stimulation of growth under salinity conditions has been reported in several species of the genus *Limonium*, such as *L. delicatulum* (Girard) Kuntze (Souid, 2016), or *L. girardianum* and *L. virgatum* (Al Hassan et al., 2017). Contrary to the strong dehydration caused by water stress, for both species leaf water content decreased only slightly in the plants subjected to salt stress, demonstrating the small contribution of water loss to the reduction of fresh weight.

The biochemical analyses revealed an increase of Pro contents in the two *Limonium* species, more pronounced in response to the water stress treatment than under salt stress. The relative increase was more accentuated in *L. albuferae* due to the very low Pro levels in the absence of stress, but higher absolute values were found in *L. dufourii* in all applied treatments. The accumulation of Pro to high levels under water deficit conditions agrees with its strong negative correlation with substrate water content, revealed by the PCA. Pro is also a reliable marker of salt stress, increasing in the plants in parallel with the increase in the external concentration of NaCl; however, Pro does not seem to be directly involved in the mechanisms of salt tolerance, as it accumulates to higher absolute levels in *L. dufourii*, the less salt-tolerant of the two species. Pro biosynthesis in salt-stressed plants of *Limonium* is a well-known phenomenon and was already reported in the early work of Cavalieri & Huang (1979). In general, plant species of a particular genus tend to use only one, or very few different compounds, as functional osmolytes; one representative example is *Plantago*: all investigated species of this genus accumulate predominantly sorbitol in response to various abiotic stresses (Flowers & Colmer, 2008). In *Limonium*, however, a large variety of chemical compounds with the function of compatible solutes have been reported in different species

including, besides Pro, quaternary ammonium compounds like β-alanine betaine, choline-0-sulfate or glycine betaine, and different soluble sugars (fructose, sucrose and glucose) and polyalcohols (e.g., inositol isomers and derivatives) (Hanson et al., 1991; Rhodes & Hanson, 1993; Morales et al., 2001; ; Tipirdamaz et al., 2006 Gagneul et al., 2007; Furtana et al., 2013; Tabot & Adams, 2014; Al Hassan et al., 2017; González-Orenga et al., 2019b). Recently, in a metabolic profiling of these two species, we reported a gradual increase in Pro concentrations in parallel to increasing salinity but also a higher accumulation of fructose and glucose in *L. albuferae*. These data are consistent with the results presented here, indicating higher values of total soluble sugars in salt-stressed plants of this latter species.

Salinity and drought, like all other types of abiotic stress, are associated with an increase in ROS production, leading to cellular damage by oxidising unsaturated fatty acids in cell membranes, amino acid residues in proteins, and DNA molecules (Aple & Hirt, 2004; Halliwel, 2006). Different biomarkers can be used for assessing the extent of the oxidative stress affecting the plants; for example, malondialdehyde (MDA), a lipid peroxidation product employed as a reliable oxidative stress marker in both animals and plants (Del Rio et al., 1996) or hydrogen peroxide (Sofo et al., 2015). In our experiments, no significant changes in MDA levels were observed in the stressed plants, except for a small (but significant) increase in plants of *L. albuferae* subjected to water stress; H_2O_2 levels even decreased in comparison to the non-stressed controls. Several studies have shown that halophytes generally do not generate ROS in excess as they are perfectly adapted to the stressful environments where they live and possess efficient mechanisms to avoid or substantially reduce oxidative stress (Bosé et al., 2014; Gil et al., 2014), and this seems to be also the case in the selected *Limonium* species. Phenolic compounds, especially the subgroup of flavonoids, include many secondary metabolites that are potent antioxidants and increase under stressful conditions in many plant species (Di Ferdinando et al., 2012). However, only small increases (Souid et al., 2016) or no significant variations (González-Orenga et al., 2019, 2020) in the concentration of these compounds have been reported in several species of this genus subjected to stress treatments. In the present work, the only significant increases in total phenolics or flavonoid levels were observed in salt-stressed *L. albuferae* plants.

Phenolic compounds (including flavonoids), as other non-enzymatic antioxidants, are regarded as a secondary line of defence against oxidative stress, activated only under severe stress conditions, whereas antioxidant enzymes constitute the first ROS scavenging system (Fini et al., 2011). The specific activities of three antioxidant enzymes were determined in control and stressed plants of the two investigated species since these antioxidant mechanisms have been reported to be important for counteracting oxidative stress in *Limonium* under salt (Souid et al., 2019) and drought (González-Orenga et al., 2019b) stress conditions. SOD is the first enzyme to be activated in response to stress as it catalyses the dismutation of superoxide radicals into O_2 and H_2O_2 (Alscher et al., 2002). CAT complements the activity of SOD by decomposing the produced H_2O_2 into O_2 and H_2O and is induced by the accumulation of its substrate (Gunes et al.,

2007). Glutathione reductase (GR) contributes to recover and maintain the adequate cellular redox state by reducing oxidised glutathione (GSSG) to its reduced form (GSH), using NADPH as a cofactor (Hameed et al., 2015). Changes in the activities of these enzymes in response to stress followed different qualitative and quantitative patterns regarding both, the stress treatment, and the species. Thus, water deficit induced SOD activity in *L. dufourii*, but no significant changes with respect to the controls were observed for the other two enzymes in this species, nor in *L. albuferae* plants for any of the three tested enzymes. Therefore, it appears that the activation of enzymatic antioxidant mechanisms against water stress is more efficient in *L. dufourii* than in *L. albuferae*, which may contribute to the relatively higher drought tolerance of the former species. Conversely, in the more salt-tolerant *L. albuferae*, the three antioxidant enzyme activities increase significantly in response to the 600 and 800 mM NaCl treatments, whereas in *L. dufourii* SOD and GR (but not CAT) activities also increased, but to lower levels and only under the presence of the highest salt concentration tested.

4.4.5 Conclusions

The field study did not reveal a clear correlation between the number of individuals censed in the analysed populations with the climatic conditions. The analysis of vegetation underlined the presence of invasive species, mostly *Spartina patens*, with a notable presence in some inventories. Although in the natural habitats, *L. albuferae* is found in sites with lower salinity, the observed changes in several growth and biochemical variables in plants of the two selected *Limonium* species subjected to stress treatments under controlled greenhouse conditions, indicated that *L. albuferae* is more salt-tolerant than *L. dufourii* but more susceptible to drought whereas, conversely, *L. dufourii* is more drought-tolerant but more salt-sensitive than *L. albuferae*. In its natural habitat in the salt marsh, *L. dufourii* appears to be sensitive to prolonged flooding. Proline was synthesised in both species, especially under water stress, whereas MDA and H_2O_2 did not show a significant variation. The activity of antioxidant enzymes plays the most important role in the mitigation of oxidative stress in both species and both stress types. Increased accumulation of phenolic compounds in the two species, and flavonoids in the more salt-tolerant *L. albuferae*, also contribute to alleviating oxidative stress in the presence of high salt concentrations.

The results presented here may be useful in conservation management of the two species. Salinity does not seem to pose a threat for future reintroduction of specimens in salt marshes, as the two species under controlled conditions tolerated salinities far beyond those in their natural environments. Water scarcity, however, may be a problem for *L. albuferae*, which proved to be more susceptible to water deficit. On the other hand, *L. dufourii* should not be introduced in sites prone to prolonged flooding. The field study also established that, besides abiotic stress factors, competition with invasive species can be a major threat to the preservation of these species in their natural habitats. These data should be considered in the design and implementation of conservation, reinforcement

or reintroduction programmes, and for the general management of the threatened populations of these rare and endemic *Limonium* species.

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Publicación V:
Subcapítulo 4.5

Salt Tolerance Mechanisms and Potential Uses of *Limonium* Species

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Salt Tolerance Mechanisms and Potential Uses of *Limonium* Species

Abstract

Limonium is one of the most interesting and biodiverse genera of halophytes, with many species adapted to saline environments. *Limonium* species have a promising potential as cultivated minor crops as many have ornamental value or are already used as medicinal plants. Other species are marketed as gourmet food or can be used for decontamination of polluted soils. Design and implementation of specific breeding programmes are needed to fully realise this potential, based on the vast genetic variation and high stress tolerance of wild species within the genus. Most *Limonium* species are halophytes, but many also resistant to drought, especially those from the Mediterranean and other arid regions. They constitute attractive models for basic research on the mechanisms of stress tolerance, both constitutive and induced. As typical recretohalopyhtes, with excretive salt glands, *Limonium* species possess remarkable morpho-anatomical traits contributing to salt tolerance, together with ion accumulation in the leaves, the concomitant use of diverse osmolytes for osmotic adjustment, and the activation of efficient antioxidant systems.

Keywords: halophytes; ornamental; recretohalophytes; osmolytes; antioxidants

4.5.1 Introduction

The genus *Limonium* is the most biodiverse within the Plumbaginaceae family, distributed throughout the world. The number of its species was estimated to be around 400 (Erben, 1993), but including the numerous microspecies recently described, endemic to small territories, the total number can reach ca. 600 species (Koutroumpa et al., 2018). In the Mediterranean region, *Limonium* is extremely rich in endemic taxa, with 70% of the total number of species endemic (Koutroumpa et al., 2018); it is the richest genus in endemic species in the vascular floras of Italy, Spain, and Greece (Buira et al., 2017). The genus includes many threatened taxa; 159 species are catalogued worldwide in red lists, red books, or lists of protected species at the national and regional levels (IUCN, 2020). However, the highest concentration of endemic and threatened species is found mainly in the Mediterranean coastal regions (Laguna et al., 2020). The exceptional biodiversity within this genus is related to the combination of sexual reproduction with apomixis, and a high frequency of polyploidisation, hybridisation and introgression (Ferrer-Gallego & Laguna, 2015, Palacios et al., 2000). Due to its complex diversity, *Limonium* has been the subject of many taxonomical studies, starting with the first infrageneric classification by Boissier (Boissier, 1848, 1859) to molecular phylogenetic analyses during the last two decades (Palacios et al., 2000; Lledó et al., 2005, 2011; Akhani et al., 2013; Malekmohammadi et al., 2017; Koutroumpa et al., 2018).

Limonium species are mostly C3 perennial herbs or shrubs, with rosulate leaves and ascendant floral scapes, simple or ramified with abundant spikelets of small flowers grouped in panicles, which make them attractive as ornamentals. *Limonium* species are found on sandy beaches, cliffs, and salt marshes in coastal areas, but they also grow in continental areas in lagoons, meadows, steppes, and deserts (Koutroumpa et al., 2018).

Species of this genus were regarded as facultative halophytes, as many have an optimal growth in the absence of salinity and appear on saline soils mostly due to biotic competition (Erben, 1978). However, many species tolerate high saline concentrations and behave as true halophytes, with optimal growth under moderate salinity (Al Hassan et al., 2017; Gonzalez-Orenga et al., 2020), and almost all are exclusive to saline habitats.

Limonium belongs to a specific category of halophytes, the so-called 'recretohalophytes', which includes around 370 species (Yuan et al., 2016), able to secrete salt from their leaves trough salt bladders and salt glands. Salt bladders consist of single epidermal cells or modified trichomes that accumulate salt on the leaf surface, whereas salt glands are stable structures, formed by two or more cells, often sunken int the epidermis, that continuously secrete toxic ions to the outside of the plant (Shabala et al., 2014; Yuan et al., 2015, 2016). The salt glands are specific to several genera (including *Limonium*) of a few families, such as Plumbaginaceae, Acanthaceae, Tamaricaceae, Frankeniaceae, Amaranthaceae or Gramineae (Lipschitz & Waisel, 1974). Salt glands play an essential role in maintaining the ion balance, contributing to the stability of osmotic pressure and enhancing salinity tolerance (Zhang et al., 2003; Grigore & Toma, 2017); they act regulating the internal ionic composition of the leaves, which, together with efficient osmotic adjustments, help avoid dehydration of leaf cells (Shabala et al., 2014). One of the main mechanisms ensuring osmotic balance under stress is the synthesis and

accumulation in the cytoplasm of compatible solutes, the so-called osmolytes. These are diverse organic compounds that, apart from their fundamental function in osmotic adjustment, play additional roles in stress tolerance mechanisms, increasing the thermodynamic stability of folded proteins and directly protecting macromolecular structures – in their role as low-molecular-weight chaperons – and also as scavengers of 'reactive oxygen species' (ROS), or as signalling molecules (Flowers & Colmer, 2008; Munns & Tester, 2008; Türkan & Demiral, 2009; Szabados & Savouré, 2010; Slama et al., 2015). However, osmolyte biosynthesis represents a high cost for the plants, since the same cellular osmolarity can be reached by ion uptake and transport with much lower energy consumption (Raven, 1985; Shabala & Shabala, 2011). In dicotyledonous halophytes, osmotic adjustment can be provided at lower costs by ion uptake and accumulation, especially of Na^+ and Cl^-, which are sequestered in the vacuoles to avoid their toxic effects in the cytosol (Wyn Jones et al., 1977; Yeo, 1983; Glenn et al., 1999).

Salinity, as well as other stressful environmental conditions, increases the production of ROS, triggering oxidative stress and the activation of antioxidant mechanisms (Sharma et al., 2012; Golldack, 2014). The most common antioxidant metabolites include phenolic compounds (especially the subgroup of flavonoids), ascorbic acid, glutathione and carotenoids, whereas catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) (and other peroxidases), or redox regulatory enzymes such as glutathione reductase (GR), are amongst the most relevant antioxidant enzymatic systems activated in plants to respond to deleterious oxidative stress effects (Apel & Hirt, 2004; Sharma et al., 2012).

The limits of plants' salt tolerance depend on their developmental stages, and younger plants are generally more susceptible to stress (Johnson et al., 1992; Houle et al., 2001; Vicente et al., 2004). Seed germination and seedling establishment represent the bottleneck of their life cycle, and even in halophytes germination is generally taking place when soil salinity is alleviated (Ungar, 1991; Gul et al., 2012).

In this paper, we review the main mechanisms of salt tolerance reported in different species of the genus *Limonium*, during seed germination and in adult plants, including responses based on anatomical adaptations, the regulation of ion uptake and transport, osmolyte biosynthesis and metabolic pathways, and the activation of antioxidant mechanisms. The wide range of salt stress responses described in *Limonium* species makes this genus an attractive model for basic studies on salt tolerance mechanisms. We also mention, briefly, potential uses of *Limonium* taxa for the development of (minor) food, medicinal and ornamental crops for a sustainable, 'saline' agriculture.

4.5.2 *Limonium*, an Infra-Utilised Reservoir of Species with Great Potential as New, Non-Conventional Crops

Halophytes represent a sustainable alternative to conventional crops in arid and salinised areas, as they are well adapted to saline wetlands and arid soils. As marginal lands are already increasing due to global warming, more practical uses of halophytes are sought. They represent a valuable resource in landscape engineering, with a potential role in desalination and erosion prevention or commercial uses as ornamental plants

(Saglam & Onder, 2018; García-Caparrós, 2020). Halophytes also constitute a group of plants of particular interest as a source of nutraceuticals and functional foods, as they generally possess robust antioxidant defence systems, based on enzymatic activities and non-enzymatic compounds, which allows them the reduction of the oxidative stress associated with salinity (Ksouri, 2007, 2008). Some secondary metabolites present in halophytes, including carotenoids, terpenes, essential oils and phenolic compounds, may delay the oxidative stress deleterious effects, facilitating repairing of tissue injuries and thus prevention of cell death (Yang et al., 2014).

Limonium includes numerous ornamental species, well known mostly in the cut-flower markets. Plants of this genus have great potential as ornamentals as their coloured calyces remain open after the flowers have senesced. As such, flowers can be used for both fresh and dry arrangements, which can be maintained for long periods. *Limonium sinuatum*, *L. latifolium*, *L. perezii*, *L. gemelinii* are only a few of the well-known cultivated species, known as 'statice' or 'sea lavender', but many additional hybrids and varieties, the product of breeding programmes, also belong to this genus. Recently, there is a growing interest in incorporating traits from less known wild species into new cultivars, as many of them inhabit marginal lands and are, therefore, more adapted to environmental stress and can grow with a low input of fertilisers and pesticides (Cervelli et al., 2012). Moreover, these wild species represent a vast genetic variation, not yet exploited; crossing different species within this genus is not complicated, and they can also be easily manipulated *in vitro* (Burchi & Mercuri, 2006; Kaninski et al., 2012).

Many *Limonium* taxa have been reported to have medicinal properties (Lin & Chou, 2000; Geng et al., 2015; Corrêa et al., 2020), such as anti-inflammatory (Medini et al., 2015), antibacterial (Blainski et al., 2017), and antiviral (Medini et al., 2014) activities. Many species contain efficient free radical scavenging compounds (Aniya et al., 2002; Murray et al., 2004; Medini et al., 2011; Rodrigues et al., 2016), and are a promising source of drugs and nutraceuticals for the pharmaceutical and food industries. Trabelsi et al., (2012) isolated powerful antioxidant flavonoids from *Limonium densiflorum*, effective against colon carcinoma cell lines and with significant anti-inflammatory activity in macrophages. *L. quesadense* extracts also exhibited an intense antioxidant activity (Ruiz Riaguas et al., 2020) and infusions and decoctions of *L. algarvense* flowers showed better antioxidant and anti-inflammatory properties than green tea (Rodrigues et al., 2016).

Although *Limonium* is less known than other halophytes as gourmet food on European markets, some species have been reported as possessing a high nutritional value. For example, *L. tetragonum* is appreciated in Korea for its salty taste and was recommended as a suitable vegetable in diets for weight reduction and obesity-related health problems (Kim et al., 2017). *Limonium vulgare*, from a dry area in Tunisia, was found to be an excellent antioxidant edible plant that could be consumed as food complement (Souid et al., 2019). Some *Limonium* species have been proposed as fodder, such as *L. stocksii* from Pakistan (Zia & Khan, 2004) or *L. pruinosum* from Egypt (El-Amier & Ejgholi, 2014).

Finally, *Limonium* species are also potential candidates for phytoremediation programmes. Halophytes are expected to respond better than glycophytes in decontamination of polluted soils. They are ideal candidates for phytoextraction, or phytostabilisation of

heavy metal-contaminated soils, especially those affected by salinity (Manousaki & Kalogerakis, 2011; Hasanuzzaman et al., 2014; Manousaki et al., 2014; Saddhe, 2020). Recrerohalophytes are of particular interest, as they may remove not only the excess of salt ions but also other toxic elements such as cadmium, zinc, lead, or copper, through the process called 'phytoexcretion' (Manousaki & Kalogerakis, 2011). Among *Limonium* species, *L. carthaginense* proved to be tolerant to trace element contamination (Martínez Sanchez et al., 2012), and *L. sinuatum* is a good candidate for lead and cadmium decontamination (Sheikh-Assadi et al., 2015).

4.5.3 Morpho-Anatomical Adaptations in *Limonium* Species

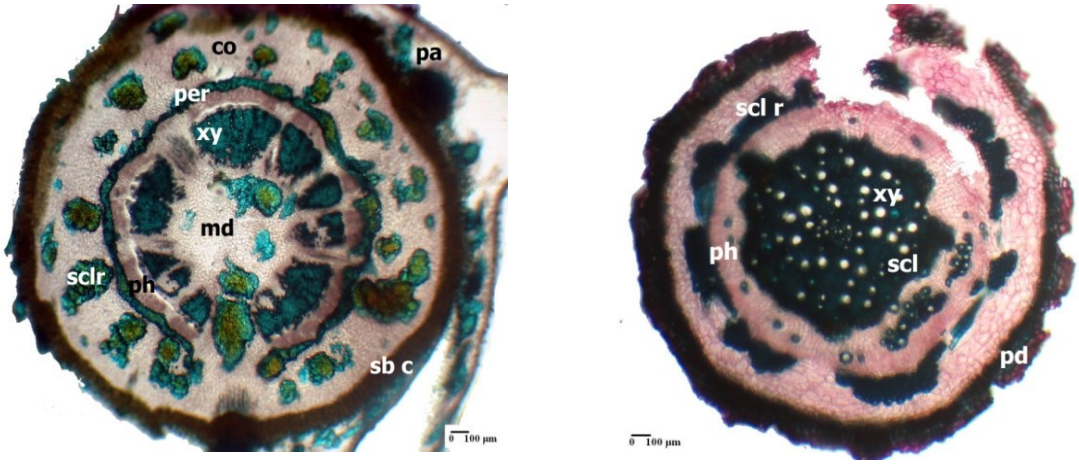
The morphology and anatomy of vegetative organs in *Limonium* species are generally well known and have been recently reviewed (Grigore & Toma, 2017; 2020a). Studies on *Limonium*'s biology have been mostly linked to the presence of salt glands ('Mettenius' or 'Licopoli' glands/organs – see Grigore & Toma, 2016), located on the stem and especially on the leaf surface. In fact, there are also some anatomical features found in *Limonium* that attest the xeromorphic nature of these halophytic species. Overall, taking into account the saline and arid environments where *Limonium* species grow, they could be considered a special case of xerophytes. This genus could serve as a model for this kind of morphological and anatomical studies (Grigore & Toma, 2020b).

Description of the morphology of the underground organs of *Limonium* in the botanical literature is sometimes confusing and contradictory. Although *Limonium* species are clearly recognised and described as perennial species (thus, the plants must possess rhizomes), there is no mention of this modified underground stem in the Romanian flora. Instead, the species are characterised as having 'tap, thick roots' (*L. gmelinii)*, 'thick, with nodes' (*L. vulgare*), 'cylindrical root, unbranched or rarely branched in its upper part' (*L. latifolium*), or 'tap root more or less thick' (*L. caspia*) (Răvăruț, 1960). The same is true for *Flora of the USSR*, where there is no data about the rhizome, and the underground system is referred to as 'taproot', with a single exception in the case of *Limonium otolepis*, which has a 'taproot (or rootstock) fairly stout' (Linchevskii, 1967). *Limonium brasiliense* is another example of this error in terming the rhizome as a root (see discussions in Antonelli-Ushirobira et al., 2015).

Nevertheless, the anatomical investigation of underground organs clarifies this confusion, as the microscopic structure reveals the typical configuration of a stem (rhizome) or a root (Grigore & Toma, 2014). Nevertheless, even with available anatomical data on the structure of underground organs in different *Limonium* species, comparisons and extrapolations must be carried out with caution. Sometimes, the anatomical description of the rhizome and the root can be accurate for a particular species, but this does not necessarily provide information on the organs' morphology unless it is clearly specified at which level the cross-sections were performed within an organ. Thus, an organ can be morphologically and anatomically identified as a root or a rhizome for a *Limonium* species but, in the absence of anatomical data, these results may not be morphologically applicable to other *Limonium* species. Some research papers contain anatomical information on the main root, lateral roots and rhizome, for example in *Limonium gmelinii* (Moțiu et al., 1987); in this case, the rhizome is defined as 'the

underground part located between the collet and the rosette of leaves that forms in its terminal side, on soil surface'. For this reason, in the absence of precise anatomical data regarding underground organs, in ecophysiological studies on *Limonium*, it is highly recommended to use the term 'underground system' or 'rhizomatous root system' (González-Orenga et al., 2020).

A common anatomical feature for the underground organs (rhizome, root) of *Limonium* species is the extensive development of sclerenchyma tissue (Solereder, 1908; Colombo & Trapani, 1992; Grigore et al., 2014). This feature has been evidenced in several Mediterranean *Limonium* species (Grigore & Toma, 2021). In the rhizome of *Limonium furfuraceum*, there is a sinuous ring of sclerenchymatous fibres that surrounds the central cylinder, thus acting as a pericycle (Fig. 1a); a similar sclerenchymatous ring occurs in the case of the aerial (flowering) stem (data not shown).
In the central cylinder of the *L. girardianum* root, at its periphery, there is a discontinuous mechanical ring, consisting of several convex arches of sclerenchymatous fibres (Fig. 1b).



(a) **(b)**

Figure 1. Cross-section through the rhizome of *Limonium furfuraceum* **(a)** and through the root of *Limonium girardianum* **(b)**; co – cortex; md – medulla; pa – parenchyma; per – pericycle; ph – phloem; xy – xylem vessel; sb c – suberified cells; pd – periderm; scl r – sclerenchyma ring; scl – sclerenchyma; sclr – sclereid.

The central cylinder in the rhizome of *L. girardianum* (Fig. 2a) presents at its periphery a thin ring of sclerenchyma, consisting of cords of periphloemic fibres of vascular bundles; the secondary xylem forms a very thick ring, with a large amount of libriform, and many vessels scattered in the interior.
In *L. girardianum*, the central cylinder of the aerial (flowering) stem has at its exterior an extremely thick ring of sclerenchyma fibres, with very thick and intensely lignified walls (Fig. 2b). A similar situation is found in the aerial stem of *L. narborense* Mill. (Fig. 2c) and *L. gmelinii* ssp. *hungaricum* (Zoric et al., 2020).

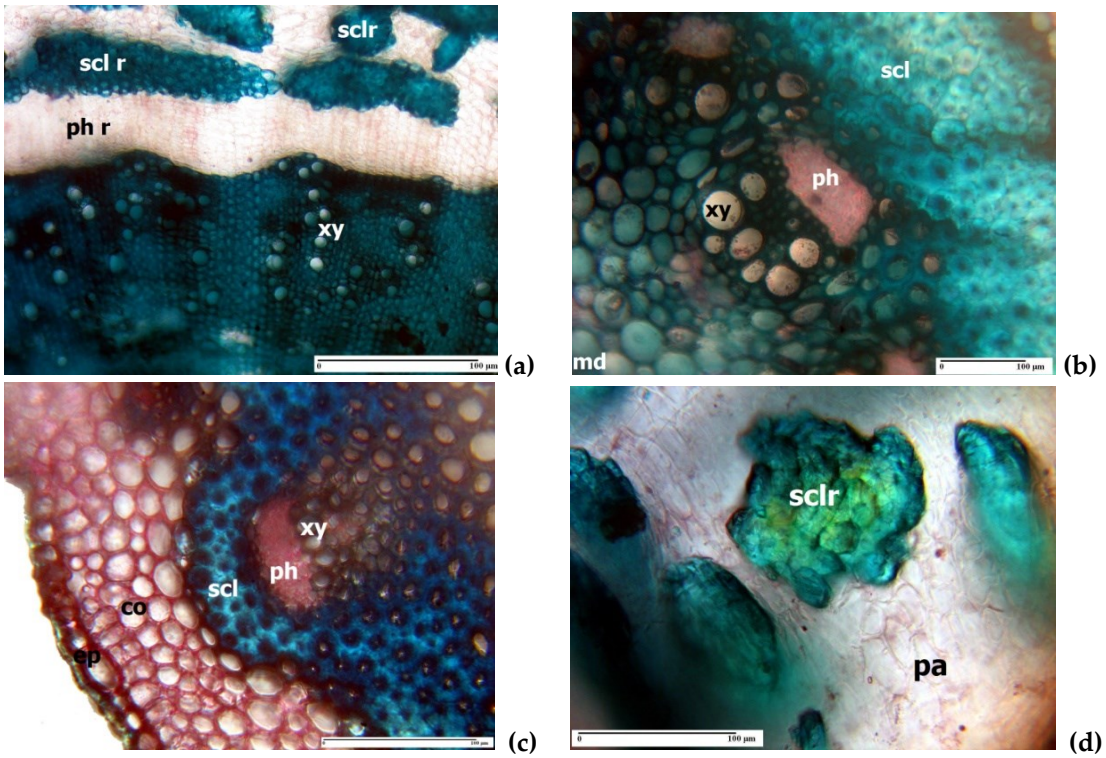


Figure 2. Cross-section through the rhizome of *Limonium girardianum* **(a)**, aerial stem of *Limonium girardianum* **(b)**, aerial stem of *Limonium narbonense* **(c)**, and groups of sclereids located in the rhizome' cortex of *Limonium girardianum* (Guss.) Fourr. **(d)**; co – cortex; ep – epidermis; pa – parenchyma; ph r – phloemic ring; scl r – sclerenchyma ring; xy – xylem vessel; sclr – sclereid; md – medulla; ph – phloem; scl – sclerenchyma; xy - xylem vessel; ph – phloem.

Groups of sclereids are present in the rhizome of several species of *Limonium*, such as *L. furfuraceum* (Fig. 1a), *L. girardianum* (Fig. 2d), *L. gmelinii* (Moțiu et al., 1987), or *L. brasiliense* (Antonelli-Ushirobira et al., 2015).

The presence of well-developed sclerenchyma in the underground organs of *Limonium* halophytic species could be interpreted as an adaptation to the harsh environmental conditions of the plants' natural habitats (Grigore & Toma, 2020a). Indeed, sclerenchymatic rings, as well as the groups of sclereids found in the rhizome, could confer to the underground system the mechanical resistance needed to penetrate the dry, compact soil to reach the water table. Also, the presence of tannin cells in the rhizome in *Limonium* species (Grigore et al., 2014) may help the plant survive under waterlogged conditions in clayed soils.

Another interesting anatomical feature in *Limonium* halophytes is the presence of mechanical idioblasts. They have been found in the lamina of *L. girardianum* (Fig. 3), *L. gmelinii* (Moțiu et al., 1987), *L. gmelinii* ssp. *hungaricum*, *L. anfractum* (Zoric et al., 2013), *L. lopadusanum* and *L. albidum* (Colombo & Trapani, 1992). These structures can be differently nominated: mechanical idioblasts (Grigore et al., 2014), spicular cells (stereids, sclereids, idioblasts) (Grigore & Toma, 2020a), sclereids in the form of idioblasts (Zoric et al., 2013), sclereidal idioblasts (Colombo & Trapani, 1992), sclereides (de Fraine, 1916), and likely play a mechanical role, conferring the coriaceous aspect of the leaves of *Limonium* species (Grigore & Toma, 2020a).

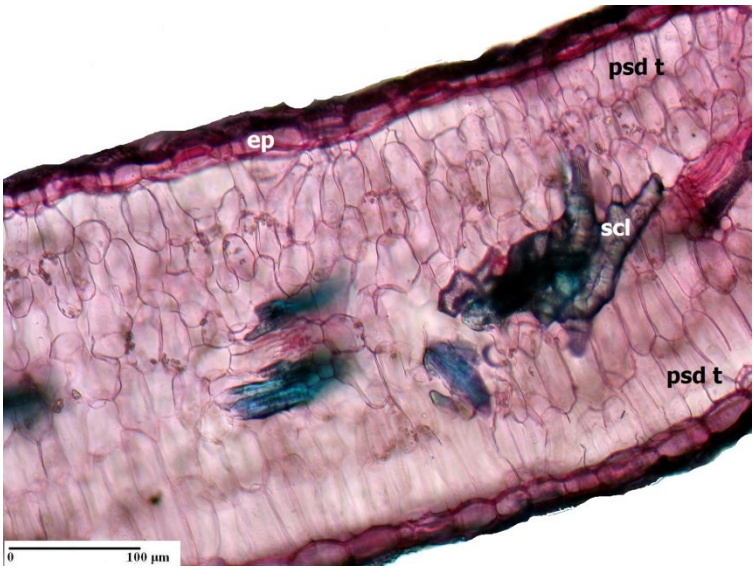


Figure 3. Branched sclereids in the lamina of *Limonium girardianum*; ep – epidermis; psd t – palisade tissue; scl – branched sclereid.

A typical anatomical trait of Plumbaginaceae is the presence of glands: chalk glands (Mettenius or Licopoli glands – salt glands – Figs. 4a-d) and mucilage glands located on leaves and stems (Grigore & Toma, 2020a). This feature classifies halophyte species from Plumbaginaceae in the group of recretohalophytes (that secrete salt via salt bladders and salt glands – the case of *Limonium* species). Salt glands are particularly well-known and of great scientific interest from the historical (see Grigore & Toma, 2016 and reference therein), anatomical (Grigore & Toma, 2010; Grigore et al., 2014; Grigore & Toma, 2017; 2020a), physiological (Yuan & Wang, 2020) and evolutionary (Caperta et al., 2020; Grigore & Flowers, 2021) points of view. Within the recretohalophytes from Plumbaginaceae, recent data report 48 species of *Limonium* to have salt glands (Caperta et al., 2020). Even though the history of anatomical description of salt glands dates back to the mid-19th century (see Grigore & Toma, 2016), there is no consensus regarding the number of cells that build up a salt gland. Caperta et al. (2020) indicated that a complex of 16-celled salt gland structure in *Limonium* species is commonly reported in the literature. However, other arrangements of 10 and 20 cells have also been described. Nevertheless, these differences in the registered number of cells of a salt gland in *Limonium* are partly due to the lack of standard definition of the cells that constitute the salt gland, and also by the fact that sometimes glands observed in front view (on the epidermis surface) do not reveal the entire architecture of the gland, as several cells remain 'hidden' below the surface.

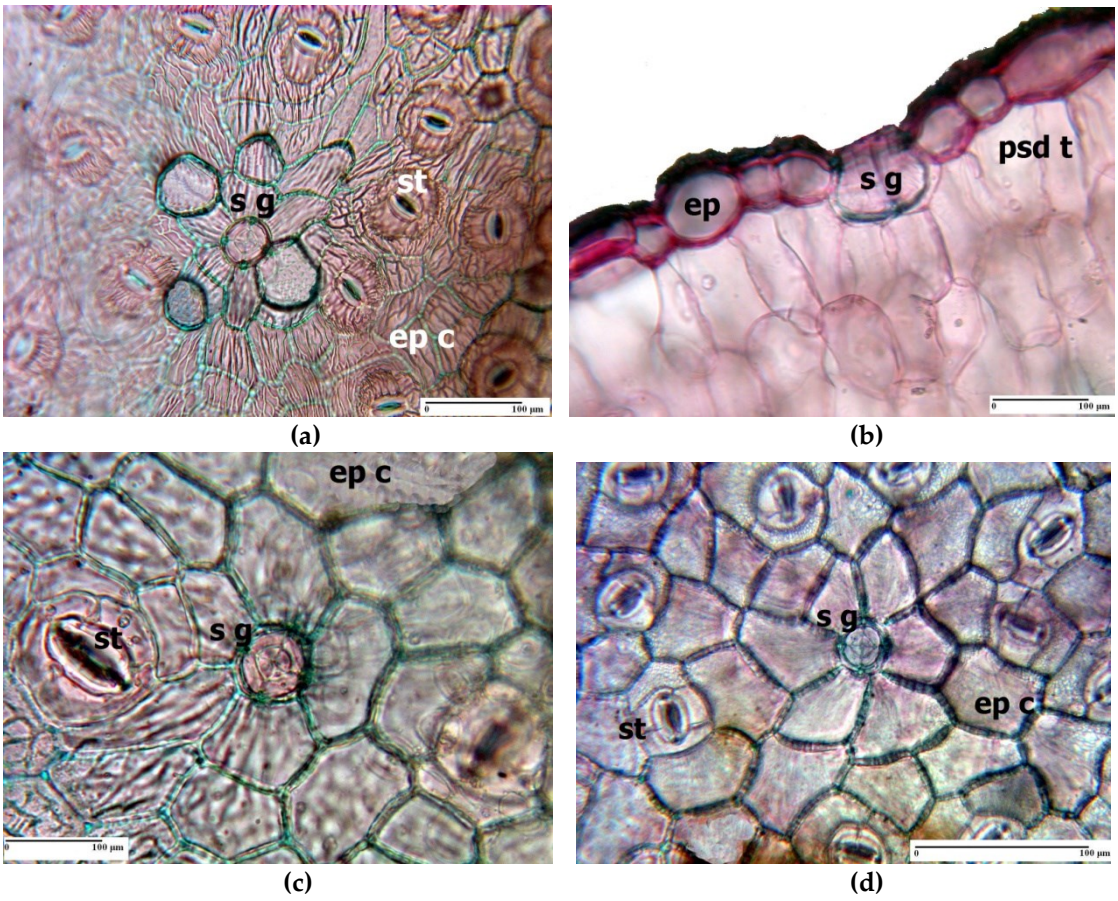


Figure 4. Salt gland in the epidermis of *Limonium furfuraceum* (surface view) **(a)**, *Limonium girardianum* (cross section) **(b)**, *Limonium narbonense* (surface view) **(c)**, and *Limonium gmelinii* (surface view) **(d)**; ep c – epidermal cells; s g – salt gland; st – stomata; psd t – palisade tissue.

4.5.4 Seed Germination under High Salinity, and Recovery of Germination

High salinities represent a constraint for the germination of the seeds of all plants, including halophytes. Although adult halophytes may withstand very high salt concentrations in their natural habitats, their seeds normally germinate when soil salinity is alleviated (Ungar, 1991, Gul et al., 2013; Kazachkova et al., 2016). Halophytes largely vary in their limits of salt concentrations suitable for germination, and many show optimal germination percentages in freshwater or at salinities below 100 mM NaCl (Ungar, 1991). However, the seeds of some highly salt-tolerant species, especially succulent halophytes, can germinate at concentrations equivalent to that of seawater or above, up to 1,7 M NaCl (Khan & Gul, 2006). There is a seasonal variation of the soil salinity in many natural environments, which is higher during summer due to accentuated evaporation. Seed germination in arid and semi-arid regions usually occurs after the rains reduce surface soil salinity (Khan, 1999; Khan & Gul, 2006). Halophytes from temperate salt marshes maintain a persistent soil seed bank and germinate in spring when salinity is alleviated (Ungar, 1995; Gul et al., 2013). At the seed germination stage, recretohalophytes generally are more susceptible to high salt concentrations than the succulent ones, and less than 20% can germinate above seawater salinity (Khan & Gul, 2006).

Several micro-endemic species of *Limonium* from SE Iberian Peninsula have been analysed, and germination percentages and rates usually decreased with increased salinity and temperature. In several species, optimal germination was registered in distilled water and at low salinity (100 mM NaCl), at 20/10 °C, with a 14/10 h light/darkness photoperiod. The percentage and rate of germination decreased with an increase in salinity and temperature *in L. cossonianum* from wetlands in SE Spain (Giménez Luque et al., 2013), *L. insigne* from coastal cliffs, littoral steppes and dry inland areas (Delgado Fernández et al., 2015), *L. tabernense*, restricted to arid environmental conditions in the Tabernas Desert (SE Spain) (Delgado Fernández et al., 2016). From the same geographic area, optimal germination was registered in distilled water at 15 ºC constant temperature and 12 h light photoperiod in *L. mansanetanum* from gypsum areas (Fos et al., 2020), and at alternating temperatures of 20 °C (under light conditions) and 10 °C (in the dark) in *L. supinum* from arid habitats of the same region (Melendo & Giménez, 2019). In all these species, an interactive negative effect of temperature and salinity on final germination percentage and germination rate was established, indicating that the germination response to salinity depends on the temperature. In two other local endemics, *L. dufourii* and the recently described *L. albuferae*, from salt marshes near Valencia in E Spain, germination was drastically reduced at 150 mM NaCl (González-Orenga et al., 2019). Maximal germination percentages and rates were also reported in control treatments in *L. emarginatum*, an endangered and endemic halophyte of the Strait of Gibraltar (Redondo Gómez et al., 2008).

In seeds of four other species sampled from the Valencia region, the temperature did not have a drastic effect on their germination (Monllor et al., 2018). In two of these species, the local endemic *L. santapolense* and the common Mediterranean *L. virgatum*, low salinity (50 mM NaCl) stimulated germination; in the latter species, an increase of 80% in the percentage of germination was observed in the presence of 100 mM salt. Seeds of the widespread *L. narbonense* showed optimal germination up to 200 mM NaCl, whereas in *L. girardianum*, present in salt marshes in E Spain and S France, significant inhibition of germination was observed already at 50 mM NaCl (Al Hasan et al., 2017). The pattern of germination of these four species was correlated with their geographic distribution. *Limonium narbonense* and *L. virgatum*, which are wide-spread throughout the Mediterranean region, have the ability to germinate at high salt concentrations and consequently can colonise habitats with higher soil salinity, avoiding competition with less tolerant species. On the other hand, the endemic *L. santapolense* and *L. girardianum* are less salt-tolerant at the seed germination stage, and more competitive only at lower salt concentrations; therefore, they are restricted to smaller areas.

These results are summarised in Figure 5. As mentioned above, the highest germination percentages were found in the seeds from control treatments or at a relatively low concentration of 50, 100 and 150 mM NaCl. With a few exceptions, 200 mM NaCl drastically inhibited germination. Complete inhibition was registered at 300-400 mM

NaCl in the most susceptible taxa, and at 500 mM practically no germination was observed in any of the analysed species.

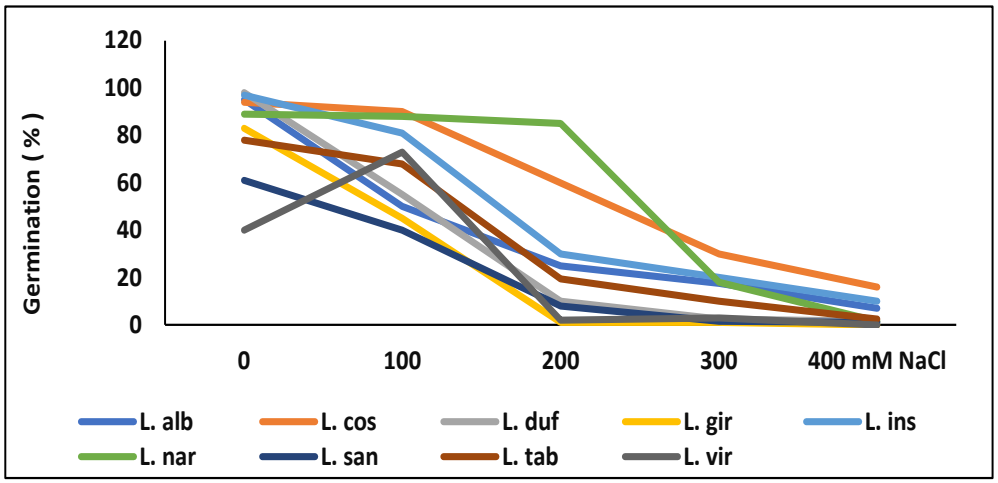


Figure 5. Final germination percentages of *Limonium* seeds after 20-25 days of exposure to the indicated salt concentrations. Abbreviations*: L. albuferae* (L. alb); *L. cossonianum* (L. cos.); *L. dufourii* (L. duf.); *L. girardianum* (L. gir.); *L. insigne* (L. ins); *L. narbonense* (L. nar); *L. santapolense* (L. san); *L. tabernense* (L. tab.); *L. virgatum* (L. vir). Based on González-Orenga et al., 2019; Delgado Fernández et al., 2015; Giménez Luque et al., 2013; Delgado Fernández et al., 2016; Al Hassan et al., 2017.

In two endemic *Limonium* species from Turkey, *L. lilacinum* and *L. iconicum*, the highest germination was obtained in distilled water at 15/25 °C and 20/30 °C, in a 12h light photoperiod. Germination was drastically inhibited by 300 mM and higher NaCl concentrations, and by the lower thermoperiod (10/20°C) in both, control and salt treatments. The germination rate increased with an increase in temperature and decreased with increasing salinity, whereas light stimulated germination in both species (Yildiz et al., 2008). Light was also reported to stimulate germination of seeds of the annual *L. lobatum* (L. fil.) Chaz, collected in Australia, where this Mediterranean species is becoming an aggressive weed. The best germination was registered in the absence of salt, in a range of temperatures between 10 and 30 °C, in the absence of salt; in the presence of 230 mM NaCl, germination was reduced by half but some seeds germinated even at 480 mM NaCl (Kleemann & Gill, 2018).

Some additional reports also indicated a great tolerance to salinity during germination in several *Limonium* species. In *L. stocksii*, from the subtropical maritime desert of Karachi, some seeds germinated even at 500 mM NaCl at the optimal temperature regime of 20/30ºC; germination was maximal in control, 100 and 200 mM NaCl, and 60% of the seeds germinated in the presence of 300 mM salt. Also, in *L. axillare*, from desert areas in Saudi Arabia, even though part of the seeds germinated at high salt concentration, a drastic reduction of germination percentages was registered at a lower concentration of 170 mM NaCl (Mahmoud et al., 1983). Germination of desert halophytes usually increases with temperature, contrary to those from humid habitats, which germinate better at a lower temperature (Khan & Gul, 2006). However, the highest tolerance to salinity during germination in *Limonium* was reported in *L. vulgare* Mill., where seed germination was still registered at the very high concentration of 1.4 M NaCl, albeit drastically reduced to 10% of the non-stressed control seeds (Woodell, 1985).

Contrary to glycophytes seeds, which cannot withstand long exposure to saline environments, halophytes possess the ability to recover from salt stress and germinate after being exposed to hyper-saline conditions, a strategy of great selective advantage. In habitats with fluctuant salinity like salt marshes, halophytes provide a viable soil seed bank, and germination occurs when there is an alleviation of salinity, generally in spring in temperate climates and after rainy periods in dessert areas (Ungar, 1982). However, this recovery capacity of halophyte seeds varies quantitatively in different species. Most halophytes show, indeed, a substantial recovery of germination when stress conditions are alleviated (Ungar, 1962; 1967; 1991; Khan & Gul, 2006, Gul et al., 2013; Yuan et al., 2019). A 60% to 70% recovery of final germination was recorded in *L. emarginated*, although seed viability decreased under hypersaline conditions (Redondo Gomez et al., 2008), as in *L. tabernense* (Delgado Fernández et al., 2016). In *L. albuferae* and *L. dufourii*, recovery of germination percentages reached 60-80% and 80-90%, respectively, whereas the rate of germination increased in both (González-Orenga et al., 2020). Recovery was complete in *L. stocksii* seeds exposed up to 500 mM NaCl (Zia & Khan, 2004). In L. *santapolense*, L. *narbonense*, *L. giradianum* and *L. virgatum*, full recovery of germination was reported, reaching values similar to those of control seeds germinated in water. Significant stimulation of germination was observed in *L. virgatum* after exposure to 150 mM NaCl and, to a lesser extent, in *L. girardianum* seeds pre-treated with 50 mM NaCl (Al Hassan et al., 2017).

4.5.5 Plant Growth under Controlled Experimental Conditions

Growth reduction is the first and most general response of plants to environmental stress conditions, such as drought or salinity, as plants use their metabolic precursors and energetic resources to activate defence mechanisms instead of biomass accumulation (Zhu, 2001; Munns & Tester, 2008). The majority of halophytes, and all glycophytes, grow optimally in the absence of salt. Only in a few, extremely salt-tolerant halophytes, growth is stimulated by low or moderate salinity, although still inhibited in the presence of salt above a higher, species-specific concentration threshold (Flowers et al., 1986). For this reason, assessment of the effect of salt stress on growth parameters in different species is a useful tool for ranking their relative degree of salt tolerance (Al Hassan et al., 2016).

Figure 6 summarises the effect of salinity and drought on the growth of six different *Limonium* species evaluated in our laboratory. Seeds of all the species, already mentioned in the previous section, were sampled in salt marshes in the Albufera Natural Park, near Valencia in eastern Spain, except for those of the endemic *L. santapolense*, present in a more southern location, in the Alicante province. *Limonium albuferae* and *L. dufourii* are endangered local endemics included in conservation programmes, whereas the remaining species have a broader distribution, *L. girardianum* in S France and E Spain, and *L. virgatum* and *L. narbonense* throughout the Mediterranean. Plants grown from the seeds were subjected to salt and water stress treatments under controlled greenhouse conditions. Growth was generally stimulated at 200 and even 400 mM NaCl and was inhibited only at higher salt concentrations. Under water stress, a significant reduction of leaf fresh weight was registered in *L. santapolense* and *L. narbonense* (González-Orenga

et al., 2019a), but mostly in plants with leaves of a larger size, such as *L. albuferae* and *L. dufourii* (González-Orenga et al., 2019b).

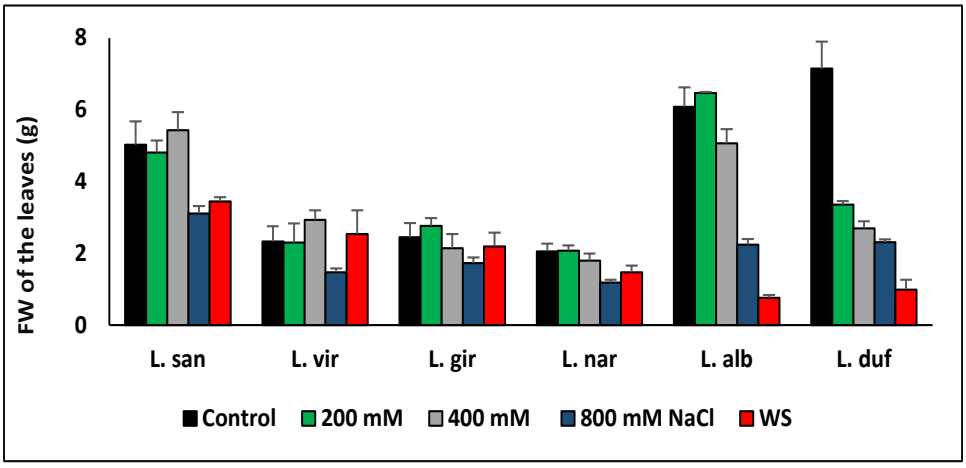


Figure 6. Mean leaf fresh weight (FW) of *Limonium* plants after one-month stress treatments. Salt treatments at the NaCl concentrations indicated above. Water stress (WS) was applied by complete withholding of irrigation- Abbreviations: *L. albuferae* (L. alb); *L. dufourii* (L. duf.); *L. girardianum* (L. gir.); *L. narbonense* (L. nar); *L. santapolense* (L. san); *L. virgatum* (L. vir). Based on Al Hassan et al., 2017 & González-Orenga et al., 2019.

Also, a remarkable salt tolerance was reported in the desert species *L. stocksii*, which maintained constant growth up to 300 mM NaCl, and only higher salt concentrations inhibited biomass accumulation (Hameed et al., 2015); a similar behaviour was observed for *L. sinense* (Ding et al., 2009). In *L. pectinatum*, a species of Canarian origin and used as ornamental worldwide, growth was stimulated at 100 mM NaCl and showed similar values to those in control plants in the presence of 200 mM NaCl (Morales et al., 2001). Similarly, the growth of *L. delicatulum* was stimulated at 50, 100 and 200 mM NaCl when compared with control plants, and drastically reduced only at salt concentrations exceeding 200 mM NaCl (Souid et al., 2016).

Lower salt tolerance was reported in a comparative study on two cultivated species, *L. perezii* and *L. sinuatum*. Growth of the former species was inhibited even at low salinities, whereas the latter showed a somewhat higher tolerance; nevertheless, both were able to complete their life cycles at concentrations above 300 mM NaCl, thus behaving as true halophytes (Grieve et al., 2005). Analysis of seedlings' responses to salt in *L. bicolor*, with a native distribution in Mongolia and NE China, showed 100 mM NaCl as the optimal salt concentration for plant growth (Li et al., 2008), and no injures were observed at concentrations up to 150 mM (Xianzhao et al., 2013). In *L. latifolium*, a native of SE Europe and W Asia and widely used as ornamental, the highest growth rate was reported in control plants, but the plants survived and remained vigorous when subjected to treatments with NaCl up to 400 mM for one month (Hamed et al., 2014). Most *Limonium* species, especially those used as ornamentals, grow better in control conditions or at low salinities, but some of the wild species are much more salt-tolerant, to a degree similar to that of extremophiles, such as *Suaeda maritima* (Flowers, 1972; Khan et al., 2000; Hameed et al., 2012).

4.5.6 Ion Transport and Accumulation

Recretohalophytes can secrete toxic ions through salt glands and trichomes, and many studies confirm this specific feature in *Limonium* (e.g., Tabot & Adams, 2014; Leng et al., 2018). In a comparative study on a wild species and a cultivated hybrid of this genus, the more tolerant wild *L. pectinatum* had higher excretion rates of Na^+ and Cl^- than the hybrid, but both secrete only small amounts of K^+ (Morales et al., 2001), as salt glands are highly selective for Na^+ and Cl^- (Ramadan, 1998).

Although it has been long known that *Limonium* species lower the ionic activity in the photosynthetic parenchyma through their excretory glands (Hill, 1967), the genus is also reported as a salt accumulator (Grieve et al., 2005; Zia et al., 2008; Al Hassan et al., 2017, among others). One of the main differences between monocotyledonous and dicotyledonous halophytes is related to control of ion transport and homeostasis. In salt-tolerant monocots, as in all glycophytes, the primary mechanism of stress resistance is based on blocking the transport of toxic ions (Na^+ and Cl^-) to the aerial part of the plants. On the contrary, in the halophytic dicots, there is an active uptake, transport to the leaves and compartmentalisation in vacuoles (Flowers & Colmer, 2008; Munns & Tester, 2008). Reports on different *Limonium* species support this 'dicot model', as concentrations of Na^+ and Cl^- increased in parallel to the concentration of NaCl applied in roots but especially in their aboveground organs (Zia et al., 2008; Al Hassan et al., 2017; Gonzalez-Orenga et al., 2019a), as reported in other stress-tolerant species (Wang et al., 2004, Lv et al., 2012; Borsai et al., 2020). Tester & Davenport (2003), considered that constant levels of NaCl in roots are maintained by its export to the shoots; this is also a mechanism to reduce the toxic effects of salt at the root level (Alarcon et al., 1999), and its use as cheap osmoticum in the shoots (Flowers & Yeo, 1986; Glenn et al., 1999). Ion toxicity in the cytoplasm is avoided by compartmentalisation of Na^+ and Cl^- in vacuoles (Munns, 2002).

Results obtained in our laboratory on the analysed species of *Limonium* are shown in Figures 7 and 8. In the six species, the contents of all quantified ions increased in parallel with the increase in external salinity in both, belowground and aboveground organs, but were significantly lower in the roots than in the leaves. The most remarkable increase was registered in the leaves of the least tolerant *L. dufourii* (Figure 7). Interestingly, this species also accumulated Na^+ in the leaves of plants subjected to a water stress treatment (Figure 7, unpublished data). Accumulation of Na^+ at low soil NaCl concentrations has been recently proposed as an essential mechanism of drought tolerance in the desert xerophyte *Zygophyllum xanthoxylum* (Xi et al., 2018).

High levels of Na^+ can be associated with toxic effects, causing a reduction in seedling emergence and survival, as well as in the contents of other ions, such as K^+ and Ca^{2+}, as shown by Carter et al. (2005) in *L. perezii*. In a comparative study on three halophytes from Brittany coast – *Limonium latifolium*, *Matricaria maritima* and *Crambe maritima* – the smallest accumulation of Na^+ was found in the most tolerant species, *L. latifolium* (Hamed et al., 2014), in agreement with the salt tolerance mechanism discussed above.

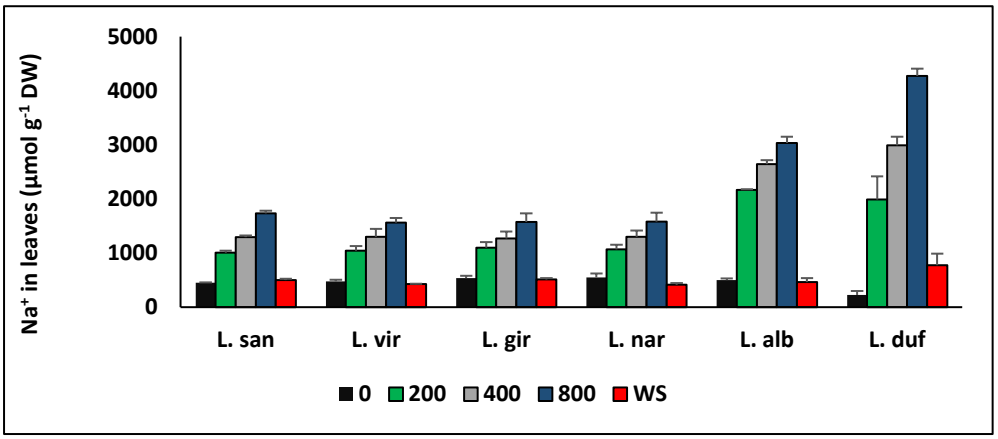


Figure 7. Accumulation of Na^+ in foliar tissue of *Limonium* plants after one-month stress treatments. Salt treatments at the NaCl concentrations indicated above. Water stress (WS) was applied by complete withholding of irrigation. Abbreviations: *L. albuferae* (L. alb); *L. dufourii* (L. duf.); *L. girardianum* (L. gir.); *L. narbonense* (L. nar); *L. santapolense* (L. san); *L. virgatum* (L. vir). Based on Al Hassan et al., 2017; González-Orenga et al., 2019; and unpublished data.

Usually, an increase in the concentration of Na^+ is associated with a reduction of K^+, as the two cations compete for the same binding sites and use the same transport proteins. Moreover, an excess of Na^+ causes the depolarisation of the plasma membrane, inducing the activation of outward rectifying K^+ channels and, therefore, the loss of cellular K^+ (Greenway & Munns, 1980). However, many *Limonium* species have the ability to activate the transport of K^+ to the leaves in response to salt treatments, leading to a smaller reduction or the maintenance of constant leaf levels of this cation (Gonzalez Orenga et al., 2019), or even an increase over the control at higher external salinity (Al Hassan et al., 2017). Similar results have also been reported in halophytes of the genus *Plantago*, both in the field (Gil et al., 2014) and in the greenhouse (Al Hassan et al., 2016b).

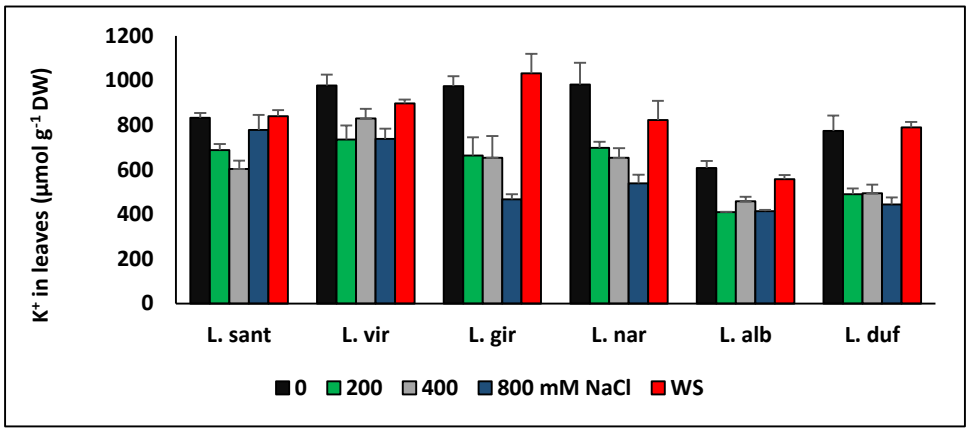


Figure 8. Accumulation of K^+ in foliar tissue of *Limonium* plants after one-month stress treatments. Salt treatments at the NaCl concentrations indicated above. Water stress (WS) was applied by complete withholding of irrigation. Abbreviations: *L. albuferae* (L. alb); *L. dufourii* (L. duf.); *L. girardianum* (L. gir.); *L. narbonense* (L. nar); *L. santapolense* (L. san); *L. virgatum* (L. vir). Based on Al Hassan et al., 2017; González-Orenga et al., 2019; and unpublished data.

As shown in Figure 8, foliar K^+ levels in *L. albuferae* and *L. dufourii* were lower in plants subjected to salt treatments than in their controls, but in the other species the reduction

was smaller. The strongest reduction in K^+ was registered in the most salt-sensitive *L. dufourii*, which also showed a relatively higher accumulation of Na^+ in salt-stressed plants, and therefore a higher Na^+/K^+ ratio in the leaves of plants submitted to the highest concentration of 800 mM NaCl). Similar findings were reported in a comparative study on two *Limonium* species, *L. perezii* and *L. sinuatum*, the latter, which is the most tolerant, showed a high selectivity of K^+ over Na^+ (Grieve et al., 2005). Maintaining a balanced cytosolic Na^+/K^+ ratio, together with high foliar levels of K^+, is regarded as an essential mechanism for salt tolerance (Percey et al., 2016; Assaha et al., 2017). In plants from the water stress treatment the variations were small in respect to controls, *in L. santapolense* K^+ values remained practically unchanged and in *L. girardianum* even increased in water-stressed plants.

4.5.7 Osmolyte Synthesis

The accumulation of inorganic ions in vacuoles is compensated by that of compatible solutes or osmolytes in the cytoplasm. Osmolytes are simple, non-toxic organic molecules, which do not interfere with cellular metabolism even at high intracellular concentrations. They play several essential roles in the responses of plants to abiotic stress, besides their primary function in osmotic adjustment, including the direct stabilisation of proteins and macromolecular structures, as 'reactive oxygen species' (ROS) scavengers or signalling molecules (Slama et al., 2015; Ejaz et al., 2020). Osmolytes are chemically diverse, including amino acids and derivatives, such as proline, methylated proline and related compounds; quaternary ammonium compounds, such as glycine betaine and other betains; soluble sugars, such as glucose, fructose, sucrose, trehalose, raffinose or fructans; and polyols or sugar alcohols, such as sorbitol, mannitol, glycerol, inositol and methylated inositols (Flowers & Colmer, 2008; Slama et al., 2015). A concomitant synthesis of different osmolytes is well-known in halophytes (Tipirdamaz et al., 2006; Gagneul et al., 2007; Slama et al., 2015; Al Hassan et al., 2016, Al Hassan et al., 2018), but the highest diversity is found within the Plumbaginaeceae family, and especially in the genus *Limonium* (Rhodes & Nadolska-Orczyk, 2002). Many species of this well-known halophytic genus have been analysed, and a wide variety of compounds has been reported as compatible solutes, which are summarised in Table 1.

Proline (Pro), one of the most common osmolytes in plants, has different biological functions in abiotic stress tolerance mechanisms, as it has also been described for other compatible solutes. Pro acts in osmotic adjustment under stress; as a low-molecular-weight chaperon, for example in the protection of plasma membrane integrity and its associated transporter proteins; as a ROS scavenger, with singlet oxygen quenching capacity; and/ or a signalling molecule in the transduction of stress signals (Szabados & Savouré, 2010; Saibi & Brini, 2020). Proline has been detected in many *Limonium* species, generally at low concentrations under control conditions but showing a significant increase in plants subjected to water deficit or salt stress treatments. However, Gagneul et al. (2007) questioned its role as compatible solute involved in salt tolerance in *L. latifolium*; these authors considered Pro, together with β-alanine betaine and choline-*O*-sulfate, rather as 'temporally associated with expression of more important traits for coping with salinity', so that the observed variations in Pro levels could be related to

successive damage and repair at the mitochondrial step of Pro oxidation (Gagneul et al., 2007).

Glycine betaine (GB) is another widely distributed osmolyte in plants, present at high concentrations in many halophytes, especially in members of the Chenopodiaceae and salt-tolerant species of the Poaceae (Rhodes & Hanson, 1993). Glycine betaine is synthesised mainly from choline, and GB accumulators have particular adaptations in choline and methyl group biogenesis that are not present in other plants (Rhodes & Nadolska-Orczyk, 2002). Choline-*O*-sulfate, reported in all Plumbaginaceae species (Hanson et al., 1994), is synthesised from choline by a salt-inducible choline sulfotransferase (Rivoal & Hanson, 1994). A possible explanation for this particularity is that binding sulphates to the choline molecule may represent a mechanism of sulphate detoxification since only chloride anions, but not sulphate, are excreted through the salt glands present in members of this family (Hanson et al., 1994). Choline-*O*-sulphate synthesis competes with that of GB for the available choline and, therefore, it may be one of the factors involved in the evolution of alternative betaines (β-alanine betaine and proline betaine) biosynthesis pathways from substrates other than choline (Hanson et al., 1994; Rhodes & Nadolska-Orczyk, 2002).

The compound β-alanine betaine, specific of the Plumbaginaceae family, is synthesised by methylation of β-alanine, a reaction that does not require oxygen and may be regarded as an adaptation to anoxic saline environments (Hanson et al., 1991, 1994; Rhodes & Nadolska-Orczyk, 2002). In agreement with this idea, *Limonium* species growing in dry, sandy or rocky soils are generally GB accumulators, whereas those typical of salt marshes synthesise predominantly β-alanine betaine (Rhodes & Hanson, 1993). Proline betaine and hydroxyproline betaine accumulate at the expense of free Pro. Proline betaine is more efficient than Pro as osmoprotectant; for this reason, it may confer increased osmotic stress resistance to *Limonium* plants using this Pro derivative as a functional osmolyte (Hanson et al., 1991, 1994; Rhodes & Nadolska-Orczyk, 2002).

In addition to these diverse metabolites, sugars and polyols have also been reported as functional osmolytes in *Limonium*. Gagneul et al. (2007), highlighted this role for free sugars, cyclitols, and organic acids previously overlooked in this genus. These authors considered that *L. latifolium*, the species they analysed, behaves as a 'glycohalophyte', with a strong ability to allocate sucrose and hexoses to counteract deleterious salinity effects, and also reported on the role of *chiro* and *mio*-ionsotiol in this species. The same compounds were also identified in *L. sinuatum* (Liu & Grieve, 2009), whereas pinitol has been reported as the main osmolyte in *L. gmelin*i (Murakeözy et al., 2002, 2003).

Table 1. Main compatible solutes reported in *Limonium* species. Only those present at high concentrations or considered as significant in the original publications are included.

Species	Treatment	Compatible solutes	Reference
L. albuferae	salt	Glu, Fru, Pro, GABA	González-Orenga et al., 2019
L. axillare	field	βAB, Pro	Youssef et al., 2003
	field	Pro	Yasseen & Abu-Al-Basal, 2008
L. anatolicum	field	βAB, Cho, Glu, Fru, Pro	Furtana et al., 2013
L. aureum	salt	βAB; COS	Hanson et al., 1991
L. diffusum	field	PB	Hanson et al., 1994

L. bicolor	salt	*Pro*	Liu et al., 2006
L. dufourii	salt	*Pro, GABA*	González-Orenga et al. 2019
L. dumosum		*βAB; COS; Pro; Tryamine*	Bouchereau et al., 1999
L. ferulaceum		*PB*	Hanson et al., 1994
L. girardianum	salt	*Pro, Fru, Suc*	Al Hassan et al., 2017
	drought	*Pro, GB, Glc*	González-Orenga et al., 2019
	field	*Pro, Fru*	Gonzalez-Orenga et al., 2020
L. globuliferum	field	*Pro, COS, βAB*	Hanson et al., 1994; Tipirdamaz et al., 2006
L. iconicum	field	*GB, COS, βAB*	Tipirdamaz et al., 2006
	field	*GB, βAB, Cho, Glu, Fru*	Furtana et al., 2013
L. latifolium	field	*βAB, COS*	Hanson et al., 1994
	Hoagland	*βAB, COS, Pro*	Bouchereau et al., 1999
	salt	*Gln, Suc, Fru, Glc, cInos, mInos*	Gagneul et al., 2007
L. lilacinum	field	*βAB, Cho, Glu, Fru, Pro*	Furtana et al., 2013
L. gmelini	Hoagland	*βAB, COS, Pro, Tryamine, Glutamate, Methionine*	Bouchereau et al., 1999
	field	*Pin*	Murakeözy et al., 2002
	field	*Pin, βAB, COS*	Murakeözy et al., 2003
L. liniifolium	drought	*Pro, oxalic acid*	Tabot et al., 2014
L. guyonianum	field	*PB, Hydroxyproline betaine*	Hanson et al., 1994
L. macrophyllum	field	*Pro, COS*	Hanson et al., 1994
L. mucrontaum	field	*Pro, COS*	Hanson et al., 1994
L. monopetalum		*Hydroxyproline betaine, PB*	Hanson et al., 1994
L. narbononense	salt	*Pro, GB, Fru*	Al Hassan et al., 2017
	drought	*R, GB*	González-Orenga et al. 2019
	field	*Pro, GB, Fru*	González-Orenga et al. 2020
L. pectinatum	field	*Pro, COS*	Hanson et al., 1994
L. perezii	salt	*GB, COS*	Hanson et al., 1991
		Glutamate, Tyrosine, Methionine, Ornithine, GB, COS	Bouchereau et al., 1999
	salt	*cInos, mInos, Fru, Glc, Suc*	Liu & Grieve, 2009
L. plumosum	field	*Pro, COS*	Hanson et al., 1994
L. puberulum	field	*Pro, COS*	Hanson et al., 1994
L. salicorniaceum	field	*Hydroxyproline betaine*	Hanson et al., 1994
L. santapolense	salt	*GB, Fru, Suc*	Al Hassan et al., 2017
	drought	*Pro, Suc, Fru, GB*	González-Orenga et al., 2019
	field	*Suc, Fru*	González-Orenga et al., 2020
L. sinuatum	salt	*GB, COS*	Hanson et al., 1991
	Hoagland	*GB, COS, Glutamate*	Bouchereau et al., 1999

	salt	*cInos, mInos Fru, Glc, Suc*	Liu & Grieve, 2009
	salt	*Pro*	Akat et al., 2020
L. tataricum	Hoagland	*βAB, COS, Pro*	Bouchereau et al., 1999
L. virgatum	salt	*Pro, GB, Suc*	Al Hassan et al., 2017
	drought	*GB*	González-Orenga et al., 2019
	field	*Fru, GB, Pro*	González-Orenga et al., 2020
L. vulgare	salt	*βAB, COS*	Hanson et al., 1991
	Hoagland	*Tryamine, Pro, βAB, COS*	Bouchereau et al., 1999

Abbreviations: Glucose (Glc), Fructose (Fru), Proline (Pro); γ-aminobutyric acid (GABA), β-alanine betaine (βAB), Choline-*O*-sulfate (COS), choline (Cho), Proline betaine (PB), Sucrose (Suc), Glycine betaine (GB); Glutamine (Gln), *chiro*-Inositol (cInos), *myo*-Inositol (mInos), Pinitol (Pin).

4.5.8 Synthesis of Antioxidant Compounds and Activation of Antioxidant Enzymes

Reactive oxygen species (ROS), generated as by-products of normal aerobic metabolism, include free radicals, highly reactive and unstable molecules with unpaired electrons – such as singlet oxygen and superoxide, hydroxyl and perhydroxyl radicals – as well as molecular oxygen, ozone or hydrogen peroxide, amongst others (Appel & Hirt, 2004). Under different biotic and abiotic stress conditions, including high salinity, ROS accumulate in excess, provoking oxidative stress in plants by oxidation of amino acid residues in proteins, the unsaturated fatty acids of cell membranes, and the nitrogenous bases in DNA. Several biochemical markers can be used to assess the level of oxidative stress affecting the plants; amongst them, malondialdehyde (MDA), a final product of polyunsaturated fatty acids peroxidation, is widely used as it is considered an excellent marker of oxidative stress (Del Rio et al., 2005). Another approach to study how stress treatments modify the cellular redox state is based on the direct quantification of specific ROS, such as hydrogen peroxide, a stable, non-radical compound mainly produced in peroxisomes chloroplasts (Cerny et al., 2018; Barsukova et al., 2019). At least theoretically, stress-induced ROS accumulation should be less pronounced in stress-tolerant than in stress-sensitive species. Therefore, when comparing the responses to salinity, or other stresses, of different related taxa, it is to be expected that higher MDA and H_2O_2 contents will be measured, under the same experimental conditions, in those more susceptible to stress.

Plants respond to oxidative stress by activating the synthesis and accumulation of antioxidant compounds and increasing antioxidant enzymes' activity. Several enzymatic systems contribute to ROS elimination and the maintenance of the appropriate cellular redox state. SOD, for example, constitutes a primary defence against ROS by catalysing the dismutation of superoxide radicals into O_2 and H_2O_2 (Alscher et al., 2002). SOD specific activity is enhanced by *de novo* synthesis of the enzyme in the presence of its superoxide substrate, which activates the transcription of the corresponding genes (Caverzan et al., 2016). H_2O_2, although not as reactive as free radicals, is still toxic, and several enzymes contribute to its elimination. The most relevant are CAT, which decomposes H_2O_2 into O_2 and H_2O and is induced by accumulation of its substrate (Gunes et al., 2007), and APX, which catalyses the reduction of hydrogen peroxide coupled to ascorbate oxidation. GR, employing NADPH as the specific cofactor,

catalyses the reduction of oxidised glutathione (GSSG) to its reduced form (GSH), thus contributing to maintaining the adequate cellular redox state (Rau & Reddy, 2008).

Under severe and/or prolonged stress, the enzymatic antioxidant machinery may be overcome, and the second line of defence is activated by the synthesis and accumulation of non-enzymatic antioxidants (Fini et al., 2011). Amongst these compounds, vitamins E and C, reduced glutathione, carotenoids and phenolic compounds, particularly the subgroup of flavonoids, have special relevance. Besides many other biological functions, these metabolites are involved in the responses of plans to all types of abiotic stresses, including salinity, through reduction of oxidative stress (Davies et al., 2018).

ROS production and toxicity is common in glycophytes and halophytes. Environmental stressful conditions disrupt plant metabolic homeostasis; as a result, as indicated above, production of ROS is drastically increased, causing oxidative stress in the cells. However, halophytes seem to be able to counteract ROS effects, maintaining them at levels that are not toxic, even at high external salinity (Jithesh et al., 2006; Ozgur et al., 2013). For example, low lipid peroxidation, assessed by quantifying MDA contents in the plants, were reported in several halophytes, both sampled in the field (Gil et al., 2014) or subjected to controlled salt stress treatments in the greenhouse (Al Hassan et al., 2017b). Antioxidant enzymes have been regarded as the essential components of the adaptive defence mechanism against oxidative stress in halophytes (Jithesh et al., 2006). Therefore, reports on oxidative stress in this category of plants have focused mostly on ROS enzymatic scavenging systems (Bosé et al., 2014). Several publications showed high constitutive levels in halophytes of antioxidant enzymes, such as SOD, CAT, different peroxidases or GR, the induction of new isoenzymes of some of them, and rapid activation of the enzymatic machinery before the accumulation of ROS to a level that can induce irreversible damage (Ozgur et al., 2013; Bosé et al., 2014).

In agreement with the above data, many studies report low levels of oxidative stress in *Limonium* plants under natural or artificial stress conditions, maintained mostly by activation of enzymatic antioxidant systems. For example, MDA contents remained low in four *Limonium* species (*L. girardianum*, *L. narbonense*, *L. santapolense* and *L. virgatum*) from eastern Spain, sampled in the field in summer (Gonzalez-Orenga et al., 2020). High temperatures and intense drought lead to the highest salinity and the lowest moisture in the soil, making summer the most stressful season under Mediterranean climate. Responses to water stress of these species were also analysed under controlled greenhouse conditions, by withholding irrigation for one month. In all cases, neither the total free radical scavenging activity of the leaf extracts nor MDA or H_2O_2 contents, differed significantly between the water-stressed and control plants, except for a slight (but statistically significant) increase in MDA levels in the leaves of *L. narbonense* (Gonzalez-Orenga et al., 2019). Furthermore, a significant increase in the specific activity of antioxidant enzymes (SOD, CAT, APX, GR) was generally observed in response to the water deficit treatment, albeit with quantitative and qualitative differences between the four species and the four enzymes, whereas no significant changes were observed in the leaf contents of antioxidant compounds (Gonzalez-Orenga et al., 2019). Similar behaviour has been observed in other species of the genus, such as *L. latifolium*, in which lipid peroxidation – assessed by MDA content measurements – did not vary significantly

in response to salt treatments (Hamed et al., 2014), or *L. bicolor*, where high SOD, POD and CAT enzyme activities were shown to ensure a low level of oxidative stress (Li, 2008). Nevertheless, under specific experimental conditions, different results have also been reported; for example, an increase in H_2O_2 and MDA concentrations was detected in *L. stocksii* and *L. deliactulum* subjected to high salinity treatments (Hameed et al., 2015; Souid et al., 2019).

The last species mentioned above, *L. delicatulum*, has been the subject of more extensive research. Plant growth was stimulated at moderate salt concentrations, up to 200 mM NaCl, compared to the control; growth was inhibited only by higher concentrations, which generated a significant increase in H_2O_2 and MDA levels. The antioxidant activity in this species is based mainly on the activation of antioxidant enzymes, SOD, APX and GPX, which increased in parallel to the salt concentration applied, whereas CAT was activated only at higher concentration. However, a low correlation was established with non-enzymatic antioxidants, such as total phenolic compounds and antioxidant flavonoids (Soiud et al., 2016). Field studies were also performed on the sabkha biotype of the same species. In plants sampled from natural environments, natural fluctuations in salinity and aridity were correlated with increased concentrations of MDA and H_2O_2, and a sharp increase of polyphenols, flavonoids, flavonols, and vitamins C and E. Regarding the antioxidant enzymes, SOD, GPX and APX showed enhanced activity and overexpression; as well as CAT but only when salinity was maximal in summer (Souid et al., 2018).

Finally, a study on eight *Limonium* species from stressful habitats in Tunisia indicated that the synthesis of secondary metabolites is enhanced with the severity of environmental conditions, especially salinity and drought. *L. vulgare* contained the highest concentrations of flavonoids, flavonols, vitamin C, vitamin E and carotenoids, and showed the highest SOD, GPX, APX and CAT activities. Enzymatic activities increased during the period when the biotopes' salinity was high (Souid et al., 2019).

Most studies on antioxidants in *Limonium*, like those described in the previous paragraphs, reveal the contribution of antioxidant enzymatic systems to the plants' responses to abiotic stress. However, *Limonium*, as other halophytes that possess efficient mechanisms of tolerance based on the control of ion transport and accumulation of osmoprotectants, may not require a high level of antioxidant activity simply because excessive ROS accumulation is prevented by those mechanisms, limiting the generation of oxidative stress (Bosé et al., 2014). Besides the salt glands and the accumulation of toxic ions in the foliar tissue vacuoles, many *Limonium* species have high constitutive concentrations of glycine betaine or polyols, which act as ROS scavengers, in addition to their role in osmotic adjustment (Bosé et al., 2014). Therefore, 'increased antioxidant activity should be treated as a damage control mechanism rather than a trait directly conferring salinity stress tolerance' (Bosé et al., 2014).

4.5.9 Conclusions

Limonium species, most highly resistant to elevated salinity and many also to drought, constitute attractive models for fundamental research on plants' responses to abiotic stress and their mechanisms of tolerance. They include morpho-anatomical

adaptations, such as salt glands, typical of recretohalophytes; the control of ion transport to accumulate toxic Na^+ (and Cl^-) in the leaf vacuoles, with the concomitant synthesis of a wide variety of osmolytes for osmotic adjustment under stress – and with additional roles as osmoprotectants; or the activation of efficient antioxidant systems. From a practical point of view, species of this genus have a great potential for the development, through appropriated breeding programmes, of (minor) commercial crops of ornamental, medicinal and gourmet food plants; and also, plants to be used in phytoremediation actions for decontamination of heavy metal-polluted soils. In this way, the enormous genetic diversity and high stress tolerance of wild *Limonium* taxa would be efficiently utilised to obtain plants that could be cultivated under harsh conditions of salinity and drought, tolerating limited irrigation and/or irrigation with brackish waters, thus contributing to a sustainable, 'saline' or 'arid' agriculture.

Author Contributions

Conceptualization, M.M. and O.V.; resources, M.N.G., M.B.; data curation, S.GO.; writing—original draft preparation, S.G.O. and M.N.G.; writing—review and editing, M.B. and O.V.; visualisation, S.G.O. amd M.N.G.; supervision, O.V.; project administration, M.B; funding acquisition, M.B.

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Publicación VI:
Subcapítulo 4.6

Responses to Increased Salinity and Severe Drought in the Eastern Iberian Endemic Species *Thalictrum maritimum* (Ranunculaceae), Threatened by Climate Change

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Responses to Increased Salinity and Severe Drought in the Eastern Iberian Endemic Species *Thalictrum maritimum* (Ranunculaceae), Threatened by Climate Change

Abstract

Thalictrum maritimum is an endangered, endemic species in East Spain, growing in areas of relatively low salinity in littoral salt marshes. A regression of its populations and the number of individuals has been registered in the last decade. This study aimed at establishing the causes of this reduction using a multidisciplinary approach, including climatic, ecological, physiological and biochemical analyses. The climatic data indicated that there was a direct negative correlation between increased drought, especially during autumn, and the number of individuals censused in the area of study. The susceptibility of this species to water deficit was confirmed by the analysis of growth parameters upon a water deficit treatment applied under controlled greenhouse conditions, with the plants withstanding only 23 days of complete absence of irrigation. On the other hand, increased salinity does not seem to be a risk factor for this species, which behaves as a halophyte, tolerating in controlled treatments salinities much higher than those registered in its natural habitat. The most relevant mechanisms of salt tolerance in *T. maritimum* appear to be based on the control of ion transport, by (i) the active transport of toxic ions to the aerial parts of the plants at high external salinity—where they are presumably stored in the leaf vacuoles to avoid their deleterious effects in the cytosol, (ii) the maintenance of K^+ concentrations in belowground and aboveground organs, despite the increase of Na^+ levels, and (iii) the salt-induced accumulation of Ca^{2+}, particularly in stems and leaves. This study provides useful information for the management of the conservation plans of this rare and endangered species.

Keywords: endangered species; water deficit; salt stress; halophytes; climate analysis; soil analysis; plant growth analysis; biochemical parameters; biodiversity; conservation programmes

4.6.1 Introduction

The study of plant responses to environmental abiotic stress conditions and stress tolerance mechanisms is one of the most active lines of research in plant biology, given its undoubted scientific interest and its practical implications in agriculture. These studies are also important to help design and implement conservation strategies for natural habitats of great ecological value, such as Mediterranean coastal salt marshes, which are highly threatened by human influence and highly sensitive to the effects of climate change (Heywood, 2011; Laguna & Ferrer-Gallego, 2015; Gómez-Mercado et al., 2017). The vegetation of these ecosystems includes different salt-tolerant species, or halophytes (Grigore, 2019; Touchette et al., 2019), some of them relatively abundant and present in many geographical areas, which constitute the typical salt marsh communities. Together with these common structural species, others, less frequent or even rare, are precisely those on which the uniqueness of each salt marsh depends and contribute substantially to increase the biodiversity of these specialised habitats. The coastal marshes near the city of Valencia, in eastern Spain, shelter a large number of such species of great ecological and conservation value, as many are endemic (including some exclusive Valencian endemics), or very rare, threatened or even extinct in recent times in part of the territory (Laguna et al., 2003). Most of these species have not been previously studied, and their limits and mechanisms of stress tolerance are virtually unknown.

Thalictrum maritimum Dufour (Ranunculaceae family), belongs to this category of species; it was identified by Jean-Marie Léon Dufour in 1860 in salt marshes near the city of Valencia, Spain (Dufour, 1860; Ferrer-Gallego et al., 2019). This species is an Eastern Iberian endemic, growing only in a few coastal areas (Aguilella et al., 2010; Ferrer-Gallego et al., 2015). Its southern limit is located within the Albufera Natural Park, the most relevant protected area of the Valencian Community (Ballester et al., 2003). It is a perennial geophyte, with an erect stem up to 80 cm. The leaves are composed of small, lanceolate and narrow leaflets. The inflorescence is loose, and flowers have yellow petals, and numerous stamens, which exceed the corolla. The fruit is a spindle-shaped achene with fine ribs and a hard shell. It flowers from July to October (Montserrat, 1986), and fruiting takes place in September–October. The radius of dispersion of the seeds is not known. The accumulated seed germination under standard controlled conditions, only imbibed with distilled water, often does not reach more than 40% (Ferrer-Gallego et al., 2013; Gil et al., 2017), but mechanical scarification and pre-treatments with sulphuric acid increase germination rate up to 80% (Lopéz & Fabregat, 2007).

The species is found worldwide only in four sites, all located in the Valencian Community. The species and its habitat are threatened by land degradation, agricultural and urban development, changes in the soil regime due to floods and increased salt levels, soil over-fertilisation, competition with invasive plant species, wildfires and touristic pressure (Aguilella et al., 2010). The species is classified at the legal rank "Vulnerable" in the Valencian Catalogue of Endangered Flora Species (Moreno, 2008) as

well as the IUCN homonym rank in the Spanish Red List of Vascular Flora (Moreno, 2008). The populations are subjected to regular monitoring, and accessions of collected seeds are long-term stored in the Valencian Wild Flora Germplasm Bank, hosted by the CIEF (Valencian Centre for Forestry Research and Experimentation), a technical facility depending on the Valencian Wildlife Service—'Servicio de Vida Silvestre', in Spanish. Because of its conservation interest, several institutions have developed propagation protocols, and population reinforcements and translocations have been performed (Aguilella et al., 2010; Curcó, 2007). However, despite these conservation efforts, many of the populations of this threatened endemic plant are in decline and the species has entirely disappeared from some locations over the last years (Servicio de Vida Silvestre, unpublished data and BDBCV). Regarding, for example, the total number of adult individuals present in the Natural Park of Albufera, it varied from 2406 plants in 2011, to 2516 in 2014 and 3333 in 2015, but decreased to 2381 in 2017 (Servicio de Vida Silvestre, unpublished data).

Unknown issues on *T. maritimum* behaviour include its tolerance limits to abiotic stress factors, as well as the mechanisms that enable this species to cope with a stressful environment. In addition to the usual factors of stress in saline habitats, salt marshes, as other wild habitats in the Mediterranean ecosystems, also suffered during the last decades from the effects of global warming, especially of increased temperatures in summer and prolonged drought throughout the year (Gómez-Mercado et al., 2017; Lionello & Scarascia, 2018). These effects of climate change will most likely also cause an increase in the salinity of these habitats in the years ahead, which could further affect the already threatened *T. maritimum* populations. The present study is based on a multidisciplinary approach, with the aims of analysing the: (i) habitat characteristics of populations of *Thalictrum maritimum* in the Natural Park of Albufera, (ii) climate analysis for the last 19 years, (iii) soil characteristics in areas where the natural populations are located, and in sites were future reintroductions are planned or proposed, (iv) limits of tolerance to drought and salinity of the species in greenhouse controlled conditions, and (v) the main mechanisms of response to abiotic stress by exploring the ion absorption and transport, synthesis of osmolytes and antioxidant responses of the plants, subjected to water deficit and salt stress treatments. The information provided by these analyses should explain the decline observed in *T. maritimum* populations over the last years and help design and implement conservation programmes of this highly endangered species.

4.6.2 Material and Methods

Study Area

The area of study is known as 'Devesa de l'Albufera', a close to 10 km long dune system, occupying 800 ha and belonging to the municipality of Valencia, in eastern Spain. This large dune complex is a sandy coastal strip that progressively closed an ancient maritime bay, nowadays converted into the Albufera lake. The Albufera, covering ca. 2000 ha, is the biggest continental lake in Spain, as well as the second most important Spanish wetland (Ballester at al., 2003). Both, sand coastal dunes and the Albufera lake, as well as a large belt of traditional rice fields, are part of the Albufera Natural Park, officially declared in 1986 by the Valencian government. This natural park

covers a surface of 21,000 ha. It is the most celebrated protected natural area in the Valencian region and one of the most important in Western Europe, mainly regarding waterfowl migrations (Ballester at al., 2003). Since 1990, the site has been included in the list of Wetlands of International Importance of the Ramsar Convention; in 1991, it was declared a Special Protection Area under the EU Directive on the Conservation of Wild Birds (79/409/EEC). In addition, the park includes habitats and refuges of species included in the EU Habitats Directive (92/43/EEC) and is also included in the Geneva Protocol on Special Protection Areas in the Mediterranean (Soria, 2006).

The populations of *Thalictrum maritimum* live in the so-called 'malladas', inter-dune depressions located in the Devesa area, acting as small salt marshes, often inundated during the winter period.

Habitat Survey

A first survey was carried out in situ to verify the existence or disappearance of the populations collected in the former literature (Mansanet, 1979; Aguilella et al., 2010) and official records on the species distribution in the natural park, kindly provided by the regional Wildlife Service (Servicio de Vida Silvestre) and the municipal Devesa-Albufera Service.

For the sites where *T. maritimum* is currently extant, vegetation relevés were carried out on each population site. Geographic location was obtained with a Garmin GPS in UTM coordinates (ETRS 89, zone 30); they have not necessarily been taken in the centre of the relevé to avoid trampling, which is aggravated when the soil is waterlogged. The phytosociological method was used to note the proportions in which the species appear (Braun-Blanquet et al., 1932). Nomenclature of the taxa follows Euro Med (2006) and the syntaxonomic nomenclature is according to Rivas-Martinez et al. (2001, 2002). In each relevé site, three measurements of soil parameters were taken: moisture (%), electric conductivity (dS m^{-1}) and temperature (°C), with a WET sensor (Delta Devices, Cambridge, UK). The relevés were carried out during the optimal phenological time, mainly from mid-June to mid-November 2019, because this is the time when *T. maritimum* individuals are easily detected; besides, this is the more recommended time to avoid disturbance to wintering birds, which are the most relevant protection object of the natural park (Ballester et al., 2003).

Climatic Analysis

Climate data were retrieved from SIAR, the Agroclimatic Information System for Irrigation of the Spanish Ministry of Agriculture, Fisheries and Food. Data on the mean, maximum and minimum temperatures, rainfall and reference evapotranspiration (ETo) were collected for the past 19 years, on a monthly basis, from the agroclimatological station Benifaio, located at 11 km from the area of study. References to bioclimatic classification were made according to the Worldwide Bioclimatic Classification System.

Soil Analysis

For a complete analysis, eight soils samples were taken from the salt marsh where the most abundant population of *T. maritimum* was found ('Mallada del Canyar'), four at 0–10 cm and four at 10–20 cm depth. The samples were air-dried at room temperature, and then crushed with a roller to break aggregates, and passed through a 2-mm sieve. Analyses were performed on fine soil (diameter <2 mm). Soil texture was analysed by the hydrometer method (Bouyoucos, 1962). Organic matter was determined by the Walkley and Black method (Walkley & Black, 1934) and carbonates by using the Bernard calcimeter (Loeppert & Suarez, 1996). The following parameters were analysed in a saturation extract: pH, electric conductivity (EC), chlorides, Na^+, K^+, Ca^{2+}, and Mg^{2+}. A Crison pH-meter Basic 20 and a Crison Conductimeter Basic 30 (Crison Instruments SA, Barcelona, Spain) were used to measure pH and EC, respectively. Sodium and potassium were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), chlorides were measured in an MKII Chloride Analyzer 926 (Sherwood, Inc., Cambridge, UK), and divalent cations, Ca^{2+} and Mg^{2+}, were measured with an atomic absorption spectrometer SpectrA 220 (Varian, Inc., CA, USA).

Plant Growth and Stress Treatments in the Greenhouse

Young plants of *T. maritimum*, provided by the 'Centre for Conservation of Freshwater Species of the Valencian Community', were transplanted individually to standard 1.6 L plastic pots, in a mixture of peat, perlite and vermiculite (2:1:1), and maintained in the greenhouse under a 12 h-light/12 h-dark photoperiod, at 23 °C during the day and 18 °C during the night. The stress treatments were initiated after three weeks of acclimatisation. The pots were divided into five batches (one per treatment) of five pots each and placed in plastic trays (55 × 40 cm), and the following treatments were applied: control (C), water stress (WS), salt treatments (100 mM, 200 mM and 300 mM NaCl), over a period of 23 days. Plants subjected to the WS treatment were not irrigated at all during this period, whereas the remaining plants were irrigated twice a week by adding to each tray 1.5 L of deionised water (for the control treatment) or NaCl aqueous solutions of the final concentrations indicated above (for the salt treatments); in the latter case, the trays were thoroughly washed with deionised water before addition of the NaCl solutions. The treatments were stopped after 23 days, when plants subjected to the water deficit treatment showed intense wilting, but before mortality was observed.

Substrate Analysis

Soil moisture, expressed as volume percentages, and soil electroconductivity (EC), expressed in dS m^{-1}, were determined in the pots substrate at the beginning, during and at the end of the treatments, with a WET-2 Sensor (Delta—T Devices, Cambridge, UK).

Plant Growth Parameters

Non-destructive plant growth parameters, such as stem length, number of leaves and number of branches, were determined at the beginning and the end of the treatments. Fresh weight of leaves, stems and roots were separately measured after 23 days of treatment, when all plants were sampled. Part of the fresh material of each organ

was weighed (fresh weight; FW), dried for four days at 65 °C, until constant weight, and then weighed again (dry weight; DW), to calculate the water content percentage (WC%) for roots, stems and leaves, according to the following formula: (Gil et al., 2014).

$$WC\% = [(FW - DW)/FW] \times 100 \quad (1)$$

Photosyntetic Pigments

Chlorophylls a and b (Chl a, Chl b) and total carotenoids (Caro) were determined as previously described (Lichtenthaler & Wellburn, 1983). Ten mL of ice-cold 80% (*v*/*v*) acetone was used to extract pigments from 0.05 g of fresh leaf material. After mixing overnight and centrifuging for 10 min at 12,000 rpm, the supernatant was collected, and its absorbance was measured at 663, 646, and 470 nm. Chl a, Chl b, and Caro concentrations were calculated using the equations below, described by Lichtenthaler et al. (Lichtenthaler & Wellburn, 1983).

$$\text{Chlorophyll a } (\mu g\ ml^{-1}) = 12.21 \times A_{663} - 2.81 \times A_{646} \quad (2)$$

$$\text{Chlorophyll b } (\mu g\ ml^{-1}) = 20.13 \times A_{646} - 5.03 \times A_{663} \quad (3)$$

$$\text{Carotenoids } (\mu g\ ml^{-1}) = (1000 \times A_{470} - 3.27 \times [\text{Chlorophyll a}] - 104 \times [\text{Chlorophyll b}])/227 \quad (4)$$

Pigment contents were finally expressed in mg g^{-1} DW.

Ion Quantification

The content of Na^+, K^+ and Ca^{2+} was quantified separately from roots, stems and leaves, according to the protocol described by Weimberg (1987), in aqueous extracts of dry plant material. Two mL of Milli-Q water were added to each sample (0.1 g), vortexed and then mixed for 24 h in a shaker. The samples were incubated in a water bath for 30 min at 95 °C, cooled on ice, and filtered through a 0.45 μm nylon filter. Sodium, potassium and calcium ions were quantified using a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), and chlorides were measured in an MKII Chloride Analyzer 926 (Sherwood, Inc., Cambridge, UK).

Osmolyte Quantification

Proline (Pro) contents were measured according to the ninhydrin-acetic acid method (Bates et al., 1973). Extracts were prepared by grinding 0.1 g of fresh leaf material in two mL of a 3% (*w*/*v*) sulfosalicylic acid solution; the samples were mixed with acid ninhydrin, placed in a water bath at 95 °C for one hour, cooled on ice for 10 min, and extracted with toluene. The absorbance of the organic phase was measured at 520 nm, using toluene as the blank. A standard curve was obtained running parallel reactions containing known Pro concentrations. Leaf Pro contents were finally expressed in μmol g^{-1} DW.

Total soluble sugars (TSS) were quantified according to the protocol described by Dubois et al. (1956). Fresh leaf material (0.1 g) was ground and extracted with two mL of 80% (*v*/*v*) methanol, by mixing the samples in a shaker for 24 h, followed by centrifugation at

12,000 rpm for 15 min. Subsequently, each supernatant, appropriately diluted with water, was mixed with 95% sulphuric acid and 5% phenol. Finally, the absorbance of the samples was measured at 490 nm. TSS concentrations were expressed as mg equivalent of glucose, used as the standard (mg eq. Gluc g^{-1} DW).

Oxidative Stress Markers and Non-Enzymatic Antioxidants

Malondialdehyde (MDA), total phenolic compounds (TPC) and total flavonoids (TF) were measured in the same 80% methanol extracts used for TSS determination. MDA contents were quantified as previously described by Hodges et al. (1999), with some modifications (Taulavuori et al., 2001). The methanol extracts were mixed with 0.5% (*w*/*v*) thiobarbituric acid (TBA) prepared in 20% (*w*/*v*) trichloroacetic acid (TCA). The samples were incubated in a water bath at 95 °C for 15 min, cooled on ice for five min and centrifuged at 12,000 rpm for 10 min at 4 °C; the absorbance of the supernatants was measured at 532 nm. After subtracting the non-specific absorbance at 440 nm and 600 nm, MDA concentrations in the extracts were calculated using the equation included in Taulavuori et al. (2001), based on the extinction coefficient of the MDA-TBA adduct at 532 nm (155 mM^{-1} cm^{-1}). Extracts mixed with TCA but without TBA, were assayed in parallel and used as controls. MDA contents were finally expressed as nmol g^{-1} DW.

Total phenolic compounds (TPC) were quantified by reaction with the Folin-Ciocalteu reagent, as previously described (Blainski et al., 2013). Leaf methanol extracts were mixed with the reagent and 15% (*w*/*v*) Na_2C0_3 and incubated at room temperature for 90 min in the dark. Finally, the absorbance of the samples was measured at 765 nm. A calibration curve was obtained from samples containing known amounts of gallic acid (GA), assayed in parallel. TPC concentrations were expressed as equivalents of gallic acid (mg eq. GA g^{-1} DW).

Total flavonoids (TF) were determined by the method described by Zhishen et al. (1999), based on the nitration with $NaNO_2$ of aromatic rings containing a catechol group, followed by reaction with $AlCl_3$ at a basic pH. After the reaction, the absorbance of the samples was determined at 510 nm. TFs concentrations were expressed as equivalents of catechin, used as the standard (mg eq. C g^{-1} DW).

Antioxidant Enzymes Assays

Enzyme specific activities were determined in crude protein extracts, prepared from leaf material stored frozen at −75 °C, as previously described (Gil et al., 2014). The protein concentration in the extracts was determined by the Bradford method (1976), using bovine serum albumin (BSA) as the standard and the Bio-Rad reagent (Bio-Rad Laboratories, Alcobendas, Spain).

Superoxide dismutase (SOD) activity was assayed following the procedure described by Beyer & Fridovich (1987), by monitoring the inhibition of nitroblue tetrazolium (NBT) photoreduction in reaction mixtures containing riboflavin as the source of superoxide radicals. After the addition of riboflavin and NBT, the samples were irradiated with 15 W fluorescent tubes for 10 min at 25 °C, and the absorbance at 560 nm was measured.

One SOD unit was defined as the amount of enzyme causing a 50% inhibition of NBT photoreduction under the assay conditions.

Catalase activity (CAT) was determined as described by Aebi (1984). The assay is based on the consumption of H_2O_2 added to the reaction mixtures by the catalase present in the extracts, which is followed by the decrease in absorbance at 240 nm. One CAT unit was defined as the amount of enzyme that breaks down one mmol of H_2O_2 per minute at 25 °C.

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

Statistical Analysis

Data were analysed using the programme Statgraphics Centurion XVII (Statgraphics Technologies, The Plains, VA, USA). All mean values throughout the text are based on five biological replicates. Significant differences between treatments were tested by one-way analysis of variance (ANOVA) at the 95% confidence level, and post hoc comparisons were made using the Tukey's HSD test at $p \leq 0.05$. A principal component analysis (PCA) was used to check the similarity between the responses to water and salt stress.

4.6.3 Results

Habitat Characteristics of the *Thalictrum maritimum* Populations in the Natural Park of Albufera

As a result of bibliographic searches and official consultations on the location of *T. maritimum* populations in the Natural Park of Albufera, a set of seven UTM 1 × 1 km squares was defined, corresponding to UTM30S YJ2961, 3061, 3062, 3156, 3157, 3256, 3257. However, after intense in situ surveys, only six of them hold current populations, having entirely vanished in the square 30S YJ3257, where the species lived in small salt marshes (Figure 1).

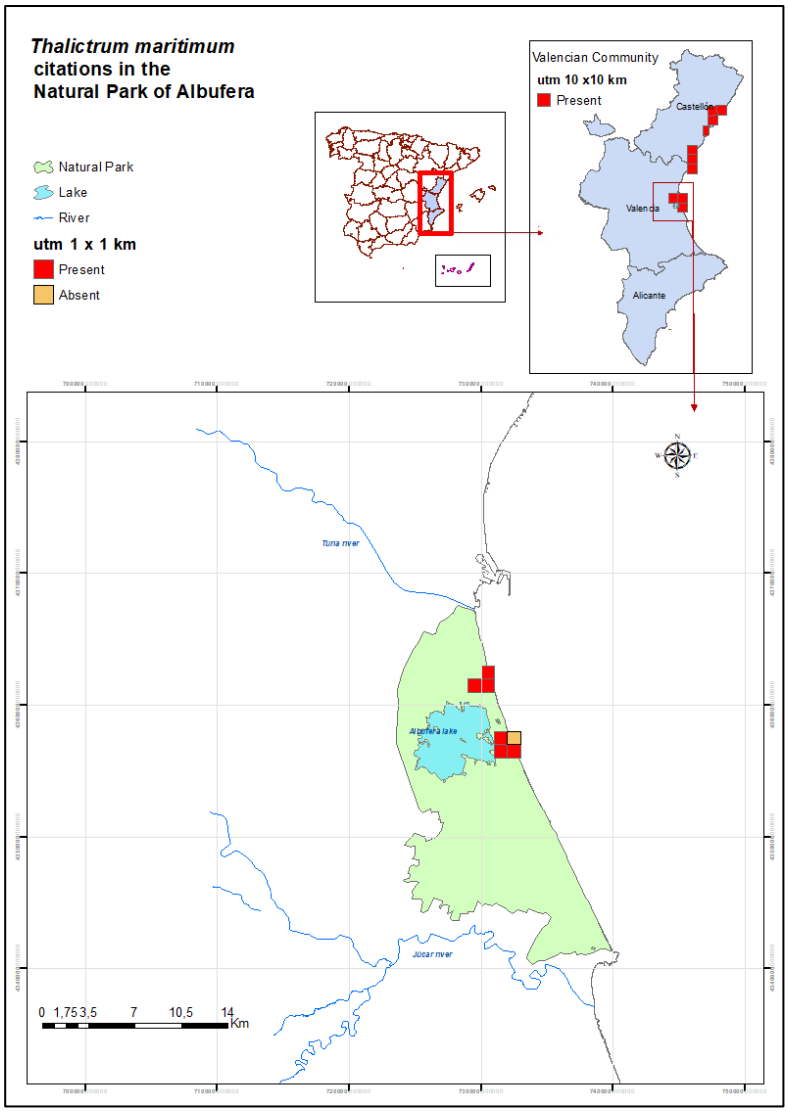


Figure 1. Location of the populations of *Thalictrum maritimum* in the Valencian Community (utm 10 × 10 km squares; upper right panel) and in the Natural Park of Albufera (utm 1 × 1 km squares; lower panel).

The phytosociological study was performed during summer, coinciding with the optimal phenological period for *T. maritimum* and other species which grow on salt marshes. The species was found in ten locations in the natural park, but some of its previously reported populations no longer exist. Table 1 synthesises the habitat characteristics of each population (the extension and coverage of the plant community, soil moisture and electric conductivity), all species present in the community with their range of cover, according to Braun-Blanquet's scale (Braun-Blanquet et al., 1932) and their corresponding vegetation classes (Rivas-Martínez et al., 2001; 2002).

Table 1. Phytosociological relevés with *Thalictrum maritimum* from the Natural Park of Albufera. The extension and vegetation coverage, as well as soil properties (moisture and electric conductivity) are shown for each of the ten relevés. Ranges of cover of the species present in each relevé are indicated, according to the Braun-Blanquet's cover-abundance scale [20]: occasional presence, <5% (+); 5–12% (**1**); 12–25% (**2**); 25–50% (**3**); 50–75% (**4**); 75–100% (**5**). The species are classified into their corresponding vegetation classes (Rivas-Martínez et al., 2001; 2002).

Relevé nº		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Area (m^2)		25	100	90	100	8	100	100	60	100	100
Vegetation coverage (%)		100	95	100	100	100	100	100	100	100	100
Moisture (%)		40.2	59.4	60.5	58.3	32.0	50.7	39.9	20.6	56.3	51.9
Conductivity (EC, dS m^{-1})		3.7	2.1	1.9	2.1	12.2	3.8	1.3	3.0	1.6	2.4
Species	**Vegetation Class**										
	Juncetea maritimi										
Thalictrum maritimum		3	3	+	3	1	1	2	2	4	2
Centaurea dracunculifolia					2			+			1
Dorycnium gracile			+				1	2			2
Elymus elongatus								1			+
Juncus acutus						2					
Juncus maritimus		2	2	3	2			1	1	1	
Linum maritimum		+	+	1	+			1			
Plantago crassifolia		+				1					
Samolus valerandi		+	+	1							
Schoenus nigricans		3	2	2	1	2		2		1	2
Scirpioides holoschoenus						2					
Spartina patens						3	5	3			2
	Phragmito-Magnocaricetea										
Cladium mariscus							+	+	4	3	2
Lythrum salicaria									1	1	1
Phragmites australis subsp. *hrysanthus*		3	3	4	1		+	+	1	2	1
Phragmites australis subsp. *australis*		+	+	+	1					1	
	Molinio-Arrhenatheretea										
Sonchus maritimus		+	1	1	1						
	Nerio-Tamaricetea										
Tripidium ravennae		+	+	+	1				1		+
Imperata cylindrica											+
	Quercetea ilicis										
Phillyrea angustifolia			+		+						

Pistacia lentiscus									+	+	1	1
Smilax aspera									+			
	Stellarietea mediae											
Lagurus ovatus		1	+	+	+					+		

In addition: *Artemisetea vulgaris*: *Ditrichia viscosa* in **3**, **4**, **5**; Galio-Urticetea: *Cynanchum acutum* in **3**, **4**; *Salicornietea fruticosae: Inula crithmoides* in **1**; *Thero-Salicornietea: Centaurium spicatum*, *Suaeda spicata* in **1**.

The soil electric conductivity and moisture were measured with a WET sensor in each population, and values shown in Table 1 represent mean values of 8–10 measurements. Out of the ten analysed locations, only one area was truly saline (relevé 5, with an EC of 12.2 dS m^{-1}), whereas in several soil samples EC was lower than 2 dS m^{-1}. Soil moisture ranged from 40 to 60%. The highest presence of *T. maritimu*m (4 in the Braun-Blanquet scale, representing a 50–75 % coverage) was found in the relevé 9, where soil salinity was the lowest (1.6 dS m^{-1})—except for relevé 7, with 1.3 dS m^{-1}—and with 56% humidity. However, in relevé 3, where the soil EC was similar and humidity even higher, over 60%, only three individuals were detected (+ according to the scale). In the relevés 1, 2 and 4, *T. maritimum* had a good presence (3 on the scale, corresponding to 25–50 % coverage). Plant communities identified in each phytosociological relevé are indicated in Table 2.

Table 2. Plant communities identified in the phytosociological analysis.

Communities	Relevé nº
Juncetalia maritimi	1, 2, 3, 4
Hydrocotylo-Mariscetum serrati	8, 9, 10
Spartino-Juncetum maritimi subass. *spartinetosum*	5, 6, 7

The relevés include some typical halophytic species, such as *Juncus acutus*, *J. maritimus*, *Plantago crassifolia* and *Schoenus nigricans*, but also many glycophytes, such as *Phillyrea angustifolia*, *Pistacia lentiscus* and *Smilax aspera* (species that characterise the vegetation class *Quercetea ilicis*). The presence of the invasive *Spartina patens* is remarkable, reaching high presence in relevés 5 and 7, and becoming a dominant species in number 6, shaping the plant community *Spartino-Juncetum maritimi* subass. *spartinetosum* O. Bolòs 1962. Other plant communities typical in the area were identified, belonging to *Juncetalia maritimi* Br.-Bl. ex Horvatic 1934, such as *Schoeno nigricantis-Plantaginetum crassifoliae* Br.-Bl. in Br.-Bl., Roussine & Nègre 1952, where the dominant species is *Juncus maritimus* (Cyperaceae family), and *Hydrocotylo-Mariscetum serrati* Rivas Goday & Mansanet.

Climate Analysis

Table 3 shows mean temperatures, mean maximal temperatures, mean minimal temperatures, mean atmospheric humidity, maximal and minimal humidity, rainfall and evapotranspiration obtained from the agroclimatological station Benifaio (at 11 km from the study area). According to the Worldwide Bioclimatic Classification System, 1996–2020, these data match the thermomediterranean climate belt, specific for coastal and low-altitude areas, with a strong water deficit in summer, often with absolutely no

rainfall for periods of one month or even longer. The evapotranspiration surpasses the rainfall amount during most months each year.

Table 3. Meteorological data for the last 19 years obtained from the agroclimatological station Benifaio; data provided by the Agroclimatic Information System for Irrigation (SIAR) [24]. T: temperature; Hum: humidity; Eto: evapotranspiration.

Year	Mean T (°C)	Max T (°C)	Min T (°C)	Mean Hum (%)	Max Hum (%)	Min Hum (%)	Rainfall (mm)	Eto (mm)
2001	17.98	29.24	7.88	65.38	93.35	19.41	313.80	1119.18
2002	17.44	29.09	7.43	67.93	94.27	21.22	322.70	1091.91
2003	17.60	29.47	6.54	67.65	93.35	18.84	289.00	1155.10
2004	17.63	28.95	7.13	69.57	96.33	18.40	594.40	1006.45
2005	16.45	29.50	4.48	68.32	95.89	16.36	377.60	1117.97
2006	17.53	29.04	6.93	69.13	95.68	18.00	464.40	1189.38
2007	16.81	29.60	5.90	68.13	95.31	16.26	894.40	1164.50
2008	16.88	29.45	5.91	68.35	95.67	17.74	674.40	1194.10
2009	17.34	29.85	6.26	68.60	97.16	19.03	446.20	1215.26
2010	16.78	29.69	5.54	68.31	97.06	19.32	565.00	1206.22
2011	17.57	30.45	6.87	70.32	96.52	18.75	472.00	1166.73
2012	17.31	30.46	5.13	67.58	98.34	18.41	503.61	1208.25
2013	17.55	29.92	6.23	63.26	95.27	16.95	263.80	1245.42
2014	18.32	30.81	8.02	65.32	95.90	15.54	224.40	1278.22
2015	17.76	30.88	7.10	70.02	98.56	17.34	401.26	1169.08
2016	17.85	29.66	6.46	68.66	98.42	20.57	259.57	1218.41
2017	17.59	29.97	6.77	68.51	97.63	18.44	307.26	1238.82
2018	17.60	29.55	6.85	68.06	97.09	22.58	684.02	1225.71
2019	17.79	31.41	7.25	66.59	97.58	19.49	427.00	1243.83
Mean	17.46	29.84	6.56	67.88	96.28	18.56	446.57	1181.82

Climate charts indicating the evolution of mean temperatures, rainfall and evapotranspiration are shown for 2015 (Figure 2a), the year when the maximal number of individuals of *T. maritimum* was censused in the territory of the natural park, and 2017 (Figure 2b), when a drastic decrease in the number of individuals was registered, as mentioned in the Introduction. The main difference between the two years was the amount of rainfall, which dropped from 401.26 mm (close to the mean value for the 19 years) in 2015 to 307.26 mm (notably lower than the mean) in 2017 (Table 3). The distribution of the rainfall also differed in the two years; 2015 was characterised by a wet

autumn (Figure 2a), contrary to 2017, when this season was exceptionally dry (Figure 2b).

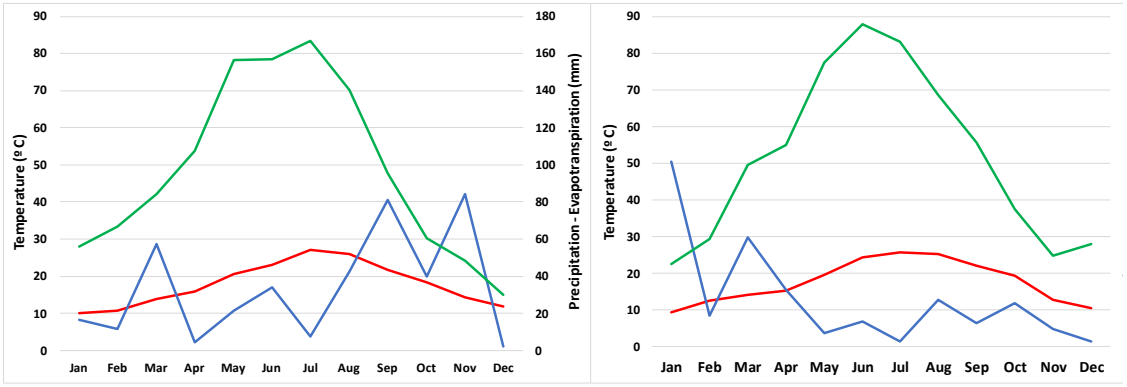


Figure 2. Climate charts including mean temperatures, rainfall and evapotranspiration during 2015 (**a**) and 2017 (**b**). Mean temperature is shown in red, rainfall in blue and evapotranspiration in green. Climatic data were from the nearest meteorological station in Benifaio, provided by the Agroclimatic Information System for Irrigation (SIAR).

Soil Characteristics

Soil characteristics were determined in the area where the most abundant population of *T. maritimum* was found in the Natural Park of Albufera, the salt marsh known as 'Mallada del Canyar'. Soil samples were taken in the vicinity of plants, at two depths, 0–10 cm and 10–20 cm. Several physical and chemical soil parameters were measured and are summarised in Table 4.

Table 4. Soil characteristics in the salt marsh 'Mallada del Canyar', where the most abundant population of *T. maritimum* is located. Values represent means followed by SE ($n = 4$).

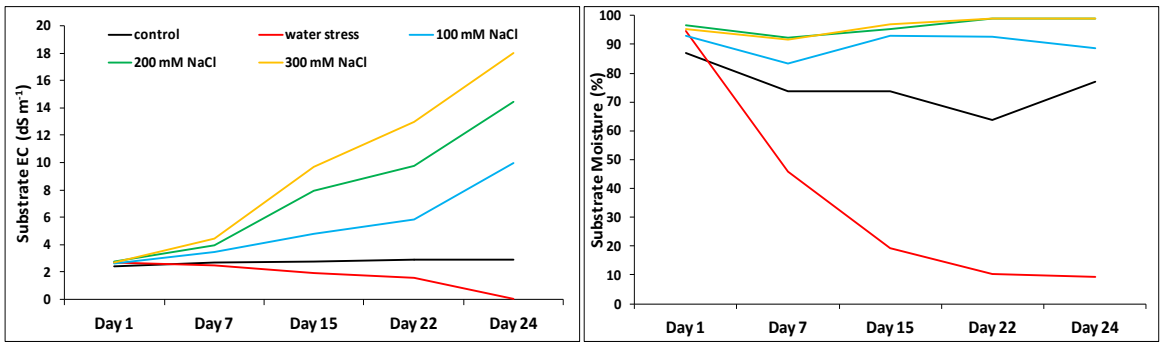
Parameter	0–10 cm Depth	10–20 cm Depth
Sand (%)	95.00 ± 0.28	93.56 ± 1.05
Silt (%)	3.50 ± 0.19	4.49 ± 0.74
Clay (%)	1.49 ± 0.08	1.92 ± 0.31
Apparent density (g cm^{-3})	1.09 ± 0.09	1.15 ± 0.07
Porosity (%)	58.86 ± 3.82	56.60 ± 2.64
Carbonates (%)	22.51 ± 4.69	28.23 ± 9.58
Organic Matter (%)	1.96 ± 0.75	1.29 ± 0.21
pH	7.30 ± 0.30	7.39 ± 0.22
EC (dS m^{-1})	5.01 ± 3.45	3.06 ± 1.32
Na^+ (meq L^{-1})	46.36 ± 6.77	42.67 ± 4.62
K^+ (meq L^{-1})	1.55 ± 0.56	1.26 ± 0.22
Cl^- (meq L^{-1})	20.55 ±5.37	28.18 ± 2.22
Ca^{2+} (meq L^{-1})	7.51 ± 2.21	6.43 ± 0.70
Mg^{2+} (meq L^{-1})	5.48 ± 3.32	3.93 ± 0.81

Soils have a sandy texture, the percentage of sand representing the major component, with low amounts of silt and clays. The pH is neutral, and the salinity at 0–10 cm depth

is low. At 10–20 cm, depth, which is the area explored by the roots of the plants, the mean value of EC is below the limit for a soil to be considered as 'saline' (4 dS m^{-1}). Amongst the analysed cations, Na^+ is found at the highest concentration, almost double than that of Cl^-. The soil samples are also characterised by a high percentage of carbonates, high concentrations of divalent cations, Ca^{2+} and Mg^{2+}, and low K^+ contents (Table 4).

Substrate Electric Conductivity (EC) and Moisture

In the greenhouse experiments, substrate EC was measured throughout the treatments in all pots. As expected, it did not vary in the control treatment and even decreased slightly under water deficit conditions, but a marked time- and concentration-dependent increase was observed in the salt treatments, reaching a maximum of 18 dS m^{-1} in the pots watered for 23 days with 300 mM NaCl, due to the progressive accumulation of salt in the substrate (Figure 3a). Regarding the moisture of the substrate, a strong reduction was registered in the water stress treatment, which could be clearly observed already after one week of the absence of irrigation. Values of substrate moisture in the salt treatments were above those in the control, due to reduced absorption of water by the plants in the presence of salt (Figure 3b).



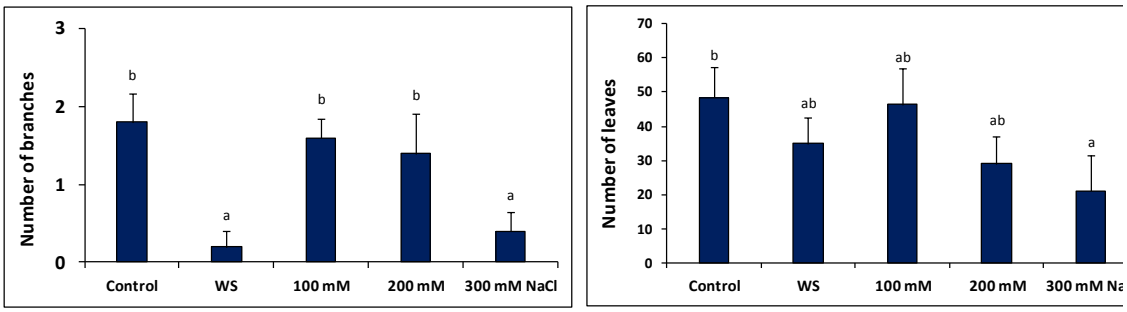
(**a**) (**b**)

Figure 3. Substrate electric conductivity (dS m^{-1}) (**a**) and moisture (%) (**b**) in the pots of the control and stressed plants of *Thalictrum maritimum* grown in controlled conditions in the greenhouse.

Plant Growth under Stress in the Greenhouse

Plants were subjected for 23 days to water deficit (completely stopping irrigation) and salt stress (100, 200 and 300 mM NaCl) treatments in the greenhouse. Determination of several morphological parameters indicated that both treatments caused inhibition of plant growth, in relation to the non-stressed controls, although in the case of salt stress a significant reduction of growth was only observed at high external salinities. For example, the number of new branches developed during the growth period, calculated as the difference between the last and first days of the treatments, decreased significantly in water-stressed plants. The mean values of new branches also decreased with increasing salinity, in a concentration-dependent manner, but the difference with the control was statistically significant only in the presence of the highest salt concentration tested, 300 mM NaCl (Figure 4a). The reduction on the average number of new leaves

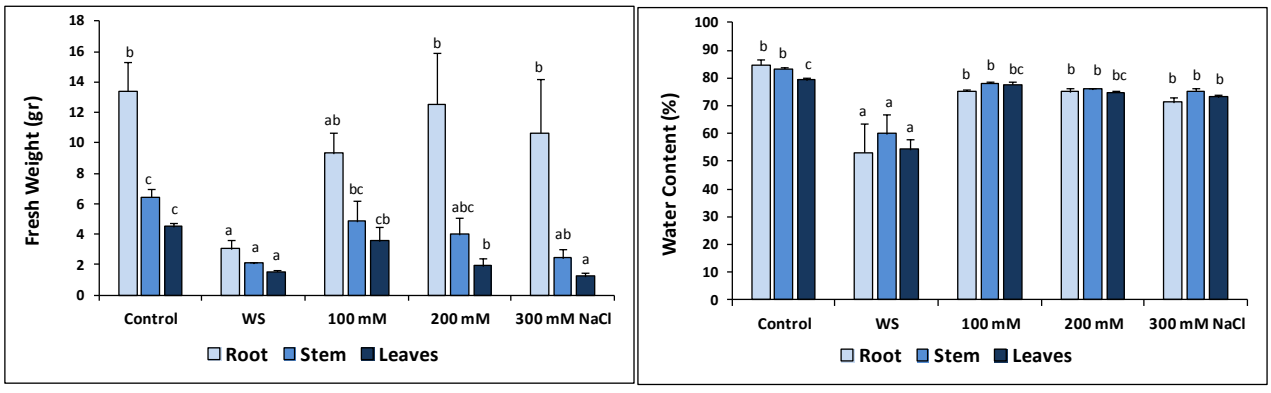
formed during the treatments showed a similar qualitative pattern as that of new branches but with quantitatively smaller differences with respect to the control, differences that were significant only for plants treated with 300 mM NaCl (Figure 4b).



(**a**) (**b**)

Figure 4. Number of new branches (**a**) and new leaves (**b**) formed during the 23 days of water stress (WS, complete withholding of irrigation) and salt stress (at the indicated NaCl concentrations) treatments, in *Thalictrum maritimum* plants grown under controlled conditions in the greenhouse. Values are calculated as the differences between the number of branches, or leaves, counted at the end and at the beginning of the corresponding treatments. Values shown are means ± SE (n = 5). Same lowercase letters over the bars indicate homogeneous groups between treatments according to the Tukey test ($p \leq 0.05$).

The same strong effect of the water stress and the 300 mM NaCl treatments was also observed, in general, on the reduction of the fresh weight (FW) of the different organs of the plants (Figure 5a). The FW of roots, stems and leaves of water-stressed plants was significantly reduced as compared to the corresponding controls; for salt-treated plants, a decreasing trend with increasing salinity was also observed for the mean values of stem and leaf FW although, here again, a significant difference with the non-treated control plants was observed only at the highest salt concentrations tested (Figure 5a). The water content of roots, stems and leaves decreased significantly in the *T. maritimum* plants subjected to water deficit conditions, whereas this species seems to be highly resistant to salt-induced dehydration: a statistically significant (albeit slight) reduction in water content was only detected in the leaves of the plants grown in the presence of 300 mM NaCl, but not at lower salinities or in roots and stems at any tested salt concentration (Figure 5b). Root length did not show any significant variation in response to the stress treatments (data not shown).



(**a**) (**b**)

Figure 5. Fresh weight (**a**) and water content percentage (**b**) of roots, stems and leaves after 23 days of water stress (complete withholding of irrigation) and salt stress (at the indicated NaCl concentrations) treatments, in *Thalictrum maritimum* plants grown in controlled conditions in the greenhouse. Values shown are means ± SE (n = 5). For each organ, roots, stems or leaves, same lowercase letters over the bars indicate homogeneous groups between treatments according to the Tukey test ($p \leq 0.05$).

Photosynthetic Pigments

In combination with the determination of growth parameters, quantification of photosynthetic pigments (chlorophylls a and b, and carotenoids) is commonly used to assess the effects of stress on plants, as inhibition of photosynthesis, which is accompanied by a decrease in pigment contents, is generally observed under stress conditions. *Thalictrum maritimum* followed this general behaviour, as the concentrations of all photosynthetic pigments decreased significantly in response to water deficit and all salt treatments—except for chlorophyll b in plants treated with 100 mM NaCl, which showed similar values as in the control (Figure 6).

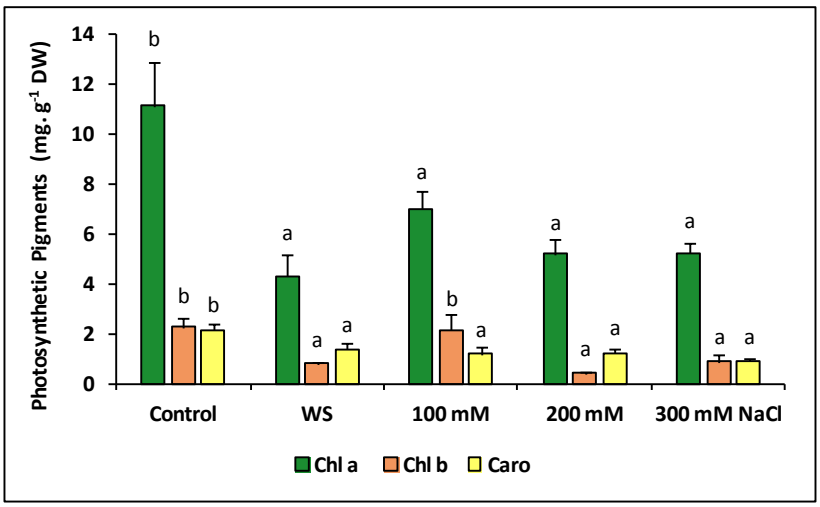


Figure 6. Photosynthetic pigments, chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Caro) after 23 days of water stress (complete withholding of irrigation) and salt stress (at the indicated NaCl concentrations) treatments, in *Thalictrum maritimum* plants grown in controlled conditions in the greenhouse. Values shown are means ± SE (n = 5). For each pigment, same lowercase letters over the bars indicate homogeneous groups between treatments according to the Tukey test ($p \leq 0.05$).

Ion Accumulation

As expected, Na^+ and Cl^- contents did not show any significant variation in roots, stem or leaves of water-stressed plants, as compared to the corresponding controls (Figure 7a, b). The concentrations of the two ions increased in the presence of salt in the three organs, although the differences with the controls were not significant for the 100 mM NaCl treatment. For example, Na^+ reached the maximum concentration (>1200 μmol g^{-1} DW) in leaves of plants treated with 300 mM NaCl, which represents a four-fold increase over control values (Figure 7a). The patterns of salt-induced variation in Cl^- contents were the same, although Cl^- concentrations were always somewhat lower than those of Na^+ under the same conditions (Figure 7b). It is worth mentioning that both, Na^+

and Cl^- accumulated at higher levels in the aboveground organs of the plants than in the roots, particularly at high external salinity (Figure 7a, b).

Regarding K^+ contents, they did not vary significantly in response to the stress treatments in any of the assayed organs, and its levels were generally higher in stems and leaves than in the roots (Figure 7c). On the contrary, the water deficit and the salt treatments induced a notable increase in the concentration of Ca^{2+} in all organs of the plants, reaching two to three-fold higher values than the controls. As for Na^+ and Cl^-, Ca^{2+} levels were higher in the aerial part than in the roots of salt-treated plants (Figure 7d).

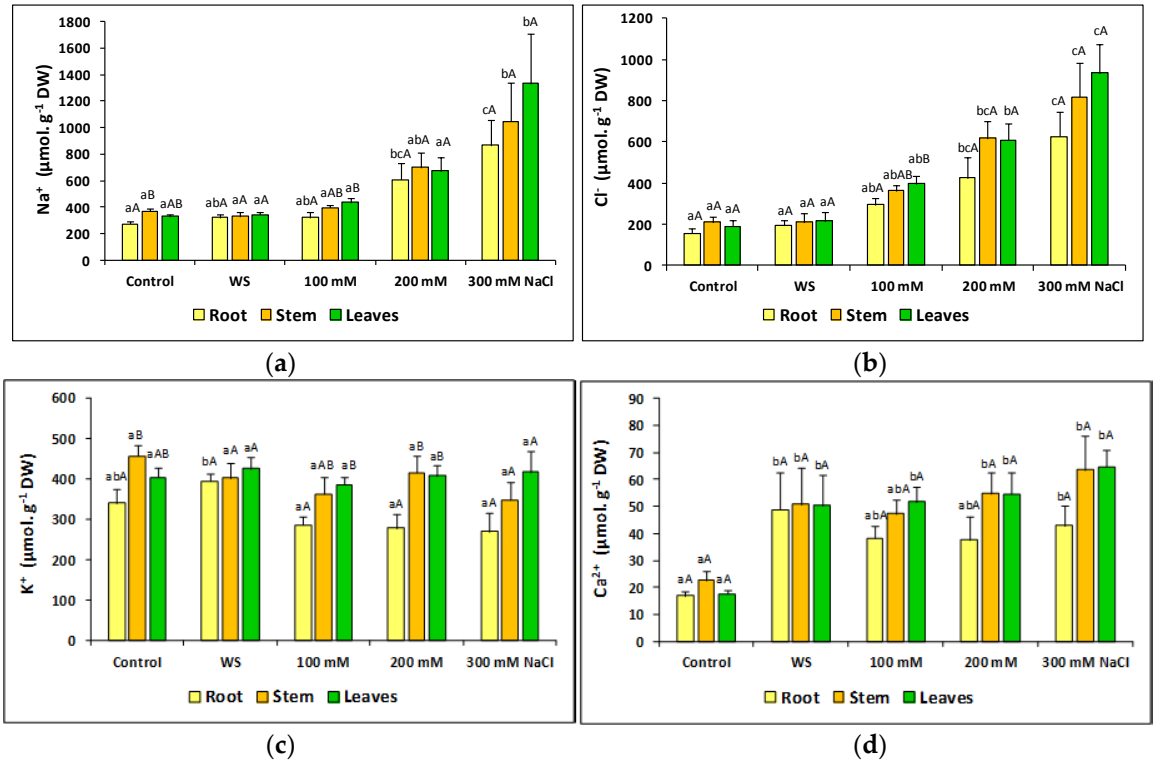


Figure 7. Ion contents, Na^+ (**a**), Cl^- (**b**), K^+ (**c**), Ca^{2+} (**d**), after 23 days of stress treatments, in roots, stems and leaves of *Thalictrum maritimum* plants grown in controlled conditions in the greenhouse. Values shown are means ± SE (n = 5). For each organ, same lowercase letters over the bars indicate homogeneous groups between treatments, whereas for each treatment same uppercase letters indicate homogeneous groups between roots, stems and leaves, according to the Tukey test ($p \leq 0.05$).

Osmolytes, Oxidative Stress Markers and Antioxidants

Proline (Pro) and total soluble sugars (TSS) are common osmolytes in plants. Leaf concentrations of Pro increased in response to water deficit and salt stress. Still, both, the relative increment over control values (less than two-fold) and the absolute concentrations reached (about 20 µmol g^{-1} DW) were low. Leaf TSS contents decreased in water-stressed plants but did not vary significantly in those subjected to the salt treatments (Table 5).

Mean values of malondialdehyde (MDA), used as a marker of oxidative stress, and of representative antioxidant compounds, total phenolic compounds (TFC) and total

flavonoids (TF), were lower in plants subjected to the water stress treatment than in the controls, but the differences with the non-stressed controls were not statistically significant. Concentrations of these compounds also showed a general decreasing trend with increasing salinity, although significant differences with the corresponding controls were observed only for TF at all salt concentrations, and for TPC in the presence of 300 mM NaCl (Table 5).

Regarding the specific activity of antioxidant enzymes, superoxide dismutase (SOD) increased significantly in the water stress treatment, but catalase (CAT) and glutathione reductase (GR) did not vary. In response to salt stress, CAT increased in the presence of 300 mM NaCl and GR in plants treated with 200 mM NaCl, whereas SOD did not show any significant variation (Table 5).

Table 5. Leaf concentrations of proline (Pro) and total soluble sugars (TSS), malondialdehyde (MDA), total phenolics (TPC) and total flavonoids (TF), and specific activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), after 23 days of stress treatments of *Thalictrum maritimum* plants grown in controlled conditions in the greenhouse. Values shown are means ± SE (n = 5). Same lowercase letters in each row indicate homogeneous groups between treatments according to the Tukey test ($p \leq 0.05$).

Biochemical Trait	Control	Water Stress	100 mM NaCl	200 mM NaCl	300 mM NaCl
Pro (μmol g^{-1} DW)	13.3 ± 1.2 ^{a}	20.7 ± 2.6 ^{b}	20.7 ± 2.5 ^{b}	15.9 ± 0.8 ^{ab}	20.5 ± 2.1 ^{b}
TSS (mg eq. Gluc g^{-1} DW)	352.2 ± 80.7 ^{bc}	233.1 ± 37.9 ^{a}	265.5 ± 6.0 ^{ab}	418.7 ± 46.0 ^{c}	401.0 ± 38.5 ^{bc}
MDA (nmol g^{-1}DW)	365.1 ± 52.9 ^{a}	268.6 ± 50.7 ^{a}	323.4 ± 28.2 ^{a}	296.1 ± 23.5 ^{a}	255.0 ± 16.7 ^{a}
TPC (mg eq GA g^{-1} DW)	26.6 ± 5.6 ^{b}	21.1 ± 2.1 ^{ab}	19.0 ± 1.4 ^{ab}	19.9 ± 2.2 ^{ab}	17.5 ± 1.7 ^{a}
TF (mg eq C g^{-1} DW)	10.7 ± 1.5 ^{c}	8.1 ± 1.7 ^{bc}	5.8 ± 0.6 ^{ab}	4.0 ± 1.2 ^{a}	4.1 ± 0.2 ^{a}
SOD (U g^{-1} protein)	13.2 ± 1.2 ^{a}	46.9 ± 8.8 ^{c}	23.6 ± 5.4 ^{a}	33.0 ± 3.6 ^{b}	30.3 ± 4.1 ^{b}
CAT (U g^{-1} protein)	228.6 ± 26.4 ^{ab}	158.3 ± 87.3 ^{a}	314.6 ± 26.9 ^{b}	272.8 ± 33.3 ^{ab}	544.5 ± 31.9 ^{c}
GR (U. g^{-1} protein)	1543.9 ± 342.0 ^{a}	1057.5 ± 249.0 ^{a}	1624.9 ± 330.0 ^{a}	2854.5 ± 137.0 ^{b}	1793.9 ± 354.0 ^{a}

Principal Component Analysis of Morphological and Biochemical Parameters Measured in Plants Grown under Experimetal Greenhouse Conditions

A principal component analysis (PCA) was performed, including all analysed traits in all individuals grown in the greenhouse (Figure 8). Nine components with an Eigenvalue greater than one were identified, which overall explained 88.7% of the total variability. The first and second principal components accounted for 29.7% and 18.1% of

the total variation, respectively. The first principal component showed positive correlations with growth parameters, such as the fresh weight of leaves (LFW) and stems (SFW); photosynthetic pigments: chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoids (Caro); malondialdehyde (MDA), or total phenolic compounds (TFC)—all of which decreased under stress—and negative correlations with Na^+ and Cl^- and catalase (CAT), which increased under salt stress. The second component was positively correlated with total soluble sugars (TSS) and water content of roots (RWC), stems (SWC) and leaves (LWC) and negatively correlated with K^+ in leaves (Kl) and roots (Kr). (Figure 8a).

The 25 individuals analysed were dispersed onto the two axes of the PCA scatterplot (Figure 8b), indicating a clear separation of the control, water stress and salt stress treatments. Plants from the control and salt treatments were distributed along the X-axis, from higher positive values (non-stressed controls) to higher negative values (300 mM NaCl), whereas those from the water stress treatment were located along the second principal component (Y-axis).

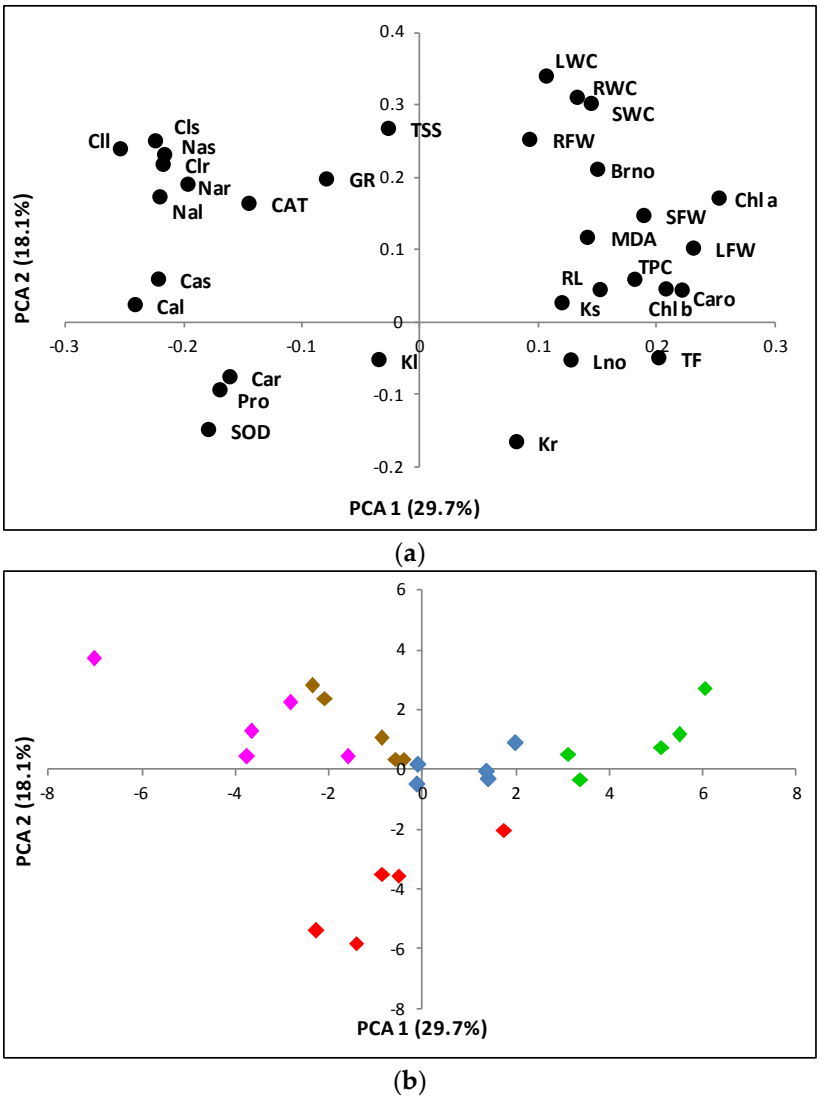


Figure 8. Loading plot (**a**) and scatterplot (**b**) of the principal component analysis (PCA) including all the analysed traits in *Thalictrum maritimum* plants subjected for 23 days to water deficit and salt treatments. The first (PC1; X-axis) and second (PC2; Y-axis) principal components accounted

for 29.7% and 18.1% of the total variation, respectively. Abbreviations in the loading plot (**a**): root length (RL), root fresh weight (RFW), root water content (RWC), stem fresh weight (SFW), stem water content (SWC), branch number increment (Brno), leaf number increment (Lno), leaf fresh weight (LFW), leaf water content (LWC), chlorophyll a (Chla), chlorophyll b (Chlb), carotenoids (Caro), sodium in roots (Nar), sodium in leaves (Nal), potassium in roots (Kr), potassium in leaves (Kl), chloride in roots (Clr), chloride in leaves (Cll), proline (Pro), total soluble sugars (TSS), malondialdehyde (MDA), total phenolic compounds (TPC), total flavonoids (TF), and superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) activities. Symbols in the scatter plot (**b**): control (green), water stress (red), 100 mM NaCl (blue), 200 mM NaCl (brown), and 300 mM NaCl (pink).

4.6.4 Discussion

Thalictrum maritimum is a rare endemic, found only in several salt marshes near Valencia in Eastern Spain. In the Natural Park of Albufera, where the species was first described, its distribution is not continuous; the plants appear spread through small populations in different salt marshes, known locally as 'malladas', occupying the inter-dune depressions. The most abundant population is that of 'Mallada del Canyar', where the soil samples were collected. As stated in the Introduction, the change of the populations in the park was followed between 2011 and 2017, reaching a maximum in 2015, but decreasing in 2017, the year of the last census, characterised by very dry summer and autumn (Servicio de Vida Silvestre, unpublished data). Dry summers represent a key trait of the Mediterranean climate, which is characterised by a pronounced water deficit in summer, often with periods of more than one month without registering any rainfall, while precipitation is concentrated in autumn and spring (Worldwide Bioclimatic Classification System, 1996-2020). The decrease in almost 1000 adult individuals from 2015 to 2017 is probably related to the exceptionally dry autumn in the second year. A wet autumn is necessary for a good regeneration of rhizomes in geophytes such as *T. maritimum,* which spend a large part of the year underground. The survey performed in this study, even though it did not include an exhaustive census, revealed that several of the small populations had disappeared, despite the conservation efforts undertaken. The climatic analysis indicated an increment of the summer temperature in the last few years and a reduction of precipitation, which may also be related to this reduction.

The range of tolerance of *T. maritimum* was checked by applying water deficit and salt stress treatments under controlled greenhouse conditions. The results revealed that the species' tolerance to salinity is far beyond that registered under natural conditions. Soil salinity, analysed in all locations where the species was present, was generally low, as previously reported in a study on soils characteristics in the habitats with *T. maritimum* and other Valencian endemics (Lidón et al., 2009); in some locations, soil was not saline at all. The vegetation accompanying *T. maritimum* includes several halophytic species, such as *Plantago crassifolia, Schoenus nigricans, Juncus acutus* and *J. maritimus*, but also glycophytes, specific of communities developed on non-saline soils like those of the tall shrublands and pinewood vegetation of the *Quercetea ilicis* phytosociological class; for example, *Phillyrea angustifolia, Pistacia lentiscus* and *Smilax aspera.* Important soil parameters related to the distribution of *T. maritimum* are moisture and organic matter content. Registered soil moisture values were above those reported in other studies in

the same area (Al Hassan et al., 2016; Koźmińska et al., 2018). The percentage of organic matter in *T. maritimum* locations was also higher than that reported for soils with sand texture from other salt marshes in the Natural Park of Albufera (González-Orenga et al., 2020).

Under greenhouse controlled conditions, *T. maritimum* proved to be more salt-tolerant than expected. Plants survived for more than three weeks at concentrations of 300 mM NaCl; the progressive accumulation of salt generated in the pot substrate an EC of 18 dS m^{-1} at the end of the treatment, substantially higher than that registered in the field. This strong saline treatment, however, was not harmless to the plants since the measurement of different morphological parameters showed inhibition of growth with respect to non-stressed controls. At lower salinity (100 mM NaCl, ca. 10 dS m^{-1} after 23 days of treatment), no significant changes were detected in the analysed growth traits, even though the substrate EC was still much higher than in most of the studied natural locations of the species. Nevertheless, to compare the effects of water deficit and salinity, all treatments had to be stopped after 23 days, due to the strong negative impact of water stress, as plants which did not receive irrigation showed intense wilting. Degradation of photosynthetic pigments was observed in all stress treatments with respect to controls, but the reduction of chlorophylls and carotenoids contents was more pronounced in plants subjected to the water stress treatment.

Therefore, we may conclude that, under the specific conditions of our experiments, water stress has a stronger negative effect on *T. maritimum* than salinity. The severe water deficit treatment applied to the plants in the greenhouse may mimic the conditions of the long and intense drought periods affecting the plants in their natural habitats during summer. In contrast, even though *T. maritimum* is present in low-salinity zones of the salt marshes, the biochemical analysis of its responses to salt stress highlighted that the species has many attributes of typical halophytes; most important, its primary mechanism of tolerance appears to be based on the active transport of toxic ions to the aboveground organs of the plants. The concentrations of Na^+ and Cl^- increased in all organs in parallel to the increase of the concentration of NaCl in the irrigation solution, but the ions accumulated mostly in the foliar tissue, as reported in many salt-tolerant plants (e.g. Zheng et al., 2009; González-Orenga, 2019, 2020). Accumulation of inorganic ions as 'cheap' osmotica (in terms of energy consumption), is a widespread strategy of tolerance in halophytes (Flowers & Yeo, 1988; Flowers & Colmer, 2008). According to the 'ion compartmentalisation hypothesis' (Flowers et al., 1977; Wyn Jones et al., 1977), Na^+ and Cl^- should be predominantly stored in leaf vacuoles, to avoid reaching toxic concentrations in the cytosol.

As Na^+ and K^+ compete for the same binding sites in proteins, including membrane transporters, accumulation of Na^+ is generally associated with a drop in cellular K^+ levels; furthermore, plasma membrane depolarisation, triggered by high Na^+ concentrations, also causes the loss of cellular K^+ by activation of outward rectifying K^+ channels (Greenway & Munns, 1980). In the present study, the homeostasis of K^+ was maintained under both types of stress, as no significant differences in K^+ concentration with respect to the corresponding controls were registered in salt and water-stressed plants. The levels of K^+ in stems and leaves were higher than in roots under all treatments, indicating

the presence of active transport of K^+ from the roots to the shoots of the plants. This mechanism partly counteracts the harmful effects of Na^+, enabling the maintenance of high leaf levels of K^+, necessary for the metabolic processes (Wu et al., 2018); this is a common feature of many halophytes, although it has also been reported for some glycophytes(Al Hassan et al., 2018; Brenes et al., 2020).

A significant increase in Ca^{2+} concentration in roots, stems and leaves was observed in the plants, in response to the water deficit and salt stress treatments. The protective effects of calcium against salt stress and sodium toxicity have been long known (Rengel, 1992; Bressan et al., 1998). Calcium is essential for plant growth under stress conditions, playing regulatory and signalling roles, for example, maintaining Na^+ and K^+ homeostasis via the SOS pathway (Hepler, 2005; Mahajan et al., 2008). Therefore, Ca^{2+} uptake and accumulation may contribute to tolerance to stress in *T. maritimum*, not only to high salinity but also to water deficit. Indeed, increased concentrations of Ca^{2+} have been reported in several typical halophytes, such as species of the genus *Limonium*, both under salt stress (González-Orenga et al., 2019a) and under water stress (González-Orenga et al., 2019b). As observed for the other analysed ions, higher Ca^{2+} concentrations have been measured in the aboveground organs of the plants than in the roots, under high salinity conditions (but not in response to water stress).

The accumulation of inorganic ions is balanced by the accumulation of compatible solutes, or osmolytes, which not only contribute to cellular osmotic adjustment under stress but are also involved in other mechanisms of stress tolerance, acting as low-molecular-weight chaperones, reactive oxygen species (ROS) scavengers or signalling molecules (Zhu, 2001; Ashraf & Foolad, 2007; Chen & Murata, 2011). Proline (Pro) is one of the most common osmolytes in plants, accumulating to high levels in many species in response to different abiotic stresses (Szabados & Savoure, 2010). In our experiments, leaf Pro contents increased in water- and salt-stressed plants, but the increase over control values and the absolute Pro concentrations reached were too low to have any relevant osmotic effect. Nevertheless, a contribution of Pro to stress tolerance in *T. maritimum*, based on its 'osmoprotectant' or signalling functions, cannot be ruled out. TSS contents did not show significant changes in response to the salt treatments, and only a slight (but significant) decrease in the water-stressed plants, suggesting that these compounds are also not involved in osmotic adjustment in *T. maritimum*. Probably, this function is mostly fulfilled by the stress-induced accumulation of inorganic ions, without excluding the possibility that some additional organic osmolyte, not identified in the present work, contributes to osmotic balance under stress in this species.

Salinity and drought are usually associated with oxidative stress, due to excessive accumulation of reactive oxygen species (ROS). Small amounts of ROS are generated during normal metabolic processes, such as photorespiration, photosynthesis and respiration, and play an essential role as signalling molecules (Apel & Hirt, 2004; Foyer & Noctor, 2005). ROS production, however, is considerably increased during abiotic stress (Asada, 2006; Gill & Tuteja, 2010; Bose et al., 2013); when in excess, ROS generate major metabolic disturbances that can lead to cell death (Demidchik et al., 2007; Shabala, 2009; Yu et al., 2011; Kumari et al., 2015) . Excessive ROS accumulation is prevented or limited by the synthesis of antioxidant compounds and the activation of antioxidant

enzymatic systems. Phenolic compounds and, particularly, the subgroup of flavonoids, are strong antioxidants in plants (Apel & Hirt, 2004), whereas some of the most common antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), or redox regulatory enzymes such as glutathione reductase (GR), among many others (Ozgur et al., 2013). At the cellular level, SOD represents the first line of defence against ROS (Apel & Hirt, 2004), catalysing the dismutation of the superoxide radical to oxygen and H_2O_2 (Apel & Hirt, 2004). SOD has been found in all aerobic organisms examined to date (Jaleel et al., 2009) and is generally activated in plants in response to different environmental stress signals, although there are also reports showing contradictory results (Szőllősi, 2014). CAT acts after SOD, catalysing the elimination of H_2O_2, which is transformed into water and oxygen (Jaleel et al., 2009).

MDA is a final product of polyunsaturated fatty acids peroxidation and is widely used as a marker of oxidative stress (Del Río et al., 2005). Therefore, an increase in MDA levels should be expected in plants that are subjected to different stress treatments, and this has indeed been reported for many species (Arbona et al., 2003; Al Hassan et al., 2017; Kozminska et al., 2017). In the present study, however, MDA contents did now show any significant change in response to water deficit or high salinity, indicating that, under the specific conditions of our experiments, high levels of oxidative stress were not generated. The lack of secondary oxidative stress associated with salt and water stress is not common in stress-sensitive plants but has been previously reported in many halophytes, both in their natural habitats (Bose et al., 2013; Gil et al., 2014; Bautista et al., 2015) and under controlled greenhouse conditions (González-Orenga et al., 2019b). In *T. maritimum*, redox equilibrium seemed to be maintained by a slight increase in the specific activity of SOD, under conditions of water deficit and high salinity, whereas activation of CAT and GR, also weak, was only detected in the presence of high salt concentrations. No additional contribution of antioxidant compounds was necessary and, consequently, we did not notice any increase in the concentrations of TPC or TF in the stressed plants.

Caution should be taken when attempting to extend the results obtained under controlled greenhouse conditions to the behaviour of the plants in the field. First, it is not possible to directly compare their responses to stress when growing in the artificial environment of a pot, or in their natural salt marsh habitat. On the other hand, plants in nature are simultaneously affected by different environmental stress conditions, which may interact in complex ways; for example, the responses to drought and soil salinity in the field cannot be considered independently of one each other, as we have done in the greenhouse experiments. Notwithstanding these limitations, we believe that the results presented here can guide the design and implementation of conservation programmes for this endangered species, helping to find the optimal sites for reintroductions or translocations from threatened areas. *Thalictrum maritimum* could grow in zones of high salinity within the salt marshes, unless it encounters strong competition from more abundant, structural halophytes. However, some local sites in the Albufera Natural Park, where *T. maritimum* vanished in the past, may not be the best ones to perform reintroduction projects, e.g., if the sites have been excessively clogged by sediments and not enough water level can be ensured. On the contrary, maybe sites closer to the Albufera lake, where the underground water table is more stable, and higher soil moisture can be maintained, could be recommended for future translocation trials.

4.6.5 Conclusions

Thalictrum maritimum is a moderate halophytic species, with optimal growth in the absence of salt, but tolerating concentrations of more than 300 mM NaCl. The primary mechanism of salt tolerance appears to be related to the active transport of ions to the aboveground organs of the plants and the maintenance of high K^+ contents with increasing Na^+ concentrations. However, the species is more sensitive to water deficit, as indicated by field and greenhouse analyses. The survival of this rare species is not threatened by increased soil salinisation in the salt marshes where it is present, an expected effect of climate change, as greenhouse experiments indicated that *T. maritimum* tolerates salinity well beyond that registered in its natural environment. However, water stress, which can also increase in the salt marsh habitat as a consequence of global warming, may endanger the populations of *T. maritimum*. Both the analyses of the changes in its populations in relation to climatic factors in the area of study and the water stress treatments performed in the greenhouse indicated that the most harmful abiotic stress factor for this species is represented by drought.

Author Contributions

Conceptualization, E.L., M.B. and O.V.; methodology, S.G.-O., J.V.L. and M.P.D.-T.; software, S.G.-O. and C.T.; validation, O.V., M.B. and P.P.F.-G.; formal analysis, S.G.-O. and F.C.; investigation, S.G.-O., C.T., F.C., J.V.L. and M.P.D.-T.; resources, M.B. and O.V.; data curation, S.G.-O.; writing—original draft preparation, S.G.-O. and M.B.; writing—review and editing, P.P.F.-G., E.L. and O.V.; visualization, M.B. and M.P.D.-T.; supervision, M.B and O.V.; project administration, M.B. and O.V.; funding acquisition, M.B. All authors have read and agreed to the published version of the manuscript.

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4.6.6 References

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Publicación VII:
Subcapítulo 4.7

Comparative Study of Stress Responses in Two Species of *Bupleurum* (Apiaceae) in Support of Conservation Programs

Este manuscrito está en preparación.

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González-Orenga, S.; Leandro, M.E.D.A.; Tortajada, L.; Llorens, J.A.; Ferrer-Gallego, P.P.; Laguna, E.; Boscaiu, M.; Vicente, O. Comparative Study of Stress Responses in Two Species of *Bupleurum* (Apiaceae) in Support of Conservation Programs. (En preparación)

4.7.1 Introduction

There is a general agreement that the study of the abiotic stress tolerance mechanisms in plants is of enormous practical importance for agriculture. However, the interest of these studies for helping design and implement conservation and regeneration strategies in natural habitats of great ecological value has been less appreciated. Mediterranean coastal salt marshes belong to this category of habitats since they are home to abundant biodiversity, are highly threatened by human influence and very sensitive to climate change. In the Valencian Community, as well as in other territories of the SE of the Iberian Peninsula, coastal marshes are frequently integrated into dune systems, forming interdune depressions that become waterlogged during rainy periods and form salt crusts in summer due to the evaporation of water. Thus, salinity gradients are established in the soil, with the most saline areas located in the centre of the marsh and the least saline at its edges. In general, it is assumed that the distribution of the different plant species in these saline areas is mainly determined by their relative salt tolerance, so that plant communities settle in concentric rings depending on soil salinity. Notwithstanding that other factors, such as nutrient supply or competition between species, may also contribute significantly to plant distribution (e.g., Barbour, 1978; Emery et al., 2001; Veldhuis et al., 2019).

The vegetation of these salt marshes includes structural species, which play a fundamental role in the structures of the plant communities; these species are relatively abundant and fairly homogeneous in the different territories (Sarika & Zikos, 2020). What provides individuality and added value to these ecosystems are less abundant, differential species, those on which the uniqueness of each salt marsh depends. In the Albufera Natural Park, located near the city of Valencia and the reference area of this study, many of these differential species are highly threatened; some have even disappeared from the Natural Park territory although they are still present in other Valencian salt marshes. Effective conservation and regeneration programmes of the salt marshes require the most profound possible knowledge of the responses of the plants to the environmental stresses affecting them in their natural habitat. The main physiological and biochemical mechanisms of tolerance to drought, salinity and other abiotic stress conditions are known for some of the structural species (Redondo-Gómez et al., 2009; Katschnig et al., 2013; Gil et al., 2011, 2014; Pardo-Domenech et al., 2015; Al Hassan et al., 2016a, 2017). However, no or only little information is available on the stress responses of differential species, which are often endemic, rare and/or endangered.

There is overwhelming evidence that all plants use the same basic mechanisms of response to adverse environmental conditions. Generally, however, these responses do not lead to stress tolerance – except for a small percentage of species adapted to different types of abiotic stress in their natural habitats, such as the salt marsh plants (Kumari e al., 2015; Al Hassan et al., 2017). These conserved responses include, amongst others, the control of ion transport, compartmentalisation of toxic ions in vacuoles with the concomitant accumulation of osmolytes in the cytosol to maintain cellular osmotic

balance, and the activation of antioxidant systems, both enzymatic and non-enzymatic (Zhu, 2001; Munns, 2002; Vinocur & Altman, 2005; Hussain et al., 2008; Munns & Tester, 2008; Pollastri & Tattini, 2011; Ozgur et al., 2013; Bose et al., 2014; Kumari et al., 2015; Volvok, 2015). However, the relevance and relative contribution of these responses to the mechanisms of tolerance of a given species to a particular stress situation is generally unknown.

A rewarding strategy to investigate stress tolerance mechanisms in plants is based on performing comparative studies of the stress responses in genetically related taxa with different stress sensitivity – for example, wild species of the same genus adapted to diverse natural habitats, different cultivars or varieties of the same cultivated species, or a crop and some of its wild relatives, which often differ in their degree of stress resistance. Information on the main tolerance mechanisms operating in the selected taxa can be obtained by correlating their relative resistance with the stress-induced changes in the levels of biochemical markers associated with particular response pathways, such as those mentioned above. Studies conducted by our research group in recent years have been based on this experimental approach and have provided several interesting and novel results, both in wild plants of the genera *Plantago* L. (family Plantaginaceae) and *Juncus* L. (family Juncaceae)(including halophytes in both cases) and in cultivated plants such as beans and eggplant (Al Hassan et al., 2016 b, c, d, 2017; Plazas el al., 2019).

In the present study, the same strategy was applied to determine the stress tolerance mechanisms of *Bupleurum tenuissiumum* L. (Apiaceae), a rare plant species with high conservation interest, as it became extinct in the territory of the Albufera Natural Park and is considered for reintroduction programmes. *Bupleurum fruticosum* L., a more common Mediterranean species, was included in the study as comparative material. This species is found in Mediterranean forests and scrublands, kermes oak (*Quercus coccifera* L.) and pine forest (*Pinus halepensis* Mill.). It is usually accompanied by very thermophilic species such as arbutus (*Arbutus unedo* L.) or oleander (*Nerium oleander* L.).

Therefore, the main objective of this work was to obtain data on the responses to salt stress and water deficit of *B. tenuissimum*, under controlled greenhouse conditions, information that could be useful for conservation and reintroduction programmes. On the other hand, comparing its stress responses to those of *B. fruticosum*, a congener species adapted to xeric conditions, should highlight the relevant mechanisms of stress tolerance in this poorly studied genus. To this aim, the plants were subjected to water stress (complete withholding of irrigation) and salt stress (watering with NaCl solutions of increasing concentration) treatments to analyse their effects on (i) plant growth, (ii) ion transport and accumulation, (iii) osmolyte biosynthesis, and (iv) activation of antioxidant systems.

4.7.2 Material and Methods

Plant Material

B. tenuissimum is distributed in the west, central and southern Europe, southern England and coastal areas of the North Sea to southern Sweden, southwest Asia and northwest Africa. It also has a scarce presence in the Iberian Peninsula and the Balearic Islands in marshes, on the borders of lagoons and watercourses, on marly, clayey or brackish soils. It can be found between sea level and 800 m of altitude. It is a recently extinct species in the Albufera and classified as vulnerable at the national level and registered as a "Non-Catalogued Protected Species" in Order 6/2013, of 25th March, of the Ministry of Infrastructure, Territory and Environment of the Generalitat Valenciana, which modifies the Valencian lists of protected species of flora and fauna. 2013/3166] (DOCV no. 6996 of 04.04.2013).

B. fruticosum is found in Mediterranean forests and bushes, on all soil types from see level to 1200 m, usually accompanied by thermophilic species such as strawberry tree or oleander.

Seeds were collected in the field in two different locations of the Valencian Community, those of *B. tenuissimum* from Villanueva de Castellón and *B. fruticosum* from the Sierra de Calderona Natural Park (Valencia), and provided by the Centre for Forestry Research and Experimentation (CIEF, Generalitat Valenciana).

Plant Growth and Stress Treatments

Seeds were sown in seedbeds with a mixture were sown on a mixture of commercial peat and vermiculite (3:1) and watered twice a week with Hoagland nutrient solution (Hoagland, 1950). One month after planting, the seedlings were transplanted into 12 cm diameter pots filled with 500 grams of the same mixture.

Treatments were started after four months when the plants were fully developed, with 7 -12 true leaves in *B. fruticosum* and 13-15 in *B. tenuissimum*. Thirty plants with uniform size for selected from each species, using 5 replicates per treatment and species. Following treatments were applied twice per week: control (irrigation with tap water), water stress (complete withholding of irrigation) and salt stress (irrigation with 75, 150, 300, and 450 mM aq. NaCl). Plant growth and stress treatments were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 h of light), temperature of 23 °C during the day and 17 °C at night, and 50–80% relative humidity.

The treatments were finalized after 21 days, when plants submitted to the water stress treatment showed pronounced wilting symptoms. At the beginning and during the treatments, the moisture and electroconductivity of the substrates were measured with a WET-2 Sensor (Delta - T Devices, Cambridge, UK). The number of leaves and the height of plants were measured at the beginning and end of the treatments. When treatments finalized, the plant material was sampled and the aerial part of the plants and their roots were weighed separately. A fraction of the material was dried in an oven at

65 °C until a constant weight was reached in order to quantify the water content according to the formula:

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

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

Photosynthetic Pigments

Leaf contents of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Caro) were determined using spectrophotometric techniques, following the method described by Lichtenthaler and Welburn (1983). Fresh plant material (50 mg) was extracted with one mL of ice-cold 80% acetone. The samples were mixed overnight (12-15 h) in a shaker in the absence of light and then centrifuged at 13,300 *g* for 10 minutes at 4 °C. The absorbance of the supernatants was measured at 470, 646 and 663 nm, and the concentrations of the pigments were calculated according to equations previously described (Lichtenthaler & Wellburn 1983) and expressed in mg g^{-1} DW.

Quantification of Ions

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

Quantification of Osmolytes

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

Total soluble sugars (TSS) were measured according to the method described by Dubois (1956). Fresh leaf material was ground in liquid N2 and extracted with 80% (v/v) methanol. After mixing in a rocker shaker for 24 h., the samples were centrifuged at 13,300 g for 10 min; supernatants were collected, appropriately diluted with water, and supplemented with concentrated sulphuric acid and 5% phenol. After 20 min incubation at room temperature, the absorbance was measured at 490 nm. TSS concentrations were expressed as equivalents of glucose, used as the standard (mg eq. glucose g−1 DW).

Determination of Oxidative Stress Markers and Antioxidant Compounds

Malondialdehyde (MDA), hydrogen peroxide (H2O2), total phenolic compounds (TPC), and total flavonoids (TF) were measured in extracts prepared in 80% (v/v) methanol from 50 mg ground fresh leaf material. For MDA quantification, the method described by Hodges et al. (1999) was followed, with the modifications introduced by Taulavouri et al. (2001). Extracts were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA) - or with 20% TCA without TBA for the controls - and then incubated at 95 °C for 20 min, cooled on ice and centrifuged at 13,300 rpm for 10 min at 4 °C. The absorbance of the supernatants was measured at 532 nm. The non-specific absorbance at 600 and 440 nm was subtracted, and the MDA concentration was determined using the equations by Taulavouri et al. (2001). MDA contents were expressed as nmol g-1 DW.

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

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

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

Activity of Antioxidant Enzymes

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

Superoxide dismutase (SOD) activity was determined following Beyer & Fridovich (1987) by monitoring spectrophotometrically at 560 nm the inhibition of nitroblue

tetrazolium (NBT) photo reduction in reaction mixtures containing riboflavin as the source of superoxide radicals. One SOD unit was defined as the amount of enzyme to cause 50% inhibition of the NBT photoreduction under the assay conditions as described in the original protocol.

Catalase (CAT) activity was measured following the consumption of H_2O_2 added to the extracts by the decrease in absorbance at 240 nm as described by Aebi (1984). One CAT unit was defined as the amount of enzyme decomposing 1 mmol of H2O2 per minute at 25 ◦C.

Glutathione reductase (GR) activity was quantified as described by Connell and Mullet (1986) following the oxidation of NADPH, the cofactor in the reaction of oxidized glutathione (GSSG) reduction, by a reduction in absorbance at 340 nm. One GR unit was defined as the amount of enzyme to oxidize 1 mmol of NADPH per minute at 25 ◦C (Connell & Mullet, 1986).

Statistical Analysis

Data were analyzed by the program Statgraph v. XVIII (Statgraphics Technologies, The Plains, VA, USA. Significant differences between treatments were tested by one-way analysis of variance (ANOVA) at the 95% confidence level, and post hoc comparisons were made using the Tukey's HSD test at $p < 0.05$. Before the analysis of variance, the Shapiro–Wilk test was used to check for the validity of normality assumption and Levene's test was used for the homogeneity of variance. If the ANOVA requirements were accomplished, the significance of the differences among treatments was tested by a one-way ANOVA at the 95% confidence level and post hoc comparisons were made using the Tukey HSD (honestly significant difference) test. A principal component analysis (PCA) was performed using means of all growth and biochemical parameters and final means of substrate moisture and electric conductivity. All the means throughout the text are followed by SE.

4.7.3 Results

Electroconductivty and Moisture of the Substrate

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

the water stress treatments, by more than 50% already during the first week, reaching final values below 1% in both species (Figures 1C, D).

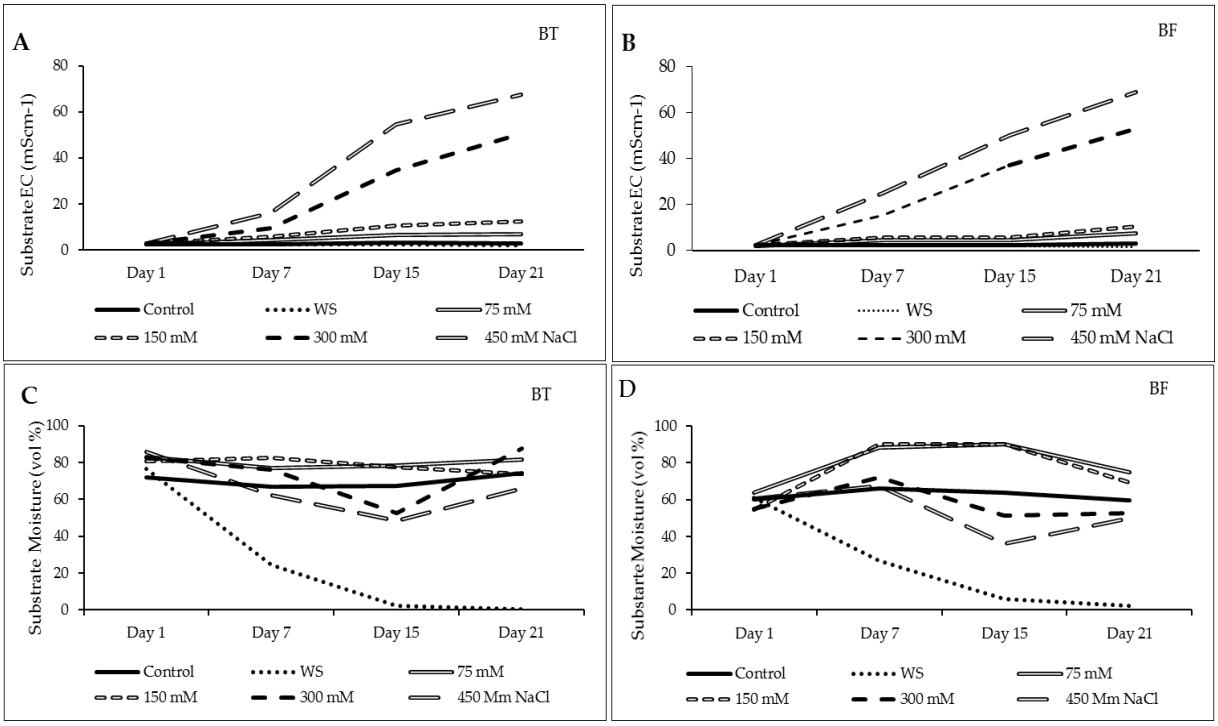

Figure 1. Electric conductivity (A and B) and moisture (C and D) of the substrate measured by a Wet Sensor twice a week during the 21 days of treatments. *Bupleurum tenuissimum* (BT, panels A and C) and *B. fruticosum* (BF, panels B and D). Values are means of five pots per treatment and species (n=5).

Analysis of Plant Growth Parameters

Both stress treatments inhibited plant growth in the two *Blupeurum* species (Table 1, Figure 2), although they were affected differently. From the observed effects of the water deficit treatment, it appears that *B. tenuissimum* is more sensitive to drought than *B. fruticosum*, as shown by a relatively stronger reduction in some of the measured growth parameters; for example, in the increment on the number of leaves during the treatment period, in relation to the non-stressed controls (Table 1). Similarly, the fresh weight of *B. tenuissimum* plants underwent a reduction of almost 90% in roots and 80% in leaves at the end of the treatment, *versus* ca. 55%, in both organs, in the case of *B. fruticosum* (Figures 2A, B). Water stress caused root dehydration in the two species, but to a lesser extent in the more tolerant *B. fruticosum* (Figure 2C), whereas no water loss was observed in the leaves of the stressed plants of this species (Figure 2D). On the other hand, the relative reduction of stem growth was the same for *B. tenuissimum* and *B. fruticosum* plants, 50% of the control (Table 1). Root length was the only parameter that increased significantly, albeit slightly, in response to the water stress treatment (Table 1).

Growth was also inhibited, in plants of the two selected species, in response to salt stress but, in contrast to the higher drought tolerance of *B. fruticosum*, *B. tenuissium* was shown to be the most salt-tolerant, particularly at high external salinities. This difference was observed, for example, in the relative salt-induced reduction of stem length increment

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

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

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

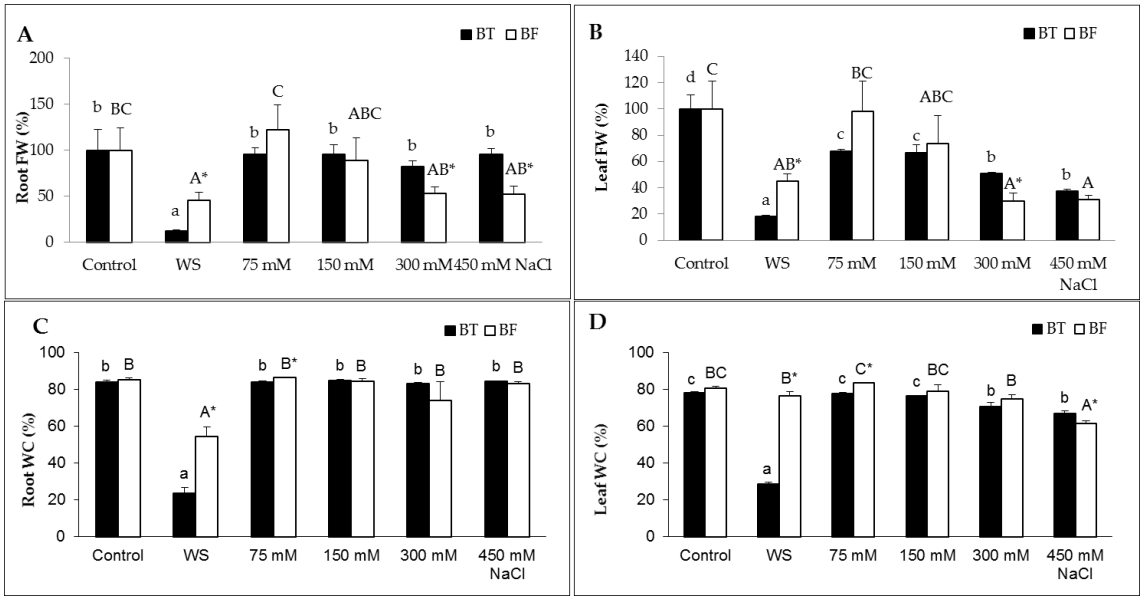

Figure 2. Effect of 21 days of treatments on fresh weight (FW) of roots (A) and leaves (B) and water content (WC) of roots (C) and leaves (D) in *B. tenuissimum* (black bars) and *B. fruticosum* (white bars). Mean values with SE are shown. Same letters (lowercase for *B. tenuissimum*, or uppercase for *B. fruticosum*) indicate homogenous groups according to the Tuckey HSD test ($p < 0.05$). An asterisk indicates significative differences between the two species.

Photosynthetic Pigments

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

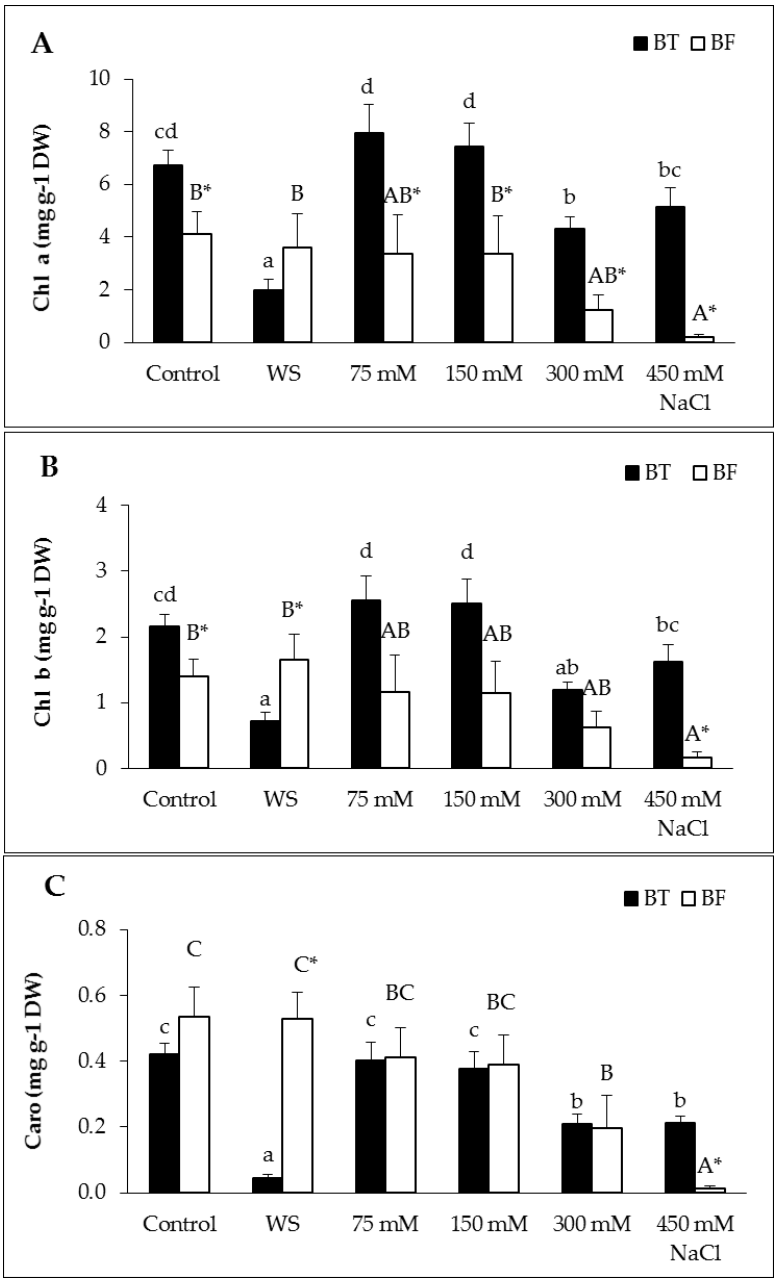

Figure 3. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments on the leaf contents of photosynthetic pigments: chlorophyll a (Chl a, A), chlorophyll b (Chl b, B) and carotenoids (Caro, C), in *B. tenuissimum*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

Quantification of Ions

It seemed logical to expect that the water stress treatment, which did not alter the electrical conductivity of the substrates in the pots, should not modify the ion contents in the plants. This expectation was confirmed in our experiments for Na^+, Cl^- and Ca^{2+} concentrations in roots and leaves of stressed plants of the two *Bupleurum* species, which

were not significantly different from those in the corresponding controls (Table 2). Changes in K^+ levels in response to water deficit stress, however, followed a different pattern, increasing more than two-fold in leaves of *B. tenuissimum*, whereas a significant reduction of the control values was observed in roots of *B. fruticosum* (Table 2). The treatments with NaCl induced a concentration-dependent increase in root and leaf levels of Na^+, Cl^- and, to a lesser extent, Ca^{2+} in plants of both *Bupleurum* species. Changes in K^+ concentrations in response to increasing external salinity were similar to those detected under water stress conditions: a significant decrease in *B. fruticosum* roots and an increase of about two-fold in *B. tenuissimum* leaves; in both cases, no significant differences were found between the different salt concentrations in irrigation water (Table 2). It should also be mentioned that, under the same experimental conditions, in roots and leaves of both species, Na^+ generally accumulated to higher levels than K^+ and Cl^-, and Ca^{2+} contents were lower than those of the other ions (Table 2).

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

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

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

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

Accumulation of Osmolytes

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

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

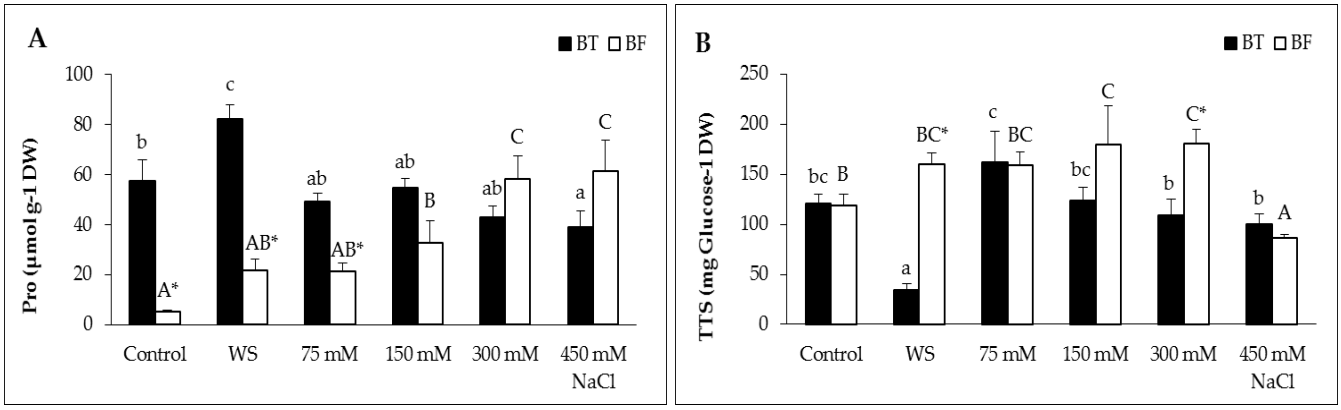

Figure 4. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments on leaf proline (Pro, A) and total soluble sugars (TSS, B) contents, in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF, white bars)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

Oxidative Stress Markers and Non-Enzymatic Antioxidants

The leaf concentration of malondialdehyde (MDA), a widely used oxidative stress marker (Del Rio et al., 2005), increased in *B. tenuissimum* plants subjected to the water stress treatments, reaching values almost double of those of the control, but no

significant changes were observed in response to increasing salinity. An opposite MDA accumulation pattern was established for *B. fruticosum* plants: a significant increase in parallel to increasing NaCl concentrations in the irrigation water, and no significant differences between the control and water-stressed plants (Figure 5A).

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

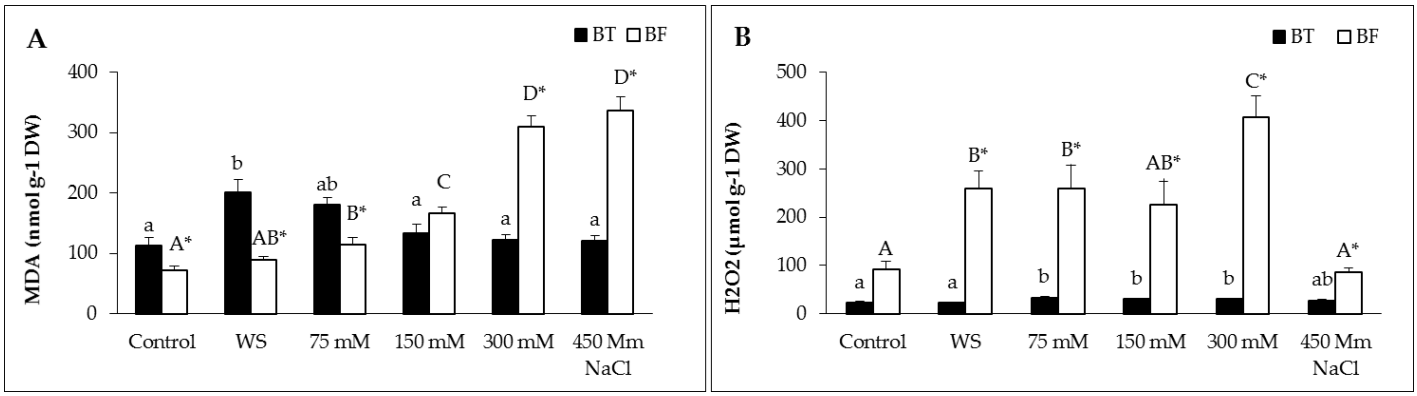

Figure 5. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments, on malondialdehyde (MDA, A) and hydrogen peroxide (H_2O_2, B), in leaves of in *B. tenuissimum* (BT, black bars) and *B. fruticosum* (BF,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

Concentrations of Total Phenolics and Antioxidant Flavonoids

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

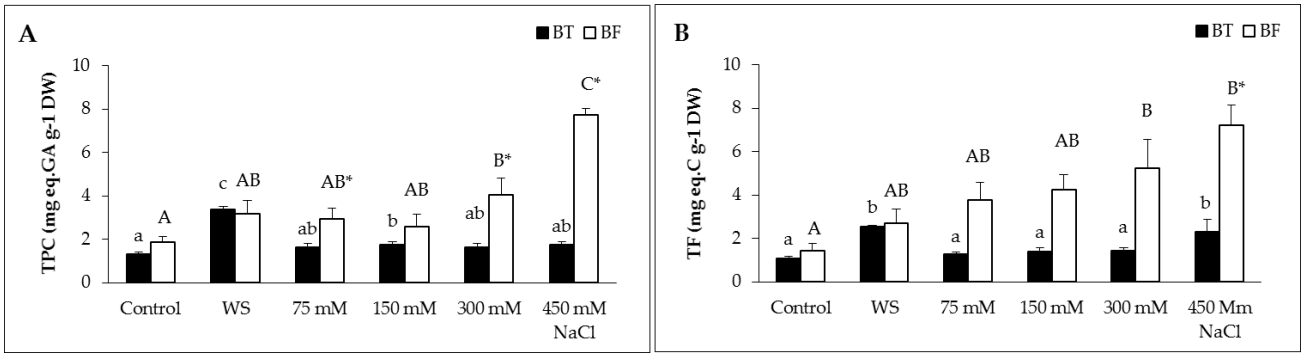

Figure 6. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments, on total phenolic compounds (TFC, A) and total flavonoids (TF, B) contents, in leaves of in *B. tenuissimum* (BT, black bars) and *B. fruticosum*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

Activity of Antioxidant Enzymes

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

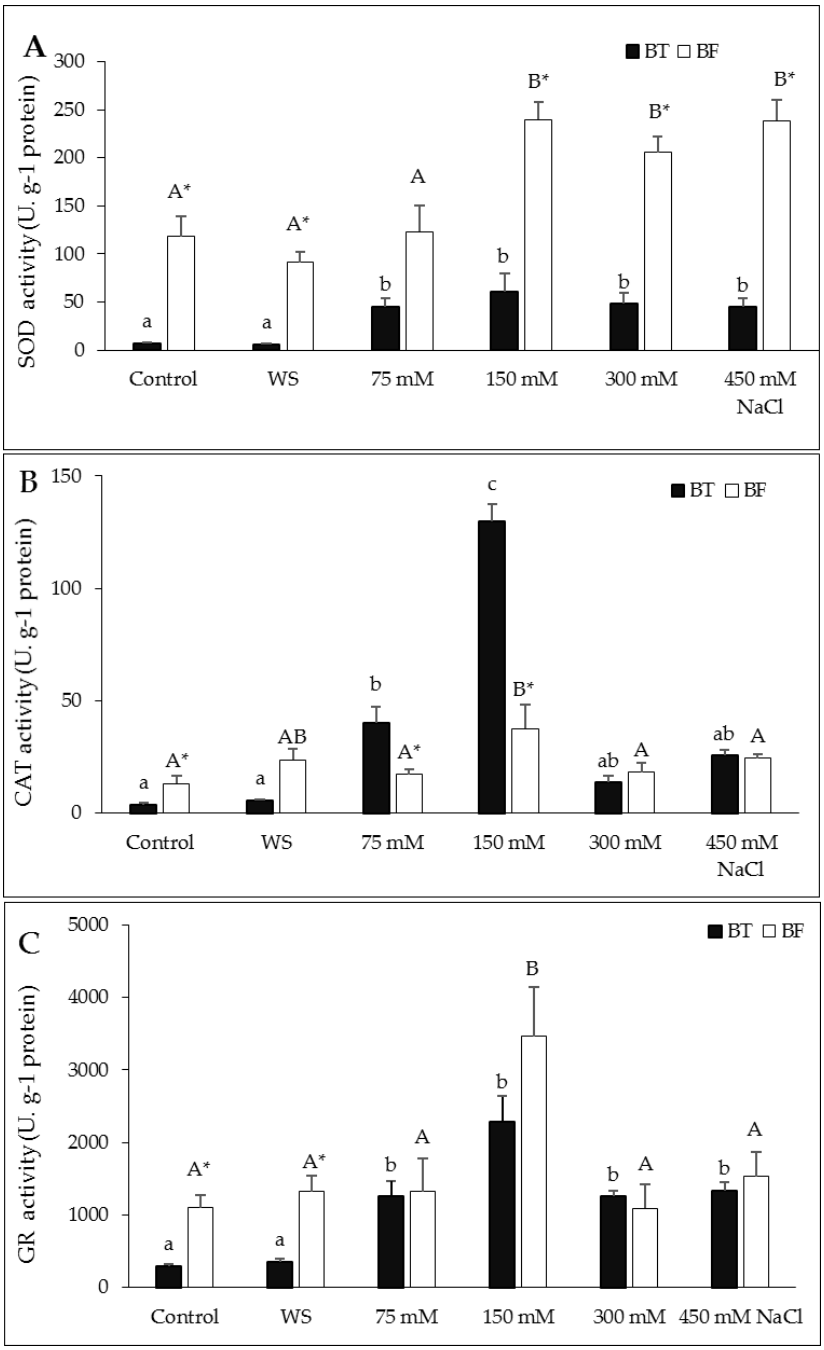

Figure 7. Effects of 21 days of water stress (WS, complete withholding of irrigation) or salt stress (watering with the indicated NaCl concentrations) treatments on the specific activity of antioxidant enzymes: superoxide dismutase (SOD, A), catalase (CAT, B) and glutathione reductase (GR, C) in leaves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

Principal Component Analysis

The PCA performed using the means of all analysed parameters, in the control, water deficit and salt stress treatments of plants of the two species, detected five components with eigenvalues greater than 1, which together explained 90% of the total variability. The first component, which covered 38.4% of the variability, was predominantly related to the salt stress treatments, whereas the second, which explained an additional 21.5%, was more related to the water deficit treatments (Figure 8). Thus, in the positive region of the X-axis, substrate EC clustered with ion contents in leaves and roots, oxidative stress markers (MDA, H_2O_2), antioxidant compounds (TF, TPC) and enzymes (especially with SOD); all these variables increased in response to salt stress. On the contrary, a negative correlation with salinity was established for growth parameters (root and stem length, leaf number, root and leaf FW) and photosynthetic pigments (Chl a, Chl b and Caro), which decreased in salt-treated plants. In the second component (Y-axis), substrate moisture correlated positively with the water content of roots (RWC) and leaves (LWC) and negatively with Pro, which increased significantly in the water stress treatment (Figure 8A).

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

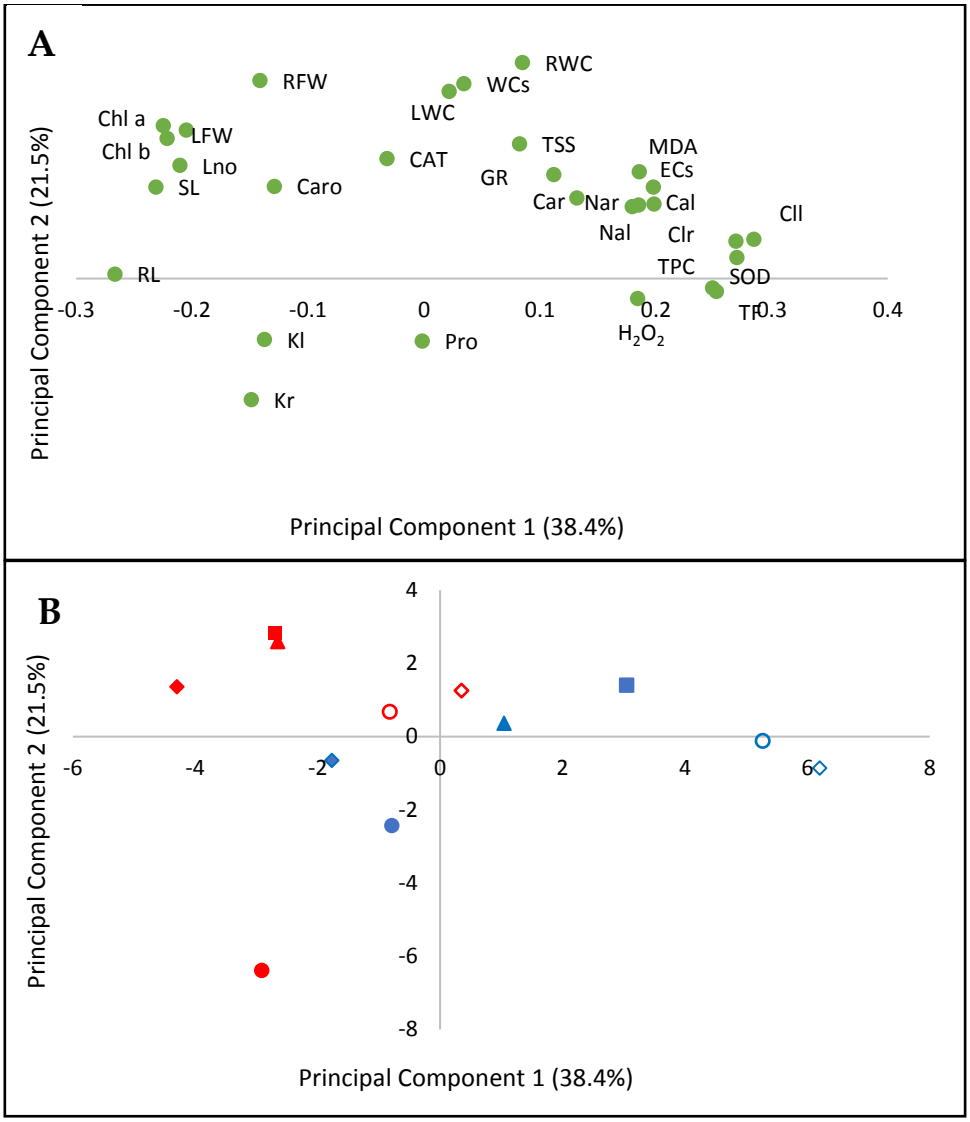

Figure 8. Loading plot (A) of the principal component analysis (PCA), conducted with the analysed substrate and plant parameters, in the control, water deficit and salt stress treatments. Substrate parameters: water content (WCs) and electroconductivity (ECs). Plant parameters: leaf number increment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

4.7.4 Discussion

Secondary metabolites in the genus *Bupleurum* have been analysed in more than 50 species and around 250 natural compounds from all major phytochemical classes were identified. This considerable interest is driven by the reason that roots of *B. sinensis* and several other species are extremely popular in oriental folk medicine (Ashour & Wink, 2011). Despite the large number of published articles and reviews on the phytochemistry of *Bupleurum* (Ashour & Wink, 2011; Yang et al., 2017; Zhu et al., 2017; Jiang et al., 2020 among others) there is very little information on responses to drought of species of this genus (Zhang et al., 2007; Yang et al., 2019, 2020) and no data are available (to our knowledge) on responses to salt stress.

The primary objective of this research was to explore the limits of salt and drought tolerance in the rare halophyte species *B. tenuissimum*, locally extinct in the Albufera Natural Park area, but by its comparison with a drought tolerant species included in the study, an insight into the responses of plants of this genus to salt and water stress and a comprehensive overview of the main mechanisms conferring tolerance to these types of stresses were attained.

B. tenuissimum may be regarded as a facultative halophyte, as it grows on moderately saline soils, but can also be found in non-saline habitats (Tyler et al., 2020), and its seeds do not germinate in the presence of salt (Al-Hawija et al., 2012). Although it has been reported to be more drought tolerant than other species of the same marsh in Central Europe (Čížková et al., 2020) when compared to *B. fruticosum*, a Mediterranean species, popular as a drought-tolerant ornamental, it was by far more susceptible to water stress imposed under greenhouse conditions, as indicated by the stronger reduction of its growth parameters after 21 days of lack of watering.

In contrast, on *B. tenuissimum*, some of its growth parameters were only affected by higher NaCl concentrations and the fresh weight of shoots and leaves showed no significant variation under salt conditions compared to the control, while *B. fruticosum* was more affected by salinity. The lesser effect of salt stress on *B. tenuissimum* is also revealed by the constant levels of chlorophylls a and b, and even a slight increase in their production under 75 and 150 mM NaCl, while *B. fruticosum* showed a significant reduction under 450 mM NaCl. Carotenoids of the former species showed a significant decrease under 450 mM NaCl, while in the latter it was also observed at 300 mM NaCl.

One of the basic mechanisms of salt tolerance that differentiate halophytes from glycophytes is ion uptake and transport. The latter are salt-exclusive, their main mechanism of resistance to salt stress is to prevent foliar accumulation of toxic ions, either by reducing root uptake or by blocking their transport to the aerial parts of the plant (Maathuis et al., 2014; Volkov, 2015). Most halophytes, except for monocots, are salt-includers, actively transporting toxic ions from roots to shoots (Flowers et al., 1977; Flower & Colmer, 2008; Maathuis et al., 2014). In the two species studied was found a significant increase in Na^+, in parallel to the applied salt concentration in both roots and leaves, indicating that there is no inhibition of Na^+ transport from roots to leaves. Toxic effects of salinity are related with increased concentrations of Na^+, which competes with

K^+ transporters and inhibits many enzymes that require K^+ (Maathuis, 2009; Maathuis et al., 2014). Therefore, increase of Na^+ under salt stress is usually associated with a reduction of K^+. This may cause an increase of Na^+/K^+ ratio at levels that exceeds the $K+/Na^+$ selectivity of many K^+ channels (Maathuis, 2014; Isayenkov & Maathuis, 2019). In both species K^+ levels were higher in leaves than in roots, but its pattern of variation under stress treatments was different. Although in control plants K^+ values in roots and leaves were higher in *B. fruticosum*, under water stress its concentration increased in *B. tenuissimum* in roots but especially in leaves, where it reached a concentration twice as high as in the control. On the contrary, in *B. fruticosum* K^+ values in roots of plants from the water stress treatment were lower than in those from control but did not show a significant variation in leaves. Under salt stress K^+ levels were constant in *B. tenuissiumum* in roots but significantly increased in leaves, while in *B. fruticosum* they significantly decreased in both roots and leaves. This finding shows that *B. tenuissimum* behaves as a salt-tolerant species, activating K^+ transport to leaves under saline conditions, while the second species does not have this ability. Interestingly, under water stress, which affects *B. tenuissimum* much more than salinity, not only the transport from roots to leaves is activated, but also K^+ uptake by roots. K^+ is an essential nutrient, playing key roles in cell elongation and maintenance of membrane integrity and stability, enzyme activation, protein synthesis, photosynthesis, photosynthesis, osmoregulation, stomatal movement, energy transfer, phloem transport, cation-anion balance, and stress resistance (Marschner, 2012). Under drought and salt stress conditions, K^+ is essential in osmotic adjustment and turgor regulation within guard cells during stomatal movement and low K_+ is often related to increased ROS (Wang et al., 2013).

An increase in Na^+ is always associated with that of Cl^- but it is generally considered that Na^+ is more toxic than Cl^-, as the cation competes with K^+ transporters and inhibits many enzymes that require K^+ (Maathuis, 2009; Maathuis et al. 2014). Most studies related soil salinity with Na^+ toxicity, and effects of Cl^- were largely overlooked and only recently its role in salt toxicity has been reconsidered (Wu & Li, 2019). Cl^- is a micronutrient but increased concentrations are toxic, and impair photosynthesis and growth (Tavakkoli et al., 2010; Tavakkoli et al., 2011). Another reason for Cl^- toxicity at high concentrations is related to the antagonism between Cl^- and NO_3^- which results in a limited nitrogen supply (Wu & Li, 2019). Although concentrations of Cl^- were lower than that of Na^+ in the two species here studied, the anion increased sharply in in roots of the plants from the stress treatments in the two species. Its increase in leaves of *B. tenuissimum* in respect to control values was lower than in roots, resulting in lower values in leaves than in roots at the higher concentrations of 300 and 450 mM NaCl. On the contrary, under the same treatments, Cl^- levels were higher in leaves than in roots in *B. fruticosum*. Foliar Cl^- in the glycophyte in plants from salt treatments was double than in the halophyte, as reported in *Juncus* when comparing salt species with different salt tolerance (Al Hassan et al., 2016c). In beans stress susceptible cultivars had a higher concentration of foliar Cl^- than the more tolerant ones (Bayuelo-Jiménez et al., 2012; Al Hassan et al., 2016d). In this species levels of Cl^- were higher than those of Na^+, as reported in other legumes or woody specie, which are better at excluding Na^+ from the leaf blades than Cl^- (Wu & Li, 2019).

The only bivalent cation analysed was calcium, which is essential for structure and functional integrity in plants, stabilising membrane and cell wall structures, regulating ion transport and selectivity, and controls cell wall enzyme activities. In condition of stress Ca^{2+} plays an important role in stress signalling involved in main tolerance strategies such as osmoprotectant accumulation, antioxidant boosting, or polyamines and nitric oxide (NO) machineries (Seifikalhor et al., 2019).

Cytosolic calcium-signal activates the calcium sensor protein SOS_3, which binds to and activates a protein kinase SOS_2. When activated, this regulates the activities of SOS_1, a plasma membrane Na^+/H^+ antiporter, and of NHX_1, a tonoplast Na^+/H^+ antiporter leading to either Na^+ efflux out of cytosol or its compartmentation in vacuole (Tester & Daveport, 2003; Hadi & Karimi, 2012). The salt-induced Ca^{2+} signalling originates in roots, and some studies revealed a most pronounced increase in cytoplasmic Ca^{2+} concentration in endodermal cells throughout the root in response to acute salt stress (Kiegle et al., 2000; Manishankar et al. 2018). In the two species analysed levels of Ca^{2+} increased significantly under salt treatments, in *B. tenuisssiumum* mostly in roots.

For compensation of osmotic balance, a general response in plants is the accumulation of compatible solutes in the cytosol. This mechanism is effective in stress tolerant plants in reducing cellular dehydration caused by different types of abiotic stresses, including salinity and drought. Besides their role in osmoregulation, osmolytes play multiple roles in stress tolerance such as acting as low-molecular weight chaperones, reactive oxygen species (ROS) scavengers or signalling molecules (Ashraf & Foolad, 2007; Sanders & Arndt, 2012; Singh et al., 2015; Slama et al., 2015). One of the most common osmolytes in plants is proline (Szabados & Savouré, 2010), which has not only a function as compatible solute, but also antioxidant as an O_2, H2O2, and OH quencher, stabilising ROS-scavenging, maintaining a low NADPH to NADP+ ratio and stabilising mitochondrial respiration enzymes (Szabados & Savouré, 2010; Bose et al., 2014; Gupta & Huang, 2014). However, its role is ambiguous as higher amount of proline in some species is related to stress tolerance whether not in others (Mansour & Ali, 2017). Increased concentrations of proline were reported the medicinal *Bupleurum chinense* in plants submitted to drought (Yang et al., 2019; 2020). Constitutively higher values of proline were found in *B. tenuissiumum*, its concentration rising only in plants from the water stress treatment, whereas in *B. fruticosum* it gradually increased under salt treatments, reaching 12-fold in respect to control in plants from the 450 mM NaCl treatment. These results indicate that proline plays an important role in stress response in *Bupleurum*, and its increase in relation to values from control plants can be used as an indicator of the level of stress suffered by plants.

More difficult is to explain the variation observed on the content of total soluble sugars as they are direct result of the primary metabolism and are involved in many physiological processes. The increased concentration of TSS observed in plants from several salt treatment of the two species, cannot be directly related to an adjustment to stress as it has been reported in other species, where they play a specific role in stress defence (Gil et al., 2013; Al Hassan et al., 2016c) as no clear pattern of variation could be fund. The pronounced reduction of TSS in plants of *B. tenuisssimum* subjected to water stress can be explained by a drastic reduction in photosynthesis as this species proved

to be drought susceptible, and its photosynthetic pigments also suffered a reduction in this treatment.

Abiotic stress is associated with increased production of reactive oxygen species (ROS). The role of ROS is twofold; they were initially considered toxic by-products of aerial metabolism that induce oxidative stress, but more recently their role as secondary signaling messengers in several key physiological processes has been established (Apel & Hirt, 2004; Foyer & Noctor, 2005; Das & Roychoudhury, 2014). ROS, such as $^1O_2$, H_2O_2, $O^{-}{}_2$, and $OH^{\bullet}$ when in excess have damaging effects on nucleic acids, lipids and proteins inducing severe dysfunctions and even cell death (Apel & Hirt, 2004). Several ROS induce lipid peroxidation, which affects the selectivity and permeability of membranes that can cause leakage of ions and other metabolites and modify proteins and affect their functions (Ozgur et al. 2013). Hydrogen peroxide (H_2O_2) along with malondialdehyde (MDA), a marker of membrane lipid peroxidation is widely used to estimate the level of oxidative stress experienced by plants and the degree of plants sensitivity to a particular type of stress (e.g., Chakraborty & Pradhan, 2012; Moussouraki et al., 2019; Zhu et al., 2020). Although the reliability of MDA as a marker of oxidative stress has recently been questioned due to methodological aspects or misinterpretation of the results (Morales & Munné-Bosch, 2019), in the two species analysed here its levels match perfectly with the other parameters. MDA increased significantly in the halophyte only in the water-stressed plants, while in the second, a drought-tolerant species, its levels increased gradually in the salt stress treatments, reaching in the plants of the 300 and 450 mM NaCl treatments levels 3 times higher than those of the control, but did not vary in the water stress treatment. Hydrogen peroxide is considered as a moderately reactive ROS formed when $O^{-}{}_2$ is non-enzymatically dismutated or mostly by a reaction catalysed by SOD. As other ROS, at low concentration has an important signaling function in essential physiological processes, but when in excess it becomes oxidises cysteine and methionine residues and inactivates many enzymes (Das & Roychoudhury, 2014). In all stress treatment levels of H_2O_2 were significantly higher in *B. fruticosum* where its levels increased up to maximum in the 350 mM NaCl treatment followed by a drop back in the 450 mM NaCl treatment back to the levels in control, which is difficult to explain. In *B. tenuissimum* H_2O_2 concentration were low and showed only a small increase in salt stress treatments.

Plants activate two main mechanisms in the defense against toxic levels of ROS, the antioxidant enzymatic machinery and the synthesis of non-enzymatic low molecular compounds such as ascorbic acid (AA), phenolics, and flavonoids among others (Gill & Tuteja, 2010; Miller et al., 2010). Some halophytes possess constitutive antioxidant defence activity, more effective than in glycophytes (Ozgur et al., 2013). Higher constitutive levels of CAT, POX, APX and GR were found in *Hordeum marinum*, in comparison to H. vulgare (Seckin et al., 2010), and higher levels of SOD gene expression under stress, and higher proline concentrations under control and salt-stressed conditions in *Thellungiella salsuginea* than in *Arabidopsis* (Taji et al., 2004). Also, higher levels of CAT were reported in drought tolerant plants in respects to related drought sensitive species.

Superoxide dismutase (SOD) is considered the first line of defence against oxidative stress in plants in (Alscher et al., 2002). It catalyses the removal of O^{2-} by dismutating it into H_2O_2 and O_2 (Das & Roychoudhury, 2014). Its main function is in the rapid conversion of $O^{-}{}^{2}$ to H_2O_2, which as discussed above, has an important signaling role. Halophytes tend to possess intrinsically higher levels of SOD, a trait that can be considered an adaptive advantage over glycophytes. Under salt stress the activity of this enzyme generally increases in this category of plants, whereas in glycophytes both increases and reductions have been reported (Bose et al., 2014). However, of the two species analysed here, higher SOD levels in all treatments, even in control plants were found in *B. fruticosum*. SOD activity increased with salt stress in the two species.

Catalase (CAT) is an enzyme thatcatalyses the conversion of H_2O_2 to H_2O_2 (Willekens et al., 1997). Having a high affinity for H_2O_2, CAT has a very fast turnover rate and is very efficient in the decomposition of peroxide (Bose et al., 2014). In both species analysed CAT activity followed the same pattern, increasing in salt treatments up to the concentration of 150 mM NaCl, where it reached considerably higher levels in *B. tenuissimum*, followed by a reduction of its activity in both species under higher salt concentrations. Most reports on salt- and drought-tolerant species indicate increased SOD activation under stress conditions (Bose et al. 2014; Laxa et al., 2019).

GR is an oxidoreductase enzyme with the main function of maintaining intracellular reduced glutathione (GSH), which is involved in a wide range of essential functions and has a strong reducing potential (Couto et al., 2016). There are many reports that activity is increased, reduced or unchanged under stress treatments, but susceptible plants are considered to predominantly activate the glutathione-dependent scavenging system (Laxa et al., 2019). The pattern of variation in GR activity was similar to that of CAT, with a peak in plants from the 150 mM NaCl treatment onwards, followed by a reduction at higher salt concentrations. In contrast to CAT, the only significant differences between the two species were found in the plants from the control and water stress treatments, with higher values in *B. fruticosum.*

In water stress treatments, the activity of the three enzymes remained virtually unchanged from the control in the two species. In *Bupleurum chinense*, a reduction of SOD, POD and CAT under drought was reported (Yang et al., 2019).

Not all ROS can be scavenged by enzymes, several highly toxic ROS, such as O2 and OH can be scavenged only by strong non-enzymatic antioxidants, such as phenolic compounds and especially flavonoids (Bose et al., 2014). The latter category is considered as a secondary ROS scavenging system in plants suffering damage to the photosynthetic apparatus due to excess excitation energy (Fini et al., 2011). In many halophytes an increase in phenolic and flavonoid components has been reported (Ksouri et al., 2012; Ozgur et al., 2013), but also in many others, such as in several *Limonium*, species no significant variation has been reported (González-Orenga et al., 2019, 2020). It is very likely that the more salt-tolerant species do not activate antioxidant systems as they do not allow excessive ROS production by possessing an efficient mechanism to prevent Na^{+} accumulation in the cytosol (Bose et al., 2014).

4.7.5 Conclusions

Overall, all the parameters analysed allowed a clear separation of the two species and their responses to the two different types of stress. *B. tenuissimum*, a moderate halophile, is clearly more susceptible to drought while, on the contrary, *B. fruticosum* is not affected by drought but by salinity, even at low NaCl concentrations.

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Capítulo 5:
Discusión General

El objetivo general de este estudio era de obtener un mejor conocimiento de los mecanismos de respuesta que contribuyen a la tolerancia al estrés hídrico y salino, muchos de los cuales son compartidos por todas las especies vegetales, con diferencias distintivas y con mayor eficiencia en las especies tolerantes a estos tipos de estrés. Debido a la complejidad del funcionamiento de estos mecanismos, parece relevante estudiar las respuestas en plantas con diferentes niveles de tolerancia, pero con características genéticas similares, como en este caso, el estudio de diferentes especies del mismo género. Esta metodología puede ayudar a establecer cuáles de las respuestas estudiadas son relevantes para la tolerancia al estrés en una determinada especie o grupo de taxones relacionados y cuáles no.

Para los estudios comparativos se seleccionaron cuatro especies del género *Limonium*, *L. santapolense, L. girardianum, L. narbonense* y *L. virgatum*, los cuáles se estudiaron tanto en su hábitat natural como bajo condiciones controladas en invernadero, y por otra parte *L. albuferae* y *L. dufourii*, con alto interés ecológico. También se analizaron dos especies del género *Bupleurum*, *B. tenuissimum* y *B. fruticosum*, con optimo ecológico diferente. Además, se ha incluido en el estudio otra especie de interés para su conservación, *Thalictrum maritimum*, en cual se han analizado las respuestas de crecimiento y bioquímicas frente a la sequía y a la salinidad del suelo.

El objetivo concreto de los estudios comparativos realizados fue, por un lado, analizar el grado relativo de tolerancia de las especies estudiadas a partir de la medición de la inhibición del crecimiento y, por otro lado, establecer la correlación de estos resultados con los niveles de marcadores fisiológicos o bioquímicos específicos de estrés asociados a vías conservadas de respuesta al estrés como la acumulación de osmolitos, el transporte iónico, la activación de sistemas antioxidantes (enzimáticos y no enzimáticos), con el fin de establecer la relevancia de las respuestas de tolerancia al estrés.

A nivel practico, los resultados obtenidos permiten mejorar las estrategias de reintroducción y conservación de las especies de interés.

A continuación, se discuten los resultados más relevantes del estudio:

5.1 Análisis Climático

En general, las plantas de la zona analizada están bien adaptadas a la sequía, ya que el clima mediterráneo se caracteriza por una drástica reducción de las precipitaciones de verano y una amplia variabilidad interanual (Lionello, 2012). Sin embargo, los efectos del cambio climático representan otra amenaza para los ecosistemas de saladar del área mediterránea. No solo los aumentos de temperatura y el riesgo de períodos de sequía más prolongados e intensos, sino también la alteración repentina de los patrones climáticos estacionales puede modificar las condiciones existentes en estos ecosistemas (Thorne et al., 2012). Además, es muy probable que los efectos del cambio climático

también provoquen un aumento en la salinidad del suelo en los próximos años, lo que podría afectar aún más a las poblaciones ya amenazadas.

Debido a esto, el clima es uno de los factores claves que afectan tanto a la distribución de las especies como a su desarrollo y supervivencia y, en consecuencia, uno de los parámetros estudiados en este trabajo.

En referencia a las especies de *Limonium* estudiadas en sus hábitats naturales se pueden diferenciar dos áreas, El Clot de Galvany, cerca de Elche (Alicante), donde se recolectó *L. santapolense* y El Saler, cerca de Valencia, donde se recolectaron las otras tres especies incluidas en el estudio (*L. narbonense, L. girardianum* y *L. virgatum*). Ambas zonas tienen un clima similar con las temperaturas más altas en verano, coincidiendo con una drástica reducción de las precipitaciones. Sin embargo, la precipitación media anual es mucho mayor en El Saler, clasificándola como semiárida mientras que la zona de El Clot de Galvany se clasificaría como árida. Pese a que *L. santapolense* presentó una menor resistencia bajo condiciones controladas en maceta, realmente era la especie más tolerante al déficit hídrico al resistir en un clima árido gracias a su gran desarrollo radicular. *L. santapolense* desarrolló bajo condiciones naturales un mecanismo morfológico de tolerancia basado en la expansión radicular para acceder al agua disponible. Este mecanismo no se observó en las condiciones del ensayo, debido al espacio limitado de crecimiento radicular en la maceta.

Por otro lado, en el estudio de las otras dos especies del género *Limonium*, *L. albuferae* y *L. dufourii*, se analizaron los datos climáticos de sus hábitats para conocer las condiciones del área de conservación y reintroducción, así como para establecer una posible relación entre los datos climáticos y la evolución del número de individuos de las poblaciones censadas. En el caso de *L. dufourii*, no se encontró una relación estadísticamente significativa, pero fue en los años más secos cuando se registró un mayor número de individuos, es decir, cuando la zona de "malladas" no se inundó o si lo hizo fue muy brevemente. Por el contrario, la población se redujo en los años de mayores precipitaciones. En cuanto a *L. albuferae*, no se han podido evaluar los posibles efectos del clima porque ha sido descrita recientemente (Ferrer-Gallego et al., 2016) y no se dispone de una serie de censos.

El estudio climático con relación a *T. maritimum*, nos ha mostrado datos destacables en cuanto al número de individuos. En 2015 fue el año en el que se censó el mayor número de individuos en el territorio del Parque Natural de l'Albufera, mientras que en 2017 se produjo un drástico descenso. Analizando los datos climáticos, la principal diferencia entre estos dos años fue la cantidad y distribución de las precipitaciones. En 2015, el valor de las precipitaciones se acercó al valor medio de los últimos 19 años (401,26 mm) y se caracterizó por un otoño húmedo, mientras que en 2017 hubo una menor cantidad de precipitaciones (307,26 mm) y la estación otoñal fue excepcionalmente seca. Estos datos indican que la disminución del número de individuos está probablemente relacionada con la cantidad y distribución de las precipitaciones, dado que para la regeneración de los rizomas en geófitos como esta especie que pasan parte del año bajo

tierra, es necesario un otoño húmedo. Además de la reducción de las precipitaciones, el análisis climático indica un aumento de las temperaturas estivales en los últimos años, que también podría estar relacionado con esta reducción poblacional.

5.2 Análisis del Suelo

El análisis del suelo, el medio en el que se desarrollan las plantas en su hábitat natural, es de gran importancia, ya que el suelo es el sustrato para su crecimiento, el medio para el desarrollo de sus raíces y la fuente para la absorción de agua y los nutrientes necesarios. Por ello, las propiedades del suelo que más van a influir en el desarrollo de las plantas son las que determinan la disponibilidad de agua y nutrientes, así como el crecimiento y la expansión de las raíces.

En el estudio realizado sobre las cuatro especies de *Limonium* en su hábitat natural, se analizaron muestras de suelo de 0 a 15 cm de profundidad de las dos zonas estudiadas. Los suelos de la zona de El Saler pertenecen a la clase de textura arenosa, mientras que los de El Clot de Galvany pertenecen a la franca arenosa, ya que tienen un menor porcentaje de arena. Esta diferencia es significativa, sobre todo después de periodos de lluvia, ya que los suelos del Clot tienen una mayor capacidad de retención de humedad que los suelos arenosos de El Saler. Aparte de estos datos de textura, los análisis del suelo no revelaron grandes diferencias entre los lugares de muestreo.

En el estudio de *L. albuferae* y *L. dufourii* se analizaron muestras de suelo de las zonas donde se encuentran las poblaciones naturales. La salinidad en las zonas donde se encontraron las dos especies fue moderada, aunque significativamente menor en la zona de *L. albuferae*, estando muy por debajo del límite de tolerancia de la especie establecido en los ensayos. La salinidad en el campo se midió durante el verano, cuando es más alta debido a la mayor evaporación, por lo que en principio la salinidad no sería un factor limitante para la especie. Sin embargo, debido a la escasez de la especie, de la que sólo se conocía una población en el momento del muestreo, los análisis sólo se realizaron en una zona y, por tanto, no son concluyentes. En el caso de *L. dufourii*, la salinidad tampoco sería un factor limitante. Otras propiedades del suelo relevantes para el desarrollo de las plantas fueron similares en todas las muestras de suelo.

En el estudio de *Thalictrum maritimum* también se realizaron análisis de suelo. Los resultados de los ensayos realizados en condiciones de estrés salino revelaron que la tolerancia de la especie a la salinidad es muy superior a la registrada en condiciones naturales, ya que los análisis muestran salinidades generalmente bajas en los hábitats donde esta especie se encuentra de forma natural. Asimismo, según el estudio se puede afirmar que los parámetros edáficos más relacionados con su distribución serían la humedad y el contenido en materia orgánica. En las zonas analizadas con presencia de *T. maritimum*, la humedad del suelo es mayor que la indicada en otros estudios en la misma zona (Al Hassan et al., 2016; Koźmińska et al., 2018), así como el contenido en materia orgánica también es mayor que en los suelos de otras zonas cercanas (González-Orenga et al., 2020).

5.3 Ensayos de Germinación

Los ensayos de germinación se realizaron únicamente en *L. albuferae* y *L. dufourii*. En las otras especies de *Limonium* estudiadas, la germinación fue analizada previamente (Al Hassan et al., 2017) y en las otras especies la cantidad de semillas disponibles ha sido insuficiente. Los porcentajes finales de germinación a los 30 días fueron muy altos para ambas especies de *Limonium* en los controles y la salinidad inhibió la germinación de las semillas en ambas especies, pero con algunas diferencias. A 300 mM de NaCl *L. albuferae* mostró un mayor porcentaje de germinación (18,5%) en relación con *L. dufourii* (2,5%), mientras que sólo unas pocas semillas germinaron a 450 mM y a concentraciones de 600 mM la germinación fue totalmente inhibida en ambas especies. A pesar de que las dos especies comparten la misma zona en el Parque Natural de l'Albufera, difieren en su nivel de tolerancia al estrés salino mostrando *L. albuferae* una mayor tolerancia a la salinidad. Este parámetro es importante ya que la germinación de las semillas y el establecimiento de las plántulas son fases críticas en el ciclo de vida de las halófitas (Houle et al., 2001) y la reducción de la salinidad en las capas superficiales del suelo para permitir la germinación es un requisito previo para su supervivencia.

Sin embargo, una característica distintiva de las especies halófilas es la recuperación de su capacidad germinativa incluso después de largos períodos de exposición a altas salinidades. Por lo tanto, se realizó adicionalmente un estudio de recuperación de la capacidad germinativa de las semillas. *L. dufourii*, a pesar de tener menores tasas de germinación en condiciones de salinidad, mostró una excelente tasa de recuperación alcanzando porcentajes de germinación del 95%, mientras que *L. albuferae*, a pesar de tener más éxito en la germinación en condiciones de salinidad, tuvo una tasa de recuperación que osciló entre el 60 y el 80%. Estos resultados indican que las semillas de ambas especies mantienen su viabilidad en presencia de altas concentraciones de NaCl, un parámetro destacable porque las altas salinidades representan una limitación para la germinación de las semillas. En las plantas halófilas, incluso aquellas que pueden soportar concentraciones de sal muy elevadas en sus hábitats naturales, normalmente sus semillas germinan cuando hay una reducción de la salinidad del suelo (Ungar, 1991; Gul et al., 2013; Kazachkova et al., 2016). La salinidad del suelo presenta una variación estacional, siendo mayor durante el verano debido a la acentuación de la evaporación, por lo que en las regiones áridas y semiáridas suele producirse después de que las lluvias reduzcan la salinidad superficial del suelo (Khan, 1999; Khan & Gul, 2006). Cabe destacar que las halófitas mantienen un banco de semillas persistente en el suelo, germinando en primavera en climas templados cuando la salinidad se alivia con las lluvias (Ungar, 1995; Gul et al., 2013).

5.4 Análisis del Sustrato

El análisis del sustrato en los tratamientos realizados en invernadero consistió en la cuantificación de la humedad y de la electroconductividad, parámetro directamente relacionado con el nivel de salinidad. Esta cuantificación se realizó durante el tiempo de aplicación de los tratamientos de forma semanal, así como al principio y al final de estos, con la sonda WET-2 Sensor (Delta—T Devices, Cambridge, UK) y, por otra parte, al final de estos, a través de la relación del peso fresco y seco del suelo, para determinar la humedad y, mediante electroconductímetro, para la determinación de la electroconductividad.

Previsiblemente, en los ensayos de estrés hídrico en *Limonium*, después de un mes de la interrupción de la irrgación, la humedad del sustrato fue significativamente menor que la registrada en los tratamientos control y no hubo diferencias entre especies dentro de cada uno de los tratamientos.

En el caso de los ensayos de estrés hídrico y salino en *Limonium*, se realizaron mediciones con la sonda al inicio de los tratamientos y después de una semana desde el comienzo, pero no fue posible realizar más mediciones debido a la alta electroconductividad alcanzada en los tratamientos de salinidad por encima de 400 mM de NaCl, ya que superaban la capacidad del dispositivo. Por lo tanto, se cuantificó la electroconductividad final en un extracto 1:5. Los resultados mostraron un aumento de la electroconductividad de forma gradual y paralela a las concentraciones de NaCl aplicadas, llegando a ser 10 veces mayor que en el control en los individuos a los que se les aplicó la mayor concentración (800 mM NaCl). En referencia a la humedad, ésta disminuyó drásticamente en el tratamiento de estrés hídrico, alcanzando valores cercanos al 3% de humedad en el sustrato en ambas especies, mientras que sólo se encontró una ligera disminución en los tratamientos de 400 mM o superiores.

En *Thalictrum* la progresiva acumulación de NaCl generó en el sustrato de las plantas sometidas a la mayor salinidad (300 mM), niveles considerablemente superiores a los registrados en campo. Como se meciona en el apartado 5.6, este tratamiento provocó inhibición del crecimiento respecto a los controles analizados. Sin embargo, en los tratamientos con 100 mM NaCl, no se detectaron cambios significativos a nivel morfológico y la salinidad al finalizar los tratamientos era mayor que la registrada en la mayoría de sus ubicaciones naturales.

En el caso de las especies de *Bupleurum*, los efectos de la salinidad fueron notables tras la primera semana en los tratamientos de mayor salinidad (300 y 450 mM), alcanzando valores en la electroconductividad muy elevados, de más de 10 veces mayores que el rango de suelo no salino (4 mS/cm). Aunque en los tratamientos de menor salinidad se alcanzaron valores de aproximadamente el doble de este rango de salinidad, las plantas no presentaron efectos notables a nivel morfológico.

El conocimiento de estos parámetros a nivel experimental nos permite hacer una extrapolación del comportamiento de las especies analizadas en sus hábitats, ya que la electroconductividad y la humedad se estudió tanto en los sustratos, como en los suelos de sus poblaciones naturales. Aunque predeciblemente, los individuos se desarrollaran mejor en campo que en maceta debido a la posibilidad de expandir su sistema radicular.

5.5 Análisis Fitosociológico

También se incluyó un estudio fitosociológico en el caso de tres de las especies, para analizar la influencia de la vegetación en sus hábitats.

En el caso de la población analizada de *L. albuferae* se observó una gran presencia de *Spartina patens*, especie invasora que podría ser la causa de la reducción de la población de la especie estudiada. Esta especie invasora ha sido señalada recientemente como una gran amenaza para las halófitas nativas de esta área (Martinez-Fort & Donat-Torres, 2020). En el caso de *L. dufourii*, las malladas donde desapareció están completamente invadidas por *S. patens*. También destaca la alta presencia de *Dittrichia viscosa*, considerada como una especie invasora autóctona, muy competitiva a salinidades bajas y moderadas de la zona (Al Hassan et al., 2016a).

Así, en las poblaciones de las dos especies de *Limonium* estudiadas, no fue posible atribuir los inventarios a asociaciones, ya que ambas se encuentran en zonas muy perturbadas, donde se ha llevado a cabo una restauración geomorfológica. Además, en estas zonas, la dinámica de la vegetación es muy rápida y está sujeta a periodos de inundación y, por tanto, a cambios en la salinidad. El régimen de lluvias de los últimos años ha sido muy variable, lo que también ha afectado a las comunidades vegetales. Sin embargo, los inventarios indicaron la presencia de halófitos típicos de las clases de vegetación *Salicornietea fruticosae*, *Thero-Suaedetea* y *Juncetea maritimae*.

En el caso del estudio de la vegetación del área natural donde se desarrolla la especie *T. maritimum*, la vegetación que le acompaña incluye varias especies halófitas, como, por ejemplo, *Juncus acutus*, *J. maritimus*, *Plantago crassifolia* y *Schoenus nigricans*, pero también incluye glicófitos específicos de comunidades desarrolladas en suelos no salinos como matorrales y vegetación típica de pinar. En este caso, no se encontró una posible vegetación invasora que pudiera causar la reducción de las poblaciones.

En un enfoque multidisciplinar, es interesante estudiar las posibles variables que podrían estar afectando y/o reduciendo el número de individuos de las poblaciones de las especies de interés, por lo que el análisis fitosociológico es clave, ya que una especie invasora podría provocar la reducción de otra por competencia lumínica o radicular, por ejemplo.

5.6 Inhibición del Crecimiento

El efecto más general del estrés abiótico, y el más fácil de cuantificar, es la inhibición del crecimiento, que permite la reducción de las necesidades de la planta y, por lo tanto, hace posible que la planta se desarrolle y viva bajo condiciones limitantes al redirigir sus recursos (precursores metabólicos y energía) desde el metabolismo normal y el crecimiento a la activación de mecanismos específicos de defensa contra el estrés (Zhu, 2001; Munns & Tester, 2008). El efecto del estrés se nota en la reducción de la tasa de crecimiento, la inhibición de la expansión foliar y el aumento del crecimiento radicular (Potters et al., 2007; Shao et al., 2008). Otro mecanismo de resistencia a nivel fisiológico es el cierre de estomas, estructuras responsables de la mayor pérdida de agua en las plantas (Taiz & Zeiger, 2006), así como cambios en un número de procesos fisiológicos y bioquímicos que rigen el crecimiento y la productividad de las plantas (Daie, 1988). La tolerancia a determinados tipos de estrés en las plantas, propiedad desarrollada evolutivamente, afecta su distribución en la naturaleza. En regiones con condiciones ecológicas difíciles han aparecido, por acción de la selección natural, plantas con alta tolerancia a factores característicos de cada región (González et al., 2002), siendo las más tolerantes las presentes en zonas semiáridas, áridas y salinas, incluyendo desiertos, dunas, salinas y otras zonas afectadas por la salinidad, donde tienen una ventaja competitiva sobre los taxones menos tolerantes (Maestre et al., 2009).

Por todo ello, el seguimiento de los parámetros de crecimiento nos permitió establecer el grado relativo de resistencia al estrés hídrico y salino de las especies estudiadas.

Entre los parámetros medidos, la pérdida de peso fresco en las hojas o en la parte aérea (dependiendo de la especie), la reducción de la longitud de la planta o del número de hojas fueron indispensables para la determinación del efecto del estrés aplicado y así definir la tolerancia relativa al estrés en las especies de interés.

Estos parámetros, por lo general, mostraron una disminución paralelamente al aumento de la concentración salina aplicada y al tratamiento de sequía en todas las especies estudiadas, en mayor o menor grado.

En referencia a las cuatro especies de *Limonium* estudiadas bajo condiciones de estrés hídrico, la ausencia total de irrigación provocó una inhibición significativa del crecimiento en dos de las especies seleccionadas, *L. santapolense* y *L. narbonense*, como muestra la reducción de su peso fresco en la parte aérea de hasta 1/3 en comparación a sus controles. Al mismo tiempo, *L. girardianum* mostró una ligera reducción (11%) y en *L. virgatum* se observó un ligero aumento bajo condiciones de estrés hídrico respecto al control, aunque este dato no fue significativo. Otro parámetro estudiado en este caso fue el área foliar, la cual también se redujo por el efecto del estrés, estadísticamente significativa solamente en *L. santapolense*, que perdió respecto al control un 40% de su superficie. Por otra parte, en cuanto al contenido hídrico, a pesar de estar un mes sin irrigiación no varió demasiado. Los datos muestran una mayor reducción en *L. santapolense* en la parte aérea, y en el caso de las raíces, una mayor reducción en *L.*

narbonense. Por lo que según estos parámetros *L. santapolense* podría considerarse la especie más afectada por el estrés hídrico bajo nuestras condiciones experimentales.

Sin embargo, al realizar el estudio de las mismas especies de *Limonium* en su hábitat natural se pudo constatar que *L. santapolense* posee mecanismos específicos que permiten su supervivencia en campo bajo condiciones áridas y esto está relacionado con el desarrollo de sus raíces. Se encontraron diferencias entre las plantas de *L. santapolense* que crecen cerca del nivel freático y las que crecen lejos de él. Las primeras tenían una raíz principal corta con nudos distribuidos de forma uniforme sobre ella, mientras que las segundas, desarrollan una raíz principal muy larga (187 cm de promedio) que solo se ramifica cuando alcanza la humedad necesaria para subsistir. En cuanto a las otras especies estudiadas, cabe destacar *L. narbonense*, que posee un sistema subterráneo muy diferente al resto, de raíces rizomatosas y más grueso en comparación con las demás, lo que explicaría la mayor reducción del contenido de agua de sus raíces bajo condiciones de estrés hídrico. Por otra parte, hay que destacar la mayor área foliar de *L. santapolense* en comparación con las otras tres especies seleccionadas, lo cual podría estar relacionado con su mayor sensibilidad al déficit hídrico bajo condiciones de invernadero en las cuales no puede desarrollar sus raíces, ya que en general este rasgo aumenta la sensibilidad al déficit de agua (Guerin & Lowe, 2012).

Por otra parte, el estrés salino inhibió el crecimiento de las otras dos especies de *Limonium* estudiadas (*L. albuferae* y *L. dufourii)* afectando principalmente a la parte aérea de las plantas como indica la disminución en el número de hojas y en el peso fresco de la parte aérea respecto a los controles. En comparación, *L. dufourii*, muestra una reducción más acentuada de estos parámetros de crecimiento en paralelo al aumento de la salinidad (Figura 21 y 22 b).

Figura 21. Detalle hojas de *L. dufourii* al final de los tratamientos: a) Control b) Estrés hídrico c) 200 mM d) 400 mM e) 600 mM f) 800 mM de NaCl.
Fuente: Propia

Figura 22. *L. dufourii* al final de los tratamientos: a) Control y Estrés Hídrico b) Control y tratamientos de salinidad.

En el tratamiento de menor salinidad (200 mM de NaCl) el peso fresco de las hojas de *L. albuferae* aumentó respecto a su control, mientras que en *L. dufourii* provocó una reducción del 50%. (Figura 23). En muchas halófitas dicotiledóneas, especialmente en las más tolerantes a la salinidad, las concentraciones moderadas de NaCl estimulan el crecimiento (Flowers et al., 1986), *L. albuferae* presentó una mayor tolerancia a la salinidad y solo se notó un efecto inhibidor contundente a partir de la concentración de 600 Mm de NaCl (Figura 24). El peso fresco de las raíces aumentó en ambas especies en salinidades moderadas (200-400 mM NaCl) disminuyendo nuevamente a salinidades mayores, pero nunca por debajo del control. Ambas especies mostraron respuestas diferentes, *L. albuferae* presentó una mayor tolerancia a concentraciones moderadas de sal. Todos los parámetros analizados indican que *L. dufourii* es más sensible durante el crecimiento vegetativo, aun así, ambas parecen ser tolerantes a salinidades del suelo mucho más altas que las de sus hábitats naturales. Por otra parte, en condiciones de estrés hídrico, ambas sufrieron una reducción fuerte en los parámetros más relevantes como son el peso fresco y el contenido de agua, especialmente *L. albuferae*, por lo que es mucho más susceptible al déficit hídrico.

Figura 23. Detalle hojas de *L. albuferae* al final de los tratamientos: a) Control b) Estrés hídrico c) 200 mM d) 400 mM e) 600 mM f) 800 mM de NaCl.

Fuente: Propia

Figura 24. *L. albuferae* al final de los tratamientos: a) Tratamiento Control y Estrés hídrico b) Tratamientos de salinidad

Fuente: Propia

En *Thalictrum maritimum*, se observó una reducción del crecimiento en el caso del estrés salino a la mayor salinidad (300 mM de NaCl), reduciéndose significativamente el número de nuevas ramificaciones y de nuevas hojas formadas, parámetro que también disminuyó bajo el estrés hídrico. El mismo efecto marcado de los tratamientos de estrés hídrico y de mayor salinidad se observó, en general, en la reducción del peso fresco de las plantas. Sin embargo, bajo salinidades inferiores a 300 mM hay ligeras diferencias respecto al control, aunque no significativas, por lo que *T. maritimum* resultó ser más tolerante a la salinidad de lo esperado.

Finalmente, en las especies estudiadas del género *Bupleurum*, los tratamientos de estrés aplicados afectaron a las dos especies de forma diferente. En *B. tenuissimum*, los parámetros de crecimiento se redujeron especialmente en las plantas sometidas a estrés hídrico, excepto en la raíz, que fue mayor en las plantas sometidas a este tratamiento (Figura 25 a). Mientras que los efectos de la salinidad sólo provocaron una reducción significativa de los parámetros medidos a partir de la mayor concentración aplicada (450 mM de NaCl) (Figura 25 b), que afectó principalmente al desarrollo de nuevas hojas, al crecimiento del tallo y, por tanto, al peso fresco de su parte aérea.

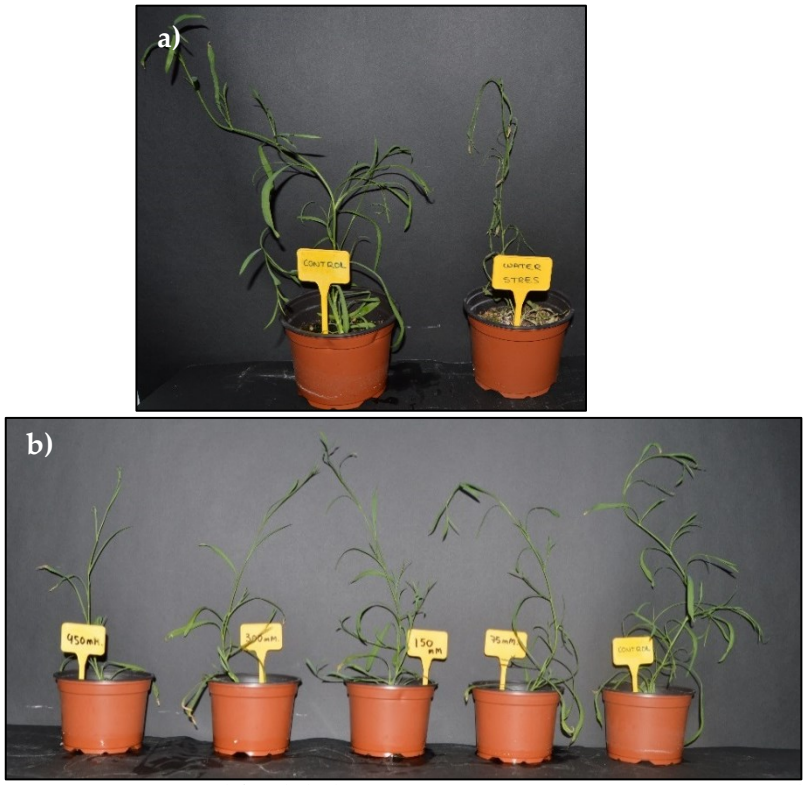

Figura 25. *B. tenuissimum* al final de los tratamientos: a) Tratamiento Control y estrés hídrico b) Tratamientos de estrés salino (de 450 a 75 mM) y Control.

En contraste, en *B. fruticosum*, las plantas sometidas al déficit hídrico experimentaron una ligera reducción del peso fresco, pero los efectos de la salinidad aumentaron en paralelo al aumentar las concentraciones de sal aplicadas (Figura 26), siendo el efecto de la salinidad evidente.

Figura 26. *B. fruticosum* al final de los tratamientos. Control, Estrés Hídrico, 75, 150, 300 y 450 Mm de NaCl.

Fuente: Propia

5.7 Inhibición de la Fotosíntesis

En todos los ensayos llevados en invernadero bajo condiciones controladas se analizaron el contenido foliar de clorofila a, clorofila b y carotenoides. En combinación con el análisis de los parámetros de crecimiento, la cuantificación de pigmentos se usa habitualmente para la evaluación de los efectos del estrés en las plantas ya que una reducción del nivel de clorofila contribuye a la inhibición de la fotosíntesis (Gummuluru et al., 1989). Por su parte, los carotenoides son compuestos multifuncionales que sirven como pigmentos accesorios de recolección de luz, que transfieren la energía de las emisiones solares a las clorofilas, ampliando así la gama de longitudes de onda de la luz para la fotosíntesis; también funcionan protegiendo las clorofilas de las reacciones destructivas de fotooxidación (Ashraf & Harris, 2013).

En el estudio comparativo de las cuatro especies de *Limonium* (*L. santapolense, L. girardianum, L. narbonense* y *L. virgatum*) el estrés hídrico no tuvo efecto sobre los niveles de pigmentos en ninguno de los taxones estudiados. Por lo que estos resultados nos indicarían que las especies estudiadas son bastante resistentes a los episodios de sequía ya que no hay inhibición de la fotosíntesis ni reducción del contenido de clorofila, efectos frecuentes de la sequía, así como de otros estreses abióticos (Kumar et al., 2017; Marček et al., 2019).

Sin embargo, la degradación de los pigmentos fotosintéticos se produjo bajo condiciones de salinidad y de estrés hídrico en *L. dufourii* mostrando diferencias significativas respecto al control en todos los pigmentos analizados, siendo estas diferencias mayores en el caso de los tratamientos salinos. En el caso de *L. albuferae*, encontramos diferencias en los contenidos de clorofila b y carotenoides de las plantas tratadas respecto al control, aunque estas diferencias no son tan elevadas como en *L. dufourii.* Estos resultados coinciden con las respuestas de las dos especies en términos morfológicos, que indican una tolerancia relativamente más alta a la salinidad en *L. albuferae.*

En el caso de *Thalictrum maritimum*, las concentraciones de todos los pigmentos fotosintéticos disminuyeron en respuesta al déficit hídrico y a la salinidad, excepto la clorofila b, que mostró en el tratamiento de menor salinidad concentraciones similares al control. La degradación fotosintética fue más pronunciada en las plantas sometidas al tratamiento de estrés hídrico.

En el estudio del género *Bupleurum*, el tratamiento de estrés hídrico tuvo un fuerte efecto de inhibición sobre la clorofila a, b y los carotenoides en *B. tenuissimum*, pero no en *B. fruticosum*. Las salinidades de 75 y 150 mM no afectaron a la concentración de pigmentos, incluso se detectó un aumento de las clorofilas en el caso de *B. tenuissimum* respecto al control. Sin embargo, en las concentraciones más altas de NaCl las concentraciones se redujeron, en especial en *B. fruticosum.* Los carotenoides en primera especie solo mostraron una disminución significativa a la mayor concentración salina, mientras que en la segunda especie la disminución de carotenoides fue ya notable a 300 mM de NaCl. En resumen, en *B. tenussimum* las concentraciones en general se reducen en el tratamiento salino, mientras que en *B. fruticosum*, aunque hay una reducción en las plantas sometidas a estrés hídrico, la reducción es más notable y gradual a medida que aumenta la salinidad aplicada.

5.8 Control del Transporte Iónico

Las dicotiledóneas halófilas tienden a acumular iones tóxicos en las partes aéreas de las plantas, que se mantienen en bajas concentraciones citosólicas por la compartimentación de las vacuolas (Blumwald et al., 2000; Hasegawa et al., 2000). Este patrón se ha detectado en las especies estudiadas en condiciones de salinidad, ya que los niveles de iones y su variación interespecífica fueron generalmente mayores en las hojas que en las partes subterráneas. Esto representa un mecanismo para aumentar la presión osmótica en los tejidos foliares, con un coste energético menor que la síntesis de solutos orgánicos para el ajuste osmótico (Raven, 1985).

Las cuatro especies de *Limonium* estudiadas bajo estrés hídrico no mostraron cambios significativos en el contenido foliar de iones. Sin embargo, si se observaron cambios en la parte subterránea, los iones tóxicos, Na^+ y Cl^-, aumentaron generalmente en respuesta al déficit hídrico. Por otro lado, los niveles de Ca^{2+} y Mg^{2+} se redujeron drásticamente en la especie más estresada, *L. santapolense*, mientras que en el resto se produjo un aumento.

Lo que sí se cumple en todas las especies, tanto en los individuos control como en los sometidos a déficit hídrico es la mayor acumulación de iones en las hojas, y esto indica que la activación del transporte iónico sea probablemente un mecanismo constitutivo de respuesta al estrés que el género *Limonium* utiliza para contribuir al equilibrio osmótico celular en las hojas. Este mecanismo constitutivo se basaría en la captación y transporte activo de Na^+ en las hojas, incluso sin condiciones de estrés o con estrés relativamente bajo, además de la acumulación de osmolitos orgánicos de acuerdo con la "preadaptación al estrés" de las halófitas (Gong et al., 2005; Sánchez et al., 2008).

Cabe destacar que los recretohalófitos pueden secretar iones tóxicos a través de las glándulas salinas y los tricomas, y muchos estudios confirman esta característica específica en el género *Limonium* (Tabot & Adams, 2014; Leng et al., 2018).

En cuanto a las especies estudiadas en su hábitat, además del transporte de los iones tóxicos a la parte aérea de las plantas, la acumulación de Ca^{2+} en las hojas también puede contribuir a los mecanismos de tolerancia a la salinidad en los taxones analizados, especialmente en *L. santapolense*, la especie que presenta mayores concentraciones foliares de este catión. El papel esencial del Ca^{2+} en el alivio de los efectos deletéreos de la salinidad ha sido analizado en muchas especies (Hasegawa et al., 2000; Hadi & Karimi, 2012).

En en el estudio realizado en *L. albuferae* y *L. dufourii*, el contenido de los iones cuantificados aumentó en paralelo a la concentración de sal aplicada, tanto en los órganos subterráneos como en los aéreos, siendo siempre significativamente menor en la parte subterránea que en las hojas. La acumulación de Na^+ está asociada a la disminución de los niveles de K^+, como se pudo observar en las dos especies de *Limonium*. Esta disminución de K^+ se debe a la competencia con el Na^+ por los mismos sitios de unión en las proteínas, incluido las proteínas de transporte de membrana. Además, un exceso de Na^+ provoca la despolarización de la membrana plasmática, lo cual induce la activación de los canales de potasio hacia el exterior y, por tanto, la perdida de K^+ celular (Greenway & Munns, 1980). La reducción de K^+ fue más notable en *L. dufourii*, que también mostró una acumulación mayor de Na^+ y por ello, la relación la relación Na^+ / K^+ aumento mucho más en esta especie. Mantener una proporción citosólica Na^+ / K^+ equilibrada se considera un mecanismo esencial para la tolerancia a la sal (Assaha et al., 2017) y por ello, las proporciones menores en el caso de *L. albuferae* podrían ser una de las responsables de su mayor tolerancia. Esto refleja su capacidad de activar el transporte de K^+ a las hojas en respuesta a la salinidad, lo cual provoca una reducción menor o mantiene los niveles de este catión en hojas. Por otra parte, el aumento que se observó en los niveles de Ca^{2+} en respuesta al aumento de salinidad, tanto en raíces como en hojas en las dos especies, probablemente también esté involucrado en los mecanismos de tolerancia a la salinidad. Como se ha mencionado anteriormente el calcio juega un papel clave en tolerancia a la salinidad, por ejemplo, el calcio extracelular es beneficioso para el mantenimiento de la homeostasis de Na^+ y K^+ a través de la vía SOS (Mahajan et al., 2008). Además, un aumento de la concentración de Ca^{2+} estimuló el desarrollo y las tasas de secreción de las glándulas salinas en otras especies de *Limonium* (Ding et al., 2010). Los datos obtenidos en estas dos especies

indican también la existencia de mecanismos constitutivos de tolerancia basados en el transporte activo de iones a las hojas para contribuir en el ajuste osmótico.

En el caso de *Thalictrum maritimum*, los contenidos de Na^+ y Cl^- no variaron en el tratamiento de estrés hídrico respecto al control, pero como era de esperar, aumentaron en presencia de sal, aunque esta variación no fue significativa en la menor concentración de sal respecto al control. Estos iones se acumularon siempre en niveles más altos en los órganos aéreos que en las raíces. Cabe destacar que tanto el déficit hídrico como la salinidad indujeron un aumento en las concentraciones de Ca^{2+} en todos los órganos de la planta. Por esto se puede considerar que su mecanismo principal de tolerancia está basado en el transporte activo de iones tóxicos a los órganos aéreos y en la mayor concentración de K^+.

En *Bupluerum*, la concentración de los iones analizados se mantuvo constante en las plantas sometidas a estrés hídrico respecto al control, a excepción de K^+ en las hojas de *B. tenuissimum*, que aumentó significativamente. En cuanto a Na^+ y Cl^-, a diferencia de las otras especies estudiadas, se encontraron valores más altos en raíces que en la parte aérea de las plantas, por lo que en este caso el mecanismo básico de tolerancia difiere, su principal mecanismo de resistencia a estrés salino es la prevención de la acumulación foliar de iones tóxicos, ya sea reduciendo la absorción de las raíces o mediante el bloqueo de su transporte a las partes aéreas (Maathuis et al., 2014; Volkov, 2015). En las dos especies estudiadas se encontró un aumento significativo de Na^+, en paralelo a la concentración de sal aplicada tanto en raíces como en hojas, lo que indica que no hay inhibición del transporte de Na^+ de las raíces a las hojas. Esto podría estar relacionado con el transporte activo de K^+, ya que e ambas especies hay un mayor contenido en hojas que en raíces, en *B. tenuissimum*, bajo estrés salino los niveles de este ion en las hojas aumentaron significativamente mientras que en la otra especie estudiada disminuyeron. Estos resultados indican que *B. tenuissimum* se comporta como una especie tolerante a la salinidad activando el transporte de potasio a las hojas bajo condiciones de salinidad, mientras que *B. fruticosum* no mostró esta capacidad.

5.9 Acumulación de Osmolitos

Uno de los principales mecanismos que aseguran el equilibrio osmótico bajo estrés es la síntesis y acumulación en el citoplasma de solutos compatibles, los denominados osmolitos, para contrarrestar la deshidratación celular causada por las situaciones de estrés. Sin embargo, es difícil establecer si el aumento dependiente del estrés en la concentración de un osmolito particular tiene un papel funcional en los mecanismos de tolerancia de una especie dada, ya que las respuestas no confieren necesariamente tolerancia.

Se trata de diversos compuestos orgánicos que, además de su función fundamental en el ajuste osmótico, juegan un papel adicional en los mecanismos de tolerancia al estrés,

aumentando la estabilidad termodinámica de las proteínas plegadas y protegiendo directamente las estructuras macromoleculares, en su función de acompañantes de bajo peso molecular, y también como carroñeros de 'especies reactivas de oxígeno' (ROS), o como moléculas de señalización (Smirnoff & Cumbes, 1989; Ashraf & Harris, 2004; Flowers & Colmer, 2008; Munns & Tester, 2008; Türkan & Demiral, 2009; Szabados & Savouré, 2010; Slama et al., 2015). Sin embargo, la biosíntesis de osmolitos representa un alto costo para las plantas, ya que se puede alcanzar la misma osmolaridad celular mediante la captación y transporte de iones, como hemos visto en el apartado anterior, con un consumo de energía mucho menor (Raven 1985; Shabala & Shabala, 2011).

Los estudios comparativos realizados deberían proporcionar evidencias a favor de la contribución a la tolerancia al estrés de osmolitos específicos, es decir, una correlación positiva entre los niveles de osmolitos y el grado relativo de tolerancia.

5.9.1 Prolina

La prolina es uno de los osmolitos más comunes en plantas, tiene diferentes funciones biológicas en los mecanismos de tolerancia al estrés abiótico. Actúa en el ajuste osmótico bajo estrés; como acompañante de bajo peso molecular, por ejemplo, en la protección de la integridad de la membrana plasmática y sus proteínas transportadoras asociadas; como captador de ROS; y / o como molécula de señalización en la transducción de señales de estrés (Vebruggen & Hermans, 2008; Szabados & Savouré, 2010, Saibi & Brini, 2020). Las variaciones observadas en los niveles de prolina podrían estar relacionadas con daños y las reparaciones sucesivas en el paso mitocondrial de la peroxidación (Gagneul et al., 2007).

En el caso de las cuatro especies de *Limonium* estudiadas bajo condiciones de estrés hídrico, a excepción de *L. narbonense*, los niveles de prolina aumentaron en comparación a los controles, siendo este incremento más pronunciado en *L. santapolense* por lo que, en este caso, el ajuste osmótico para proteger las plantas de la deshidratación se basa en la acumulación de prolina, entre otros, que mostraremos más adelante.

Cuando se estudiaron estas mismas cuatro especies en sus hábitats naturales, los individuos de *L. santapolense*, mostraron diferencias con las otras tres analizadas, mostrando concentraciones más bajas de prolina. Por lo que la acumulación de prolina como mecanismo de defensa parece solo activarse bajo condiciones de estrés hídrico muyaccentuado.

La variación del contenido de prolina en *L. albuferae* y *L. dufourii*, siguió un patrón similar en ambas especies, aumentando en paralelo a las concentraciones externas de cloruro de sodio, y especialmente bajo estrés hídrico. El aumento respecto a los valores del control en plantas sometidas a estrés fue mayor en ambos tipos de estrés para *L. albuferae*, pero esto se debe a los niveles relativamente bajos de prolina en el tratamiento control. En condiciones de estrés hídrico se registró una acumulación de prolina en ambas especies. Sin embargo, este osmolito no parece estar directamente involucrado en los mecanismos

de tolerancia a la salinidad, ya que se acumula a niveles absolutos más altos en *L. dufourii*, la especie menos halotolerante.

La concentración de prolina foliar en *T. maritimum* aumentó en respuesta al déficit hídrico y al estrés salino, pero las concentraciones absolutas de prolina alcanzadas fueron demasiado bajas como para tener un efecto osmótico relevante, aunque no se puede descartar la contribución de la prolina a la tolerancia a los diferentes tipos de estrés en esta especie, basándonos en sus funciones "osmoprotectoras" o de señalización.

En las especies estudiadas del género *Bupleurum*, las concentraciones de prolina y el patrón de acumulación fueron diferentes en las dos especies. *B. tenuissimum* presentó altos niveles de prolina en las plantas control, y su concentración no varió en los tratamientos de estrés salino, pero su concentración aumentó en el tratamiento de estrés hídrico. Por el contrario, *B. fruticosum* presentó valores más bajos en las plantas control y su nivel aumentó en ambos tipos de estrés, aunque especialmente en los tratamientos de salinidad. Estos resultados indican que este osmolito tiene un papel importante en la respuesta al estrés en este género, y su incremento se puede considerar como un indicador del nivel de estrés.

Se puede concluir que el contenido de prolina aumenta generalmente en respuesta a la salinidad y al estrés hídrico y puede utilizarse como marcador del nivel de estrés que afecta a las plantas de las especies estudiadas, con la excepción de *Thalictrum maritimum* en la que las concentraciones de prolina acumuladas fueron muy bajas.

5.9.2 Azúcares Solubles

La evaluación del papel de los azúcares solubles en los mecanismos de tolerancia al estrés es difícil debido a las múltiples funciones que presentan como, por ejemplo, productos directos de la fotosíntesis, componentes primarios del metabolismo y moléculas reguladoras (Gil et al., 2013).

L. santapolense mostró un contenido de azúcares totales foliar mayor en el control que las otras tres especies estudiadas, pero los niveles no variaron bajo condiciones de estrés hídrico para ninguno de los cuatro taxones. Por otra parte, se observaron tres picos principales en los cromatogramas, los cuales correspondían a glucosa, fructosa y sacarosa, pero los patrones de sus concentraciones difieren para las cuatro especies, destacando la acumulación de fructosa y de sacarosa en *L. santapolense* bajo condiciones de estrés hídrico. *L. santapolense* también presentó diferencias con las otras tres especies analizadas bajo las condiciones de sus hábitats naturales, mostrando concentraciones foliares de fructosa menores que el resto de las especies, por lo que el ajuste osmótico parece que se logre mediante acumulación de sacarosa ya que está presente en niveles mayores que en los otros tres taxones.

Bajo estrés hídrico, los azúcares solubles totales aumentaron en *L. dufourii* y disminuyeron en *L. albuferae*, sin embargo, a la menor concentración de sal (200 mM), el contenido de azúcares aumentó en *L. albuferae*; por encima de esta salinidad no se

encontraron diferencias significativas, por lo que esto podría significar que la acumulación de azúcares estaba actuando como mecanismo de defensa frente al déficit hídrico en *L. dufourii* y frente al estrés salino en *L. albuferae*, al menos en condiciones de salinidad moderada, ya que a concentraciones de 400 mM o superiores no se encontraron diferencias significativas entre ambas especies, que podrían estar utilizando otros mecanismos de respuesta a la alta salinidad.

Para obtener mayor información sobre las dos especies de mayor interés de conservación, *L. albuferae* y *L. dufourii*, se analizaron los perfiles metabólicos, detectándose tres categorías de metabolitos primarios: carbohidratos, ácidos orgánicos y aminoácidos. El contenido de los diferentes carbohidratos fue similar en ambas especies, excepto la rafinosa, más abundante en *L. albuferae*, y la fructosa y la glucosa, más abundante en *L. dufourii*. Se detectaron ácidos orgánicos en las dos especies, pero sin diferencias marcadas entre las especies. Respecto al contenido de aminoácidos, se observaron diferencias cuantitativas entre las dos especies destacando el ácido glutámico para *L. albufera*. Se detectaron cambios en el contenido relativo de algunos metabolitos en respuesta al estrés salino. Respecto al contenido de azúcares y polioles, en ambas especies el eritriol, la ramnosa y la sacarosa aumentaron con la salinidad aplicada mientras que el mioninositol disminuyó en respuesta al estrés salino. Los niveles de ácido fosfórico y alguno ácidos orgánicos aumentaron en las plantas estresadas de ambas especies. Respecto a los aminoácidos, se detectó una tendencia general de aumento bajo condiciones de estrés salino, especialmente en *L. albuferae*, donde encontramos los contenidos máximos en las concentraciones de sal más altas.

En *T. maritimum*, el contenido de azúcares disminuyó en las plantas sometidas a estrés hídrico respecto al control, pero no varió en las sometidas a los tratamientos salinos, lo que sugiere que estos compuestos no están involucrados en el ajuste osmótico en esta especie.

En las especies analizadas del género *Bupleurum*, la concentración de azúcares fue similar en las plantas control de ambas especies, pero su patrón de variación fue diferente bajo condiciones de estrés hídrico, con una reducción fuerte en *B. tenuissimum*, pero sin variación significativa en *B. fruticosum.* En plantas sometidas a bajas salinidades aumentó en ambas especies, disminuyendo en el tratamiento de 450 mM. Al no mostrar un patrón de variación claro, no se puede afirmar que el aumento o disminución de azúcares tenga un papel especifico en la respuesta frente al estrés en estas especies. Por otra parte, la marcada reducción en el contenido de azúcares en el tratamiento de estrés hídrico en *B. tenuissimum*, se explica por la drástica reducción de la fotosíntesis, ya que en esta especie en este tratamiento disminuyeron también los pigmentos fotosintéticos.

5.10 Activación de los Sistema Antioxidantes

Las especies reactivas de oxígeno (ROS) se producen constantemente en las plantas debido al metabolismo celular normal en cloroplastos, mitocondrias, peroxisomas otras

partes de la célula como resultado de los procesos metabólicos vitales como la fotosíntesis (Martínez et al., 2001; Mittler, 2002; Uchida et al., 2002), incluyen radicales libres, moléculas altamente reactivas e inestables con electrones desapareados, como el oxígeno singlete y superóxido, radicales hidroxilo y perhidroxilo, así como oxígeno molecular, ozono o peróxido de hidrógeno, entre otros (Appel & Hirt, 2004). Todos los organismos tienen mecanismos de defensa antioxidante, los cuales incluyen componentes enzimáticos y no enzimáticos, para minimizar los efectos de las ROS. Bajo condiciones de estrés la producción de ROS aumenta pudiendo llegar a acumularse en exceso, lo que resulta en estrés oxidativo por oxidación de los residuos de aminoácidos en las proteínas, los ácidos grasos insaturados de las membranas celulares y las bases nitrogenadas en el ADN.

Se pueden utilizar diversos marcadores bioquímicos para la evaluación del nivel de estrés oxidativo; entre ellos, el malondialdehído (MDA), producto final de la peroxidación de ácidos grasos poliinsaturados, el cual es ampliamente utilizado por ser considerado un excelente marcador de estrés oxidativo (Del Rio et al., 2005). Otro enfoque, se basa en la cuantificación directa de ROS específicos, como el peróxido de hidrógeno, un compuesto estable y no radical producido principalmente en peroxisomas de cloroplastos (Cerny et al., 2018; Barsukova et al., 2019).

Por otra parte, la acumulación de compuestos antioxidantes no enzimáticos y la activación de enzimas antioxidantes, es una vía esencial para la tolerancia al estrés abiótico (Blokhina et al., 2003; Parida & Das, 2005; Parvaiz & Satyawati, 2008). Estudiándose los compuestos fenólicos, y especialmente el subgrupo de flavonoides, metabolitos secundarios importantes debido a su actividad antioxidante (Farah & Donangelo, 2006) y las enzimas antioxidantes como catalasa (CAT), superóxido dismutasa (SOD), ascorbato peroxidasa (APX) y glutatión reductasa (GR). La actividad específica de SOD se potencia mediante la síntesis de la enzima en presencia de su sustrato superóxido, que activa la transcripción de los genes correspondientes (Caverzan et al., 2016). El H_2O_2, aunque no es tan reactivo como los radicales libres, sigue siendo tóxico y varias enzimas contribuyen a su eliminación, siendo las más relevantes, la CAT, que descompone el H_2O_2 en O_2 y H_2O y es inducido por la acumulación de su sustrato (Gunes et al., 2007) y APX, que cataliza la reducción del peróxido de hidrógeno acoplado a la oxidación del ascorbato. El GR, empleando NADPH como cofactor específico, cataliza la reducción del glutatión oxidado (GSSG) a su forma reducida (GSH), contribuyendo así a mantener el estado redox celular adecuado (Hameed et al., 2015).

5.10.1 Marcadores de Estrés Oxidativo

Los niveles de MDA aumentaron tras el tratamiento de estrés hídrico en las cuatro especies de *Limonium,* pero de forma significativa solo en *L. narbonense*. Además, los niveles de H_2O_2 foliar y la actividad de eliminación total de radicales libres no mostraron variación marcada por lo que esto indica que el tratamiento de estrés hídrico no condujo a un grado detectable de estrés oxidativo, lo que probablemente fue resultado de la

activación de sistemas antioxidantes eficientes, que analizaremos en los siguientes apartados.

Las mediciones de estos parámetros en estas mismas cuatro especies en sus hábitats naturales no revelaron diferencias significativas, a pesar de la diferencia de aridez del hábitat de *L. santapolense* en comparación con el resto de las especies.

El contenido de MDA no varió bajo ninguno de los estreses aplicados en *L. albuferae* y mostró un incremento significativo, aunque pequeño, en *L. dufourii* bajo condiciones de estrés hídrico, mientras que el peróxido disminuyó con respecto a los controles correspondientes. Diversos estudios han demostrado que las halófitas, en general, no generan un exceso de ROS porque están adaptadas a los ambientes estresantes que habitan y poseen mecanismos que evitan o reducen sustancialmente el estrés oxidativo (Bosé et al., 2014; Gil et al., 2014), lo que parece ser el caso de las seis especies de *Limonium* estudiadas.

En *T. maritimum*, el contenido de MDA no mostró ningún cambio significativo en respuesta al déficit hídrico y a la alta salinidad, indicando que en estas condiciones no se generaron altos niveles de estrés oxidativo debido al mantenimiento del equilibrio redox por la actividad específica de ciertos compuestos antioxidantes.

En el caso de *B. tenuissimum*, los niveles de MDA aumentaron en el tratamiento de estrés hídrico, indicando una mayor susceptibilidad de esta especie a este tipo de estrés que a la salinidad. Por el contrario, en *B. fruticosum*, los niveles de este marcador aumentaron gradualmente y en paralelo al incremento de la salinidad externa aplicada. El nivel de estrés oxidativo también se evaluó analizando la concentración de peróxido de hidrógeno, en este caso, los niveles no variaron en ninguno de los tratamientos de la primera especie, pero aumentaron en la segunda para todos los tipos de estrés aplicados, aunque curiosamente mostró una disminución en la salinidad más alta.

En conclusión, la acumulación de ROS inducida por el estrés ha sido menos pronunciada en las especies tolerantes al estrés que en las sensibles. Por lo tanto, cuando se comparan las respuestas a la salinidad, u otros estreses, de diferentes taxones relacionados, es de esperar que se midan mayores contenidos de MDA y H_2O_2, en las mismas condiciones experimentales, en aquellas más susceptibles al estrés. En los casos en los que no se han observado diferencias significativas en los niveles de estos marcadores bioquímicos, en los géneros *Limonium* y *Thalictrum*, se justifica debido a la eficiencia en el balance redox de los sistemas antioxidantes, ya sean enzimáticos o no enzimáticos, que analizaremos en los siguientes apartados.

5.10.2 Mecanismos Antioxidantes No Enzimáticos

En las cuatro especies de *Limonium* estudiadas bajo condiciones de estrés hídrico los mecanismos antioxidantes no enzimáticos no se activaron, la activación de enzimas antioxidantes, como se detalla en el siguiente apartado, fue suficiente para contrarrestar el estrés oxidativo.

Los compuestos fenólicos totales mostraron un patrón similar de variación en respuesta al estrés salino aplicado en las dos especies de *Limonium*, con pequeños cambios, por lo general no significativos, mientras que el contenido de flavonoides aumentó de forma significativa en *L. albuferae* a concentraciones de 400 mM o superiores, contribuyendo por lo tanto al alivio del estrés oxidativo bajo condiciones de alta salinidad.

En *Th. maritimum*, los niveles de fenoles y flavonoides tienden a decrecer bajo situaciones de estrés, por lo que en esta especie no se están activando esta clase de mecanismos como defensa frente al estrés oxidativo.

Los niveles de fenoles y flavonoides en *B. tenuissimum* siguieron un patrón similar, encontrándose concentraciones mayores de los mismos en el tratamiento hídrico, el más estresante para esta especie. En *B. fruticosum* los niveles de estos compuestos fueron más altos a elevadas concentraciones de NaCl. En este caso, es muy probable que las especies más tolerantes a la salinidad no activen los sistemas antioxidantes ya que no permiten la producción excesiva de ROS al poseer un mecanismo eficiente para prevenir la acumulación de Na^+ en el citosol (Bose et al., 2014).

Los compuestos fenólicos (incluidos los flavonoides), como otros antioxidantes no enzimáticos, se consideran una línea secundaria de defensa contra el estrés oxidativo, que se activa solo en condiciones de estrés severo, mientras que las enzimas antioxidantes, que analizamos en el siguiente apartado, constituyen el primer sistema de eliminación de ROS (Fini et al., 2011).

5.10.3 Mecanismos Antioxidantes Enzimáticos

Las enzimas antioxidantes se han considerado como los componentes esenciales del mecanismo de defensa adaptativo contra el estrés oxidativo en halófitos (Jithesh et al., 2006).

Las actividades específicas de las cuatro enzimas estudiadas (SOD, CAT, APX y GR) mostraron patrones diferentes en las cuatro especies de *Limonium* tanto para los valores en los controles como para los valores en la respuesta al déficit hídrico. *L. santapolense* mostró la respuesta más fuerte ya que la actividad de las cuatro enzimas estudiadas aumentó en las plantas estresadas frente a los controles.

En el ensayo de *L. albuferae* y *L. dufourii* las tres enzimas analizadas mostraron diferentes patrones en respuesta a los diferentes tipos de estrés aplicados. En *L. albuferae*, en comparación con los niveles medidos en el control, la actividad de las tres enzimas aumentó al estar sometidos a salinidades muy altas (600-800 mM de NaCl). En *L. dufourii* la enzima SOD aumentó en la salinidad más alta y en el tratamiento de sequía, GR tan solo en el tratamiento con la mayor concentración de sal aplicada y los niveles de CAT no variaron. Por lo que hubo mayor activación de CAT y SOD bajos condiciones salinas en *L. albuferae* y mayo activación de SOD y GR bajo condiciones de estrés hídrico en *L. dufourii*. La ctivación de los mecanismos antioxidantes enzimáticos frente a estrés hídrico es más eficiente en *L. dufourii*, lo que puede contribuir a una mayor tolerancia a estas

condiciones, mientras que en *L. albuferae*, la actividad enzimática aumenta en altas salinidades, contribuyendo a la resistencia a la salinidad en esta especie.

En *Thalictrum* la actividad de SOD aumentó en el tratamiento de déficit hídrico y la de CAT y GR en los tratamientos salinos. No fue necesaria un aporte adicional de compuestos antioxidantes y por ello, no se observó un aumento de compuestos antioxidantes no-enzimáticos.

La actividad de la SOD aumentó en condiciones de estrés salino, en las concentraciones más bajas en *B. tenuissimum* y a partir de 150 mM en *B. fruticosum*, manteniéndose constante en plantas sometidas a la ausencia de irrigación. La CAT se activó solo en la especie *B. tenuissimum*, en el tratamiento de menor salinidad, teniendo el pico máximo de actividad bajo concentraciones de sal de 150 mM. La actividad de la GR también mostró un pico máximo en el tratamiento de 150 mM en ambas especies. El tratamiento de déficit hídrico no provocó la activación de ninguna de los sistemas antioxidantes enzimáticos.

En conclusión, de acuerdo con los datos anteriores, los niveles bajos de estrés oxidativo observados mediante la cuantificación de MDA y H_2O_2, observados en *Limonium* y *Thalictrum* bajo condiciones de estrés natural o artificial, estarían mantenidos principalmente por la activación de sistemas antioxidantes enzimáticos.

Capítulo 6:
Conclusiones

Las conclusiones de este trabajo con un enfoque multidisciplinar son relvantes tanto a nivel de reintroducción y conservación de las especies estudiadas como a nivel bioquímico:

1) Las cuatro especies de *Limonium (L. santapolense, L. girardianum, L. narbonense* y *L. virgatum)* mostraron una buena tolerancia al déficit hídrico basadas en los mecanismos de defensa constitutivos, como el transporte activo de iones y la actividad de las enzimas antioxidantes. Las respuestas inducidas por el estrés hídrico contribuyeron a la tolerancia a la sequía, destacando la acumulación de osmolitos y la activación de sistemas antioxidantes enzimáticos.

2) El comportamiento de *Limonium santapolense* bajo estrés hídrico en condiciones controladas fue diferente al de las otras tres especies. Aunque esta especie se encuentra en un hábitat natural más árido, fue la más afectada por la ausencia de riego. Esto se debe a que su mecanismo de defensa fundamental observado en el campo está basado en la expansión de las raíces para alcanzar capas de suelo más profundas y húmedas, lo que no pudo desarrollarse en los experimentos en maceta. La morfología de las raíces es un rasgo que debería considerarse con más frecuencia en los estudios sobre las respuestas de las plantas a la sequía y la salinidad, ya que el crecimiento de las raíces es un mecanismo funcional esencial en la adaptación de las plantas a sus entornos naturales.

3) El análisis de las cuatro especies de *Limonium* en sus hábitats naturales sugirió que sus mecanismos de tolerancia al estrés se basan principalmente en el transporte iónico junto con la síntesis y acumulación de solutos compatibles.

4) El estudio de germinación, los parámetros de crecimiento, el contenido de pigmentos fotosintéticos y la relación Na^+/K^+ indicó que *L. albuferae* es más tolerante a altas salinidades que *L. dufourii*. Mientras que *L. dufourii* es más tolerante a la sequía.

5) La fructosa y la glucosa, osmolitos principales en este género, se acumularon solo en *L. albuferae*, que también mostró niveles estables de ácidos cítrico y málico. La prolina y el ácido γ-aminobutírico (GABA), ambos con funciones osmorreguladoras, aumentaron en *L. dufourii* y en *L. albuferae*.

6) La prolina se sintetizó en *L. albuferae* y *L. dufourii*, pero la actividad de las enzimas antioxidantes tuvo el papel más importante en la mitigación del estrés oxidativo en ambas especies, tanto en condiciones de estrés hídrico como salino.

7) La salinidad no sería un factor limitante para la reintroducción de las dos especies de *Limonium* de interés en el Parque Natural de l'Albufera, ya que las dos especies toleran salinidades mucho más altas en condiciones controladas que las de sus hábitats naturales. Sin embargo, la escasez de agua podría ser un problema para *L. albuferae*, mientras que *L. dufourii* no debería introducirse en zonas propensas a inundaciones prolongadas.

8) El estudio de campo reveló la presencia de especies invasoras y por lo tanto la competencia, mayoritariamente con *Spartina patens*, en los hábitats de *L. albuferae* y *L. dufourii*, la cual puede ser una gran amenaza.

9) *Thalictrum maritimum* se comportó como una especie halofítica moderada, con un crecimiento óptimo en ausencia de salinidad pero que tolera concentraciones de al menos 300 mM de cloruro de sodio, mucho más altas que las de sus hábitats naturales. Sin embargo, mostró ser sensible al déficit hídrico, dato que corroboran tanto los ensayos realizados como el análisis de los cambios en sus poblaciones en relación con los datos climáticos.

10) El mecanismo principal de *Thalictrum maritimum* de tolerancia a la salinidad está relacionado con el transporte activo de iones a la parte aérea y el mantenimiento de K^+ ante el aumento de Na^+ en las hojas y la activación de sistemas antioxidantes enzimáticos.

11) *Bupleurum tenuissimum*, halófilo moderado, mostró ser más sensible al estrés hídrico mientras que *B. fruticosum* fue más susceptible a la salinidad incluso en concentraciones bajas.

12) A diferencia del resto de especies, en *Bupleurum* el principal mecanismo de resistencia a estrés salino es la prevención de la acumulación foliar de iones tóxicos.

13) La acumulación de prolina tiene un papel importante en ambas especies de *Bupleurum* como respuesta al estrés. Fue el único género estudiado en el cual fue evidente el estrés oxidativo, cuantificado por los niveles de MDA, y por lo tanto podríamos afirmar que el menos tolerante a las condiciones estudiadas. Para conseguir el equilibrio celular activó sus sistemas antioxidantes enzimáticos y no enzimáticos.

14) El crecimiento de las plantas en condiciones de estrés depende de la eficacia de los mecanismos de tolerancia de cada especie en particular. La estrategia de realizar estudios comparativos en taxones genéticamente relacionados con diferentes grados de tolerancia demostró ser un enfoque valioso, especialmente en lo que respecta a distinguir entre las respuestas relevantes para la tolerancia y las que no lo son.

15) Estos datos ayudaran en el diseño e implementación de programas de conservación, refuerzo o reintroducción, y para el manejo general de las poblaciones amenazadas de estas especies raras y endémicas.

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Apéndice

Effects of Drought and Salinity on European Larch (*Larix decidua* Mill.) Seedlings

Autores: Plesa, I.O.; González-Orenga, S.; Al Hassan, M.; Sestras, A.F.; Vicente, O.; Prohens, J.; Sestras, R.E.; Boscaiu, M.

Publicado en la revista Forests (2018), 9: 320.

Resumen: *Larix decidua*, the European larch, is not normally affected by drought or salinity in its natural habitats, but it may be when grown as an ornamental tree, by the widespread practice of winter de-icing of mountain roads with NaCl, and because of global warming-induced environmental changes. The responses of two-month-old larch seedlings to 30 days water deficit (withholding irrigation) or salt stress (150 mM NaCl) treatments were studied by determining stress-induced changes in several growth parameters and biochemical markers (ion and osmolyte contents, level of oxidative stress, activation of enzymatic and non-enzymatic antioxidant systems). Both treatments caused the inhibition of growth, degradation of photosynthetic pigments, a small increase in malondialdehyde (MDA, an oxidative stress biomarker), and the activation of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). In all cases, salinity appeared to have stronger effects on the seedlings than water deficit. The presence of relatively high concentrations of glycine betaine, both in control and stressed plants, may represent a constitutive mechanism of defence against stress in European larch. Additionally, other responses were specific for salt stress and included the activation of K^+ transport from roots to shoots and the accumulation of Pro as an osmoprotectant.

Responses to Drought in Seedlings of European Larch (*Larix decidua* Mill.) from Several Carpathian Provenances

Autores: Plesa, I.O.; Al Hassan, M.; González-Orenga, S.; Sestras, A.F.; Vicente, O.; Prohens, J.; Boscaiu, M.; Sestras, R.E.

Publicado en la revista Forests (2019), 10: 511.

Resumen: European larch (*Larix decidua* Mill.) has been reported either as more tolerant or as more sensitive to drought than conifers with perennial leaves. Previous studies have revealed that Carpathian populations of European larch display a high genetic variability. A comparative study of the responses of these populations to drought stress at the seedling stage might allow the identification of drought tolerant genotypes and reliable drought stress biomarkers, which could be eventually used for the early detection of drought effects in larch, not only under control greenhouse conditions, but also in their natural stands. Growth responses were analyzed in larch seedlings from six Romanian Carpathian populations, submitted to one month of mild drought stress under controlled conditions. Levels of photosynthetic pigments (chlorophylls a and b, and carotenoids), osmolytes (proline and total soluble sugars), monovalent cations (Na^+ and K^+), and malondialdehyde (MDA) and non-enzymatic antioxidants (total phenolics and flavonoids) were compared with control treatments and between populations. Growth and the pattern of the biochemical responses were very similar in the six populations. Drought stress lead to stem length decrease in all population, whereas reduction of fresh weight of needles was significant only in one population (BVVC), and reduction of water content of needles in two populations (BVVC and GuHo). The optimal biochemical traits for an early detection of drought symptoms in this species is the increase—in most populations—of total soluble sugars, MDA, and total phenolic compounds, whereas K^+ reduction was significant in all populations. Photosynthetic pigments remained unchanged, except for the Anin population where they were reduced under stress. Multivariate principal component and hierarchical clustering analyses confirmed the impact of drought in the growth and physiology of European larch, and revealed that the humidity of the substrate was positively correlated with the growth parameters and the levels of K^+ in needles, and negatively correlated with the levels of MDA, total soluble sugars, total phenolic compounds, and flavonoids in needles.

Comparative Analysis of the Responses to Water Stress in Eggplant (*Solanummelongena*) Cultivars

Autores: Plazas, M.; Nguyen, H.T.; González-Orenga, S.; Fita, A.; Vicente, O.; Prohens, J.; Boscaiu, M.

Publicado en la revista Plant Physiology and Biochemistry (2019), 143: 78-82.

Resumen: Little information is available on the physiological and biochemical responses to water stress in eggplant (*Solanum melongena*). We evaluated four genetically diverse eggplant varieties (MEL3-MEL6) under control and water stress conditions. Measurements were taken for plant growth, tissue water content, levels of chlorophylls *a* and *b*, carotenoids, proline, malondialdehyde, total phenolics, total flavonoids, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities. For most traits, the water stress treatment had a greater contribution than the variety effect to the total sums of squares in an ANOVA analysis, except for total flavonoids, SOD, APX, and GR. The water stress treatment had a strong effect on plant growth and tissue water content. In general, water stress reduced the three photosynthetic pigments, increased proline, malondialdehyde, total phenolics, and total flavonoids, although some varietal differences were observed.

Different patterns were also detected in the activities of the four enzymes evaluated, but few differences were observed for individual varieties between the control and water stress treatments. Many significant phenotypic correlations were observed among the traits studied, but only eight environmental correlations were detected. A PCA analysis distinctly separated individuals according to the treatment, and revealed a clearer separation of varieties under water stress than under control conditions, pointing to varietal differences in the responses to stress. Our results suggest that proline could be used as a marker for drought stress tolerance in this species. The information obtained provides new insight on the physiological and biochemical responses of eggplant to drought stress.

Comparative Studies on the Physiological and Biochemical Responses to Salt Stress of Eggplant (*Solanum melongena*) and Its Rootstock *S. torvum*

Autores: Breves, M.; Pérez, J.; González-Orenga, S.; Solana, A.; Boscaiu, M.; Prohens J.; Fita, A.; Vicente, O.

Publicado en la revista Agriculture (2020), 10: 328.

Resumen: This study investigated the physiological and biochemical responses to salinity stress of *Solanum melongena* and its wild relative, *Solanum torvum*, commonly used as eggplant rootstock. Young plants of both species were watered during 25 days with NaCl aqueous solutions at the following four final concentrations: 0 (for the controls), 100, 200, and 300 mM. Plant growth parameters, photosynthetic pigments content, monovalent ion concentrations in roots and leaves, leaf levels of osmolytes (proline and total soluble sugars), oxidative stress markers (MDA and H_2O_2), non-enzymatic antioxidants (total phenolic compounds and total flavonoids), and enzymatic antioxidant activities (superoxide dismutase, catalase, glutathione reductase) were determined after the stress treatments. Salt-induced growth reduction was more significant in *S. melongena* than in *S. torvum*, especially at high salt concentrations, indicating a (slightly) higher salt tolerance of the wild species. The mechanisms of tolerance of *S. torvum* were partly based on the active transport of toxic ions to the leaves at high external salinity and, presumably, a better capacity to store them in the vacuoles, as well as on the accumulation of proline to higher concentrations than in the cultivated eggplant. MDA and H2O2 contents did not vary in response to the salt treatments in *S. torvum*. However, in *S. melongena*, MDA content increased by 78% when 300 mM NaCl was applied. No activation of antioxidant mechanisms, accumulation of antioxidant compounds, or increase in the specific activity of antioxidant enzymes in any of the studied species was induced by salinity. The relatively high salt tolerance of *S. torvum* supports its use as rootstock for eggplant cultivation in salinized soils and as a possible source of salt-tolerance genes for the genetic improvement of cultivated eggplant.

Screening for Salt and Water Stress Tolerance in Fir (*Abies alba*) Populations

Autores: Todea, I.M.; González-Orenga, S.; Plazas, M.; Sestras, A.F.; Prohens, J.; Vicente, O.; Sestras, R.E.; Boscaiu, M.

Publicado en la revista Notulae Botanicae Horti Agrobotanici Cluj-Napoca (2019), 47 (4): 1063-1072.

Resumen: Drought periods are becoming more frequent and intense, due to the effects of climate change, threatening natural hábitats worldwide, including European forests. Forest trees can also be affected by high soil salinity, because of the common practice of de-icing of mountain roads with NaCl in winter. We have evaluated the responses to salt and water stress of silver fir (*Abies alba*), an important forest species for which very limited information is available. One year-old fir seedlings, with origin in seven different locations in Romania, were subjected to salt (watering with NaCl solutions of increasing concentrations) and water deficit (complete withholding of irrigation) treatments in the greenhouse. After one month, plant material was harvested and different morphological parameters were determined in the stressed and control plants. Both stress treatments inhibited growth of fir seedlings from all seven provenances, although quantitative differences in the responses to stress were observed between populations. Growth inhibition was established by the relative reduction – as compared to the non-stressed controls - in several parameters, such as stem elongation, root length, number of needles, or fresh weight and water content of roots and needles. Statistical multivariate analysis of the results suggested that seedlings from Valea Morii (population 6) were the most tolerant to both, water deficit and high (300 mM NaCl) salt concentrations. These results support the possibility to screen a large number of individuals from different populations, at the seedling stage, to select *Abies alba* genotypes with enhanced drought and/or salinity tolerance.

Responses to Water Deficit and Salt Stress in Silver Fir (*Abies alba* Mill.) Seedlings

Autores: Todea, I.M.; González-Orenga, S.; Boscaiu, M.; Plazas, M.; Sestras, A.F.; Prohens, J.; Vicente, O.; Sestras, R.E.

Publicado en la revista Forests (2020), 11 (4): 395.

Resumen: Forest ecosystems are frequently exposed to abiotic stress, which adversely affects their growth, resistance and survival. For silver fir (*Abies alba*), the physiological and biochemical responses to water and salt stress have not been extensively studied. Responses of one-year-old seedlings to a 30-day water stress (withholding irrigation) or salt stress (100, 200 and 300 mM NaCl) treatments were analysed by determining stress-induced changes in growth parameters and different biochemical markers: accumulation of ions, different osmolytes and malondialdehyde (MDA, an oxidative stress biomarker), in the seedlings, and activation of enzymatic and non-enzymatic antioxidant systems. Both salt and water stress caused growth inhibition. The results obtained indicated that the most relevant responses to drought are based on the accumulation of soluble carbohydrates as osmolytes/osmoprotectants. Responses to high salinity, on the other hand, include the active transport of Na^+, Cl^- and Ca^{2+} to the needles, the maintenance of relatively high K^+/Na^+ ratios and the accumulation of proline and soluble sugars for osmotic balance. Interestingly, relatively high Na^+ concentrations were measured in the needles of *A. alba* seedlings at low external salinity, suggesting that Na^+ can contribute to osmotic adjustment as a 'cheap' osmoticum, and its accumulation may represent a constitutive mechanism of defence against stress. These responses appear to be efficient enough to avoid the generation of high levels of oxidative stress, in agreement with the small increase in MDA contents and the relatively weak activation of the tested antioxidant systems.

Effects of Drought and Salinity on Two Commercial Varieties of *Lavandula angustifolia* Mill.

Autores: Szekely-Vargas, Z.; González-Orenga, S.; Cantro, M.; Jucan, D.; Boscaiu, M.; Vicente, O.

Publicado en la revista Plants (2020), 9: 637.

Resumen: Global warming is not only affecting arid and semi-arid regions but also becoming a threat to agriculture in Central and Eastern European countries. The present study analyzes the responses to drought and salinity of two varieties of Lavandula angustifolia cultivated in Romania. Lavender seedlings were subjected to one month of salt stress (100, 200, and 300 mM NaCl) and water deficit (complete withholding of irrigation) treatments. To assess the effects of stress on the plants, several growth parameters and biochemical stress markers (photosynthetic pigments, mono and divalent ions, and different osmolytes) were determined in control and stressed plants after the treatments. Both stress conditions significantly inhibited the growth of the two varieties, but all plants survived the treatments, indicating a relative stress tolerance of the two varieties. The most relevant mechanisms of salt tolerance are based on the maintenance of foliar K^+ levels and the accumulation of Ca^{2+} and proline as a functional osmolyte in parallel with increasing external salinities. Under water stress, significant increases of Na^+ and K^+ concentrations were detected in roots, indicating a possible role of these cations in osmotic adjustment, limiting root dehydration. No significant differences were found when comparing the stress tolerance and stress responses of the two selected lavender varieties.

Antioxidant Responses to Drought and Salinity in *Lavandula angustifolia* Mill.

Autores: Szekely-Vargas, Z.; González-Orenga, S.; Cantor, M.; Boscaiu, M.; Vicente, O.

Publicado en la revista Notulae Botanicae Horti Agrobotanici Cluj-Napoca (2020), 48 (4): 1980-1992.

Resumen: Drought and salinity are amongst the most damaging environmental stressors that can affect a plant's life cycle, from germination to senescence. In the present study were analysed the responses to salinity and drought in greenhouse-controlled conditions of two varieties of *Lavandula angustifolia*. Three-month-old lavender seedlings were subjected to water deficit and salt stress (100, 200 and 300 mM NaCl) during a 30-day period. Complementing a previous analysis focused on stress tolerance mechanisms based on the regulation of ion transport and the synthesis of osmolytes, we have now evaluated the effects of the water deficit and salt treatments on the generation of secondary oxidative stress, by measuring malondialdehyde levels, and the activation of antioxidant systems, both non-enzymatic and enzymatic, determining total phenolic compounds and flavonoids contents and calculating superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase specific activities, respectively, in extracts of control and stressed plants. The results obtained confirm that both lavender varieties react in the same way to the applied stress treatments, activating the same antioxidant responses. However, some differences were observed when comparing the specific mechanisms triggered by each type of stress. Thus, the oxidative stress induced under drought conditions was counteracted by accumulation of phenolic compounds and flavonoids, without apparent involvement of antioxidant enzymes. Salt stress, on the other hand, in addition to an increase in flavonoid levels also induced superoxide dismutase and catalase activities. These antioxidant responses are likely to contribute to the relatively high tolerance (as compared to most crops) of lavender to drought and salinity.