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Comparative Analyses of Plant Responses to Drought and Salt Stress in Related Taxa: A Useful Approach to Study Stress Tolerance Mechanisms

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Alas, my 3 years journey has come to end, and I find myself in front of the challenging task to summarize my gratitude to those who made this experience end happily.

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Abstract

Introduction

Salinity and drought are the most important environmental stress conditions reducing crop yields worldwide and limiting the distribution of wild plants in nature. Soil salinity, especially secondary salinity caused by anthropogenic practices, such as prolonged irrigation, lead to substantial agricultural yield losses, especially in arid and semiarid regions. Drought, caused by reduced water content in the soil, occurs due to disorders in nature´s water cycle, chiefly when evapotranspiration exceeds precipitation in a certain area, to the point where soil water reserves can no longer support plant growth.

Drought and salt stress trigger the activation of a series of basic stress mechanisms that includes among others, the control of ion transport, exclusion and compartmentalization, as well as the accumulation of compatible solutes ('osmolytes'), and the activation of antioxidant systems. These mechanisms are conserved in all plants, stress tolerant and sensitive alike, and don't necessarily confer tolerance.

To decipher those mechanisms and have a better understanding on the contribution of different stress responses to the stress tolerance of a given species, we have carried out comparative studies on the responses to drought and salinity in a number of genetically related taxa with different tolerance potentials.

Methodology

The work concentrated on studying the responses to salt and drought stress in genetically related plants with different tolerance to abiotic stress. The studied taxa included salt-tolerant (halophytes) and salt-sensitive (glycophytes) wild species of two

different genera: *Juncus* (monocotyledonous) and *Plantago* (dicotyledonous), as well as plants of crop species: *Solanum lycopersicum* var. cerasiforme (cherry tomatoes) and different *Phaseolus* cultivars, one of *P. coccineus* and three of *P. vulgaris*. .

The experimental approach was mostly based on *i)* establishing the relative tolerance to water and salt stress in the studied species from their distribution in nature (in the case of wild species) and through the relative inhibition of growth in the presence of stress, and *ii)* correlating changes in the levels of biochemical 'stress markers' associated to specific response pathways (ion transport, osmolyte accumulation…) upon stress treatments, with the already established relative tolerance to stress. This strategy proved to be appropriate to distinguish mere *general responses to stress* from those mechanisms relevant for *stress tolerance* of the investigated species and cultivars.

The work also sheds light on other aspects affected by salt stress, specifically regarding germination and reproductive success or anatomical changes in salt-stressed plants. The expression patterns of the gene *NHX1*, encoding a vacuolar Na^+/H^+ antiporter were also studied in the *Plantago* taxa, as a first step in the full characterisation of this ion transporter, that appears to play an important role in the mechanisms of salt tolerance in this genus.

Results and discussion

Through the results attained from this work, we have been able to establish which general stress responses are relevant for tolerance in the investigated species or cultivars, and which are not. Thus, we provide clear evidence that, although all plants seem to activate the same mechanisms of defense in response to abiotic stress, their relative contribution to stress tolerance differs widely in different genera and species.

Moreover, in general, the relative tolerance of the investigated species and cultivars were the same, when referring to salt stress and to water stress, and the same mechanisms – except some related to ion transport and homeostasis – were relevant for tolerance to both stresses.

In the studied *Phaseolus* cultivars, *P. vulgaris* cv. 'Maxidor' showed the smallest growth inhibition under salt and water stress conditions, and therefore was defined as the most tolerant. 'Maxidor' accumulated lower levels of toxic ions and proline, and recorded higher levels of *myo*-inositol than the other cultivars. We concluded that blocking ion transport from the roots to the leaves and *myo*-inositol accumulation, were the mechanisms most relevant for stress tolerance in *Phaseolus*. Proline is a reliable stress biomarker in this genus, indicating the degree of stress affecting the plants, but is not directly involved in tolerance mechanisms.

In the studied *Plantago* species it was found that the more tolerant taxa transported Na^+ and Cl^- to the leaves more efficiently than the most sensitive *P. major*, and tended to accumulate large amounts of proline, albeit only under extreme stress conditions; these responses appear to be the most relevant for tolerance in *Plantago*. I Toxic ions transported to the leaves are presumably accumulated in vacuoles, which gave incentive to isolating, sequencing and studying the expression of the Na^+/H^+ vacuolar antiporter gene *NHX1* in the studied species. Upon short-term treatments with high NaCl concentrations, the more tolerant species showed higher salt-induced expression of the aforementioned gene, supporting the contribution of the *NHX1* antiporter to salt tolerance in *Plantago*.

Meanwhile, the tolerant *Juncus* species were able to partly inhibit ion transport from the roots to the plants aerial parts and recorded a much larger increment (about 60-fold over the controls) in proline contents, as compared to the stress-sensitive congener.

Therefore, blocking accumulation of toxic ions and inducing accumulation of proline in the culms appear to be the most important mechanisms of tolerance in *Juncus*. On the other hand, we did not detect significant stress-induced anatomical differences when comparing salt tolerant and sensitive *Juncus* taxa.

Conclusion

The results obtained in this work contribute to a better understanding of general stress tolerance mechanisms in plants, and provides clear insights into the mechanisms conferring tolerance, specifically, to drought and salt stress in some wild species and crops. This work also shed more light on the highly efficient responses to stress in halophytes, plants that could be viewed as nature´s answer to the aforementioned adverse environmental conditions via evolution and adaptation. Halophytes can therefore be considered as a suitable source – underutilized at present, in our opinion – of knowledge, genetic resources and biotechnological tools for the needed improvement of stress tolerance in crops.

This work has yielded eight 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) Al Hassan, M., Pacurar, A., Gaspar, A., Vicente, O., Boscaiu, M. (2014). Growth and reproductive success under saline conditions of three *Plantago* species with different levels of stress tolerance. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 42(1): 180-186.

2) Al Hassan, M., Fuertes, M., Sánchez, F., Vicente, O., Boscaiu, M. (2015). Effects of Salt and Water Stress on Plant Growth and on Accumulation of Osmolytes and Antioxidant Compounds in Cherry Tomato. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(1): 1-11.
3) Al Hassan, M., Gohari, G., Boscaiu, M., Vicente, O., Grigore, M. (2015). Anatomical modifications under salt stress in two ecologically distinct *Juncus* species. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(2): 501-506.
4) Al Hassan, M., Morosan, M., Lopez-Gresa, M.P., Boscaiu, M., Vicente, O. (2015). Selection and characterisation of salt and drought-resistant *Phaseolus* cultivars: a 'proof-of-concept' study. (Under revision).
5) Al Hassan, M., Pacurar, A., Lopez-Gresa, M.P., Llinares, J., Boscaiu, M., Vicente, O. (2015). Effects of Salt and Water Stress on Three Ecologically Distinct *Plantago* Species. (Under revision).
6) Al Hassan, M., Lopez-Gresa, M.P., Boscaiu, M., Vicente, O. (2015). Stress tolerance mechanisms in *Juncus*: Responses to salinity and drought in three *Juncus* species adapted to different natural environments. (Under revision).
7) Al Hassan, M., Cortes, J., Gaspar, A., Boscaiu, M., Vicente, O. (2015). Differential anti-oxidative responses under salinity and drought challenges in two halophytes and one glycophyte of the genus *Juncus*. (In preparation).
8) Al Hassan, M., Daniso, E., Martinelli, F., Boscaiu, M., Vicente, O. (2015). Expression of the vacuolar Na^+/H^+ antiporter gene (*NHX1*) in three *Plantago* species differing in salt tolerance (In preparation).

Other manuscripts, published or submitted 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.

Resumen

Introducción

La salinidad y la sequía son las condiciones de estrés ambiental más importantes, que reducen los rendimientos de los cultivos en todo el mundo y que limitan la distribución de las plantas silvestres en la naturaleza. La salinidad del suelo, especialmente la salinización secundaria causada por prácticas antropogénicas, como la irrigación prolongada, conducen a pérdidas importantes de rendimiento agrícola, especialmente en las regiones áridas y semiáridas. La sequía, provocada por la reducción de contenido de agua en el suelo, se produce debido a alteraciones en el ciclo del agua en la naturaleza, principalmente cuando la evapotranspiración excede la precipitación en un área determinada, hasta el punto que las reservas de agua del suelo ya no pueden soportar el crecimiento de la planta.

La sequía y el estrés salino desencadenan la activación de una serie de mecanismos básicos de respuesta, que incluyen entre otros el control del transporte, la exclusión y la compartimentación de iones, así como la acumulación de solutos compatibles ('osmolitos'), y la activación de sistemas antioxidantes. Estos mecanismos están conservados en todas las plantas, tolerantes y sensibles a estrés por igual, y no confieren necesariamente tolerancia.

Para descifrar estos mecanismos y conseguir una mejor comprensión de la contribución de diferentes respuestas a estrés a la tolerancia al estrés en una especie dada, hemos llevado a cabo estudios comparativos sobre las respuestas a la sequía y la salinidad, en un número de taxones relacionados genéticamente con diferentes potenciales de tolerancia.

Metodología

El trabajo se ha centrado en el estudio de las respuestas a la sal y la sequía en plantas genéticamente relacionadas pero con diferente tolerancia al estrés abiótico. Los taxones estudiados incluyeron plantas tolerante a sal (halófitas) y sensibles a sal (glicófitas) de especies silvestres de dos géneros diferentes: *Juncus* (monocotiledóneas) y *Plantago* (dicotiledóneas), así como plantas de especies de cultivo: *Solanum lycopersicum* var. cerasiforme (tomates cherry) y diferentes cultivares de *Phaseolus*, uno de *P. coccineus* y tres de *P. vulgaris*.

El enfoque experimental se basó principalmente en *i)* establecer la tolerancia relativa al estrés hídrico y al estrés salino en las especies estudiadas, a partir de su distribución en la naturaleza (en el caso de especies silvestres) y atendiendo a la inhibición relativa de su crecimiento en presencia de estrés, y *ii)* correlacionar cambios en los niveles de 'marcadores bioquímicos de estrés' asociados a vías específicas de respuesta (transporte de iones, acumulación de osmolitos ...) inducidos por los tratamientos de estrés, con la tolerancia relativa a estrés de las plantas, previamente establecido. Esta estrategia ha resultado ser apropiada para distinguir meras *respuestas generales a estrés* de los mecanismos relevantes para la *tolerancia a estrés* de las especies y cultivares investigados.

El trabajo también arroja luz sobre otros aspectos afectados por el estrés salino, específicamente en relación con la germinación y el éxito reproductivo, o cambios anatómicos en las plantas tratadas con sal. También se estudiaron los patrones de expresión del gen *NHX1*, que codifica un antiportador vacuolar Na^+/H^+, en las especies de *Plantago*, como un primer paso en la caracterización completa de este transportador de iones, que parece desempeñar un papel importante en los mecanismos de tolerancia a sal en este género.

Resultados y discusión

A partir de los resultados obtenidos en este trabajo, hemos podido establecer qué respuestas generales a estrés son relevantes para la tolerancia en las especies o cultivares investigados, y cuáles no. Hemos podido aportar pruebas claras de que, a pesar de que todas las plantas parecen activar los mismos mecanismos de defensa en respuesta a estrés abiótico, su contribución relativa a la tolerancia a estrés difiere ampliamente en diferentes géneros y especies. Por otra parte, en general, la tolerancia relativa de las especies y cultivares investigados fueron los mismos, por cuando se refiere al estrés salino y al estrés hídrico, y los mismos mecanismos – excepto algunos relacionados con el transporte y la homeostasis de iones – son relevantes para la tolerancia a ambos tipos de estrés.

En los cultivares estudiados de *Phaseolus*, *P. vulgaris cv*. 'Maxidor' mostró la menor inhibición del crecimiento en condiciones de estrés salino e hídrico, y por lo tanto se definió como el más tolerante. 'Maxidor' acumula los niveles más bajos de iones tóxicos y de prolina, y registra los más altos de *myo*-inositol, en comparación con los otros cultivares. Llegamos a la conclusión que el bloqueo de transporte de iones desde las raíces hasta las hojas y la acumulación de *myo*-inositol, son los mecanismos más importantes para la tolerancia a estrés en *Phaseolus*. La prolina es un biomarcador de estrés fiable en este género, que indica el grado de estrés a que están sometidas las plantas, pero no está directamente implicada en los mecanismos de tolerancia.

En las especies de *Plantago* estudiadas se encontró que los taxones más tolerantes transportaban Na^+ y Cl^- a las hojas más eficientemente que el más sensible, *P. major*, y tendían a acumular grandes cantidades de prolina, aunque sólo en condiciones

de estrés extremo; estas respuestas parecen ser las más relevantes para la tolerancia a estrés en *Plantago*. Los iones tóxicos transportados a las hojas se acumulan presumiblemente en las vacuolas, lo que dio incentivo para aislar, secuenciar y estudiar la expresión del gen del antiportador vacuolar Na^+/H^+, *NHX1*, en las especies investigadas. En tratamientos a corto plazo con altas concentraciones de NaCl, las especies más tolerantes mostraron una mayor expresión inducida por sal del gen mencionado anteriormente, en apoyo a la contribución del antiportador NHX1 a tolerancia a sal en *Plantago*.

Mientras tanto, las especies de *Juncus* tolerantes fueron capaces de inhibir parcialmente el transporte de iones de las raíces a la parte aérea de las plantas, y registraron un incremento mucho mayor (aproximadamente 60 veces con respecto a los controles) en el contenido de prolina, en comparación con el congénere sensible a estrés. Por lo tanto, el bloqueo de acumulación de iones tóxicos y la inducción de la acumulación de prolina en las cañas parecen ser los mecanismos más importantes de tolerancia en *Juncus*. Por otro lado, no detectamos diferencias anatómicas importantes inducidas por el estrés al comparar los taxones tolerantes y el sensible a sal de *Juncus*.

Conclusión

Los resultados obtenidos en este trabajo contribuyen a una mejor comprensión de los mecanismos generales de tolerancia al estrés en plantas, y proporcionan ideas claras sobre los mecanismos que confieren tolerancia, en concreto, a la sequía y al estrés salino, en algunas especies silvestres y cultivadas. Este trabajo también arroja más luz sobre las respuestas a estrés altamente eficientes en halófitas, plantas que podrían ser vistas como la respuesta de la naturaleza a las condiciones ambientales adversas antes mencionadas, a través de la evolución y la adaptación. Por lo tanto, las halófitas pueden

ser consideradas como una fuente adecuada – infrautilizada en la actualidad, en nuestra opinión – de conocimiento, recursos genéticos y herramientas biotecnológicas para la necesaria mejora de la tolerancia al estrés en plantas cultivadas.

Este trabajo ha dado lugar a ocho 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) Al Hassan, M., Pacurar, A., Gaspar, A., Vicente, O., Boscaiu, M. (2014). Growth and reproductive success under saline conditions of three *Plantago* species with different levels of stress tolerance. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 42(1): 180-186.
2) Al Hassan, M., Fuertes, M., Sánchez, F., Vicente, O., Boscaiu, M. (2015). Effects of Salt and Water Stress on Plant Growth and on Accumulation of Osmolytes and Antioxidant Compounds in Cherry Tomato. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(1): 1-11.
3) Al Hassan, M., Gohari, G., Boscaiu, M., Vicente, O., Grigore, M. (2015). Anatomical modifications under salt stress in two ecologically distinct *Juncus* species. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(2): 501-506.
4) Al Hassan, M., Morosan, M., Lopez-Gresa, M.P., Boscaiu, M., Vicente, O. (2015). Selection and characterisation of salt and drought-resistant *Phaseolus* cultivars: a 'proof-of-concept' study. (Under revision).
5) Al Hassan, M., Pacurar, A., Lopez-Gresa, M.P., Llinares, J., Boscaiu, M., Vicente, O. (2015). Effects of Salt and Water Stress on Three Ecologically Distinct *Plantago* Species. (Under revision).

6) Al Hassan, M., Lopez-Gresa, M.P., Boscaiu, M., Vicente, O. (2015). Stress tolerance mechanisms in *Juncus*: Responses to salinity and drought in three *Juncus* species adapted to different natural environments. (Under revision).
7) Al Hassan, M., Cortes, J., Gaspar, A., Boscaiu, M., Vicente, O. (2015). Differential anti-oxidative responses under salinity and drought challenges in two halophytes and one glycophyte of the genus *Juncus*. (In preparation).
8) Al Hassan, M., Daniso, E., Martinelli, F., Boscaiu, M., Vicente, O. (2015). Expression of the vacuolar Na^+/H^+ antiporter gene (*NHX1*) in three *Plantago* species differing in salt tolerance (In preparation).

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

Resum

Introducció

La salinitat i la sequera són les condicions d'estrès ambiental més importants, que redueixen els rendiments dels cultius a tot el món i que limiten la distribució de les plantes silvestres en la naturalesa. La salinitat del sòl, especialment la salinització secundària causada per pràctiques antropogèniques, com la irrigació perllongada, condueixen a pèrdues importants de rendiment agrícola, especialment en les regions àrides i semiàrides. La sequera, provocada per la reducció de contingut d'aigua en el sòl, es produeix a causa d'alteracions en el cicle de l'aigua en la naturalesa, principalment quan la evapotranspiració excedeix la precipitació en un àrea determinada, fins al punt que les reserves d'aigua del sòl ja no poden suportar el creixement de la planta.

La sequera i l'estrès salí desencadenen l'activació d'una sèrie de mecanismes bàsics de resposta, que inclouen entre uns altres el control del transport, l'exclusió i la compartimentació d'ions, així com l'acumulació de soluts compatibles ('osmolits'), i l'activació de sistemes antioxidants. Aquests mecanismes estan conservats en totes les plantes, tolerants i sensibles a estrès per igual, i no confereixen necessàriament tolerància.

Per a desxifrar aquests mecanismes i aconseguir una millor comprensió de la contribució de diferents respostes a estrès a la tolerància a l'estrès en una espècie donada, hem dut a terme estudis comparatius sobre les respostes a la sequera i la salinitat, en un nombre de taxons relacionats genèticament amb diferents potencials de tolerància.

Metodologia

El treball s'ha centrat en l'estudi de les respostes a la sal i la sequera en plantes genèticament relacionades però amb diferent tolerància a l'estrès abiòtic. Els taxons estudiats van incloure plantes tolerant a sal (halòfites) i sensibles a sal (glicòfites) d'espècies silvestres de dos gèneres diferents: *Juncus* (monocotiledóneas) i *Plantago* (dicotiledònies), així com plantes d'espècies de cultiu: *Solanum lycopersicum* var. cerasiforme (tomaques xerry) i diferents conreus de *Phaseolus,* un de *P. coccineus* i tres de *P. vulgaris*. .

L'enfocament experimental es va basar principalment en *i)* establir la tolerància relativa a l'estrès hídric i a l'estrès salí en les espècies estudiades, a partir de la seua distribució en la naturalesa (en el cas d'espècies silvestres) i atenent a la inhibició relativa de el seu creixement en presència d'estrès, i *ii)* correlacionar canvis en els nivells de 'marcadors bioquímics d'estrès' associats a vies específiques de resposta (transport d'ions, acumulació d'osmolits ...) induïts pels tractaments d'estrès, amb la tolerància relativa a estrès de les plantes, prèviament establert. Aquesta estratègia ha resultat ser apropiada per a distingir meres *respostes generals a estrès* dels mecanismes rellevants per a la *tolerància a estrès* de les espècies i conreus investigats.

El treball també llança llum sobre altres aspectes afectats per l'estrès salí, específicament en relació amb la germinació i l'èxit reproductiu, o canvis anatòmics en les plantes tractades amb sal. També es van estudiar els patrons d'expressió del gen *NHX1*, que codifica un anti-portador vacuolar Na^+/H^+, en les espècies de *Plantago*, com un primer pas en la caracterització completa d'aquest transportador d'ions, que sembla exercir un paper important en els mecanismes de tolerància a sal en aquest gènere.

Resultats i discussió

A partir dels resultats obtinguts en aquest treball, hem pogut establir què respostes generals a estrès són rellevants per a la tolerància en les espècies o conreessis investigats, i quins no. Hem pogut aportar proves clares que, a pesar que totes les plantes semblen activar els mateixos mecanismes de defensa en resposta a estrès abiòtic, la seua contribució relativa a la tolerància a estrès difereix àmpliament en diferents gèneres i espècies. D'altra banda, en general, la tolerància relativa de les espècies i conreus investigats van ser els mateixos, per quan es refereix a l'estrès salí i a l'estrès hídric, i els mateixos mecanismes – excepte alguns relacionats amb el transport i l'homeòstasi d'ions – són rellevants per a la tolerància a tots dos tipus d'estrès.

En els conreus estudiats de *Phaseolus*, *P. vulgaris cv*. 'Maxidor' va mostrar la menor inhibició del creixement en condicions d'estrès salí i hídric, i per tant es va definir com el més tolerant. 'Maxidor' acumula els nivells més baixos d'ions tòxics i de prolina, i registra els més alts de *myo*-inositol, en comparació dels altres conreus. Arribem a la conclusió que el bloqueig de transport d'ions des de les arrels fins a les fulles i l'acumulació *de myo*-inositol, són els mecanismes més importants per a la tolerància a estrès en *Phaseolus*. La prolina és un biomarcador d'estrès fiable en aquest gènere, que indica el grau d'estrès al fet que estan sotmeses les plantes, però no està directament implicada en els mecanismes de tolerància.

En les espècies de *Plantago* estudiades es va trobar que els taxons més tolerants transportaven Na^+ i Cl^- a les fulles més eficientment que el més sensible, *P. major*, i tendien a acumular grans quantitats de prolina, encara que només en condicions d'estrès extrem; aquestes respostes semblen ser les més rellevants per a la tolerància a estrès en *Plantago*. Els ions tòxics transportats a les fulles s'acumulen presumiblement en els vacúols, la qual cosa va donar incentiu per a aïllar, seqüenciar i estudiar l'expressió del

gen del antiportador vacuolar Na^+/H^+, *NHX1*, en les espècies investigades. En tractaments a curt termini amb altes concentracions de NaCl, les espècies més tolerants van mostrar una major expressió induïda per sal del gen esmentat anteriorment, en suport a la contribució del antiportador NHX1 a tolerància a sal en *Plantago*.

Mentrestant, les espècies de *Juncus* tolerants van ser capaces d'inhibir parcialment el transport d'ions de les arrels a la part aèria de les plantes, i van registrar un increment molt major (aproximadament 60 vegades pel que fa als controls) en el contingut de prolina, en comparació del congènere sensible a estrès. Per tant, el bloqueig d'acumulació d'ions tòxics i la inducció de l'acumulació de prolina en les canyes semblen ser els mecanismes més importants de tolerància en *Juncus*. D'altra banda, no detectem diferències anatòmiques importants induïdes per l'estrès en comparar els taxons tolerants i el sensible a sal *de Juncus*.

Conclusió

Els resultats obtinguts en aquest treball contribueixen a una millor comprensió dels mecanismes generals de tolerància a l'estrès en plantes, i proporcionen idees clares sobre els mecanismes que confereixen tolerància, en concret, a la sequera i a l'estrès salí, en algunes espècies silvestres i conreades. Aquest treball també llança més llum sobre les respostes a estrès altament eficients en halòfites, plantes que podrien ser vistes com la resposta de la naturalesa a les condicions ambientals adverses abans esmentades, a través de l'evolució i l'adaptació. Per tant, les halòfites poden ser considerades com una font adequada – infrautilitzada en l'actualitat, en la nostra opinió – de coneixement, recursos genètics i eines biotecnològiques per a la necessària millora de la tolerància a l'estrès en plantes conreades.

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

1) Al Hassan, M., Pacurar, A., Gaspar, A., Vicente, O., Boscaiu, M. (2014). Growth and reproductive success under saline conditions of three *Plantago* species with different levels of stress tolerance. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 42(1): 180-186.
2) Al Hassan, M., Fuertes, M., Sánchez, F., Vicente, O., Boscaiu, M. (2015). Effects of Salt and Water Stress on Plant Growth and on Accumulation of Osmolytes and Antioxidant Compounds in Cherry Tomato. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(1): 1-11.
3) Al Hassan, M., Gohari, G., Boscaiu, M., Vicente, O., Grigore, M. (2015). Anatomical modifications under salt stress in two ecologically distinct *Juncus* species. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(2): 501-506.
4) Al Hassan, M., Morosan, M., Lopez-Gresa, M.P., Boscaiu, M., Vicente, O. (2015). Selection and characterisation of salt and drought-resistant *Phaseolus* cultivars: a 'proof-of-concept' study. (Under revision).
5) Al Hassan, M., Pacurar, A., Lopez-Gresa, M.P., Llinares, J., Boscaiu, M., Vicente, O. (2015). Effects of Salt and Water Stress on Three Ecologically Distinct *Plantago* Species. (Under revision).
6) Al Hassan, M., Lopez-Gresa, M.P., Boscaiu, M., Vicente, O. (2015). Stress tolerance mechanisms in *Juncus*: Responses to salinity and drought in three *Juncus* species adapted to different natural environments. (Under revision).

7) Al Hassan, M., Cortes, J., Gaspar, A., Boscaiu, M., Vicente, O. (2015). Differential anti-oxidative responses under salinity and drought challenges in two halophytes and one glycophyte of the genus *Juncus*. (In preparation).
8) Al Hassan, M., Daniso, E., Martinelli, F., Boscaiu, M., Vicente, O. (2015). Expression of the vacuolar Na^+/H^+ antiporter gene (*NHX1*) in three *Plantago* species differing in salt tolerance (In preparation).

Altres manuscrits, publicats o enviats per a la seua publicació durant el període de treball, més o menys relacionats amb el seu tema però no inclosos en la Tesi, s'enumeren en l'apèndix al final d'aquest document.

Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Winston Churchill

Table of Contents

Chapter 1:
General Introduction

This chapter begins with a brief insight into salt and water stress, and the damage they cause to crop production worldwide; this is followed by a description of the deleterious effects of these two stress conditions on plants. Then, we highlight the usefulness of performing comparative studies on the responses to salt and water stress of genetically related taxa with different degrees of stress resistance – such as halophytes (salt tolerant plants) and glycophytes (salt sensitive plants) belonging to the same genus, or different cultivars of the same species – as this experimental approach can provide novel and interesting information on the relevant mechanisms of stress tolerance in plants.

1.1. Drought and soil salinity, and their effects on crop production worldwide

Abiotic stress is defined as any non-living factor diminishing the plant´s ability to thrive through photosynthesis, and converting its harvested energy into biomass accumulation (Grime, 1977) and successful propagation. These factors include salinity, drought, heat, cold, alkalinity, UV radiation, heavy metals and other environmental stresses (Van Breusegem *et al*., 2001). Yet, among those mentioned, drought and soil salinity are the two factors that impose the greatest threat to world´s crop production and, in addition, are largely responsible for the distribution patterns of wild plants in nature according to their tolerance (Boyer, 1982; Owens, 2001; Bartels and Sunkar, 2005; Flowers and Flowers, 2005; Zhang, 2011).

Compared to salt stress, drought is more common and damaging (Boyer, 1982), as it is a natural disaster whose occurrence cannot be predicted. The damage caused by drought is considered to be worth billions of dollars (Riebsame *et al*., 1990), and in the past few decades has occurred in large areas in all continents (Ding *et al*., 2011) with extensive damage and massive death toll. Drought is the single abiotic stress condition most devastating for agriculture: insufficient rainfall brings about a progressive reduction of the amount of water

available for plants in the soil, affecting their growth and development and reducing crop productivity, or even causing premature plant death and the loss of the whole crop, if drought is prolonged in time (Osakabe *et al*., 2014). Irrigation systems are necessary to maintain acceptable yields in those regions with low rainfall. In fact, irrigated land is much more productive than rain fed cropland: irrigation is currently used to grow crops in about 280 million hectares of arable land; this represents just below 20% of the total cultivated land, but produces more than 40% of world food supplies (Munns and Tester, 2008). Yet, intensive and prolonged irrigation causes another serious problem for agriculture: soil salinization.

Soil salinization is the accumulation of water-soluble salts in the soil in levels impacting agricultural production, environmental health, and economic welfare (Rengasamy, 2006). Salinity resulting from natural causes is known as 'primary salinity'. It affects large areas of land worldwide, which are as such never used for agricultural activities since all major crops are salt sensitive. The term "secondary salinity" was coined for soil salinization due to human activities, especially prolonged irrigation schemes without sufficient drainage and the massive and uncontrolled use of fertilizers (Flowers, 2004; Parihar *et al*., 2015).

Salt-affected soils occur in more than 100 countries of the world with a variety of extents, nature, and properties. Although arid and semi-arid regions are most affected with salinization; no climatic zone is free from it (Rengasamy, 2006). About 20-30% of irrigated croplands is affected by salt (Owens, 2001; Unesco Water Portal, 2007), an area that is approximately 400 million hectares of land (Al-Sadi *et al*., 2010); nearly the size of the Indian subcontinent; and this area is increasing by 1 to 2% on a yearly basis (FAO, 2002). The situation is further aggravated by the forecasted effects of global climate change (Hillel and Rosenzweig, 2002) including an increase in average temperatures worldwide, changes in rainfall patterns, longer, more frequent and more intense extreme weather phenomena such as droughts, 'heat waves' or floods, and more importantly due to the increasing demand for food

resources as a results of the ever expanding human population, that global food production will need to increase by 38% by 2025 and by 57% by 2050 (Wild, 2003) if food supply to the growing world population is to be maintained at current levels. However, what must be noted is that, most of the suitable lands have been already cultivated and expansion into new areas to increase food production is rarely possible or desirable. The aim, as such, should be an increase in yield per unit of land rather than in the area cultivated (Rengasamy, 2006). Therefore, an effective approach to increase crop yields, and hence food production, in the next decades could be based on the biotechnological improvement, through genetic engineering methods, of the abiotic stress tolerance of our major crops (Fita *et al*., 2015). This, in turn, requires a profound knowledge of the intricate physiological, biochemical and molecular networks underlying plant stress tolerance mechanisms. For this reason – apart from the academic interest of this topic – the study of the responses of plants to abiotic stress has become an active area of research in plant biology.

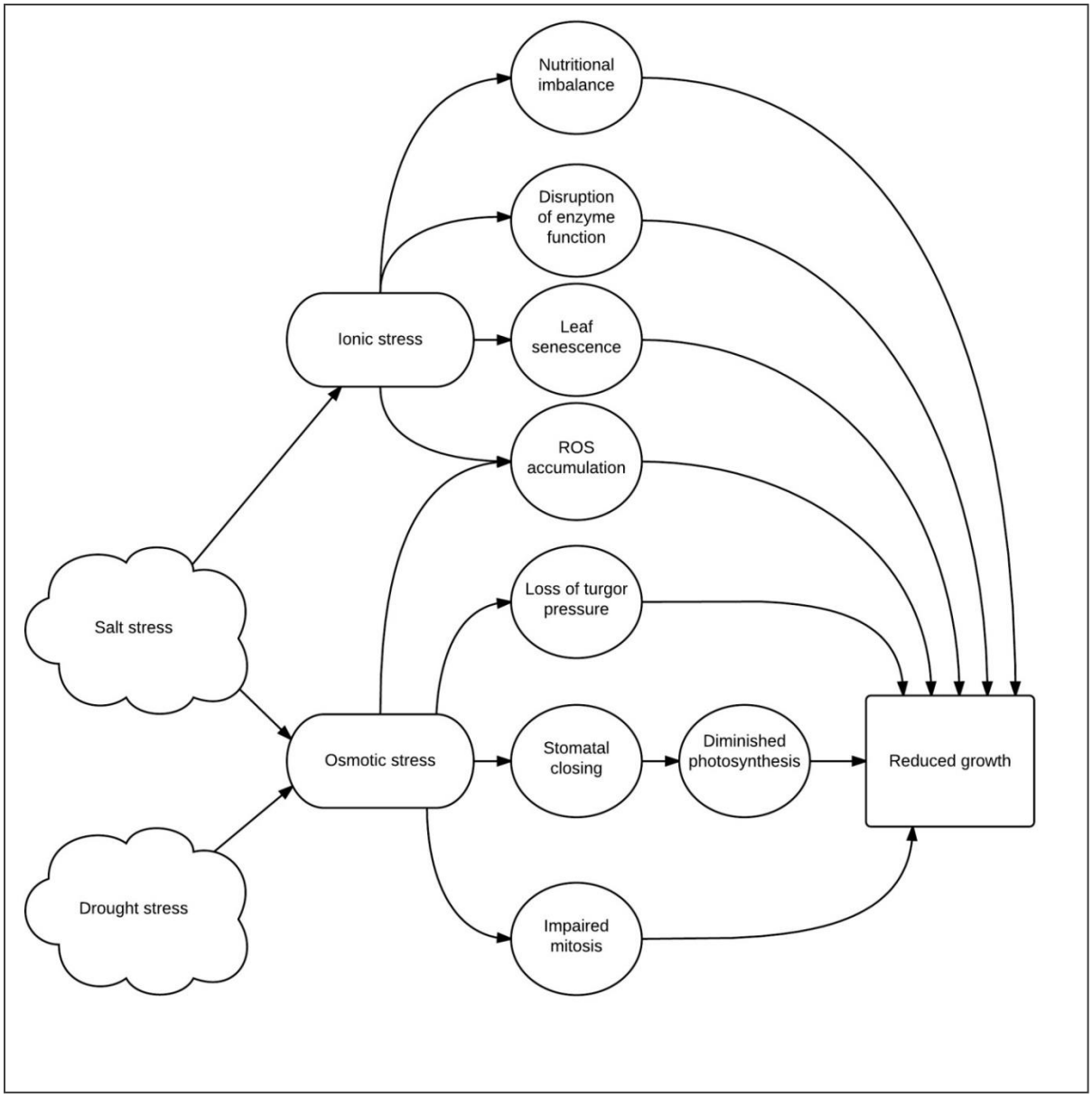
1.2. Effects of salt and water stress on plants

Early responses to water and salt stress in plants have been considered to be almost identical (Munns, 2002), due to the fact that drought and salinity share a physiological water deficit that affects, more or less intensely, all plant organs. Salinity however inhibits plant´s growth and can lead to plant death for two main reasons, since salt stress has two different components: osmotic stress and ion toxicity (Munns and Tester, 2008), the latter being specific only for salt stress, where firstly, the elevated concentration of ions in the soil cause a low water potential in the root cells, leading to reduced ability of the plant to uptake water resulting in a curtailed vegetative growth, and secondly ions enter the plant root cells and are

transported to its aerial parts, causing ionic imbalance and toxicity and further reduced plant growth (Greenway and Munns, 1980).

Osmotic stress, or reduced turgor pressure especially in root cells, due to the high concentration of ions in the soil (salinity) or scarcity of water (drought), is a common effect of water and salt stress, which causes reduced water uptake, thus inducing a cascade of effects leading to inhibition of plant growth and, eventually, to plant death (Munns, 1993; Li *et al*., 2009) (Fig. 1). These effects include reduced mitosis in roots (Sharp *et al*., 1988) and leaves (Spollen and Nelson, 1994; Durand *et al*., 1995), stomatal closure and thus reduced gas exchange, reactive oxygen species (ROS) accumulation (Osakabe *et al*., 2014), and disrupted ability to detoxify ROS (Munns and Tester, 2008).

Ionic stress, also termed salt-specific effect of salinity (Greenway and Munns, 1980) normally follows osmotic stress in plants affected by salt stress. This stress commences when excessive ions enter plant tissue (through the roots) and cause a range of disturbances, starting from ion homeostasis disturbance and nutritional balance impairment (altering the uptake of K^+ and Ca^{2+}, for example) (Epstein, 1980; Serrano *et al*., 1997), inhibition of the activity of many enzymes (Na^+ can compete – with Mg^{2+} which is required for the activity of many enzymes as a cofactor; e.g., Albert *et al*., 2000) and direct inactivation of proteins and other macromolecular structures, ROS accumulation (Apel and Hirt, 2004; Mahajan and Tuteja, 2005; Ahmad, 2010; Ahmad and Prasad, 2012), chlorophyll degradation (Kato and Shimizu, 1987), and tissue injury (leaf senescence) (Allu *et al*., 2014) (Fig. 1), all contributing to further reduce vegetative growth.



Source: Author's own illustration.

Fig. 1 Effects of salt stress and drought leading to reduced vegetative growth in affected plants.

1.3. Glycophytes and halophytes: the importance of comparative studies.

Understanding how plants respond to drought, salt and co-occurring stresses plays a major role in stabilizing crop performance under drought and saline conditions and in the protection of natural vegetation via adequate management techniques and plant genetic

breeding which would prove to be essential tools to improve resource use efficiency (including water) by plants under stress.

Plants are categorized into salt sensitive glycophytes and salt resistant halophytes; this classification however is not absolute as plants have a continuous range of tolerance/sensitivity to salt from extremely sensitive glycophytes to highly salt tolerant halophytes. Nearly 98% of terrestrial plant species are considered as glycophytes, including all major crops (Flowers *et al*., 1986). Unlike halophytes, they cannot complete their life cycle in soils with a salt concentration higher than 200 mM NaCl – the somewhat arbitrary threshold that is generally accepted as the limit to separate halophytes and glycophytes.

Halophytes are a versatile set of plants that can flourish in salt affected soils where glycophytes usually cannot, by maintaining an osmotic pressure lower than that of the salinized soil, a feature that can hardly be attained in the salt sensitive glycophytes. Even though halophytes evolved from polyphyletic origins (Flowers *et al*., 2010), they seem to activate a series of basic, conserved mechanisms in response to high soil salinity (conserved in glycophytes as well, but with major differences that we will discuss during the course of this work) (Fig. 2), although the relative contribution of these responses to salt tolerance may differ in different halophytes. In addition to these conserved mechanisms of response, there are in some halophytes more specific morphological and anatomical adaptations (succulence, salt glands, salt bladders…) that may be important for tolerance in those taxa (Flowers and Colmer, 2008; Flowers *et al*., 1977).

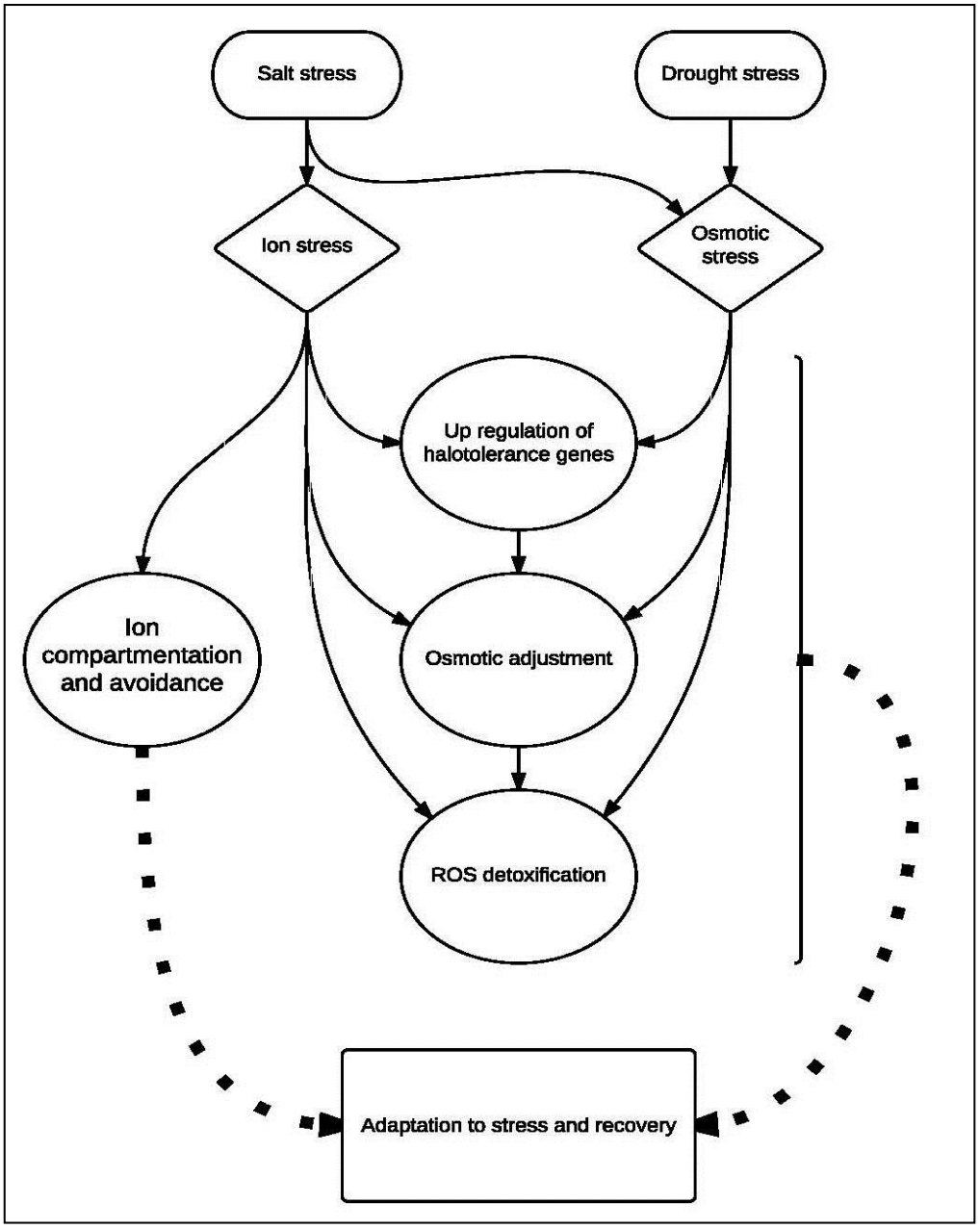
As mentioned previously, stress tolerance in plants relies on the activation of a series of conserved response pathways, some of them common to different abiotic stresses. One of these basic response mechanisms is the control of ion homeostasis and maintenance of osmotic balance, to counteract cellular dehydration caused by soil salinity, drought, cold or high temperatures, among other stressful conditions. The synthesis and accumulation of

compatible solutes ('osmolytes') in the cytoplasm (Rhodes and Hanson, 1993), in general, together with compartmentalization of toxic ions in the vacuole (overexpression of tonoplast antiporters (*NHX1* gene, *NHX2* gene, …) when referring specifically to salt stress, are essential for osmotic adjustment (Munns and Termaat, 1986; Zhu, 2001; Munns and Tester, 2008), other mechanisms include salt avoidance (Allen *et al*., 1994), ion transport and others…. Overexpression of genes encoding antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), polyphenol peroxidase (POD), or ascorbate peroxidase (APX) which are vital in the detoxification of ROS (Mittler, 2002; Rahnama and Ebrahimzadeh, 2005) is yet another conserved mechanism dealing however with the effects of the arising osmotic stress.

The activation of these processes however is not specific for halophytes, but shared by all plants, and does not necessarily result in salt tolerance (Zhu, 2001); in fact, as mentioned above, most species are sensitive to salinity. Therefore, the relative resistance to salt stress of plants, which varies widely among taxa, should be attributed to quantitative as well as qualitative differences in their mechanisms of response, which only in halophytes are efficient enough to confer salt tolerance, always within species-specific limits (Greenway and Munns, 1980; Zhu, 2001; Grigore *et al*., 2011; Gil *et al*., 2013).

Besides the quantitative edge that halophytes attain over the glycophytes in terms of efficiency of their mechanisms of response, they also retain some qualitative distinctions as well, like the presence of tonoplast transporters (Shabala and Mackay, 2011) and the probable presence of modified lipid composition in their vacuole membranes that prevent Na^+ leakage back to the cell´s cytoplasm, allowing effective compartmentalization of inflowing ions in vacuoles (Flowers and Yeo, 1986; Cheeseman, 1988), besides the aforementioned anatomical differences (succulence , salt glands, and salt bladders), all of which contribute in the elevated tolerance to salt stress in halophytes and its absence in glycophytes.

Comparative studies concerning the effects of salt stress and the stress response mechanisms in related taxa with different levels of tolerance have been carried out extensively in the last years. Due to which several authors discussed that halophytes possess constitutive mechanisms which enable their "stress-anticipatory preparedness" or a metabolic anticipation of stress (Gong *et al*., 2005; Sanchez *et al*., 2008). However, the pre-adaptation hypothesis is still under debate as other studies showed contrary results. On the other hand such comparative studies of the responses to drought and salt stress in genetically related halophytes and glycophytes proved to be essential to understand the response mechanisms conveying tolerance in some species, as well as the function of certain osmolytes that in some cases appear to be species-specific or genus-specific, all of which would contribute greatly to our understanding of the naturally designed tolerance of some plants (halophytes and xerophytes) to drought and salt stress; an key factor in the global effort to produce more food resources via stress tolerant crops.



Source: Author's own illustration.

Fig. 2 Salt and drought tolerance mechanisms in plants.

Chapter 2:
Objectives

We aimed in this work to distinguish between the concepts of responses to stress *vs*. stress tolerance, concerning specifically water and salt stress conditions. In order to tackle this issue, we tried to explore and analyze conserved responses to drought and salinity in genetically related plants with different stress tolerance potential. The methodology focused on *i)* establishing the relative tolerance to water and salt stress in the studied species via their distribution in nature (in the case of wild species) and through the relative inhibition of growth after controlled stress treatments, and *ii)* correlating stress-induced changes in the levels of biochemical 'stress markers' associated to specific response pathways (ion transport, osmolyte accumulation…), with the previously established relative tolerance of the taxa under study.

The investigation performed at the biochemical level is pivotal in our work; however some anatomical and molecular aspects were included as well. We attempt to list our objectives as:

1- Diagnosis and characterization of the effects of salt and water stress on the vegetative growth of the studied plants.

2- Assessment of the relative tolerance to water and salt stress in the investigated species according to the inhibition of their growth upon controlled stress treatments, estimated by determination of different growth parameters, and from their distribution in nature.

3- Analysis of conserved stress response pathways like ion transport, accumulation of specific osmolytes, or activation of antioxidant systems.

4- Identification of the function of the major accumulated osmolytes (response *vs*. tolerance), considering their concentration, variation under stress, and their correlation with the established relative tolerance in species.

5- Testing the validity of the "stress-anticipatory preparedness" theory – or presence of constitutive stress response mechanisms – concerning halophytes, stating the presence of relatively high contents of biomarkers involved in stress tolerance, even in control conditions, in non-stressed plants.

6- Assessment of the influence of salt stress on one of the most critical phases of the life cycle: seed germination and seedling establishment and growth, in three *Plantago* species.

7- Isolation of the vacuolar Na^+/H^+ antiporter gene *NHX1* from the three *Plantago* species, and comparing its expression patterns in the presence of salt, in the *Plantago* halophytes, their salt-sensitive congener and the glycophyte *Arabidopsis thaliana*.

8- Checking whether the anatomical changes in two *Juncus* species under salt stress, represent a mere alteration due to stress or, on the contrary, may be considered as true adaptations to salinity.

Chapter 3:
Plant Material

3.1. *Solanum lycopersicum*

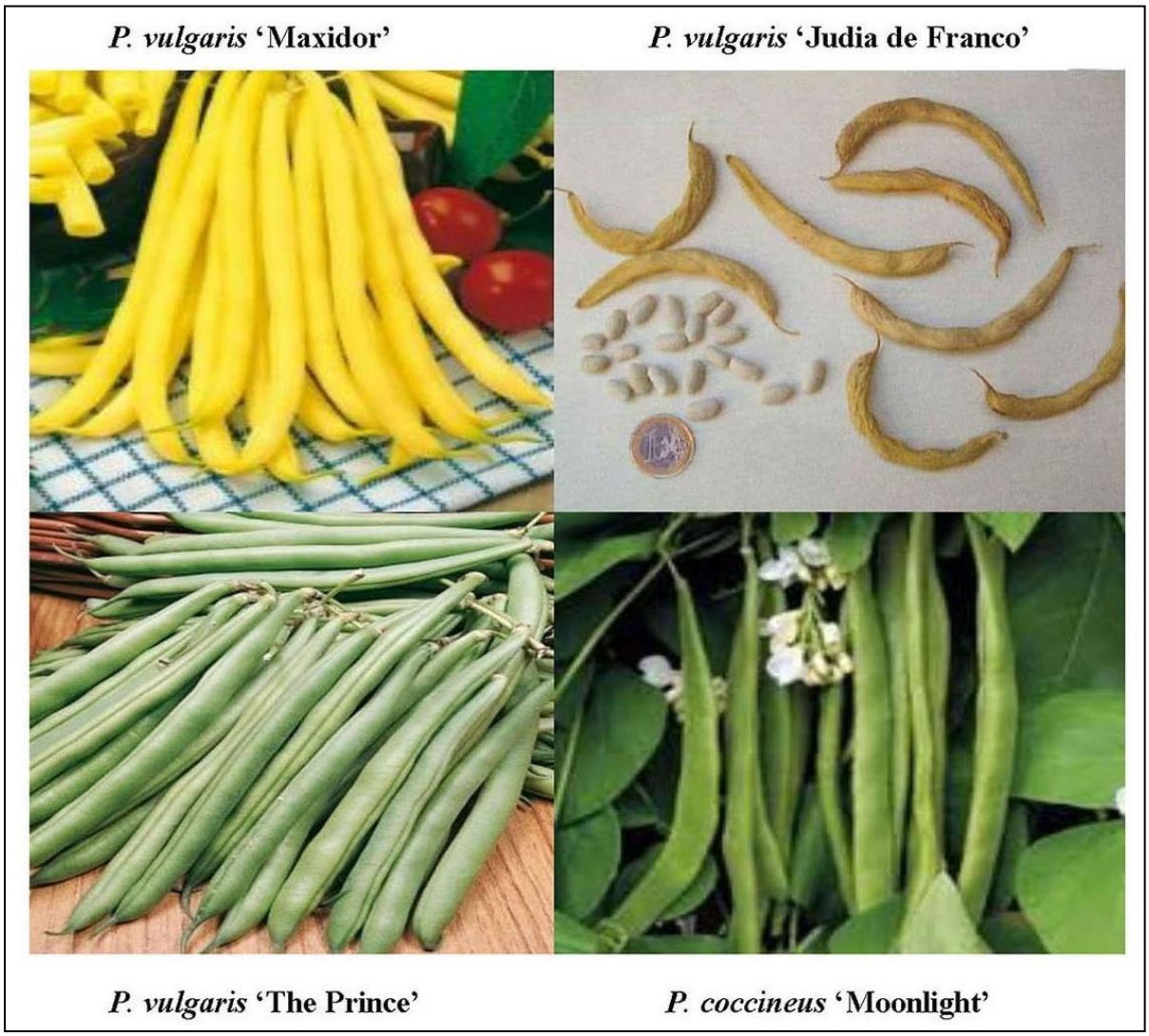
Cherry tomato (*Solanum lycopersicum* L. var. cerasiforme) is clearly differentiated from other tomatoes by the smaller size of its fruits (Fig. 3). Plants of this variety are vigorous in their growth and have a higher leaves/fruits ratio; therefore, the fruits receive more photoassimilates, in comparison to larger-size varieties, and have higher sugar and acid contents. Thus, cherry tomatoes are more flavored and are used mostly for fresh consumption. Seeds of cherry tomato were kindly provided by Pilar Corella, from Rijk Zwaan Ibérica S.A. (Almería, Spain).

Source: uniprot (http://www.uniprot.org/taxonomy/4081).

Fig. 3 *S. lycopersicum* L. var. *cerasiforme* (cherry tomato).

3.2. Phaseolus species

- *Phaseolus vulgaris* L. (Fig. 4).



Source: 'Maxidor' (http://www.seeds-gallery.eu/en/vegetable-seeds/dwarf-bean-seeds-maxidor.html);
'Judía de Franco' ("Variedades autóctonas de legumbres españolas", p. 251);
'The Prince' (http://www.diynetwork.com/outdoors/legumes-beans/pictures/page-7.html);
'Moonlight' (http://www.shootgardening.co.uk/plant/phaseolus-coccineus-moonlight).

Fig. 4 Pods of the selected Phaseolus taxa.

a- *Phaseolus vulgaris* L. 'Maxidor'

'Maxidor' is also a dwarf French bean cultivar. It has a bushy growth, reaching in average 35-40 cm, with precocious flowering. Pods are round and yellow, 12 cm long with relatively large seeds; the 100-seed mass is about 26 g. It is considered as an early or

medium-early variety; pods can be harvested 60-65 days after germination and dry beans after 100-110 days. 'Maxidor' is cultivated in several countries in Europe, and is one of the most commonly grown in Western Romania (Madoşă, 2013).

b- *Phaseolus vulgaris* L. 'The Prince'

'The Prince' is a dwarf French bean cultivar, with a bushy growth and an average height of 35-45 cm. Pods are green and flat, with a length of about 20 cm and large seeds (average weight: ca. 0.5 g per seed). It is an early cultivar; it takes 60-70 days for snap pods to form, and 90 days for dry seeds. *Cv*. 'The Prince' has been reported as drought sensitive (Boutraa and Sanders, 2001). The cultivar is one of the most commonly used in Europe.

c- *Phaseolus vulgaris* L. 'Judía de Franco'

'Judía de Franco' is a local landrace of *P. vulgaris* from the province of Teruel (E. Spain). It has an indefinite growth, surpassing 3 m high. It reaches maturity after 96 days. Pods are 9 cm long, flat and green. The average weight of 100 seeds is about 30 g.

Seeds of *P. vulgaris* L. 'Maxidor' and 'The Prince' were purchased from S.C. AGROSEM IMPEX S.R.L., Targu Mures (Romania). Seeds of *P. vulgaris* L. 'Judía de Franco' were obtained from the Germplasm Bank of COMAV (Institute for Conservation and Improvement of Valencian Agrodiversity, Polytechnic University of Valencia), Valencia, Spain.

- *Phaseolus coccineus* L.

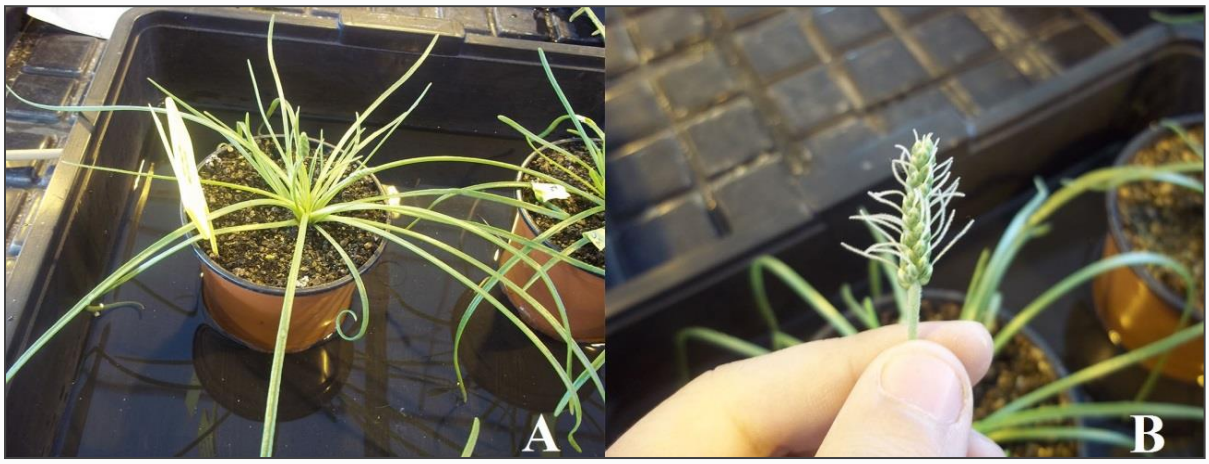
Seeds of *P. coccineus* L. 'Moonlight' were obtained from AJP Garden & Crafts, Chew Valley, United Kingdom. Seeds originated from Mexico. It reaches 3 m high. It has a longer biological cycle of 120-125 days. Pods are green and long, reaching 25 cm.

3.3. *Plantago* species

- *Plantago crassifolia*

Seeds of *P. crassifolia* were harvested from a salt marsh located in the Natural Park of La Albufera (Province of Valencia, Spain).

P. crassifolia Forssk. is a perennial Mediterranean, succulent halophyte belonging to the family Plantaginaceae, specific for sandy littoral soils and salt marshes (Chater and Cartier, 1976). Its strap-like leaves are formed in a basal rosette and grow in length up to 20 cm (Fig. 5A). The tiny flowers of *P. crassifolia* are clustered densely on erect spikes, carried upon smooth green stalks (Fig. 5B) (Grigore *et al.*, 2014).



Source: Author's own photo.

Fig. 5 *P. crassifolia*: (A) overview of leaves´ rosettes formation, and (B) flowers; of *P. crassifolia*.

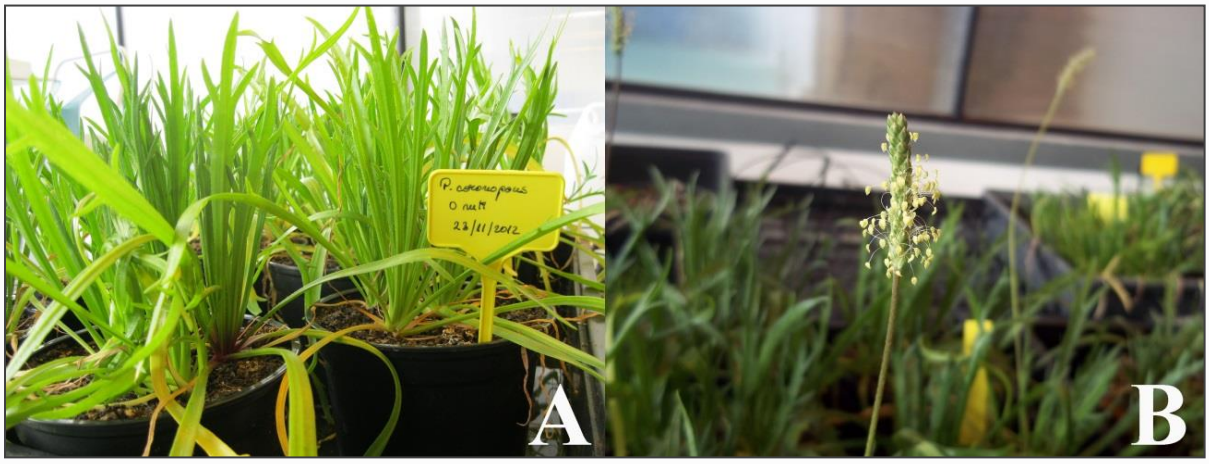
- *Plantago coronopus*

Seeds of *P. coronopus* were harvested from a salt marsh located in the Natural Park of La Albufera (Province of Valencia, Spain).

P. coronopus is a perennial flowering plant belonging to the family Plantaginaceae. Its leaves are lance shaped reaching up to 25 cm long, and are formed in rosettes (Fig. 6A),

the flowers are small on long ramified spikes (Fig. 6B), and the petals of its tiny flowers are hairy on the outside, and the flowering spike often bends or nods prior to flowering.

P. coronopus is a weed common on all continents found on disturbed ground, waste places and on chalky banks, especially near the sea (Yoshida and Tanaka, 1997; Glen, 1998). It is able of surviving conditions such as poor drainage, brackish swamps and compacted droughty grasslands, and is used as an indicator species for highly saline soils, acting as an alert for land managers in irrigation regions (Koyro, 2006).



Source: Author's own photo.

Fig. 6 *P. coronopus*: (A) overview of leaves´ rosettes formation and lance shape, and (B) flowers; of *P. coronopus*.

- *Plantago major*

Seeds of *P. major* were procured from B and T World Seeds sarl, Paguignan, Aigues-Vives, France.

P. major L. is a perennial plant that belongs to the Plantaginaceae family. It can reach about 15 cm high; the size however differs from one habitat to another. The leaves grow in rosettes (Fig. 7A), with parallel venation (5–9) (Fig. 7B). The flowers are small, brownish-green on long non-ramified spikes (Fig. 7C) (Samuelsen, 2000). *P. major* is pollinated by wind, and large amounts of seeds are produced, up to 20,000 per plant (Fægri, 1970; Tutin *et*

al., 1976). The seeds are located in capsules (8–16 per capsule) and become sticky in humid weather due to the swelling of the polysaccharides present in the seed coat (Qadry, 1963).

P. major L. is widely distributed in Europe, Asia and north of Africa, and throughout the world. Although a few *P. major* subspecies are adapted to saline environments (Chater and Cartier, 1976), the common taxon *P. majo*r subsp. *major* included in this work is frequent in humid areas but never found in salty soils, and it is therefore considered as a glycophyte.

Source: herbario virtual del mediterraneo occidental.

Fig. 7 *P. major*: (A) overview of rosette leaf formation, (B) leaf venation, and (C) flowers; of *P. major*.

3.4. *Juncus* species

- *Juncus maritimus*

Seeds of *J. maritimus* were harvested in a salt marsh located in the Natural Park of La Albufera (Province of Valencia, Spain).

J. maritimus (Fig. 8, Fig. 11A) are monocot perennials that belong to the family Juncaceae, which can grow up to 1 meter high, that inhabit coastal salt marshes, saline meadows, and sand dunes, and thus are considered as halophytes.

Source: herbario virtual del mediterraneo occidental.

Fig. 8 *Juncus maritimus*.

- *Juncus acutus*

Seeds of *J. acutus* were harvested in a salt marsh located in the Natural Park of La Albufera (Province of Valencia, Spain).

J. acutus (Fig. 9, Fig. 11B) are monocot perennials that belong to the family Juncaceae, which can grow up to 1 meter) high, that inhabit salt marshes, moist saline habitats and alkaline soils and thus are considered as halophytes.

Source: herbario virtual del mediterraneo occidental.

Fig. 9 *Juncus acutus*.

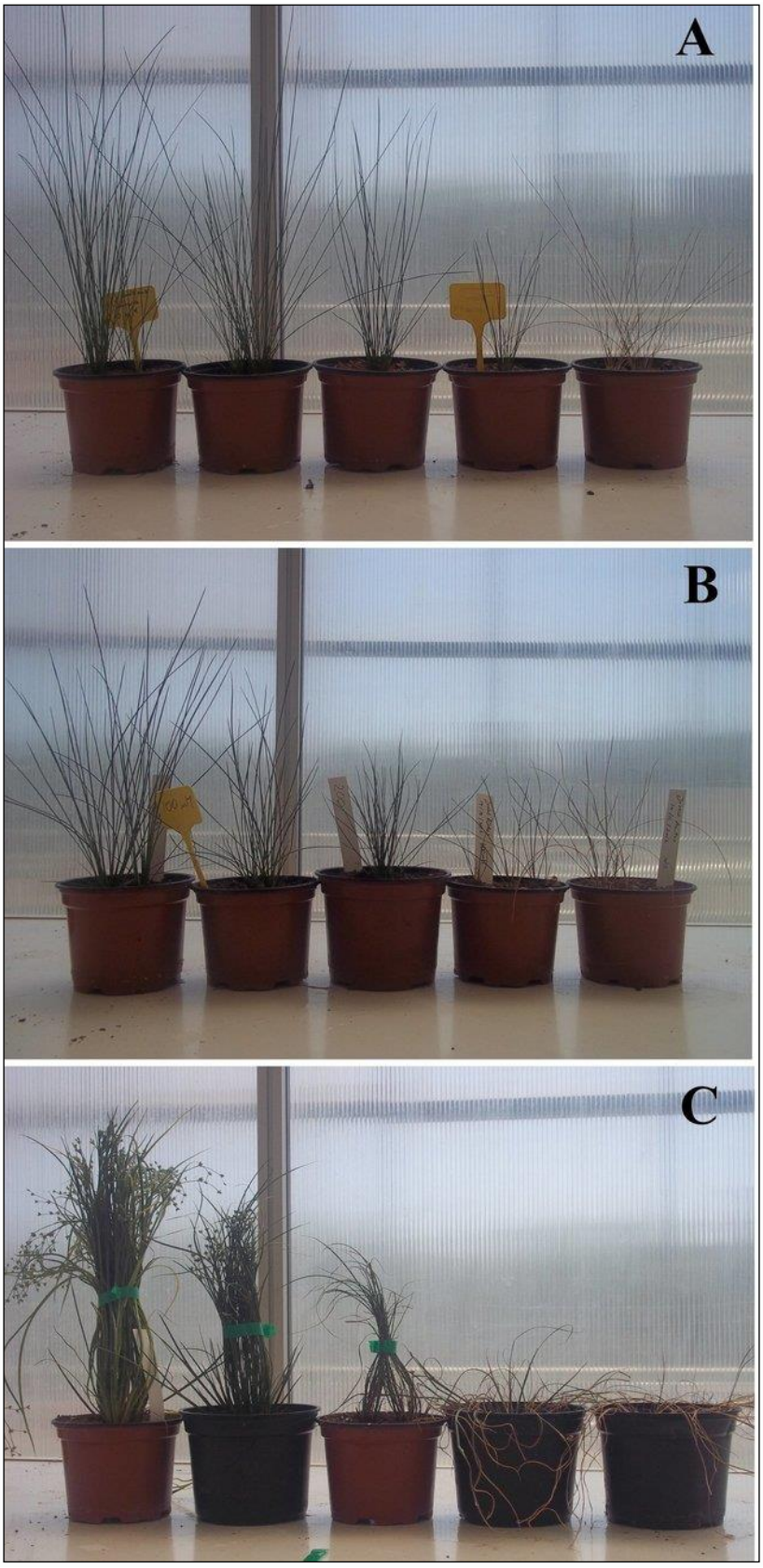
- *Juncus articulatus*

Seeds of *J. articulatus* were harvested from Natural Park of La Albufera (Province of Valencia, Spain).

Source: The Botanical Gardens of Charles University.

Fig. 10 *Juncus articulatus*.

J. articulatus (Fig. 10, Fig. 11C) are monocot perennials that belong to the family Juncaceae, which can grow up to 1 meter high. *J. articulatus* L. is frequent in the northern hemisphere and in Australia in different humid areas such as wetlands, but also along the creeks and rivers (Albrecht, 1994). No previous study on its tolerance to salt and drought stress was reported to have been carried out on this fresh water growing *Juncus* species (Chambers *et al.*, 1995).



Source: Author's own photo.

Fig. 11 (A) *Juncus maritimus*, (B) *J. acutus*, and (C) *J. articulatus*; Under treatment (from left to right: Control, 100 mM NaCl, 200 mM NaCl, 400 mM NaCl, and water stress plants, after 8 weeks of treatments).

Chapter 4:
Results

Publication I:

Subchapter 4.1.

Effects of salt and water stress on plant growth and on accumulation of osmolytes and antioxidant compounds in cherry tomato

Reference:

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Effects of Salt and Water Stress on Plant Growth and on Accumulation of Osmolytes and Antioxidant Compounds in Cherry Tomato

Abstract. The effects of salt and water stress on growth and several stress markers were investigated in cherry tomato plants. Some growth parameters (stem length and number of leaves) and chlorophyll contents were determined every third day during plant growth, and leaf material was collected after 25 and 33 days of treatment. Both stresses inhibited plant growth; chlorophyll levels, however, decreased only in response to high NaCl concentrations. Proline contents largely increased in leaves of stressed plants, reaching levels high enough to play a major role in cellular osmotic adjustment. Despite reports indicating that tomato does not synthesize glycine betaine, the stress-induced accumulation of this osmolyte was detected in cherry tomato, albeit at lower concentration than that of proline. Therefore, it appears that the plants are able to synthesise glycine betaine as a secondary osmolyte under strong stress conditions. Total sugars levels, on the contrary, decreased in stress-treated plants. Both stress treatments caused secondary oxidative stress in the plants, as indicated by a significant increase in malondialdehyde (MDA) contents. Water stress led to an increase in total phenolics and flavonoid contents and a reduction of carotenoid levels in the leaves; flavonoids also increased under high salinity conditions.

***Keywords*:** proline; glycine betaine; carbohydrates; MDA; carotenoids; phenolics; flavonoids

4.1.1. Introduction

Tomato is grown in a wide range of climatic conditions, from the tropics to subarctic regions, but its optimal cultivation areas are found in warm and rather dry regions, such as

Mediterranean countries (Cuartero and Fernández Muñoz, 1999). In these arid or semi-arid zones, water stress and high soil salinity are common environmental factors that can significantly reduce crop yields. One of the most general responses to drought and salinity – as well as to other abiotic stress conditions also causing cellular dehydration in plants, such as cold, elevated temperatures or exposure to heavy metals – is based on the synthesis and cytoplasmic accumulation of osmolytes, a conserved phenomenon observed in all plants, tolerant as well as sensitive to stress (Parida and Das, 2005; Munns and Tester, 2008; Parvaiz and Satyawati, 2008). Osmolytes are 'compatible solutes', very soluble, low-molecular-weight organic compounds that do not interfere with normal metabolism even when present at high concentrations. While toxic inorganic ions are sequestered in vacuoles, organic osmolytes accumulate predominantly in the cytoplasm, preventing or limiting cellular dehydration (Stewart and Lee, 1974; Handa *et al.*, 1986; Büssis and Heineke, 1998). Reduction of the osmotic potential due to accumulation of osmolytes in response to stress improves the ability of the plant cells to maintain turgor pressure at low water potentials, which is essential for biological processes such as photosynthesis or cell expansion, as well as for maintaining enzymatic activities (Tyree and Jarvis, 1982). Besides their role in osmotic adjustment, osmolytes act as osmoprotective substances, directly stabilising proteins and cell membranes under dehydration conditions. Osmolytes also protect cells from oxidative stress by inactivating 'reactive oxygen species' (ROS) (Hare *et al*., 1998; Szabados and Savouré, 2010).

The amino acid proline (Pro) and glycine betaine (GB), a quaternary amine, are probably the most common compatible solutes synthesised by plants as a response to abiotic stress (Ashraf and Foolad, 2007; Chen and Murata, 2008; Verbruggen and Hermans, 2008). As for other osmolytes, besides their role in osmoregulation, both compounds can act as 'low-molecular-weight chaperons', contributing to maintain the active conformation of

macromolecules in stressed plants, and participate in detoxification of ROS. Moreover, Pro and GB seem to be involved, directly or indirectly, in the regulation of gene expression as signalling molecules, and also serve as metabolites for the cellular storage of carbon and nitrogen during stress, which would be used by the cell once stress has ceased (Munns and Tester, 2008; Szabados and Savouré, 2010). Compatible solutes also include soluble carbohydrates, such as sugars (e.g. sucrose, glucose, fructose or trehalose), sugar alcohols (sorbitol, mannitol, and different inositol isomers and derivatives), and the raffinose family of oligosaccharides (Parida *et al*., 2002; Gavaghan *et al*., 2011). Although sugars have been shown to act as functional osmolytes in several species, it is not so easy to assess their specific functions in the responses to stress, which can be masked by their multiple additional roles as direct products of photosynthesis, components of the primary metabolism and regulatory molecules (Gil *et al*., 2013).

A secondary effect of abiotic stresses, including drought and salinity, is the increased generation of 'reactive oxygen species' (ROS), including highly reactive free radicals such as superoxide, singlet oxygen, hydroxyl or perhydroxyl radicals, as well as hydrogen peroxide, molecular oxygen, ozone and other strong oxidant molecules (Apel and Hirt, 2004). ROS are continuously generated by plants as by-products of different metabolic pathways, but under stress their production increases leading to oxidative damage of cellular membranes, proteins, carbohydrates and DNA (Van Breusegem and Dat, 2006). In response to stress, plants activate powerful antioxidant systems, both enzymatic (e.g., superoxide dismutase, catalase, glutathione reductase, several peroxidades) and non-enzymatic (vitamins C and E, carotenoids, flavonoids and other phenolic compounds, etc.) (Apel and Hirt, 2004).

Carotenoids are pigments with several functions in plants, besides their direct role in photosynthesis, including their involvement in the mechanisms of oxidative stress tolerance (Gill and Tuteja, 2010). Phenolic compounds also fulfill multiple roles in plants, as structural

components of cell walls, participating in the regulation of growth and developmental processes, as well as in the mechanisms of defence against herbivores and pathogens; in addition, they are involved in the responses of plants to practically to all types of abiotic stress: UV radiation, extreme temperatures, mineral nutrient imbalance, drought, salinity, heavy metals and herbicides among others (Gould and Lister, 2006; Cheynier *et al*., 2013). Flavonoids represent the main and more complex subgroup of polyphenols, including more than 9000 different compounds with a wide array of biological functions (Winkel-Shirley, 2002; Treutter, 2005; 2006; Pollastri and Tattini, 2011; Di Ferdinando *et al*., 2012). In the last decade, they have been the object of many studies, not only due to the academic interest in elucidating their multiple functions in plants, but also because their alleged beneficial effects for human health, as powerful dietary antioxidants (Wiseman, 2006).

Responses to salinity in tomato, within its salt-tolerance range, have been extensively studied, but only few authors have analysed its responses to high salt concentrations (Maggio *et al.*, 2004). In addition, there are only a few papers reporting the effects of both salinity and drought on the same plant material (Giannakoula and Ilias, 2013). These two adverse environmental conditions, which will worsen as a consequence of climate change, could seriously affect tomato production in Mediterranean countries in the coming years. The aims of the present study were to analyse the effects of water stress and of high salinity, beyond the tolerance threshold, on the growth of tomato plants, and to assess their responses to these conditions in terms of accumulation of the main osmolytes (proline, glycine betaine and total soluble sugars) and of some non-enzymatic antioxidants (total carotenoids, phenolics and flavonoids). The experiments were carried out in cherry tomato, a variety that has not been extensively studied despite its growing commercial interest.

4.1.2. Material and Methods

Plant material

Cherry tomato (*Solanum lycopersicum* L. var. *cerasiforme*) is clearly differentiated from other tomatoes by the smaller size of its fruits. Plants of this variety are vigorous in their growth and have a higher leaves/fruits ratio; therefore, the fruits receive more photoassimilates, in comparison to larger-size varieties, and have higher sugar and acid contents. Thus, cherry tomatoes are more flavoured and are used mostly for fresh consumption. Seeds of cherry tomato were kindly provided by Pilar Corella, from Rijk Zwaan Ibérica S.A. (Almería, Spain).

Plant growth

The seeds were sown in seed trays containing a mixture of commercial peat and vermiculite (1:1), and were placed in a greenhouse with regulated temperatures ranging between 17 and 23°C, under a long-day photoperiod (16 h light / 8 h dark). Young plants were transferred to individual pots (11 cm in diameter) on the same substrate 36 days after sowing and were watered for 26 additional days with a standard nutritive solution. Then, salt and water stress treatments were started, by watering the plants with increasing NaCl concentrations (150, 300 and 450 mM NaCl in nutritive solution) and by stopping irrigation altogether, respectively; 10 plants were used per treatment. Watering was carried out twice a week by adding 1.5 L of the salt solutions to each tray, which contained 12 standard pots. Control plants were grown in parallel, maintaining the standard irrigation regime with nutritive solution. After starting the treatments, plant height was measured and the number of leaves per plant was counted every three days, to assess the effect of stress on vegetative plant growth. Chlorophyll content was also determined at the same times, using a portable 'Chlorophyll meter SPAD-502Plus' (Konica Minolta).

For each stress treatment, including non-treated controls, leaves in equivalent positions were detached from all plants, 25 and 33 days after starting the treatments, and weighed on a precision balance to calculate the average leaf fresh weight (FW) corresponding to each treatment. Part of the leaf material was stored at -75ºC until further use, and part was dried at 65ºC until constant weight, to measure the average leaf dry weight (DW) for each treatment. Average leaf 'water content' after each treatment, expressed in percentage, was calculated as (FW - DW / FW) x 100.

Electric conductivity of the substrate

Soil $EC_{1:5}$ was checked at the beginning and at the end of the treatments. Soil samples were taken from three pots per treatment, air-dried and then passed through a 2-mm sieve. A soil: water (1:5) suspension was prepared in Milli Q water and stirred for one hour at 600 u/min at 21ºC. Electric conductivity was measured with a Crison Conductivity meter 522 and expressed in dS/m.

Osmolyte contents

Pro contents were determined according to the method of Bates *et al*. (1973) with minor modifications (Vicente *et al*., 2004). Frozen plant material (100 mg) was ground to a fine powder in a mortar, in the presence of liquid nitrogen. Extraction was carried out with 3% sulfosalicylic acid, and cell debris was removed by filtration. One volume of the filtrate was mixed first with one volume of freshly prepared acid ninhydrin (25 mg/mL ninhydrin in 10.44 M acetic acid and 2.4 M phosphoric acid); then one volume of glacial acetic acid was added, the sample was mixed and incubated at 95ºC for 1 h. After stopping the reaction by cooling the samples on ice, they were extracted with two volumes of toluene. The absorbance of the organic phase was determined at 520 nm, using toluene as a blank. A calibration curve

was obtained for each assay, using solutions of Pro in 3% sulfosalicylic acid of known, increasing concentrations and subjected to the same treatment as the samples. Pro contents in the plant samples were expressed as 'µmol Pro per gram of dry weight'.

The extraction and quantification of glycine betaine was performed following the method of Grieve and Grattan (1983) with the modifications proposed by Nawaz and Ashraf (2010). Frozen plant material (100 mg) was ground, using a homogenizer (Ultraturrax), in a volume of 2 mL Milli-Q water, and centrifuged for 10 minutes at -4°C and 13000 rpm. One mL was removed from the supernatant of each extract and mixed with 400 µL HCl 2N. After stirring, 200 µL of each tube was mixed with 80 µL of potassium iodide and brought to 100 mL with Milli-Q water. The tubes were quickly placed in ice and stirred every 20 seconds for 15 min. After 90 min, 800 µL of cold Milli-Q water and 4 mL of 1.2-dichloroethane – kept in the -20ºC freezer until used – were added to each tube. The samples were vortexed for one minute and allowed to settle until the water and the organic phases separated; finally, 1 mL of the lower aqueous phase was aspirated and its absorbance at 265 nm was measured in a spectrophotometer. A standard curve was obtained for each assay, using glycine betaine solutions in Milli-Q water of increasing concentration subjected to the same treatment as the plant samples. Glycine betaine contents in the plant samples were expressed as 'µmol GB per gram of dry weight'

Total sugars were quantified according to the method described by Dubois *et al.* (1956). Dried material (0.1 g) was crushed and diluted with 3 mL of 80% methanol, left on an agitator for 20–48 h, and then mixed with concentrated sulfuric acid and 5% phenol; finally, absorbance of the samples at 490 nm was measured in a spectrophotometer. Glucose solutions were used as standard, and the amount of total soluble sugars in each sample was calculated as 'mg. equivalent of glucose per gram of dry weight'.

Malondialdehyde and non-enzymatic antioxidants

Malondialdehyde (MDA), total phenolics and flavonoids contents were measured in the same extracts used for total soluble sugars determination (0.1 g of dried material in 3 mL of 80% methanol). MDA, a product of membrane lipid peroxidation, is considered as an excellent marker of oxidative stress (Del Rio *et al.*, 2005) and is routinely used to assess the degree of oxidative damage induced in plants by different types of stress (e.g. Aghaleh *et al.*, 2009; Li *et al.*, 2010). MDA content was determined by the thiobarbituric acid-reactive-substances (TBARS) assay, modified to correct for the presence of interfering compounds, as described by Hodges *et al.* (1999).

Total phenolic compounds were quantified by reaction with the Folin-Ciocalteu reagent, according to Blainski *et al.* (2013). Absorbance was measured at 765 nm, and the results expressed in equivalents of gallic acid (mg eq. GA g^{-1} DW), used as standard. Flavonoid contents were determined following the method described by Zhishen *et al.* (1999); the absorbance was measured at 510 nm, and the amount of flavonoids was expressed in equivalents of catechin (mg eq. C g^{-1} DW), used as standard.

Total carotenoids were measured and quantified following Sims and Gamon (2002): 0.2 g fresh material was crushed and diluted in 80% ice-cold acetone/Tris buffer for 1 hour on a shaker at 4ºC, then centrifuged for 15 min at 3000 x *g*. The supernatant was separated and optical density was measured at 663 nm, 646 nm, and 470 nm in disposable plastic cuvettes. Total carotenoids values were then converted into µg g^{-1} DW.

Statistical analysis

Data were analysed using the Statgraphics Centurion XVI programme for Windows. Prior to the analysis, the Levene test was applied to check the ANOVA requirements. The significance of differences between treatments was assessed using one-way ANOVA, at 95%

confidence level. The post-hoc Tukey test was used to estimate homogeneous groups when more than two samples were compared.

4.1.3. Results

Substrate electric conductivity

The electric conductivity ($EC_{1:5}$) of the plants' substrate was determined before and after the stress treatments (Tab. 1). In the control pots, not treated with NaCl, the $EC_{1:5}$ value at the end of the experiment was significantly higher than the initial one; this can be easily explained by accumulation of ions present in the nutritive solution. As expected, $EC_{1:5}$ increased even more after watering the plants with NaCl solutions of increasing concentration, in a concentration-dependent manner. The mean $EC_{1:5}$ also increased slightly in the substrate of water stressed plants, as compared to the initial value – most likely due to concentration of ions from the nutritive solution – but the difference was not statistically significant.

Stress-induced inhibition of plant growth

Both salt and water stress negatively affected vegetative plant growth, which was estimated by the increase in plant length and in the number of leaves during the 33 days of treatment (Fig. 1). As compared to the unstressed controls, all NaCl concentrations tested totally blocked growth of tomato plants, with a clear concentration effect: the higher the salt concentration used, the shorter the lag period before growth inhibition was observed (Figs. 1A and C). Water stress also stopped stem growth and the increase in leaf number; this effect was clearly observed 9 days after the last irrigation with nutritive solution, once the substrate had dried (Figs. 1B and D).

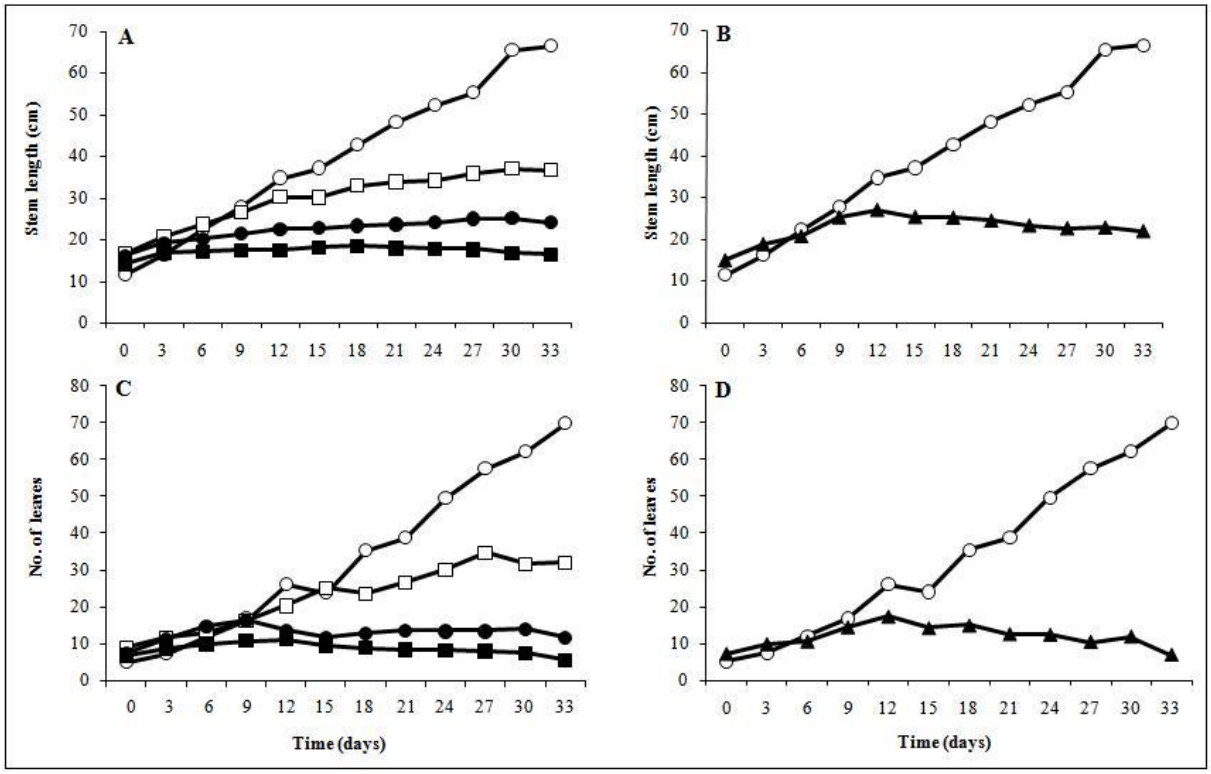

Fig. 1 Stem length (A, B) and number of leaves (C, D) changes during the salt (A, C) and water (B, D) stress treatments. Control, non-stressed plants (-○-, all panels). 50 mM (-□-), 100 mM (-●-) and 150 mM (-■-) NaCl (panels A, C). Water-stressed plants (-▲-, panels B, D).

Apart from the growth parameters measured continuously, stress-induced inhibition of plant growth was also assessed by determining the mean fresh weight (FW) and dry weight (DW) of the tomato leaves after 25 days (sampling 1) and 33 days (sampling 2) of salt or water stress treatment (Fig. 2). A significant, concentration-dependent decrease of FW was observed in salt-treated plants, reaching approximately a 75% reduction in those irrigated with 450 mM NaCl, with respect to the non-treated controls. In the presence of this high salt concentration, the plants were already strongly affected after 25 days of treatment and no significant differences were found between the two samplings; at lower salt levels – 150 or 300 mM NaCl – reduction of FW increased with the duration of the treatment (Fig. 2A). Similar qualitative results were obtained when the plants were submitted to water stress, although with stronger differences between the two samplings: after 25 days without

irrigation plant FW was reduced by ca. 25%, as average, but by almost 90% after 33 days of treatment (Fig. 2B). The relative decrease in FW under water stress conditions – as compared with the control plants – was not only a consequence of the inhibition of growth, but also due to a significant loss of water, as shown by the calculated water content of the tomato leaves: it was only 50% of the FW for the plants maintained for 33 days without water, while for those irrigated normally with nutritive solution water content was about 85% of the total leaf weight (Fig. 2D).

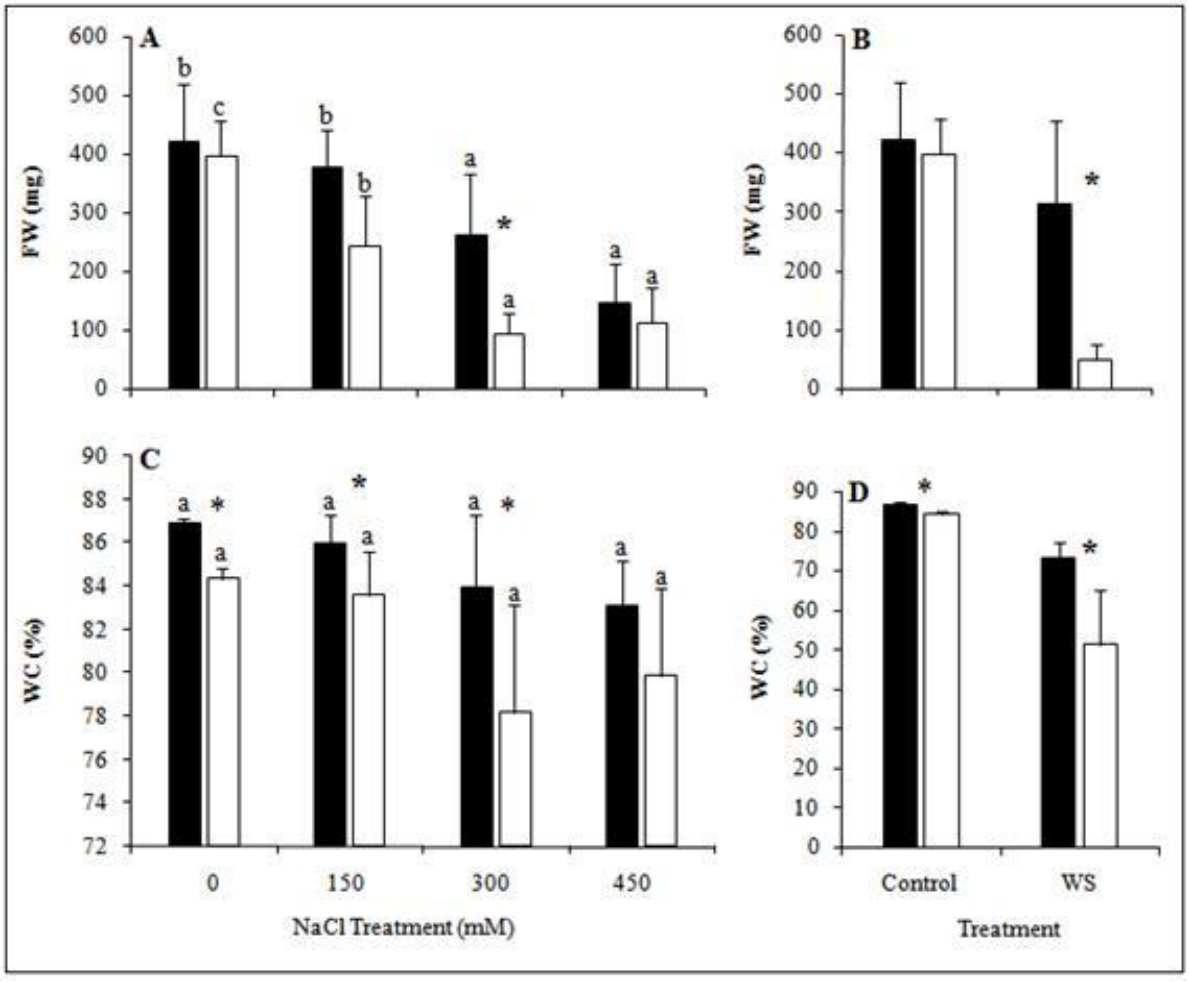

Fig. 2 Average fresh weight (A, B) and relative water content (WC) as percentage of fresh weight (C, D), of tomato leaves after 25 days (sampling 1, black bars) or 33 days (sampling 2, white bars) of salt (A, C) or water (B, D) stress treatments (means ± SD, *n* = 3). Different lower case letters indicate significant differences between treatments, for the same sampling, according to Tukey test (α = 0.05); asterisks indicate significant differences between sampling 1 and sampling 2, for each treatment.

In the presence of salt, a general but not statistically significant decrease of the mean leaf water content was observed with increasing NaCl concentrations, both after 25 and 33 days of treatment, whereas significant differences were detected between the samples collected at different times for each NaCl concentration, up to 300 mM (Fig. 2C).

Chlorophyll content

The chlorophyll content of crop plants is positively correlated with their photosynthetic activity (Gummuluru *et al.,* 1989) and a reduction of chlorophyll level contributes to the inhibition of photosynthesis observed under abiotic stress conditions. A decrease in chlorophyll levels with respect to the controls was indeed detected in plants irrigated with high salt concentrations – 300 and 450 mM NaCl – for two weeks or longer times (Fig. 3A). On the other hand, no significant differences with non-stressed plants were observed when a moderate – 150 mM – NaCl concentration was used (Fig. 3A) or when the tomato plants were subjected to the water stress treatment (Fig. 3B); this latter observation was consistent with the visual appearance of the plants, which were clearly wilted but had not lost their green colour (data not shown).

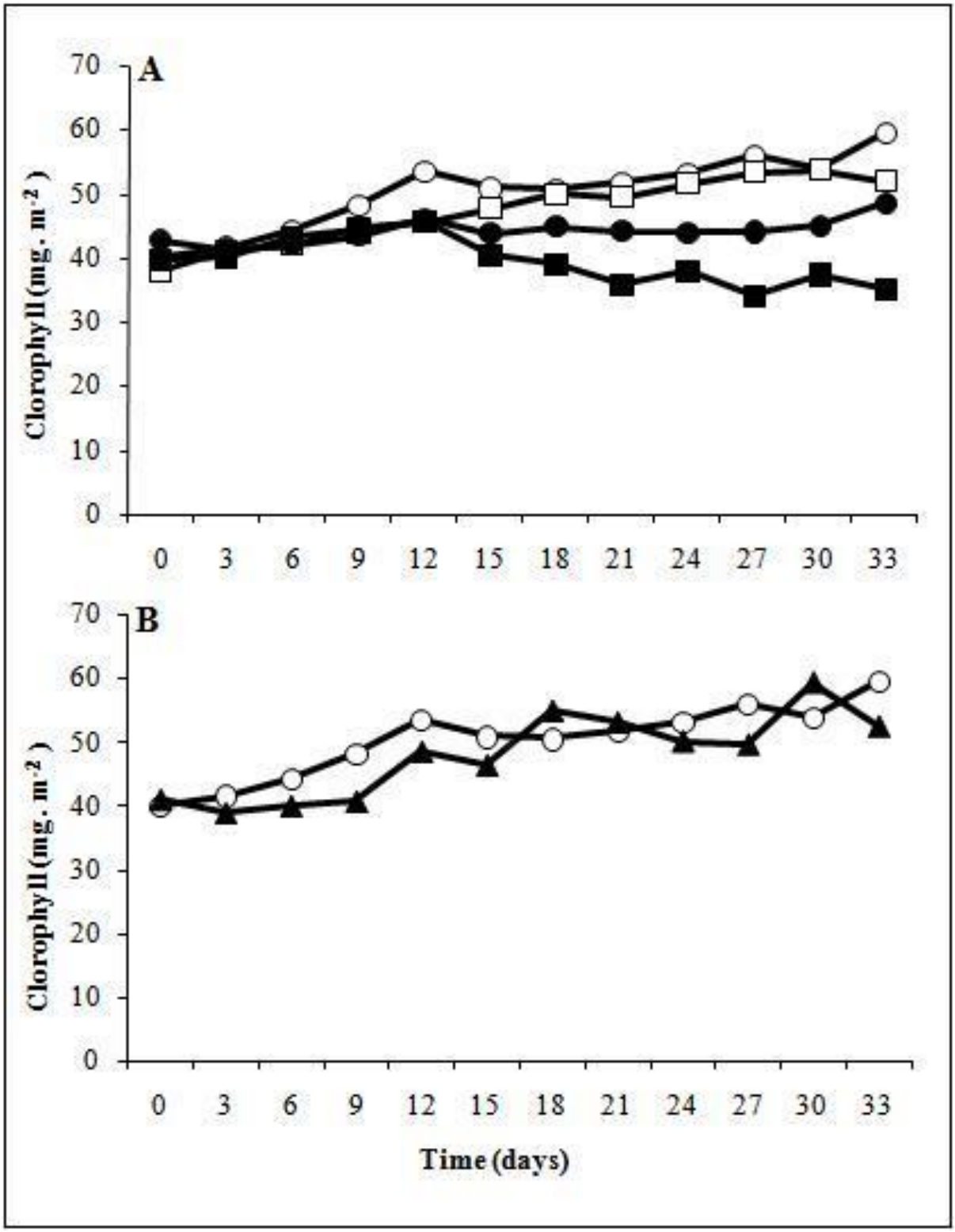

Fig. 3 Chlorophyll contents (mg per square meter) in leaves of tomato plants during the salt (A) and water (B) stress treatments. Control, non-stressed plants (-○-, in both panels). 50 mM (-□-), 100 mM (-●-) and 150 mM (-■-) NaCl (panel A). Water-stressed plants (-▲-, panel B).

Proline content

Application of salt treatments significantly increased Pro levels in tomato leaves, in a concentration-dependent manner. Mean Pro values in the presence of 300 mM and 450 mM external NaCl also increased with longer times of treatment although the differences observed between the two samplings of plant material were significant only at 450 mM NaCl. Under the strongest salt stress conditions tested, Pro levels reached about 350 µmol per gram DW, which represented an increase of 16-fold with respect to the non-treated controls (Fig. 4A). Pro accumulation was more clearly observed in plants kept without irrigation, with increases of about 20-fold and 36-fold after 25 and 33 days, respectively, of water stress (Fig. 4B). Pro contents in the plants subjected to the strongest water stress treatment (33 days without irrigation), in terms of dry weight (ca. 800 µmol g^{-1}) were about double than those determined in plants treated for the same time with 450 mM NaCl (Figs. 4A and B); however, considering the drastic reduction of water content in non-irrigated plants, it can be said that both treatments induced Pro accumulation to similar levels, in absolute terms.

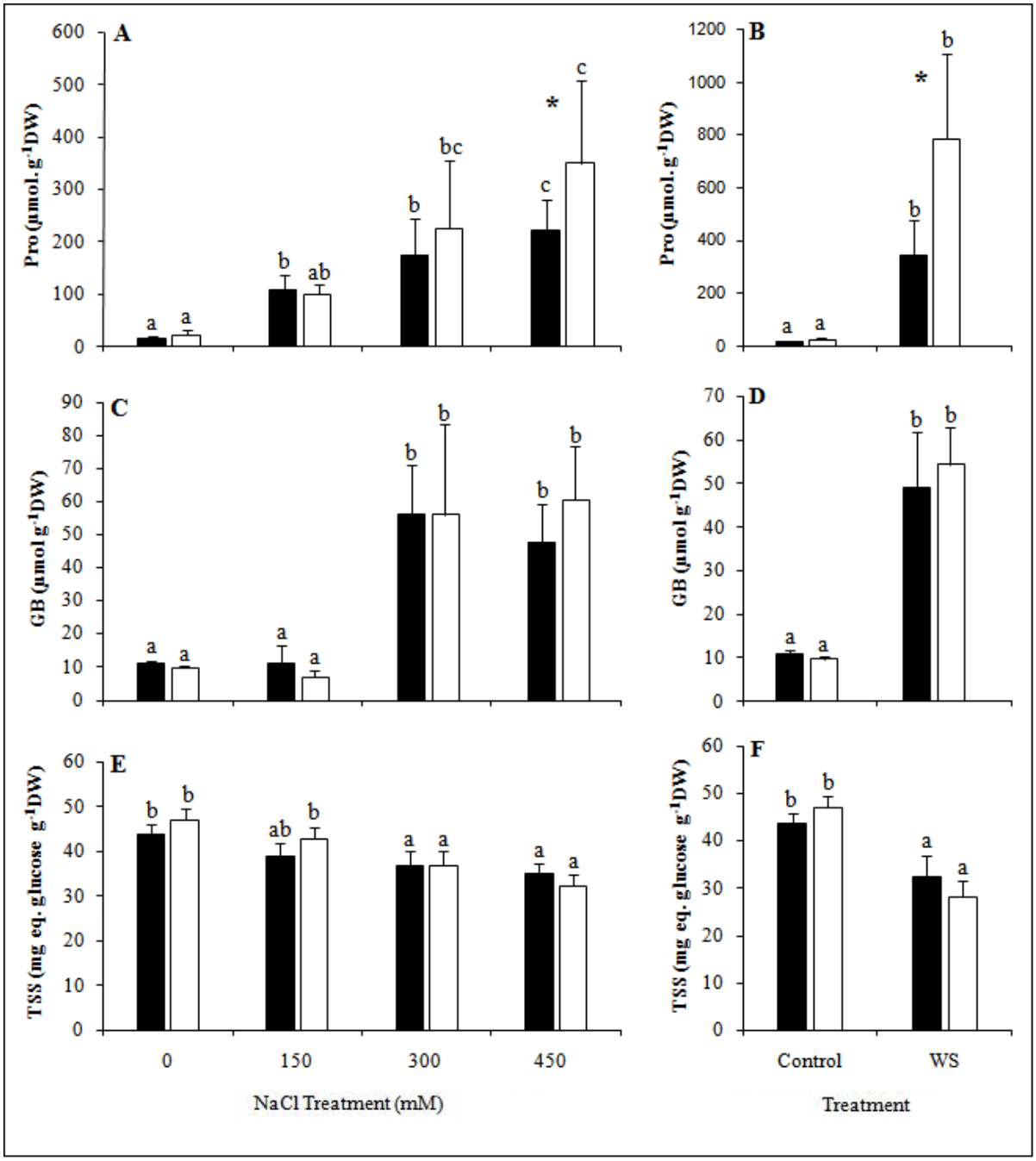

Fig. 4 Proline (Pro, µmol per gram DW) (A, B), glycine betaine (GB, µmol per gram DW) (C, D), and total soluble sugars (TSS, mg equivalent of glucose per gram DW) (E, F) in tomato leaves after 25 days (sampling 1, black bars) or 33 days (sampling 2, white bars) of salt (A, C, E) or water (B, D, F) stress treatments (means ± SD, *n* = 3). Different lower case letters indicate significant differences between treatments, for the same sampling, according to Tukey test (*α* = 0.05); asterisks indicate significant differences between sampling 1 and sampling 2, for each treatment.

Glycine betaine

Salt and water stress also induced the synthesis and accumulation of GB in tomato (Figs. 4C and D). Yet the absolute values of GB content reached in plants watered with 300 or 450 mM NaCl (50-60 µmol per gram DW) and its relative increase as compared to the

control plants (ca. 5-fold) were lower than those of Pro. Moreover, no time-dependent increase in GB levels was detected in this experiment, as the values measured for the two samplings of plant material were not significantly different (Fig. 4C). The same results were obtained for plants subjected to 25 or 33 days of water stress (Fig. 4D).

Total soluble sugars

The total amount of soluble sugars decreased slightly in salt-stressed plants, but significant differences were registered only starting with 300 mM NaCl in both samplings (Fig. 4E). Under water stress conditions the decrease was also significant, and more accentuated after a longer drought treatment (Fig. 4F).

Malondialdehyde

MDA is a product of membrane lipid peroxidation and, as mentioned above, is considered a reliable marker of oxidative stress; thus, higher MDA contents should correspond to a higher degree of oxidative stress. As expected, MDA levels increased with all treatments, mostly after longer exposure to stress, so higher values were registered in the second sampling, after 33 days of treatment (Tab. 2 and 3). The plants most affected by oxidative stress were those treated with 450 mM NaCl, in which the highest MDA contents were measured (Tab. 2).

Total carotenoids, phenolics and flavonoids

Carotenoid levels in leaves decreased in all treatments with respect to the control. In plants treated with 450 mM NaCl, this reduction was of about 50%. When comparing the two samplings, lower values were registered in plants exposed for a longer time to stress in all treatments (Tab. 2 and 3). Contrary to the pattern of variation of carotenoids, total phenolics

increased in plants under stress, especially in those treated with high salt concentrations. Values were significantly higher in plants from the second sampling than in those from the first one in all stress treatments (Tab. 2 and 3). A different response to the type of stress was observed in total flavonoid contents, which increased significantly in the presence of NaCl (Tab. 2) but decreased under drought conditions (Tab. 3).

4.1.4. Discussion

All our major crops and most wild species are relatively sensitive to environmental stress conditions such as drought or salinity, but tomato is moderately resistant to salt, withstanding a soil EC_{sat} up to 6 dS m^{-1}, so that it can be cultivated in regions exposed to a certain degree of soil salinisation (Maggio *et al.,* 2004). Yet high soil salinity inhibits seed germination and plant growth, and causes reduction in crop yields (Dalton *et al*., 1997; Romero-Aranda *et al*., 2002). In this study, young tomato plants have been grown in the presence of increasing concentrations of NaCl, and a concentration- and time-dependent inhibition of vegetative growth has been observed, which was better shown by the reduction of the fresh weight of the plants; this was not unexpected, since inhibition of growth is probably the most general response of plants to stress (Munns, 2002; Munns and Tester, 2008). Despite the fact that high salinity should cause cellular dehydration, in the experimental conditions used here the water content of the plants was reduced very slightly in the presence of salt, suggesting that tomato plants activate relatively efficient mechanisms to cope with the osmotic component of salt stress, and that growth inhibition is mostly due to the ionic toxicity of the salt. Water stress also inhibited growth but, contrary to salinity, in this case a severe dehydration of the plants was observed.

Another difference between salt and water stress treatments refers to changes in chlorophyll contents. In plants treated with high NaCl concentrations, a significant reduction in chlorophyll levels, up to ca. 40% of the non-treated controls, was observed. The decrease in chlorophyll levels in plants affected by salt is due to the inhibition of chlorophyll synthesis, together with the activation of its degradation by the enzyme chlorophyllase (Santos, 2004). Yet reduction of chlorophyll contents is not the only reason for the inhibition of photosynthesis in the presence of salt, since NaCl also inhibits key enzymes involved in this process such as Rubisco and PEP carboxylase (Soussi *et al*., 1998). Although water stress should also reduce chlorophyll levels and photosynthetic activity (Alberte *et al*., 1977; Sanchez *et al*., 1983; Mafakheri *et al*., 2010), this was not observed in the present study, probably because the time that the plants were maintained without irrigation was not long enough to detect these effects.

Proline is generally considered as a good indicator of environmental stress in tomato (Clausen, 2005), and there are many reports describing an increase in Pro contents as a response to water or salt stress in this species (Handa *et al*., 1986; Inal, 2002; Nahar and Gretzmacher, 2002; Yokas *et al*., 2008; Umebese *et al*., 2009; Babu *et al*., 2012; Ghorbanli *et al*., 2013; Giannakoula and Ilias, 2013), although it should be mentioned that data specific for cherry tomatoes are rather scarce (Maggio *et al*., 2007; Rosales *et al*., 2007). What is not so clear is the possible contribution of Pro accumulation to the (relative) resistance of tomato to salinity. When comparing tomato cultivars with differences in their degree of salt tolerance, in some cases the more tolerant cultivars were found to synthesise higher amounts of Pro under stress (Ali *et al*., 2011), but in others there was no correlation between tolerance and Pro levels (Alian *et al*., 2000). In the present study, a clear positive correlation between Pro accumulation and the intensity of the applied stress treatments has been established; that is, of Pro levels with the electric conductivity of the substrate – reflecting increasing NaCl

concentrations in the nutritive solution – and with the time of exposure to stress. Moreover, Pro reached levels high enough to play a significant role in cellular osmotic adjustment under these stress conditions. Taken together, these data strongly support the notion that Pro is the major physiological osmolyte in cherry tomato, as it has been suggested for other tomato varieties (Nahar and Gretzmacher, 2002).

Usually, a given plant species accumulate preferentially a particular type of osmolyte in response to environmental stress; there have even been attempts to use this preference for one specific type of compatible solute as a taxonomic criterion in wild species (Gorham *et al*., 1980; Tipirdamaz *et al*., 2006). In agreement with this idea, and since Pro appears to be the major osmolyte in tomato, it is generally accepted that tomato does not accumulate glycine betaine in natural conditions; in fact, it has been reported that this species lacks two enzymes required for GB biosynthesis (Goel *et al*., 2011). Nevertheless, exogenous application of GB to tomato plants improves their resistance to drought (Rezaei *et al*., 2012) and salinity (Makela *et al*., 1998; Heuer, 2003; Chen *et al*., 2009). On the other hand, there are some examples of plant species that, under severe stress conditions, can activate the simultaneous synthesis of several osmolytes to help alleviate the detrimental effects of stress, by contributing to osmotic adjustment and/or acting as 'osmoprotectants', as it has been recently discussed (Tipirdamaz *et al*., 2006; Gil *et al*., 2013). This seems to be the case also for tomato, at least for the cherry variety: contrary to the general assumption, in the present study the accumulation of GB, in response to water or salt stress treatments, has been detected in tomato plants. It is true that the maximum levels of GB measured (ca. 50 µmol g^{-1} DW) were about 10-fold lower than those of Pro in the same plants – and also much lower than those measured in taxa that are clear GB accumulators (Boscaiu *et al.*, 2011; Gil *et al.*, 2014). Therefore, GB will have only a modest effect on osmotic balance in stressed tomato plants, but may still contribute significantly to stress resistance due to its putative functions as

low-molecular-weight chaperon and/or ROS scavenger. Most published data support the absence of detectable GB in tomato; yet the present results are not the only evidence for accumulation of endogenous GB in response to stress, which has also been reported in plants submitted to chilling, where a threshold level ensures sufficient protection to low temperatures (Park *et al*., 2006).

The increase of sugars after mild salt and water stress is well-known in tomato fruits, including the cherry variety and moderate abiotic stress was even recommended as a strategy for improving the quality of tomatoes (Ullah *et al*., 1994; Fernández-García *et al*., 2004; Sgherri *et al.,* 2008). Salt stress was found to produce an increase in carbohydrates accumulation, in the form of starch, in the early stages of fruit development, but as soluble sugars in ripe fruits, thus improving the flavour of tomatoes (Yin *et al*., 2010). There are also several reports of increased amount of sugars in tomato berries under drought (e.g., Nahar and Grezmacher, 2002). However, there are relatively few data published on stress-induced sugar accumulation in vegetative organs in this species, and they generally indicate a reduction of total carbohydrates in leaves (e.g., Amini and Ehsanpour, 2005; Li, 2009), as it has been found in the present study.

In addition to sugars, tomato fruits are rich in several compounds considered as 'health-promoting', such as carotenoids, flavonoids and other phenolics. These secondary metabolites play multiple roles in plants, including scavenging of ROS induced under different stress conditions and causing oxidative stress. A clear symptom of oxidative damage is cell membrane degradation; therefore, MDA – a product of membrane lipid peroxidation – is an excellent marker of oxidative stress (Del Rio *et al*., 1996). In this study a significant increase of MDA levels in tomato leaves upon salt and water stress treatments of the plants was obseved, in agreement with previous reports showing a salt-induced increase of MDA

contents in tomato leaves (Shalata and Thal, 1998), roots (Shalata *et al.*, 2001) and fruits (Rosales *et al*., 2006).

Mild and moderate saline and waters stress produce also an increase in carotenoids levels in tomato fruits. Lycopen, the main carotenoid in tomato, is known as an important natural antioxidant with anti-carcinogenic properties (Krauss *et al.,* 2006). In addition, lycopen is responsible for the red colour of tomatoes, aspect of great economic importance and therefore salt treatments are recommended as an alternative strategy to transgenic crops for obtaining tomatoes with higher amounts of carotenoids (Borghesi *et al*., 2011). In the present work, carotenoid levels were measured in tomato leaves (not fruits), and a reduction was detected under stress conditions; this is in agreement with previous reports that found a negative correlation between Na^+ and carotenoid contents in tomato leaves (Juan *et al.,* 2005; Tuna *et al*., 2014).

Salt stress led to a significant increase of phenolics and flavonoids in leaves of plants submitted to the salt treatment and an enhancement of the former in water-stressed plants. There are many publications reporting an increase in the levels of phenolics and flavonoids in tomato fruits in conditions of abiotic stress, which is a topic of direct interest for human health (e.g., Krauss *et al.,* 2006; Ali and Ismail, 2014); in fact, consumption of tomatoes has been recommended to reduce the risk of cardiovascular diseases and cancer (Holiman *et al.*, 1996). Similar studies on leaf material are much scarcer; for example, Sánchez-Rodriguez *et al.* (2011) found an enhancement of some phenolics and flavonoids under moderate water stress (50% of the field capacity) in the more tolerant cultivars of cherry tomatoes but a reduction in the more sensitive ones, in partial agreement with the data presented here.

4.1.5. Conclusions

Salt and water stress treatments inhibited vegetative growth in cherry tomato, a variety that has not been extensively studied yet, despite its growing commercial interest. In non-irrigated plants, a strong dehydration was partly responsible for reduction of leaf fresh weight, an effect not detected in the presence of NaCl. Both stresses led to the accumulation in the leaves of high levels of Pro, which functions as the major osmolyte in cherry tomato, responsible for osmotic adjustment under stress conditions. Glycine betaine also accumulated as a response to salt and water stress – albeit at lower levels than Pro – despite numerous publications reporting its absence in tomato plants; therefore, GB appears to act as a secondary osmolyte that could contribute to stress tolerance in cherry tomato. Both stress treatments caused secondary oxidative stress in the plants, as indicated by a significant increase in malondialdehyde (MDA) contents. The increase in antioxidant phenolic compounds levels in leaves can be considered as part of the response induced to cope with oxidative stress. Contrary to what has been reported for tomato fruits, other metabolites such as total soluble sugars or carotenoids do not increase, but rather decrease in leaves in response to the stress treatments.

4.1.6. References

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Tab. 1 Substrate electric conductivity $EC_{1:5}$ (dS/m) at the beginning (day 0) and at the end (day 33) of the stress treatments. Different lower case letters indicate significant differences between treatments.

Treatment	(day 0)	0 mM NaCl	150 mM NaCl	300 mM NaCl	450 mM NaCl	Water Stress
$EC_{1:5}$	0.21 ± 0.05a	0.94 ± 0.08b	2.56 ± 0.39c	3.37 ± 0.35d	4.78 ± 0.51e	0.38 ± 0.18a

Tab. 2 Effects of increasing salt concentration on MDA and non-enzymatic antioxidant levels in plants after 25 days (sampling 1) and 33 days (sampling 2) of treatment. Values shown are means followed by ± SD (n = 5). Different lower case letters in a file indicate significant differences between treatments, for each compound and sampling. 'GA', gallic acid; 'C', catechin.

	Sampling	Salt treatments (mM NaCl)			
		0	150	300	450
MDA (nmol g^{-1}DW)	1	145.07 ± 13.00a	163.45 ± 8.60ab	169.03 ± 10.00ab	214.16 ± 2.90b
	2	149.30 ± 7.00a	188.24 ± 10.00b	206.02 ± 4.00bc	237.56 ± 20.00c
Total carotenoids (µg g^{-1} DW)	1	648.02 ± 31.00b	613.48 ± 31.00b	546.00 ± 42.00ab	344.37 ± 19.00a
	2	659.53 ± 36.00c	473.01 ± 27.00b	352.98 ± 16.00ab	306.17 ± 18.00a
Total phenolics (mg eq. GA g^{-1} DW)	1	9.71 ± 0.17a	11.64 ± 1.60ab	13.05 ± 0.90b	14.70 ± 0.83c
	2	12.41 ± 0.60a	17.09 ± 0.30b	15.85 ± 3.30b	17.64 ± 2.20b
Total flavonoids (mg eq. C g^{-1} DW)	1	10.19 ± 0.30a	11.24 ± 0.80b	12.24 ± 1.10b	12.87 ± 0.50b
	2	8.74 ± 0.60a	11.03 ± 1.00b	11.64 ± 0.50b	13.74 ± 0.50c

Tab. 3 Effects of drought stress on MDA and non-enzymatic antioxidant levels in plants after 25 days (sampling 1) and 33 days (sampling 2) of treatment. Values shown are means followed by ± SD (n = 5). Different lower case letters in a file indicate significant differences between treatments, for each compound and sampling. 'GA', gallic acid; 'C', catechin

	Sampling	Treatments	
		Control	Water stress
MDA (nmol g^{-1}DW)	1	145.07 ± 13.00a	173.18 ± 5.20b
	2	149.30 ± 7.00a	198.35 ± 7.60b
Total carotenoids (µg g^{-1} DW)	1	648.02 ± 31.00b	474.73 ± 11.00a
	2	659.53 ± 36.00b	357.15 ± 3.60a
Total phenolics (mg eq. GA g^{-1} DW)	1	9.71 ± 0.17a	12.10 ± 1.90b
	2	12.41 ± 0.60a	19.48 ± 0.90b
Total flavonoids (mg eq. C g^{-1} DW)	1	10.19 ± 0.30b	7.03 ± 0.20a
	2	8.74 ± 0.60b	5.10 ± 0.50a

Publication II:

Subchapter 4.2.

Selection and characterisation of salt and drought-resistant *Phaseolus* cultivars: a 'proof-of-concept' study

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Selection and characterisation of salt and drought-resistant *Phaseolus* cultivars: a 'proof-of-concept' study

Abstract Given the increasing demand for food, as a result of the ever rising human population, and the spreading of aridity and soil salinisation in the context of global climate change, stress tolerance studies in crops are of great importance. As a 'proof-of-concept' for a long-term project aimed at the selection and characterisation of stress tolerant bean varieties, in the present work responses to salt and water stress have been analysed in young plants of three *Phaseolus vulgaris* and one *P. coccineus* cultivars. Determination of several growth parameters was used to establish the relative tolerance to stress of the studied cultivars. Measurements of ion (Na^+, K^+, Cl^-) and osmolyte (proline, glycine betaine, total soluble sugars) contents were performed in leaves of plants harvested after three weeks of treatment; individual soluble carbohydrates in the plant extracts were also quantified after HPLC fractionation. The major aim of this study was to identify the specific responses to stress that are the most relevant for tolerance in the analysed *Phaseolus* cultivars. Salt stress resistance in beans is mostly based on restriction of Na^+ (and, to a lesser extent, also of Cl^-) transport to shoots, and on the accumulation of *myo*-inositol for osmotic adjustment; the increase of *myo*-inositol levels is also important under water stress conditions; glycine betaine biosynthesis may also contribute to drought tolerance in *P. coccineus*, but not in *P. vulgaris*. These responses to both, salt and water stress during vegetative growth appear to be more efficient in *P. vulgaris cv*. 'Maxidor', the most tolerant of the tested *Phaseolus* genotypes. Proline accumulation is a reliable marker of the level of stress affecting the plants, but does not seem to be directly related to stress tolerance mechanisms.

***Keywords*:** glycine betaine; *myo*-inositol; osmotic adjustment; *Phaseolus*; Proline; stress tolerance.

4.2.1. Introduction

Drought and soil salinity are the most important environmental stress factors that reduce crop yields worldwide (Boyer, 1982; Bartels and Sunkar, 2005). Drought is the single abiotic stress condition most devastating for agriculture: insufficient rainfall brings about a progressive reduction of the amount of water available for plants in the soil, affecting their growth and development and reducing crop productivity, or even causing premature plant death and the loss of the whole crop, if drought is prolonged in time. Irrigation systems are necessary to maintain acceptable yields in those regions with low rainfall. In fact, irrigated land is much more productive than rain fed cropland: irrigation is currently used to grow crops in about 280 million hectares of arable land; this represents just below 20% of the total cultivated land, but produces more than 40% of world food supplies (Munns and Tester, 2008). Yet, intensive and prolonged irrigation causes another serious problem for agriculture: soil salinisation. After some decades of continuous irrigation, toxic ions dissolved in irrigation water progressively accumulate in the soil, leading to the so-called 'secondary salinisation' – of anthropic origin – of arable land (Flowers, 2004). At present, more than 20% of irrigated cropland is affected by salt, to a greater or lesser extent (Flowers and Flowers, 2005), and more of 10 million hectares are lost for agriculture every year due to soil salinisation (Owens, 2001).

Average yields of all major crops are only a fraction, somewhere between 20% and 50%, of record yields, depending on the species. Adverse environmental conditions affecting the plants growing in the field – mostly drought and soil salinity, as mentioned above – are

the major cause of these differences. These losses are expected to increase in the near future, at least in arid and semiarid regions, due to the forecasted effects of climate change, which include an increase in average temperatures worldwide and the occurrence of more frequent, longer and more intense drought periods (IPCC, 2014), and consequently a shortage of water available for irrigation, which will especially affect subsistence farming in developing countries (Morton, 2007). Therefore, an effective approach to increase crop yields, and hence food production, in the next decades could be based on the biotechnological improvement, through genetic engineering methods, of the abiotic stress tolerance of our major crops (Fita *et al.*, 2015). This, in turn, requires a profound knowledge of the intricate physiological, biochemical and molecular networks underlying plant stress tolerance mechanisms. For this reason – apart from the academic interest of this topic – the study of the responses of plants to abiotic stress has become an active area of research in plant biology.

In addition to some species-specific responses, which may be important for the stress tolerance of particular taxa, all plants appear to react against adverse environmental conditions by activating a series of conserved response mechanisms, which are common to, or overlapping for different abiotic stresses. One of these basic stress responses involves the control of ion homeostasis and the maintenance of cellular osmotic balance: water transport into the cell, compartmentalisation of toxic ions in the vacuole, and synthesis and accumulation of compatible solutes or osmolytes in the cytoplasm, to counteract cellular dehydration caused by high soil salinity and drought – but also by other stressful conditions such as cold or high temperatures (Munns and Termaat, 1986; Zhu, 2001; Munns and Tester, 2008). Apart from contributing to osmotic adjustment, osmolytes play 'osmoprotectant' roles by acting as low-molecular-weight chaperons that stabilise proteins, membranes and other macromolecular structures under cellular dehydration conditions, and also as scavengers of 'reactive oxygen species' (ROS) (Chen and Murata, 2008; Flowers and Colmer, 2008;

Hussain *et al*., 2008; Szabados and Savouré, 2010). Obviously, these mechanisms are responsible, at least in part, for the tolerance of plants to abiotic stress. However, the relative contribution of different stress responses to stress tolerance, which may vary widely among plant species, remains largely unknown. Comparative studies correlating stress responses, such as ion and osmolyte accumulation, with the relative tolerance of genetically related taxa – different species of the same genus, different varieties or cultivars of the same species – may be extremely useful to define those mechanisms that are most relevant for the resistance to drought and/or salinity in a given species (Grigore *et al*., 2011; Gil *et al*., 2013). It seems logical to assume that enhancement of these particular responses by genetic engineering will be an effective strategy to improve the abiotic stress tolerance of particular crops.

Conventional breeding, based on sexual crosses and selection, can also contribute significantly to the improvement of crop abiotic stress tolerance. This approach has had limited success in the past, but now new biotechnological tools are available that make the breeding process much more efficient and rapid: the use of molecular markers in 'marker assisted selection' (MAS), high-throughput DNA sequencing technologies ('next generation sequencing', NGS) and other breeding applications of genomics, or different *in vitro* culture methods (Varshney *et al*., 2009; Fita *et al*., 2015). Since the current industrialised agriculture is based in a very limited number of varieties of each crop, it will be also important to screen minor commercial cultivars, local and neglected varieties and landraces stored in seed banks, as well as wild relatives, as a source of genetic variability to be used in breeding of abiotic stress tolerance of our crops (Ceccarelli *et al*., 1998; Blair *et al*., 2012).

The genus *Phaseolus* includes more than 50 species that spread naturally from North to South America, of which five are cultivated (Broughton *et al*. 2003). *Phaseolus vulgaris* L., the common bean, occurs in the wild from northern Mexico to north-western Argentina. It was independently domesticated in Central America and in the Andes (Gepts and Debouck,

1991), but is now cultivated all over the world. The common bean is consumed as both dry beans and green pods, and represents the most important legume for human nutrition (Broughton *et al*., 2003). Beans are essential components of people's diet, mostly in developing countries where they are a major source of protein, vitamins, minerals and fibre (Bellucci *et al*., 2014). *Phaseolus coccineus* L., the runner bean, was domesticated more than 2,000 years ago in Mexico where it is, at present, the second most important legume in the local diet after the common bean (Vargas Vázquez *et al*., 2012); it is also cultivated worldwide, albeit at a much smaller scale than the common bean. *Phaseolus vulgaris* is a typical rain fed crop, cultivated under diverse environmental conditions, including relatively dry areas (Graham and Ranalli, 1997; Singh, 2007), although it is not considered very tolerant to water stress (Molina *et al*., 2001; Cuellar-Ortiz *et al*., 2008). Actually, only about 7% of the bean cultivated area receives adequate rainfall (Broughton *et al*., 2003), and in some regions drought may cause the loss of most of the crop (Bellucci *et al*., 2014). For example, a drastic reduction of 80% seed yield has been reported in Romania, under natural drought conditions in the field, due to the combined effects of the reduction in the number of pods per plant, the number of seeds per pod and the average seed weight (Szilagyi, 2003). In Sub-Saharan Africa, about 40% of bean growing areas are affected by drought, and average yields there reach only 400 kg ha^{-1} $year^{-1}$, as compared to 1,500 kg ha^{-1} $year^{-1}$ reported from less drought-prone areas in eastern Africa (Namugwanya *et al*., 2014). Although water deficit has a general negative effect on crop productivity, large quantitative differences (between ca. 20% and 70% yield reduction) have been reported for different bean genotypes subjected to experimental drought conditions (Ramirez-Vallejo and Kelly, 1998).

As all major crops, the common bean is a glycophyte, sensitive to salt. Even relatively low soil salinity levels, below 2 dS m^{-1}, will significantly reduce crop productivity (Maas EV and Hoffman, 1977), although some cultivars appear to be more resistant to salt stress

(Szilagyi, 2003; Gama *et al*., 2007; Cuellar-Ortiz *et al*., 2008; Kaymakanova and Stoeva, 2008). About 20 to 30% of the bean-production areas in the Middle East are affected by soil salinity (Bayuelo-Jiménez *et al*., 2002) which causes a drastic yield reduction. At salinity level equal to 100 mM NaCl, pod yield per plant in this species decreased by 85% (De Pascale *et al*., 1997).

Considering the need to increase food production to feed a growing human population and the importance of beans in people's diet in many countries, on the background of global climate change, the genetic improvement of bean stress resistance is a matter of much interest. We have undertaken the screening of a large number of *Phaseolus* commercial cultivars and landraces of different origins, under standardised conditions, with the final aim of selecting several varieties with increased tolerance, which could be eventually included in breeding programmes for the enhancement of salt and water stress tolerance of beans. In addition, the characterisation of the physiological, biochemical and molecular mechanisms of stress response in those relatively tolerant cultivars will provide useful information for selecting the most appropriate approach for engineering stress tolerance in transgenic *Phaseolus* plants.

In the present work, designed as a 'proof-of-concept' of the aforementioned long-term project, we have analysed the relative tolerance to water and salt stress during vegetative growth in young plants of three *P. vulgaris* cultivars and in one cultivar of *P. coccineus*, a species that have been previously reported as being more stress-tolerant than common bean (Subbarao and Johansen, 1994). Responses to stress are dependent on the plant developmental stage, as it has been shown in different species (Läuchli and Epstein, 1990; Johnson *et al*., 1992; Vicente *et al*., 2004); this specific growth phase was selected because during vegetative development seedlings and young plants are generally more sensitive to

stress than adult plants and it should be easier to detect differences in the relative tolerance of the investigated cultivars.

The major aim of this comparative study was to get information on the biochemical mechanisms underlying plant tolerance to environmental stress and, specifically, to establish which responses to water and salt stress are the most relevant for tolerance in *Phaseolus*. We assumed that differences in the stress resistance of the selected cultivars could be explained by differences in the efficiency of the aforementioned general stress response mechanisms; that is, control of ion transport and maintenance of cellular osmotic balance. Therefore, we tried and correlated the degree of salt and/or water stress resistance of the different cultivars with the levels of ***i)*** toxic ions (Na^+ and Cl^-), and ***ii)*** different osmolytes (proline, glycine betaine and soluble carbohydrates) in the plants subjected to the salt and water stress treatments.

4.2.2. Materials and methods

4.2.2.1. Plant material

Two commercial cultivars (‘Maxidor’ and ‘The Prince’) and one Spanish local variety (‘Judía de Franco’) of *Phaseolus vulgaris* L. and one cultivar of *Phaseolus coccineus L.* (‘Moonlight’) were used in the present study. Seeds of ‘Judía de Franco’ were obtained from the Germplasm Bank of COMAV (*Institute* for Conservation and Improvement of Valencian Agro diversity, Polytechnic University of Valencia); *P. coccineus* seeds were obtained from Thompson and Morgan, AJP Garden and Crafts (UK), and those of *cvs*. 'The Prince' and 'Maxidor' were purchased from S.C. AGROSEM IMPEX S.R.L., Targu Mures (Romania).

4.2.2.2. Characterization of cultivars

'The Prince' is a dwarf French bean cultivar, with a bushy growth. Pods are green and flat, with a length of about 20 cm and large seeds (average weight: ca. 0.5 g per seed). It is an early cultivar; it takes 60-70 days for snap pods to form, and 90 days for dry seeds. *Cv*. 'The Prince' has been reported as drought sensitive (Boutraa and Sanders, 2001). The cultivar is one of the most commonly used in Europe.

'Maxidor' is also a dwarf French bean cultivar. It has a bushy growth, with precocious flowering. Pods are round and yellow, 12 cm long with relatively large seeds; the seed mass is about 0.26 g. It is considered as an early or medium-early variety; pods can be harvested 60-65 days after germination and dry beans after 100-110 days. 'Maxidor' is cultivated in several countries in Europe, and is one of the most commonly grown in Western Romania (Madoşă *et al*., 2013).

'Judía de Franco' is a local landrace of *P. vulgaris* from the province of Teruel (Spain). It has an indefinite growth, surpassing 3 m high. It reaches maturity after 96 days. Pods are 9 cm long, flat and green. The average weight of a seed is about 0.3 g.

Cv. 'Moonlight' belongs to the species *P. coccineus*. Seeds originated from Mexico. It reaches 3 m high. It has a longer biological cycle of 120-125 days. Pods are green and long, reaching 25 cm, and seed mass is about 0.45 g.

4.2.2.3. Growing conditions and stress treatments

Seeds were sterilised with a 0.3% (v/v) solution of sodium hypochlorite for 5 min, rinsed in distilled water and then sown on a moistened mixture of peat, perlite and vermiculite (2:1:1) in 0.5 L pots (Ø = 11 cm). One seed was placed per pot on the substrate surface. Throughout the germination process, the substrate was kept moderately moisten using Hoagland nutritive solution. Water and salt stress treatments (50, 100 and 150 mM NaCl) were started 16 days after germination, when the first trifoliate leaves had already

appeared. The control plants were watered twice a week with Hoagland nutritive solution (one litre per three plants), and for the salt stress treatments the plants were watered with the same volume of nutritive solution supplemented with the appropriate NaCl amounts prior to irrigation. Drought treatments were carried out by completely preventing irrigation. All the experiments were conducted for 3 weeks, including five plants per treatment, in an environment chamber under the following controlled conditions: long day photoperiod (16 hours of light and 8 hours of darkness), temperature (23ºC during the day and 17ºC at night), CO_2 level (≈ 300 ppm). Humidity ranged between 50-80% while all the treatments were underway.

4.2.2.4. Soil analysis

The electrical conductivity ($EC_{1:5}$) of the substrate was checked at the end of the treatments. Soil samples were taken from five pots per treatment, air-dried and then passed through a 2-mm sieve. A soil: water (1:5) suspension was prepared in distilled water and stirred for 1 h at 600 rpm and 21ºC. EC was measured with a Crison Conductivity meter 522 and is expressed in dS m^{-1}.

4.2.2.5. Plant growth parameters

After three weeks of salt or water stress treatment, the aerial part of each plant was collected and the following growth parameters were determined: stem length, leaf number, fresh and dry weight, and percentage of water content. Fresh material was stored at -20°C for further studies. To compare the degree of stress-induced inhibition of growth of the different cultivars, which differ in plant size, stem length and fresh weight were expressed as percentage of the values corresponding to the non-stressed controls. To determine water content, part of each sample was weighed (FW), dried at 65°C until constant weight (48-72

h), and then reweighed (DW); water content of each sample (%) was calculated as indicated in Gil *et al*. (2014).

4.2.2.6. Ion content measurements

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

4.2.2.7. Osmolyte quantification

Osmolyte contents (proline, glycine betaine and total soluble sugars) were determined in the stressed and control plants. Proline (Pro) was extracted with 3% (w/v) sulfosalicylic acid, from 0.2 g of frozen plant material in liquid nitrogen, and was quantified according to the acid-ninhydrin method described by Bates *et al*. (1973). Pro concentration was expressed as µmol g^{-1} DW.

Glycine betaine (GB) was determined from 0.1 g dry material according to Grieve and Grattan (1983). GB concentration was expressed as µmol g^{-1} DW.

Total soluble sugars (TSS) were measured in 0.1 g dry plant material suspended in 3 ml 80% (v/v) methanol, following the phenol / sulphuric acid method, as described by Dubois *et al*. (1956). TSS contents were expressed as ‘mg equivalent of glucose’ per g DW.

4.2.2.8. HPLC analysis of carbohydrates

The soluble sugar fraction (mono and oligosaccharides) was analysed using a Waters 1525 high performance liquid chromatography coupled to a 2424 evaporative light scattering detector (ELSD). The source parameters of ELSD were the following: gain 75, data rate 1 point per second, nebulizer heating 60%, drift tube 50ºC, and gas pressure 2.8 kg cm^{-2}. The analysis was carried out injecting 20 µL aliquots of the samples with a Waters 717 autosampler into a Prontosil 120-3-amino column (4.6 x 125 mm; 3 µm particle size) maintained at room temperature. An isocratic flux (1 mL/min) of 85% acetronitrile (J.T. Baker) during 25 minutes was applied in each run. Standards of glucose, fructose, sucrose and *myo*-inositol served to identify peaks by co-injection. Sugars were quantified with peak integration using the Waters Empower software and comparison with glucose, fructose, sucrose, and *myo*-inositol standard calibration curves.

4.2.2.9. Statistical analysis

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

4.2.3. Results

4.3.3.1. Electric Conductivity of substrates

Electric conductivity ($EC_{1:5}$) was recorded in samples of the pot substrates after three weeks of salt and water stress treatments (Table 1). For all cultivars, a similar increase in

$EC_{1:5}$ was detected in parallel to the increase of NaCl concentrations, which confirms the high correlation between EC and the concentration of the saline solutions used in the treatments. Nearly no statistical significant differences were found between different cultivars for each treatment. The EC of the pot substrates in the water stress treatments were lower than in the controls watered with Hoagland solution (except in 'The Prince' where it increased slightly), which can be explained by accumulation of ions present – at low concentrations – in the nutritive solution.

4.3.3.2. Effect of salt stress on plant growth

The stem length of five plants of each cultivar was measured at the end of the treatments. The average stem length of the control plants was considered as 100% for each cultivar (absolute values are specified in the legend of Fig. 1 for each control), and decreased in a concentration-dependent manner upon salt treatment. In the presence of 150 mM NaCl the stem length was reduced by 40% in *P. coccineus* and *P. vulgaris cv*. 'The Prince', by more than 60% in *cv*. 'Judía de Franco', but by only 20% in *cv*. 'Maxidor' (Fig. 1a).

The number of trifoliate leaves also decreased during the salt treatments, with some differences among cultivars (Fig. 1b). 'The Prince' appeared to be the most affected by salt, as the number of leaves already dropped by more than 50% at the lower concentration tested, 50 mM NaCl, and by ca. 75% in the presence of 150 mM NaCl, whereas the reduction of average leaf number in *P. coccineus* and *P. vulgaris cv*. 'Maxidor' under the same conditions was less than 5% – a not significant difference – and less than 50%, respectively. An intermediate behaviour was observed in *cv*. 'Judía de Franco' (Fig. 1b).

The fresh weight (FW) of five harvested plants per treatment and cultivar was measured, and the mean of the control plants was taken as the reference (100%) for each cultivar. The greatest reduction of fresh mass of the aerial part of the plants was registered in

cv. 'The Prince', with values of ca. 80% after treatment with 100 mM NaCl and 95% in the presence of 150 mM NaCl. The least affected by salt stress appeared to be *cv*. 'Maxidor' – about 63% decrease in FW in the 150 mM NaCl treatment – followed by 'Judía de Franco', and *P. coccineus* (Fig. 1c).

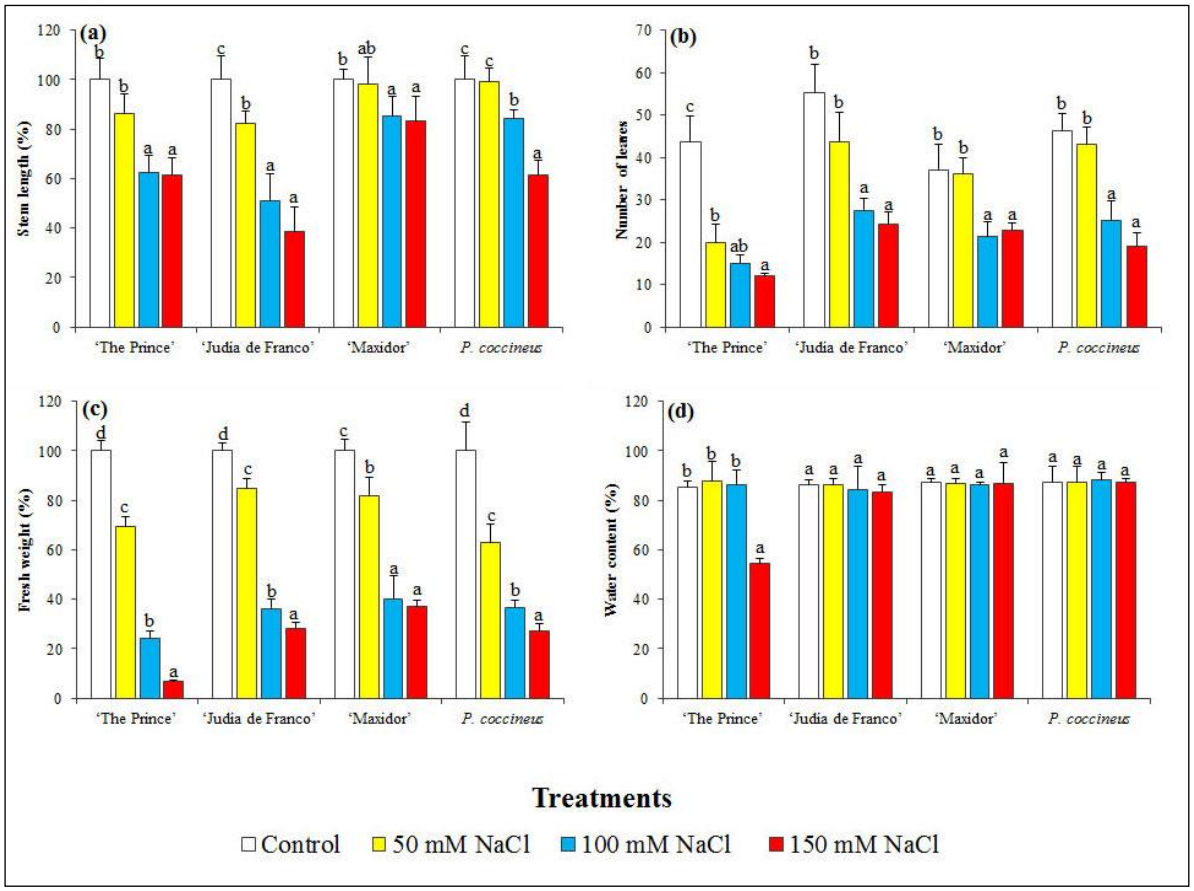

Fig. 1 Salt stress-induced changes in: (a) stem length (%), with the stem lengths of control, non-treated plants (*Phaseolus vulgaris*, cv. 'The Prince': 60.00 cm; cv. 'Judía de Franco': 174.61 cm; cv. 'Maxidor': 44.16 cm; *Phaseolus coccineus*: 219.00 cm) considered as 100% for each cultivar; (b) number of leaves; (c) Fresh weight (%), with the fresh weight of control plants (*Phaseolus vulgaris*, cv. 'The Prince': 31.54 g; cv. 'Judía de Franco': 34.87 g; cv. 'Maxidor': 17.17 g; *Phaseolus coccineus*: 30.26 g) considered as 100% for each cultivar; (d) water content (%). Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

A decrease in water content would reflect the loss of water in the plants as a result of the salt treatments. Yet, beans appear to have relatively efficient mechanisms to avoid dehydration of their aerial parts since, in general, no significant reduction of water content was observed in the salt-treated plants. Only in *P. vulgaris cv*. 'The Prince', and only at the

highest NaCl concentration tested (150 mM), a significant decrease in water content, of nearly two-fold, was detected (Fig. 1d); this may partly explain the higher sensitivity to salt of this cultivar indicated by the aforementioned growth measurements, as compared to the other varieties.

4.3.3.3. Ion contents

The patterns of sodium accumulation in the aerial part of the plants upon salt stress treatment were different for the *P. vulgaris* cultivars: Na^+ levels rose four to five-fold in the plants treated with 100 and 150 mM NaCl in *cv*. 'The Prince'; a two-fold increase was measured in 'Judía de Franco', but only at the highest salt concentration, whereas no statistically significant Na^+ variation was detected in *cv*. 'Maxidor'; in *P. coccineus*, a very small, but significant increase in Na^+ content was observed in the presence of 150 mM NaCl (Fig. 2a). Contrary to sodium, the four tested cultivars showed a similar qualitative pattern regarding chloride accumulation: in all of them Cl^- contents increased significantly, roughly in parallel with increasing external NaCl concentrations. In the presence of 150 mM NaCl, Cl^- levels rose up to 10 to 15-fold in *P. coccineus* and *P. vulgaris cvs*. 'The Prince' and 'Judía de Franco', or ca. five-fold in *cv*. 'Maxidor', as compared to the control, non-treated plants (Fig. 2b). Comparing the levels of both cations, it is clear that the salt-treated plants accumulated much more Cl^- than Na^+ in their aerial parts, again with quantitative differences among cultivars: in the presence of high NaCl concentrations, Cl^-/Na^+ rations of, approximately, 5, 10, 10 and more than 15 were calculated for 'The Prince', 'Judía de Franco' Maxidor' and *P. coccineus*, respectively (Fig. 2a, b). Plant potassium contents showed some variations some cultivars under study, however non that can be correlated with the increased salt concentrations (Fig. 2c).

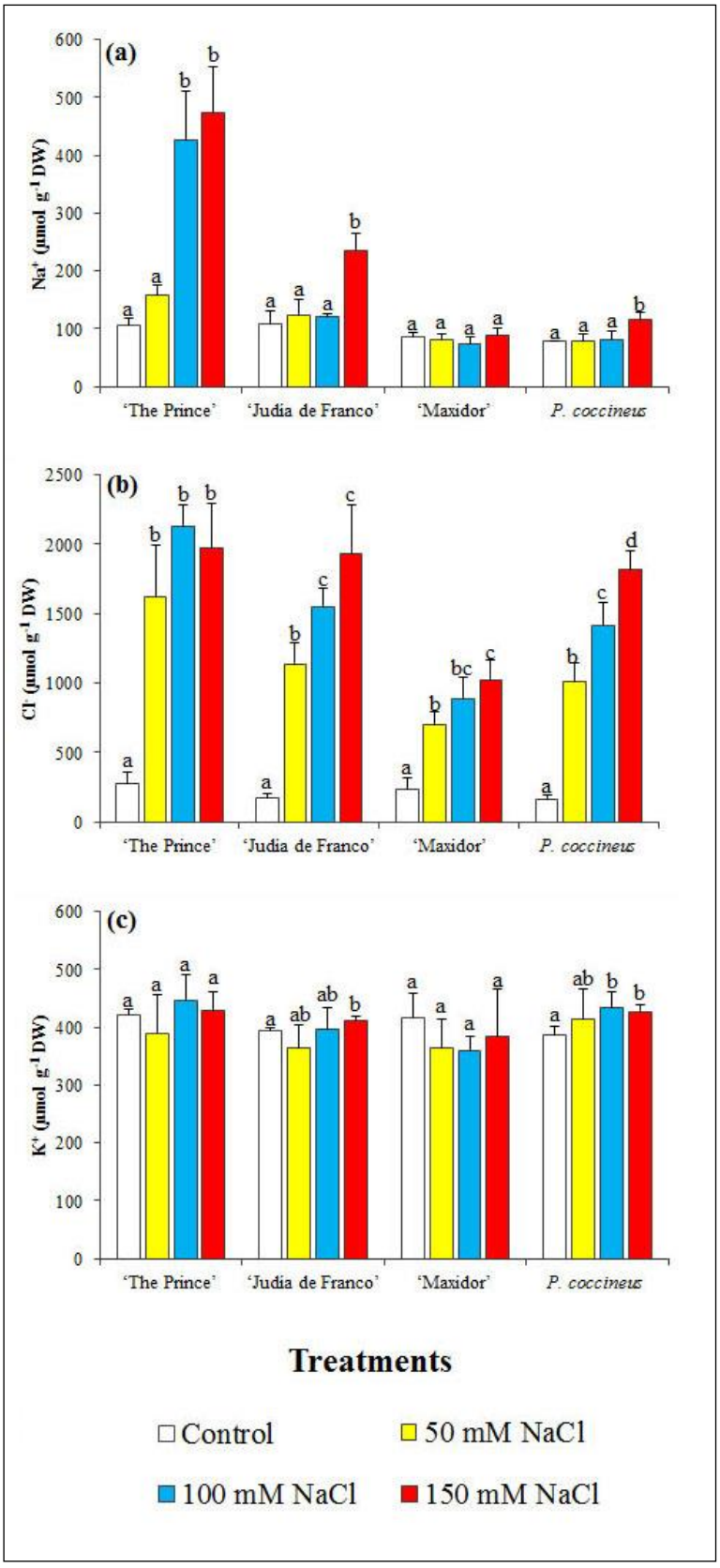

Fig. 2 Salt stress-induced changes in: (a) sodium, (b) chloride, and (c) potassium contents in leaves of *Phaseolus* plants of the studied cultivars. Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

4.3.3.4. Osmolyte contents

Proline contents in the control plants were relatively low and similar in all cultivars (2-4 µmol g^{-1} DW), but increased significantly in the presence of 100 and 150 mM NaCl, for all cultivars except 'Maxidor'; the highest Pro concentrations were reached in *cv*. 'The Prince', 12 and 42 µmol g^{-1} DW in plants treated with 100 and 150 mM NaCl, respectively; the corresponding values for 'Judía de Franco' and *P. coccineus* were 8 and 22 µmol g^{-1} DW. In *cv*. 'Maxidor', a significant increase in Pro levels was only detected in the presence of 150 mM NaCl, reaching a concentration of only 9 µmol g^{-1} DW, much lower than in the other cultivars (Fig. 3a).

Apparently, glycine betaine plays no role in responses to salt stress of the investigated *Phaseolus* cultivars, since no significant salt-induced changes in the levels of this osmolyte were detected in any of the treatments (Fig. 3b). The absolute GB concentrations were similar for *P. coccineus* and *P. vulgaris*, *cvs*. 'The Prince' and 'Maxidor', between 15 and 20 µmol g^{-1} DW, and slightly higher in *cv*. 'Judía de Franco' (Fig. 3b).

Mean values of total soluble sugars did not show a clear pattern of variation in response to the salt treatments for any of the selected cultivars, and the observed differences, in general, were not statistically significant, possibly due in part to the variability in the TSS contents of individual plants, reflected in relatively large SD of the mean values (Fig. 3c). Here again, 'Judía de Franco' presented slightly higher TSS levels under stress than those measured in the other studied cultivars (Fig. 3c).

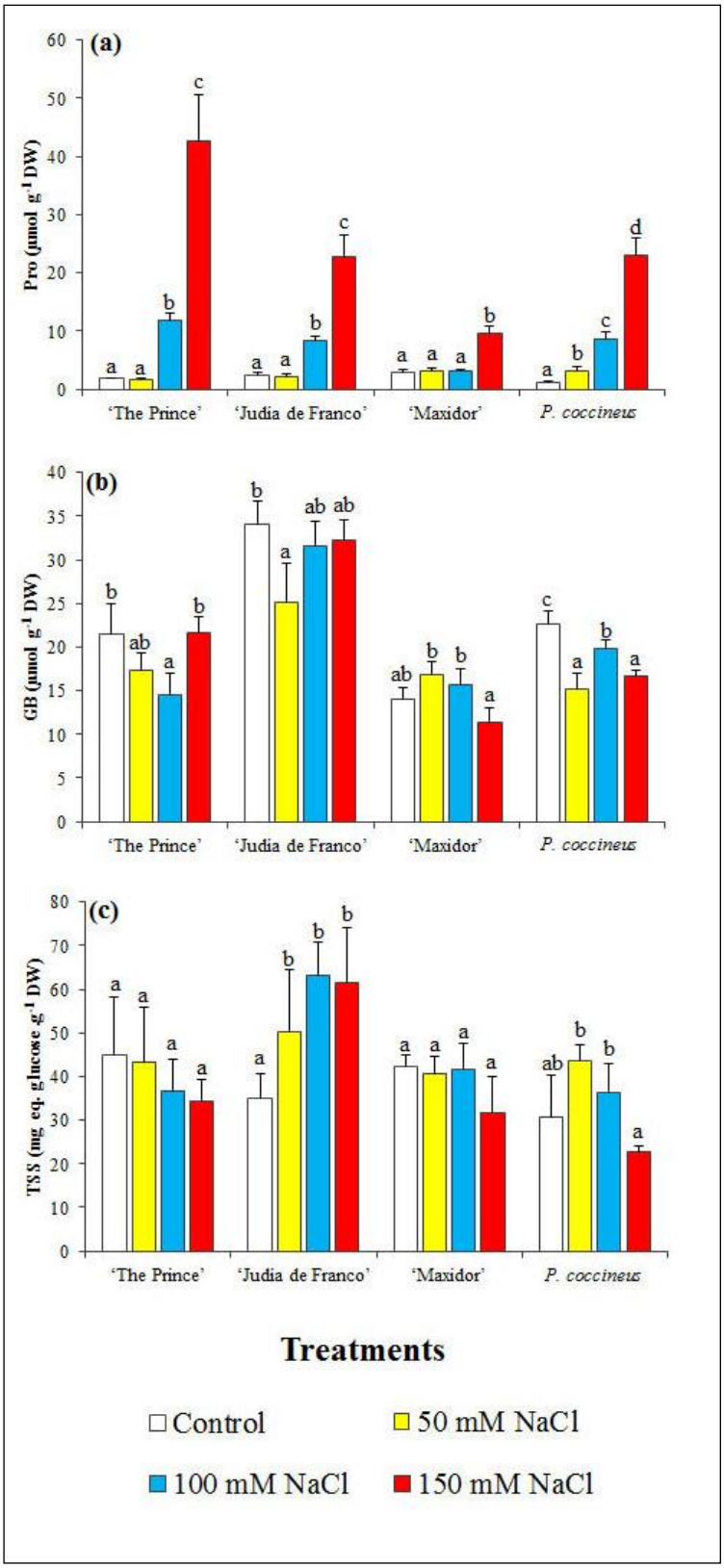

Fig. 3 Salt stress-induced changes in the levels of: (a) proline (Pro), (b) glycine betaine (GB), and (c) total soluble sugars (TSS) in the same samples as in Fig. 2. Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

The major soluble carbohydrates in the extracts were identified and quantified by HPLC, and corresponded to fructose, sucrose and *myo*-inositol. Their pattern of variation was different in the four cultivars analysed. A clear salt-dependent increase in fructose and sucrose was only detected in *cv*. 'The Prince', whereas in the other cultivars changes in the concentrations of both sugars were not statistically significant or did not correlate with external salinity (Figs. 4a and b). A considerable increase of *myo*-inositol was detected in *P. vulgaris cv*. 'Maxidor' and, to a lesser extent, in *P. coccineus*, in parallel with increasing salt concentrations in the watering solution – reaching in the presence of 150 mM NaCl about three-fold and two-fold, respectively, higher levels than in the non-stressed controls – but not in the other two studied varieties (Fig. 4c).

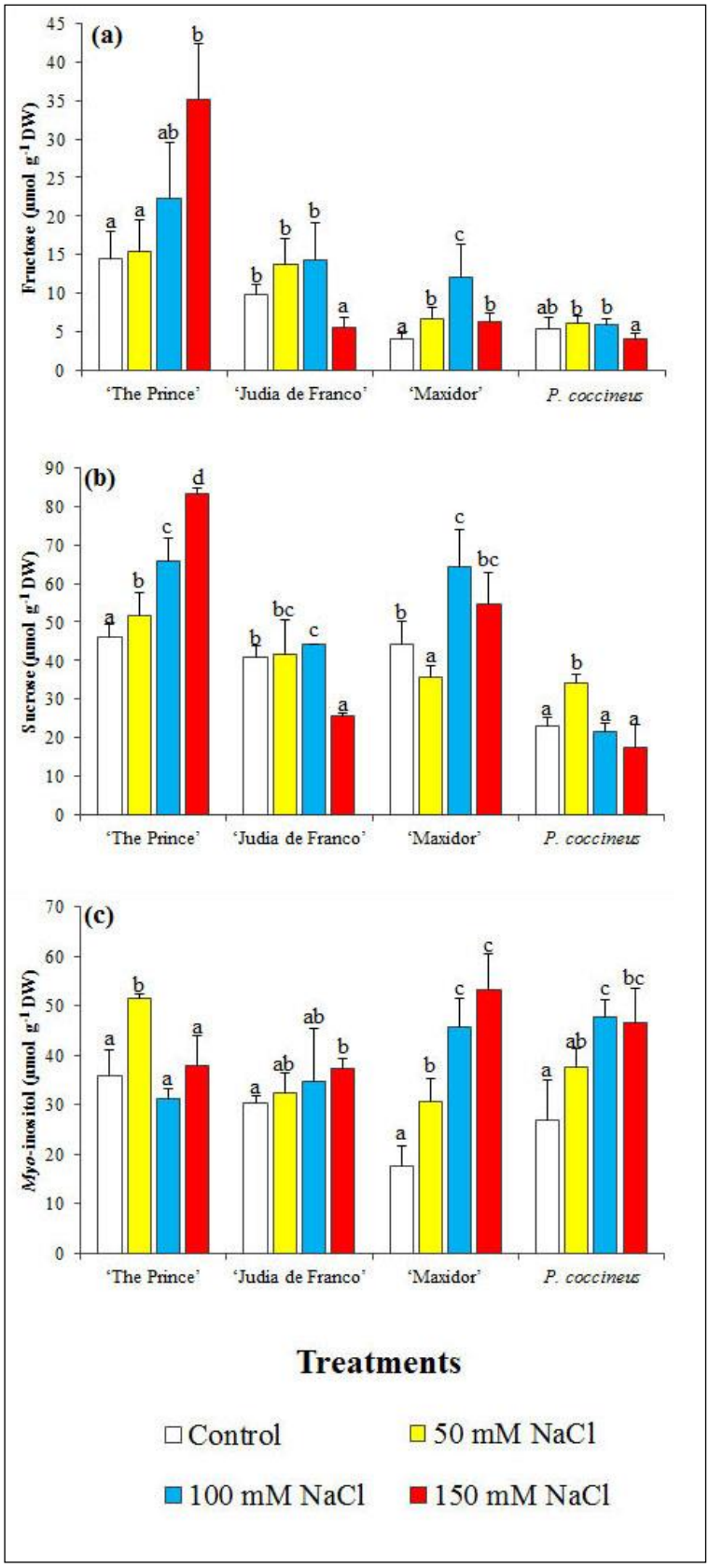

Fig. 4 Salt stress-induced changes in the levels of: (a) fructose, (b) sucrose, and (c) myo-inositol, separated by HPLC, in the same samples as in Fig. 2. Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

4.3.3.5. Effect of water stress on growth and osmolyte contents

Lack of irrigation inhibited growth in all selected cultivars (Fig. 5), which showed a relative resistance to water stress similar to that observed under salt stress conditions (Fig. 1). Thus, regarding the relative decrease of stem length, the most affected cultivars were 'The Prince' and 'Judía de Franco', with a 60% reduction, followed by *P. coccineus* with only a 40%, while *cv*. 'Maxidor' plants did not show any reduction of stem length under water stress conditions (Fig. 5a). The number of trifoliate leaves was reduced in all cultivars after the stress treatment, mostly in 'The Prince' (ca. 4.5-fold), followed by 'Judía de Franco' (2.7-fold) and *P. coccineus* (2.2-fold), with the smallest decrease, about 1.7-fold, observed again in *cv*. 'Maxidor' (Fig. 5b). The fresh weight of the plants of the four cultivars was strongly affected by drought, even more than by salt stress; a reduction of 97% was registered in 'The Prince', around 90% in 'Judía de Franco' and *P. coccineus*, and 67% in 'Maxidor' (Fig. 5c). In this case, however, the decrease of fresh weight was partly due to loss of water, which was significant, albeit quantitatively different, for all cultivars. Again, the strongest reduction of water content, ca. 85% was measured in *cv*. 'The Prince', in comparison with 32% in 'Judía de Franco', 20% in *P. coccineus* and 18% in 'Maxidor' (Fig. 5d). These data confirm that the mechanisms to avoid cellular dehydration under stress are much less efficient in *cv*. 'The Prince' than in the other studied varieties, as shown also for the salt treatments.

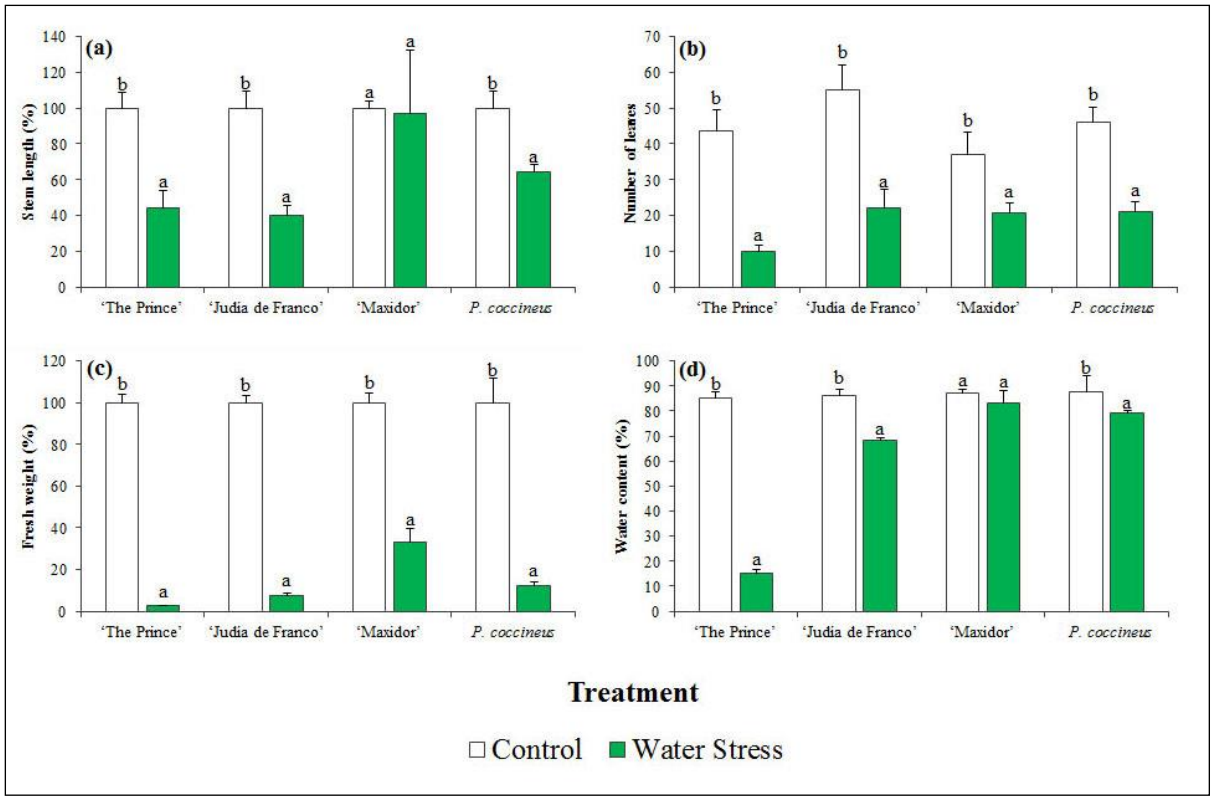

Fig. 5 Water stress-induced changes in: (a) stem length (%), with the stem lengths of control, non-treated plants (mentioned in the legend of Fig. 1) considered as 100% for each cultivar; (b) number of leaves; (c) Fresh weight (%), with the fresh weight of control plants (mentioned in the legend of Fig. 1) considered as 100% for each cultivar; (d) water content (%). Stem lengths and fresh weights of control plants as in Fig. 1. Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

As expected, in all cultivars, ion levels (Na^+, Cl^-, K^+) did not significantly change in water-stressed plants when compared to the corresponding controls (Table 2).

Proline levels increased under drought stress by 12 to 15-fold in plants of *P. vulgaris cvs*. 'The Prince' and 'Judía de Franco', and in *P. coccineus* plants, but by only four-fold in those of *cv*. 'Maxidor' (Fig. 6a). Glycine betaine showed no significant variation under water stress conditions in the three cultivars of *P. vulgaris*, but an almost half-fold increase in *P. coccineus* (Fig. 6b). The measured mean amounts of total soluble sugars did not vary significantly in any of the investigated *Phaseolus* varieties (Fig. 6c).

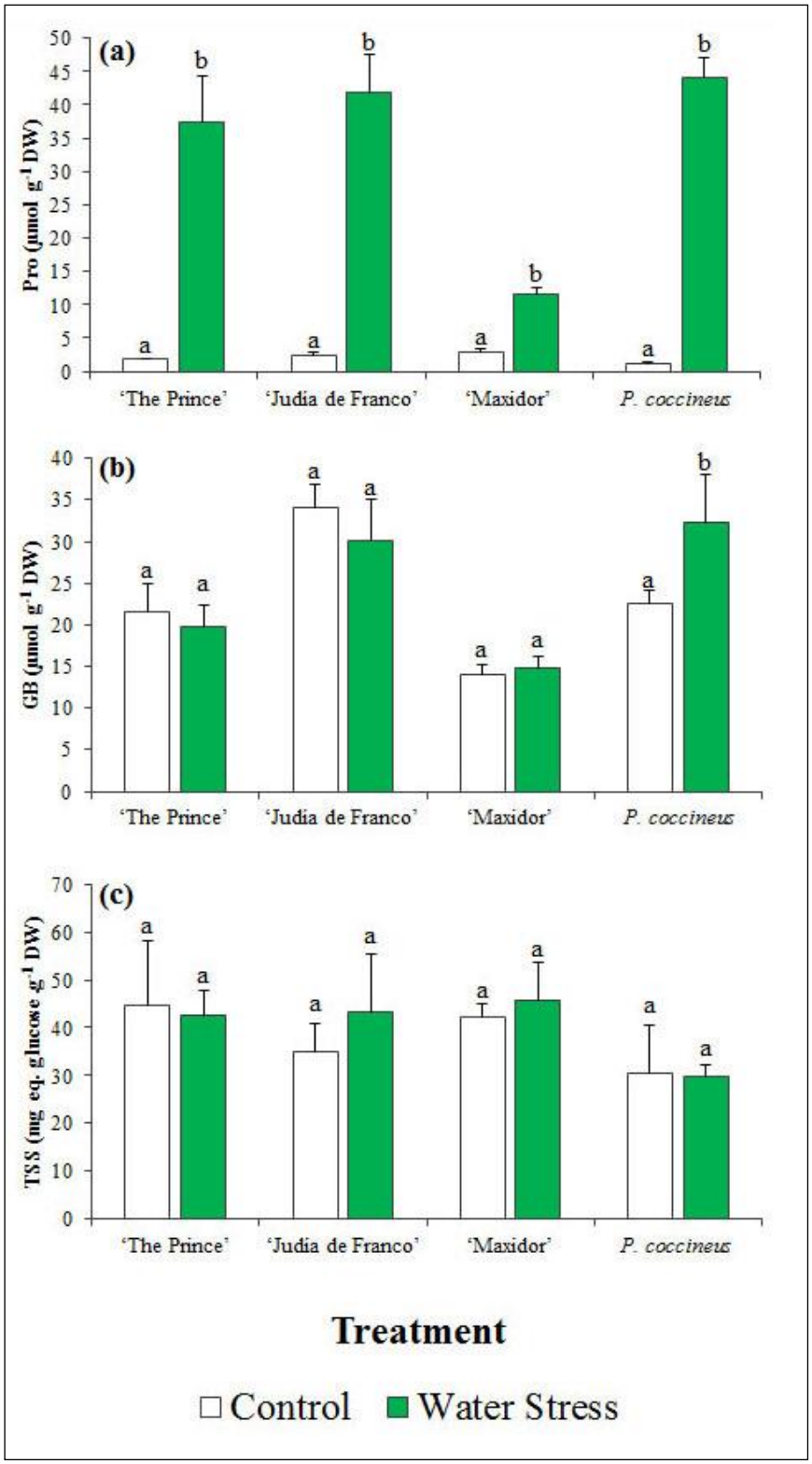

Fig. 6 Water stress-induced changes in the levels of: (a) proline (Pro), (b) glycine betaine (GB), and (c) total soluble sugars (TSS) in leaves of *Phaseolus* plants of the studied cultivars. Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

However, some differences between stressed and control plants were found when analysing individual soluble sugars, separated by HPLC. Fructose levels increased significantly in *P. vulgaris cv*. 'Judía de Franco', but differences were not detected in *cv*. 'The Prince', nor in *P. coccineus* (Fig. 7a), while a decrease in sucrose contents was observed in all varieties, except 'The Prince' (Fig. 7b). Yet, the most interesting finding was the large increase in *myo*-inositol contents measured in plants of *cv*. 'Maxidor' and, to a lesser extent, of *P. coccineus* subjected to water stress (Fig. 7c).

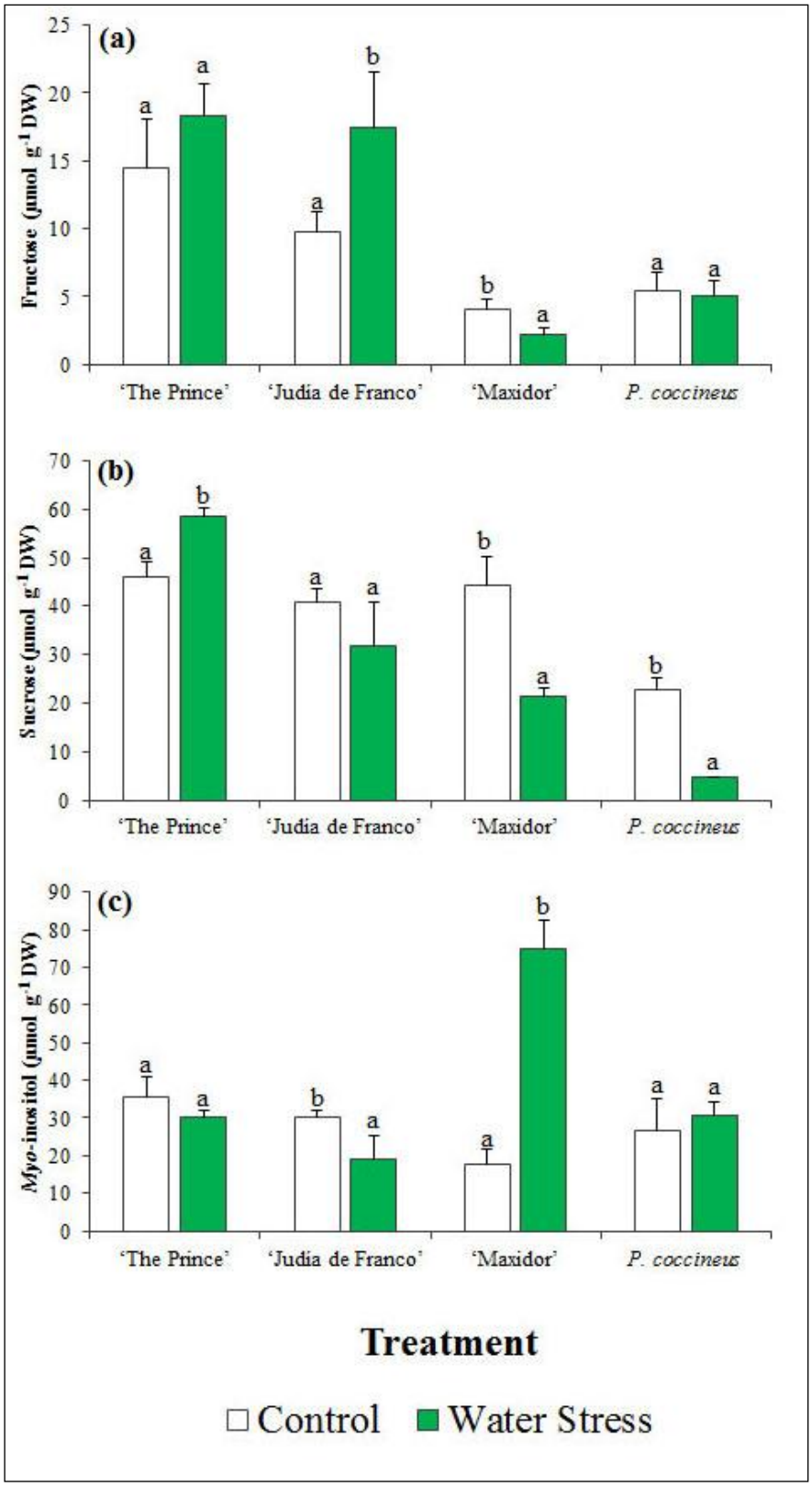

Fig. 7 Water stress-induced changes in the levels of: (a) fructose, (b) sucrose, and (c) myo-inositol, separated by HPLC, in the same samples as in Fig. 6. Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

4.2.4. Discussion

Water deficit in the soil due to lack of rain or irrigation (drought stress), or high concentration of salts in the soil solution (salt stress) both cause lowering of the water potential, leading to cellular dehydration (osmotic stress). Apart from this osmotic component, high soil salinity has a number of additional deleterious effects for plants due to salt toxicity, mostly of Na^+ ions, which when present at too high levels inhibit many enzymatic activities and basic cellular processes, such as mRNA processing and protein synthesis; moreover, the high concentration of Na^+ and Cl^- results in nutrient imbalance through the reduction of uptakes of K^+, NO_3^- and PO_4^{3-} (Greenway and Munns, 1980; Forment *et al*., 2002; Bartels and Sunkar, 2005; Munns and Tester, 2008). Therefore, plant responses to water and salt stress are similar and differ mostly in ion transport-related processes. It has even been considered that the initial response to salt is identical to that induced by drought, and is triggered by its osmotic component, while salinity-specific responses due to ion toxicity develop only over time with longer exposure to stress (Munns, 2002). This overlap explains that often the physiological effects of water stress and high salt concentrations are the same, as we have observed in the present work with different *Phaseolus* cultivars. There are numerous papers reporting a wide range of salt (Gama *et al*., 2007; Kaymakanova and Stoeva, 2008) and drought tolerance (Cuellar-Ortiz *et al*., 2008; Santos *et al*., 2009; Omae *et al*., 2012) within this genus, supporting the possibility of selecting the most resistant genotypes to be used in breeding programmes to improve the stress tolerance of beans.

The most general effect of abiotic stress, and the easiest to quantify, is inhibition of growth, which allows plants to survive under adverse conditions by re-directing their resources (metabolic precursors and energy) from normal metabolism and growth to the activation of specific stress defence mechanisms (Zhu, 2001; Munns and Tester, 2008).

Determination of several growth parameters allowed us to establish the relative degree of resistance to water and salt stress of the investigated bean varieties. *Phaseolus vulgaris cv*. 'The Prince' was the most sensitive, followed by 'Judía de Franco', while *cv*. 'Maxidor' was the most stress-resistant of the selected common bean cultivars – even more than the tested *P. coccineus* cultivar, although this species has been previously reported as being more tolerant than *P. vulgaris* (Subbarao and Johansen, 1994; Gutierrez *et al*., 2009).

It is well established that all plants share a series of basic, conserved mechanisms of response to abiotic stress, including for example the control of ion transport and the accumulation of different osmolytes in the cytoplasm to help maintain cellular osmotic balance (Zhu, 2001; Munns and Tester, 2008). Yet, it is not so clear the relative importance and contribution of those response mechanisms to the stress tolerance of a particular species. The comparative analysis of related genotypes, such as that reported here for several *Phaseolus* cultivars, may help to answer this question, by correlating their relative degree of resistance to stress with changes in the levels of stress markers associated to specific responses. Our results indicate, for example, that one of the main reasons behind salt resistance in *Phaseolus* is the presence of mechanisms that restrict the transport of Na^+ to the aerial part of the plants, mechanisms that are more efficient in the relatively more tolerant cultivars.

It is known that plants of the genus *Phaseolus* are able to exclude sodium from the shoots, even in the presence of relatively high NaCl concentrations in the soil (Seemann and Critchley, 1985). Earlier autoradiography studies on *P. vulgaris* indicated that Na^+ is retained in roots by binding at sites in the stele or at those bordering it (Jacobi, 1964). This was confirmed later-on by Kramer *et al*. (1977) using X-ray microanalyses in *P. coccineus*, which also excluded Na^+, but not Cl^-; these authors proposed that in this species Na^+ is also reabsorbed from vessels by xylem parenchyma cells. In more recent studies, a higher Na^+

concentration in roots than in leaves was found in *P. vulgaris* and *P. latifolius*, showing that, within this genus, the basic sodium exclusion mechanisms are related to its transport from roots to shoots, rather than to absorption by roots (Bayuelo-Jiménez *et al*., 2012). The anion Cl^-, on the other hand, displays high mobility within the plant and is not effectively compartmentalised in cells: chloride concentration was high in both the vacuole and the chloroplast-cytoplasm in salt-stressed plants of *P. vulgaris* (Seemann and Critchley, 1985). The salt-sensitive *Phaseolus* genotypes analysed by Bayuelo-Jiménez *et al*. (2012) gave a higher Cl^- concentration in leaves than more tolerant ones. In all cultivars analysed in the present work, Na^+ concentration in leaves was clearly lower than that of Cl^-, under the same external conditions, thus confirming this mechanism. Yet, the pattern of ion accumulation in response to increasing NaCl concentration in the nutritive solution varied, according to the relative salt tolerance of the different cultivars, with the lowest ion contents measured in the most tolerant, *cv*. 'Maxidor', and the highest in the most sensitive, *cv*. 'The Prince'.

Since Na^+ can compete with K^+ for the same transporters, a common effect of salt stress is to lower K^+ levels (Munns and Tester, 2008); mechanisms able to increase K^+ contents in leaves, thus maintaining relatively low Na^+ / K^+ ratios, would therefore contribute to salt tolerance. Increased K^+ in foliar tissue upon salt treatments has been reported in beans by Seeman and Critchley (1985), while Bayuelo-Jiménez *et al*. (2012) observed similar values in plants at moderate salinity levels and in the controls. These latter authors also reported reduced K^+ contents in roots and stems of salt-treated plants, and explained the differences in the distribution of this cation in the plants by the translocation of K^+ from roots and stems to leaves due to activation of highly selective K^+ transporters. Maintenance of constant K^+ concentration in leaves was considered as an important criterion to assess the drought tolerance of alfalfa genotypes (Benabderrahim *et al*., 2015). The levels of K^+ in leaves were similar for all four *Phaseolus* cultivars tested here, and did not vary significantly

in the presence of salt; therefore, Na^+ / K^+ ratios in leaves were dependent on Na^+ contents, and lower in the more tolerant than in the more sensitive cultivars.

Another general response to abiotic stress is the synthesis and accumulation of specific osmolytes in the stressed plants. Yet, it is often difficult to establish whether the stress-dependent increase in the concentration of a particular osmolyte has a functional role in the mechanisms of tolerance of a given species. When accumulated at high enough levels, osmolytes will contribute to cellular osmotic balance and therefore to stress tolerance (Ashraf and Foolad, 2007) – although the concentration required to have a relevant osmotic effect will also depend on the intracellular distribution of the compound and the relative volume of the cytoplasm in the cell. However, osmolytes may significantly contribute to stress tolerance based on their additional roles as 'osmoprotectants': low-molecular-weight chaperons, ROS scavengers, or as signalling molecules involved in regulation of gene expression and metabolic processes (Smirnoff and Cumbes, 1989; Ashraf and Harris, 2004; Szabados and Savouré, 2010). Comparative studies on the stress responses of related taxa should provide evidence in favour of the contribution to stress tolerance of osmolyte accumulation, if a positive correlation between osmolyte levels and the relative degree of tolerance can be found.

Proline is one of the commonest osmolytes in plants. Many reports have shown significant increases in Pro contents in *Phaseolus* plants submitted to either salt stress (Kaymakanova and Stoeva, 2008; Nagesh-Babu and Devaraj, 2008) or water stress (Ashraf and Iram, 2005; Svetleva *et al*., 2012). Pro seems to be a reliable marker of stress in *Phaseolus*, as in many other genera, but the correlation between Pro accumulation and stress tolerance remains unclear, since apparently contradictory results have been obtained when comparing different varieties. Higher free Pro levels have been reported in more drought-tolerant (Ghanbari *et al*., 2013) or salt-tolerant (Cárdenas-Avila *et al*., 2006) bean cultivars

than in less tolerant ones. However, there are also reports of higher Pro levels in sensitive than in more resistant cultivars, in *P. vulgaris* (Jiménez-Bremont *et al*., 2006; Rosales *et al*., 2012; Zadehbagheri *et al*., 2012), as well as in many other plant genera where no positive correlation between Pro contents and tolerance has been found (Lutts *et al*., 1996; Guerrier, 1998; Ashraf and Foolad, 2007; Chen *et al*., 2007).

Our experimental approach should help to clarify the confusion often found in the literature between the concepts of 'stress responses' and 'stress tolerance'. The present study revealed that Pro accumulation is a common response to salt and water stress in the four cultivars analysed here, in agreement with the aforementioned published results. Yet, Pro biosynthesis cannot contribute significantly to their stress tolerance, since the levels reached in the most tolerant variety, *cv.* 'Maxidor', were by far lower than in the other cultivars. In this case, Pro should be considered as a marker of the level of stress affecting the plants, and these results simply reflect the fact that 'Maxidor' plants were less stressed than the others.

Glycine betaine is another osmolyte synthesised in response to salt and water stress in many different plant groups (Hanson and Scott, 1980; Rhodes and Hanson, 1993; Ashraf and Foolad, 2007). There are only a handful of references describing the presence of this osmolyte in *Phaseolus* (Ashraf and Iram, 2005; Ali and Abdel-Fattah, 2006), although at concentrations lower than those reported here, which are in turn much lower than the GB levels recorded in real GB accumulator species (Khan *et al*., 2000; Tipirdamaz *et al*., 2006). However, the applied stress treatments did not lead to significant increases of GB contents in the analysed *Phaseolus* varieties, with the exception of water-stressed *P. coccineus* plants, suggesting that GB may contribute to drought tolerance in this species.

Assessing the role of soluble sugars (e.g. sucrose, glucose or fructose) in the mechanisms of stress tolerance has the added difficulty that their function as compatible solutes may be masked by their multiple functions in plants, as direct photosynthesis

products, components of primary metabolism and regulatory molecules (Gil *et al*., 2013). There are several papers dealing with the variation of soluble carbohydrate contents in beans under stress conditions (Vassey and Sharkey, 1989; Tazuke *et al*., 2009; Sassi *et al*., 2010), but it has been recently reported that salt stress-induced sugar accumulation in the genus *Phaseolus* barely contributes to the leaf osmotic potential (Bayuelo-Jiménez *et al*., 2012). We did not detect significant changes, correlated with the stress treatments, in the levels of total soluble sugars in the analysed *Phaseolus* cultivars. However, after separation of the carbohydrate fraction by HPLC, strong increases in *myo*-inositol contents were observed in *cv*. 'Maxidor', the most tolerant cultivar of *P. vulgaris*, and to a lesser extent also in *P. coccineus*; *myo*-inositol accumulation was induced in the presence of NaCl, in a concentration-dependent manner, but mostly in water-stressed plants. Therefore, this polyalcohol appears to play a significant role in the stress tolerance mechanisms in *Phaseolus* taxa. To our knowledge, publications on the presence of *myo*-inositol in this genus are scarce; for example, Bahena-Betancourt *et al*. (2006) reported glucose and inositol as the major protectant sugars in salt stressed beans, but we did not detect a significant variation of glucose in the cultivars analysed here.

4.2.5. Conclusions

We have determined the relative tolerance to salt and water stress of four *Phaseolus* cultivars during the stage of vegetative growth; resistance to both stresses is highest in *P. vulgaris cv*. 'Maxidor', followed by *P. coccineus*, while the cultivar most sensitive to stress appears to be *P. vulgaris cv*. 'The Prince'. Correlation of these data with changes in the levels of ions and several osmolytes, allowed establishing which mechanisms of response to stress that are the most relevant for tolerance in *Phaseolus*. In conditions of high soil salinity,

resistance to stress is mostly based on restriction of Na^+ (and, to a much lesser extent, also of Cl^-) transport to shoots, and on the accumulation of *myo*-inositol as the major functional osmolyte. Under water stress conditions, the most relevant response is the increase of *myo*-inositol levels, although in *P. coccineus* accumulation of glycine betaine may also contribute to drought tolerance. Similar comparative analyses of additional bean varieties with different degrees of stress resistance, selected after screening of a much larger number of *Phaseolus* cultivars and landraces, will help to confirm the conclusions of the present work.

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Tab. 1 Variation in Electric conductivity (EC1:5, dS m^{-1}) of the substrate samples after 3-week treatments. Electric conductivity (EC1:5, dS m^{-1}) of the substrate samples after 3-week treatments of the *Phaseolus* plants with the indicated NaCl concentrations or subjected to water stress.The values shown are means with SD (n= 5). Different lower case letters in each row indicate significant differences between cultivars experiencing the same treatment according to the Tukey test (α = 0.05).

Treatment	*P. vulgaris cv.* 'The Prince'	*P. vulgaris cv.* 'Judía de Franco'	*P. vulgaris cv.* 'Maxidor'	*P. coccineus cv.* 'Moonlight'
Control	0.34 ± 0.06a	0.62 ± 0.04b	0.75 ± 0.10b	0.69 ± 0.08b
50 mM NaCl	1.84 ± 0.38ab	1.85 ± 0.33ab	1.66 ± 0.27a	2.18 ± 0.04b
100 mM NaCl	2.59 ± 0.46a	2.63 ± 0.28a	2.61 ± 0.18a	2.57 ± 0.20a
150 mM NaCl	3.62 ± 0.50a	3.74 ± 0.61a	3.07 ± 0.93a	3.75 ± 0.07a
Water stress	0.43 ± 0.03ab	0.41 ± 0.01a	0.44 ± 0.06ab	0.50 ± 0.05b

Tab. 2 Drought stress-induced changes in ions (Na+, Cl-, and K+). Drought stress-induced changes in sodium, chloride, and potassium contents in leaves of plants of the studied *Phaseolus* cultivars. Measurements were performed after three weeks of treatment. The values shown are means with SD (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

Ion (µmol.g^{-1} DW)	Treatment	*P. vulgaris* *cv.* 'The Prince'	*P. vulgaris* *cv.* 'Judía de Franco'	*P. vulgaris* *cv.* 'Maxidor'	*P. coccineus* *cv.* 'Moonlight'
Na^+	Control	106,14 ± 12,3a	108,25 ± 21,93a	85,82 ± 6,48a	77,82 ± 1,14a
	WS	109.32 ± 4.03a	114.65 ± 10.57a	86.68 ± 4.31a	86.74 ± 7.56a
Cl^-	Control	273.19 ± 82.02a	175.28 ± 30.73a	231.76 ± 89.22a	158.5 ± 31.16a
	WS	285.63 ± 27.96a	188.99 ± 55.32a	244.91 ± 30.06a	175.31 ± 24.54a
K^+	Control	422.63 ± 7.72a	394.51 ± 4.52a	415.76 ± 43.24a	387.68 ± 13.01a
	WS	446.23 ± 33.05a	401.67 ± 14.08a	452.06 ± 8.1b	386.42 ± 21.5a

Publication III:

Subchapter 4.3.

Effects of Salt and Water Stress on Three Ecologically Distinct Plantago Species

This manuscript has been submitted to the Journal; *Frontiers in Plant Science*, and is currently under review.

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Effects of Salt and Water Stress on Three Ecologically Distinct *Plantago* Species

Abstract. Comparative studies on the responses to abiotic stress of taxonomically related taxa should help to elucidate relevant mechanisms of stress tolerance in plants. We have applied this strategy to three *Plantago* species adapted to different natural habitats, *P. crassifolia* and *P. coronopus* – both halophytes – and *P. major*, considered as salt-sensitive. Stress-induced growth inhibition and accumulation of monovalent ions and specific osmolytes were determined in plants after four weeks to salt or water stress treatments. Our results confirm that salt tolerance in this genus is mostly dependent on transport of toxic ions to the leaves – a process more efficient in the halophytes than in *P. major* – and the use of sorbitol as the major compatible solute for osmotic adjustment. In addition, specific mechanisms characteristic of the more tolerant species, *P. crassifolia* and *P. coronopus*, have been observed, such as the capacity to use inorganic ions as osmotica, even under low salinity conditions. In the presence of very high NaCl concentrations the salt-tolerant taxa strongly activate the synthesis of proline, as a secondary osmolyte, and the transport of K^+ to the leaves, thus avoiding a drastic reduction in K^+/Na^+ ratios. This suggests the existence in the *Plantago* halophytes of built-in mechanisms to rapidly adapt to increases in soil salinity in their natural habitats. The succulent *P. crassifolia* is the most drought-tolerant of the selected species, apparently due to its extreme resistance to dehydration, although accumulation of inorganic and organic solutes is also involved in water stress tolerance. Some of these mechanisms appear to be constitutive, as monovalent cations and sorbitol leaf contents are relatively high even in the absence of stress, with species-specific quantitative differences. Therefore, our results also support the hypothesis of a 'pre-adaptation' or 'metabolic preparedness' to stress in tolerant species of the genus *Plantago*.

***Keywords*: *Plantago crassifolia*, *P. Coronopus*, *P. Major*,** abiotic stress, sorbitol, proline, ion transport.

4.3.1. Introduction

Drought and soil salinity belong to the environmental factors most adverse for plants, which cause the biggest losses in agricultural production throughout the world and determine to a large extent the distribution of wild species in nature (Boyer, 1982; Bartels and Sunkar, 2005). Although the vast majority of plants, including all major crops, are glycophytes (that is, salt sensitive), there are also some species naturally adapted to saline environments, named halophytes. They represent only about 0.25% of total angiosperm taxa, but are very diverse from a taxonomic point of view, as tolerance to salinity seems to have evolved independently in different plant lineages (Flowers *et al.*, 2010).

Stress tolerance in plants relies on the activation of a series of conserved response pathways, some of them common to different abiotic stresses. One of these basic response mechanisms is the control of ion homeostasis and maintenance of osmotic balance, to counteract cellular dehydration caused by soil salinity, drought, cold or high temperatures, among other stressful conditions. The synthesis and accumulation of compatible solutes ('osmolytes') in the cytoplasm, in general, together with compartmentalisation of toxic ions in the vacuole when referring specifically to salt stress, are essential for osmotic adjustment (Munns and Termaat, 1986; Zhu, 2001; Munns and Tester, 2008). Yet, the activation of these processes is not specific for halophytes, but shared by all plants, and does not necessarily result in salt tolerance (Zhu, 2001); in fact, as mentioned above, most species are sensitive to salinity. Therefore, the relative resistance to salt stress of plants, which varies widely among taxa, should be attributed to quantitative rather than qualitative differences in their mechanisms of response, which only in halophytes are efficient enough to confer salt

tolerance, always within species-specific limits (Greenway and Munns, 1980; Zhu 2001; Grigore *et al*., 2011; Gil *et al*., 2013).

Several authors have proposed that halophytes possess constitutive mechanisms which enable their 'stress-anticipatory preparedness' or a metabolic anticipation of stress (Gong *et al*., 2005; Sanchez *et al*., 2008), a hypothesis supported by several experimental results. In a Mediterranean salt marsh in SE Spain no significant seasonal changes were detected in the levels of Na^+, Cl^- or the osmolyte glycine betaine in two highly tolerant succulent halophytes, *Sarcocornia fruticosa* and *Inula crithmoides*, despite strong oscillations in soil water content and salinity during the two-year field study (Gil *et al*., 2014). Moreover, the profiles of some major organic solutes in *Limonium latifolium* (a halophyte of the Plumbaginaceae family) upon NaCl treatments suggested that they pre-accumulate in the plants in prevention to stress (Gagneul *et al*., 2007). Similarly, metabolite profiling indicated several-fold higher pre-stress concentrations of the major compounds related to salt tolerance in the halophyte *Thellungiella halophila* than in *Arabidopsis thaliana* (Gong *et al*., 2005); the two species are somewhat related genetically, as both genera belong to the same family, Brassicaceae. Yet, the pre-adaptation hypothesis is still under debate; for example, comparative ionomic and metabolomic studies in three species of genus *Lotus* (Fabaceae), one of which is a moderate halophyte and two are typical glycophytes, did not reveal any 'preparedness to stress' in the halophyte (Sanchez *et al*., 2011). For this reason, further studies on taxonomically related, stress tolerant and stress sensitive taxa – such as congener species; the closer the relation, the better – are of great interest and should be extended and diversified. In addition, these comparative studies would help to establish the relative contribution of different stress responses to the stress tolerance of a given species, by correlating the relative degree of stress resistance with the levels of biochemical markers associated to distinct stress response pathways – specific osmolytes, for instance (Boscaiu *et al*., 2013). One of the genera most

appropriate for such comparative studies is probably *Plantago* L., with more than 200 species, including about 20 halophytes. The genus is well represented in the Mediterranean region, and 27 species are present in the Iberian Peninsula alone (Pedrol, 2009). A few of the Iberian species are halophytes, but most grow in arid habitats, tolerating strong summer droughts, which are common in this region and are characterised by a drastic rainfall reduction, combined with increased temperature and evapotranspiration (Rivas-Martínez and Rivas-Saenz, 1996–2009).

Three *Plantago* species, presumably with different degrees of stress resistance, were chosen for the present study. Two of them are considered stress tolerant, but have different ecological requirements and occupy different habitats. *P. crassifolia* Forssk. is a Mediterranean, succulent halophyte specific for sandy littoral soils and salt marshes. *P. coronopus* L. has a broader distribution, reaching the Atlantic region of Europe and west Asia, where it grows in habitats with a variable degree of salinity, and often on degraded soils; its presence is considered as an indicator of salinisation of marginal lands. This study also included *P. major* L., widely distributed in Europe, Asia and north of Africa, and naturalised throughout the world. Although a few *P. major* subspecies are adapted to saline environments (Chater and Cartier, 1976), the common taxon *P. majo*r subsp. *major* included in the present study is frequent in humid areas but never found in salty soils, and it is therefore considered as a glycophyte. Molecular taxonomy of *Plantago*, based on nuclear ribosomal ITS and plastid *trnL-F* sequences, recognises several clades with category of subgenera (Rønstead *et al*., 2002). *P. crassifolia* and *P. coronopus* are closely related, belonging to subgenus *Coronopus* (Lam. & DC.) Rahn, whereas *P. major* is included in subgenus *Plantago*.

Different aspects of the responses to salinity have been previously investigated in a few *Plantago* species, especially in the halophyte *P. maritima*, often in comparison with non-

halophytes from the same genus (Erdei and Kuiper, 1979; Königshofer, 1983; Staal *et al.*, 1991). *P. crassifolia* has already been one of the target species of our studies (Vicente *et al.*, 2004; Boscaiu *et al.*, 2005; Gil *et al.*, 2011; Al Hassan *et al.*, 2014; Gil *et al.*, 2014; Pardo-Domenech *et al.*, 2015), but we are not aware of any report of comparative studies on species of this genus including the analysis of the responses to both, salinity and drought stress conditions.

In this paper we describe the effects of salt and water stress on the aforementioned *Plantago* species under controlled experimental conditions, regarding growth inhibition and the activation of basic stress responses based on the control of ion homeostasis and osmolyte bioynthesis. 'Shock' treatments with very high NaCl concentrations, beyond those the plants will normally encounter in the field, were included in the study to detect potential mechanisms which could be activated in response to an increase in the degree of environmental stress affecting the plants in their natural habitats – due, for example, to the foreseeable effects of climate change in the Mediterranean region. The specific aim of the work was to investigate the mechanisms underlying the tolerance to drought and salinity in *Plantago*, by correlating the relative sensitivity to stress of the investigated species – estimated from their distribution in nature and growth inhibition measurements under controlled conditions – with the levels of ions and specific osmolytes accumulated in the leaves of the plants upon the stress treatments.

4.3.2. Material and Methods:

4.3.2.1. Plant material and experimental design

Seeds of P. crassifolia and P. coronopus were harvested from the Natural Park of 'La Albufera' (Province of Valencia, Spain), and those of P. major were obtained from a

commercial supplier (Spicegarden, EU); all were stored at room temperature until used. Previous to germination, seeds were sterilised in a 5% hypochlorite solution (commercial bleach) for five minutes, and then washed thoroughly with distilled water. Germination was carried out on standard Petri-dishes at 25°C and 16 hours photoperiod. After three weeks, seedlings were transplanted onto a moistened mixture of peat (50%), perlite (25%) and vermiculite (25%) in 1 litre pots. During the entire course of plant growth the substrate was kept moderately moist, using Hoagland's nutritive solution. The stress treatments started three weeks after seedling transplantation, and were carried out for four weeks. For the salt stress experiments, plants were watered twice a week with freshly prepared aqueous NaCl solutions of increasing concentration (100, 200, 400, 600, and 800 mM), using 1.5 l per tray containing 12 pots. The control, non-treated plants were watered in parallel with distilled water. Drought treatments were performed by completely ceasing irrigation. All experiments were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 hours of light), temperature of 23°C during the day and 17°C at night, and CO_2 level ≈300 ppm. Relative humidity ranged between 50 and 80% during the course of the treatments (four weeks).

4.3.2.2. Soil analysis

Electrical conductivity ($EC_{1:5}$) of the substrate was measured after the four weeks of treatment. Soil samples were taken from pots of the same treatment, air-dried and then passed through a 2-mm sieve. A soil: water (1:5) suspension was prepared using deionised water at 20°C and mixed for one hour at 600 u/min. Electric conductivity was determined using a Crison Conductivity meter 522 and expressed in dS m^{-1}.

4.3.2.3. Plant growth parameters

At the end of the treatments, the aerial part of each plant – constituted only by its rosette leaves, since during the course of the experiment the plants remained in the vegetative growth phase, before development of the reproductive stem – was collected and the following growth parameters were measured: number of leaves (NL), fresh weight (FW), and water content percentage (WC%). NL was recorded by simply counting the number of leaves of the plants under study. FW was measured by weighing the total mass of the leaves after harvesting. A fraction of the fresh material was dried in an oven at 65 $^{\circ}$C until constant weight, to obtain the dry weight (DW), which was used to calculate the leaf water content, in percentage, for each plant: WC = [(FW – DW)/ FW] x 100.

4.3.2.4. Ion content measurements

Concentrations of potassium, sodium and chloride were measured in leaves of plants sampled after the stress treatments, and in the corresponding non-stressed controls. Extraction of K^+ and Na^+ were performed according to Weimberg (1987), by heating the samples (0.15 g of dried, ground plant material in 25 mL of water) in a water bath, for 1 h at 95ºC, followed by filtration through a filter paper (particle retention 8-12 µm); these cations were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, USA). Chlorides were determined with the Spectroquant chloride test (Merck, Darmstadt, Germany).

4.3.2.5. Osmolyte quantification

Proline (Pro) content was determined in fresh tissue by the ninhydrin-acetic acid method of Bates *et al.* (1973). Free Pro was extracted in 3% aqueous sulfosalicylic acid, the extract was mixed with acid ninhydrin solution, incubated at 95ºC for 1 h, cooled on ice and then extracted with two volumes of toluene. The absorbance of the organic phase was

determined at 520 nm using toluene as a blank. Glycine betaine (GB) was determined according to Grieve and Grattan (1983) using dried tissue. Plant material was ground in a mortar, suspended in 2 ml of Milli-Q water and extracted with 1.2-dichlorethane; the absorbance of the solution was measured at a wavelength of 365 nm. Total sugars (TSS) were quantified according to the technique described by Dubois *et al*. (1956). Dried material was ground and mixed with 3 ml of 80% methanol on a rocker shaker for 24–48 h. Concentrated sulphuric acid and 5% phenol was added to the sample and the absorbance was measured at 490 nm.

Sorbitol (Sor) was analysed using a Waters 1525 HPLC system coupled to a 2424 evaporative light scattering detector (ELSD). The source parameters of ELSD were the following: gain 75, data rate 1 point per second, nebulizer heating 60%, drift tube 50ºC, and gas pressure 2.8 Kg/cm^2. Plant dry material was boiled in milliQ water for 10 minutes and then filtered using 0.22 micrometer filters. Analysis was carried out injecting 20 µL aliquots with a Waters 717 autosampler 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 during 25 minutes was applied in each run. Sor was identify and quantified by peak integration using the Waters Empower software and comparison with the standard calibration curve.

4.3.2.6. Statistical analysis

Data were analysed using the programme Statgraphics Centurion v.16. Before the analysis of variance, the Shapiro-Wilk test was used to check for validity of normality assumption and Levene´s test for the homogeneity of variance. If ANOVA requirements were accomplished, the significance of the differences among treatments was tested by one-way

ANOVA at a 95% confidence level and *post hoc* comparisons were made using the Tukey HSD test. All means throughout the text are followed by SD.

4.3.3. Results

4.3.3.1. Electric conductivity of the soil

Soil electrical conductivity ($EC_{1:5}$) values at the end of each stress treatment were similar for the three studied *Plantago* species, and in all of them salinity in the pot soil increased in parallel to the NaCl concentrations applied. In the case of water stress treatments, no significant change in $EC_{1:5}$ was detected in comparison with the pots of control plants, as it should be expected (Table 1).

4.3.3.2. Effect of salt stress on plant growth

The most general, and the easiest to assess effect of stress on plants is inhibition of growth, which can be quantified by different measurements. A reduction in the number of leaves with increasing salt concentrations was detected in all selected *Plantago* species. The average number of leaves per plant varied from 12 (in *P. major*) to 45 (in *P. crassifolia*) for control, non-stressed plants, while at the highest salt concentration tested (800 mM NaCl) this number was reduced by 43% in *P. coronopus*, by 51% in *P. crassifolia* and by 54% in *P. major* (Fig. 1). Therefore, according to this criterion, *P. coronopus* appears to be the most salt-tolerant of the three species, which is also supported by the fact that a significant decrease in the mean leaf number was only observed in this species at high salt concentrations (≥ 400 mM NaCl, Fig. 1).

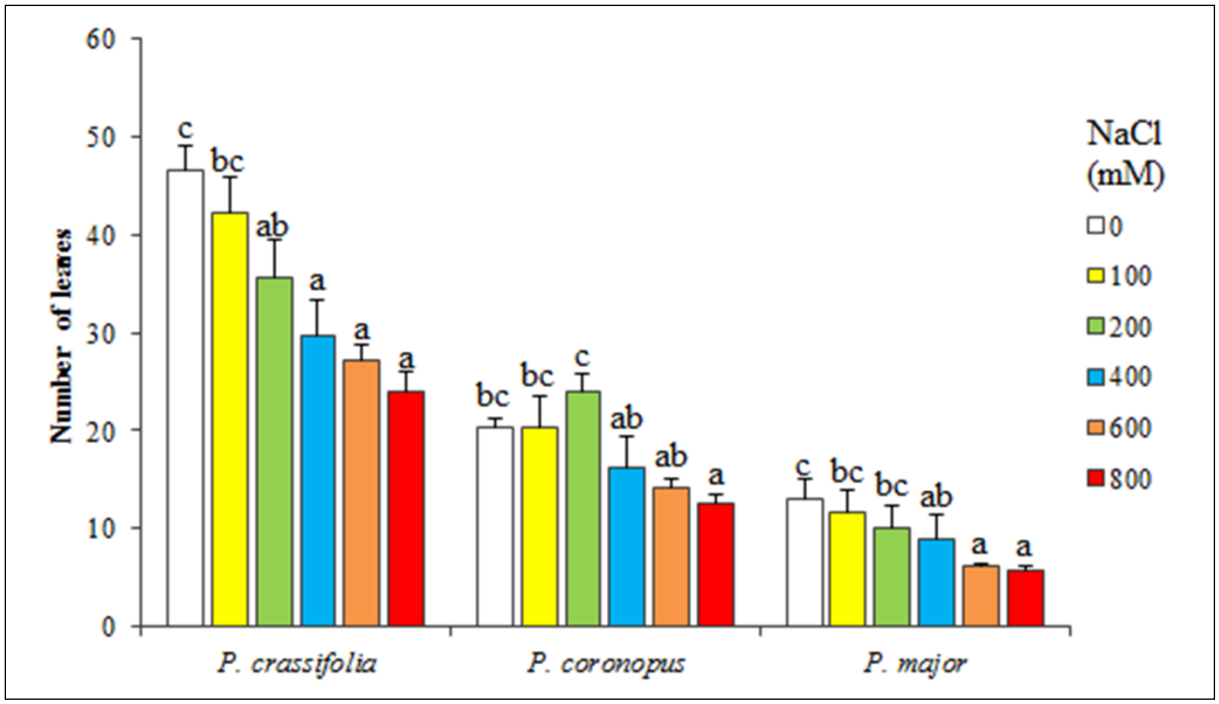

Fig. 1 Number of leaves of *Plantago* plants after four weeks of treatement with the indicated NaCl concentrations (means ± SD, *n* = 5). Different lower case letters within each species indicate significant differences between treatments, according to Tukey test (α = 0.05).

The relative tolerance to salt of the three species was confirmed, and more clearly established by measuring the fresh weight of the leaves (after the four-week salt treatment), which also decreased in a concentration-dependent manner. In *P. crassifolia* and *P. major*, FW was significantly reduced already at 200 mM NaCl to ca. 45% and 20%, respectively, of the mean value of the corresponding controls; in *P. coronopus*, on the contrary, this salt concentration did not affect the fresh weight, and a significant decrease was only detected in the presence of 400 mM NaCl. Higher salt concentrations caused a stronger growth inhibition, but the relative sensitivity to NaCl of the three *Plantago* taxa was maintained (Fig. 2), as it was after eight weeks of salt treatments – although no plants survived this time in the presence of the highest concentrations tested, 600 or 800 mM NaCl (data not shown).

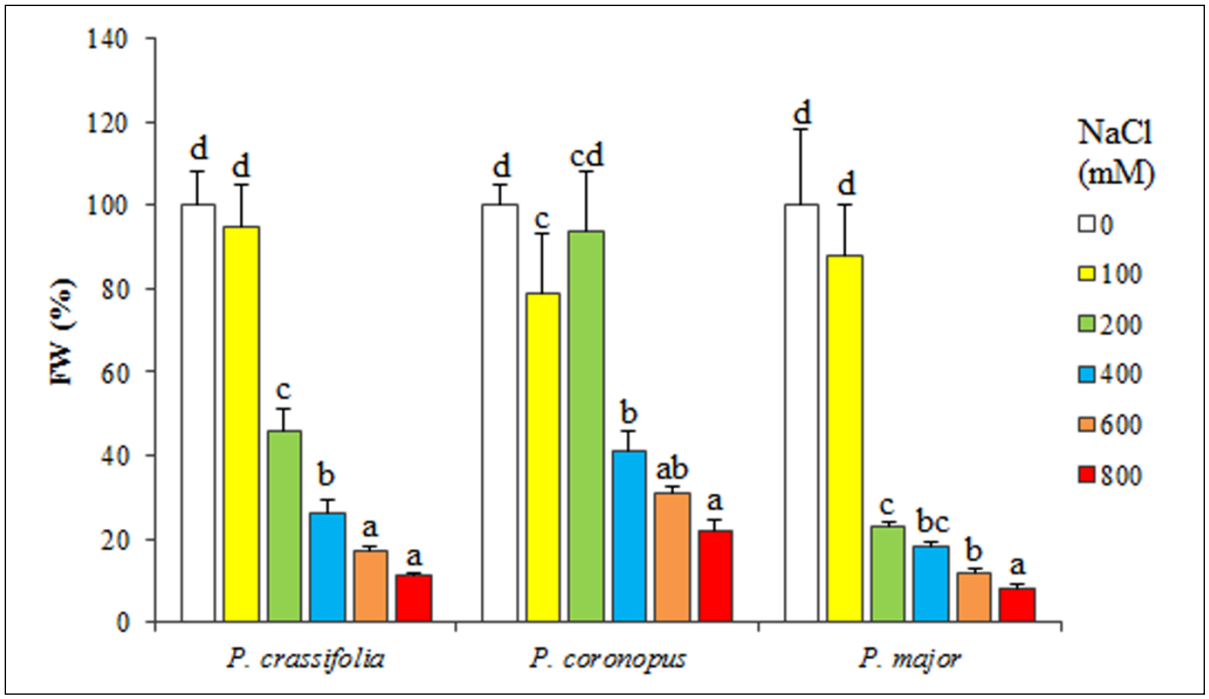

Fig. 2 Leaf fresh weight after four weeks of treatment with the indicated NaCl concentrations, for the three tested *Plantago* species. For each species, values (means ± SD, *n* = 5) are shown as percentages of the mean FW of the control plants, considered as 100% (absolute values: 11.35 ± 1.84 g, 10.61 ± 0.86 g, and 25.83 ± 3.51 g for *P. crassifolia*, *P. coronopus* and *P. major*, respectively). Different lower case letters within each species indicate significant differences between treatments according to Tukey test (α = 0.05).

In the absence of salt, leaf water content – expressed as percentage of fresh weight (WC %) – varied from 85% in *P. mayor* to more than 95% in the succulent *P. crassifolia*, and decreased to some extent as a result of increasing external NaCl concentrations, with a pattern similar for the three species (Fig. 3). Although the relative loss of water was slightly higher in *P. major* than in the other two species (19% *vs*. 13-14%), all three appear to possess efficient mechanisms to avoid dehydration of the leaves, even in the presence of very high salt concentrations. These data also indicate that the salt-dependent reduction of plant fresh weight in the three *Plantago* species was indeed due, mostly, to inhibition of growth, and that there were no important differences in their degree of water loss.

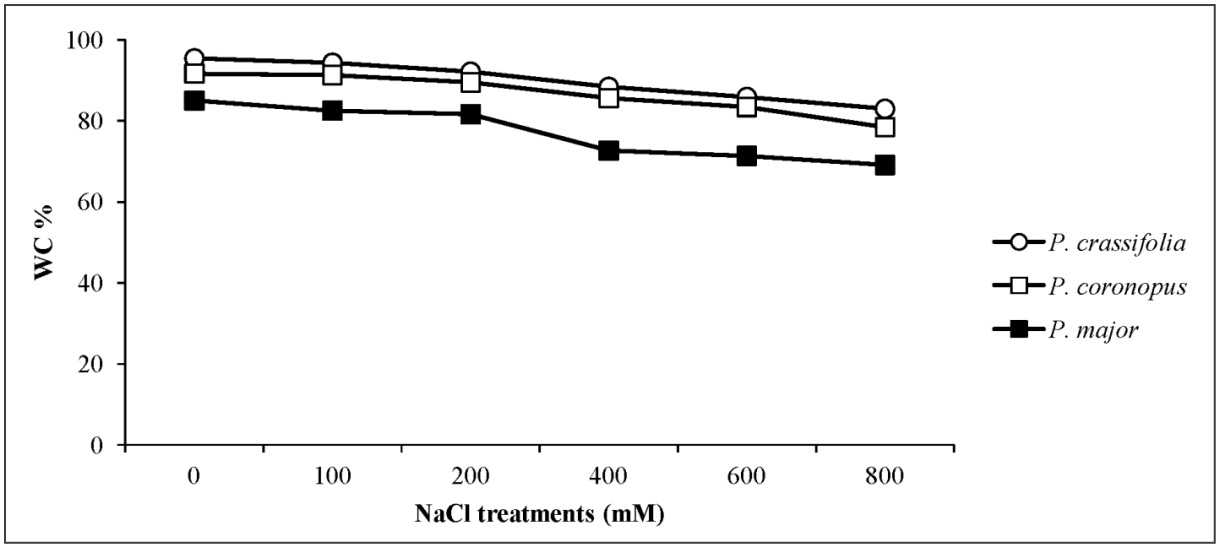

Fig. 3 Leaf water content (%) after four weeks of treatment with increasing NaCl concentrations, in the three selected *Plantago* species (*n* = 5).

4.3.3.3. Effects of salt stress on ion accumulation

One common mechanism of salt tolerance in dicotyledonous halophytes is based on the transport of toxic Na^+ and Cl^- ions to the leaves, where they accumulate in the vacuoles to avoid their deleterious effects in the cytosol, according to the so-called 'ion compartmentalisation hypothesis' (Flowers *et al*., 1977; Wyn Jones *et al*., 1977; Glenn *et al*., 1999). Sodium levels in the leaves of NaCl-treated plants showed a concentration-dependent increase in the three species, although the absolute value reached in *P. major* in the presence of the highest salt concentration tested, 800 mM NaCl, (ca. 1.3 mmol/g DW) was less than half of those measured in the more tolerant taxa (2.8 – 3.0 mmol/g DW) (Fig. 4A). It should be mentioned, however, that Na^+ contents in *P. crassifolia* and *P. coronopus* untreated controls were relatively high, over 1 mmol/g DW, as compared to ca. 250 µmol/g DW in *P. major*; as a consequence, when comparing leaf Na^+ levels at 800 mM external NaCl with the corresponding controls, the *relative* increase was higher in *P. major* (ca. 5.3-fold) than in *P. crassifolia* (2.7-fold) or *P. coronopus* (2.1-fold) (Fig. 4A). The patterns of Cl^- accumulation in leaves and the absolute values reached at the highest salt concentrations tested were similar to those of Na^+, in the three species; the only remarkable difference was that only *P.*

crassifolia – but not *P. coronopus* – showed high Cl^- contents in the control plants not subjected to salt stress (Fig. 4B).

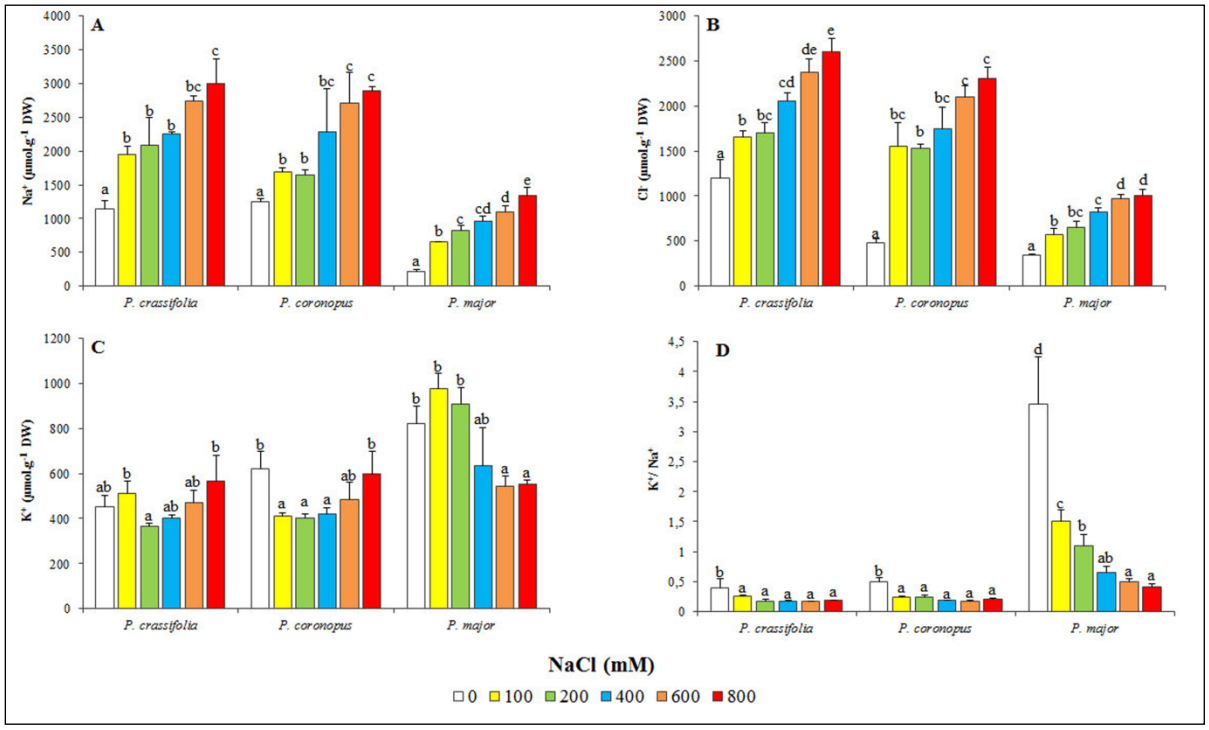

Fig. 4 Leaf contents of (A) sodium (Na^+), (B) potassium (K^+), and (C) chloride (Cl^-), and (D) K^+/Na^+ ratios, in the selected *Plantago* species, after four weeks of treatment with the indicated NaCl concentrations (means ± SD, *n* = 5). Different lower case letters within each species indicate significant differences according to Tukey test (α = 0.05).

An increase in Na^+ contents is generally accompanied by a concomitant decrease in K^+ levels, as both cations compete for the same transport systems (Rodriguez-Navarro and Rubio, 2006; Adams *et al.*, 2014;). This general trend was observed in the three *Plantago* species, but with clear differences depending on their relative stress tolerance. In *P. crassifolia* and *P. coronopus*, a reduction of the mean K^+ levels was observed at low and moderate NaCl concentrations, to increase again under high salinity conditions; *P. major*, on the other hand, showed relatively higher K^+ contents in the absence of salt or below 400 mM NaCl, and a significant decrease was only observed at higher salt levels. In any case, variation of K^+ concentrations in the plants was relatively small, with all values included in the range 0.4 – 1.0 mmol/g DW (Fig. 4C). The relative levels of K^+ and Na^+ in each species

led to two clearly different patterns of variation in K^+/Na^+ ratios. In control *P. major* plants, K^+/Na^+ was found to be quite high (ca. 3.5) and this value was progressively reduced, in parallel with the increase of salt concentrations, to 0.4 – that is, almost 9-fold – in the presence of 800 mM NaCl. In *P. crassifolia* and *P. coronopus*, K^+/Na^+ ratios were maintained below 0.5, with a reduction to about 50% of the controls in all salt treatments (Fig. 4D).

4.3.3.4. Salt stress-induced osmolyte accumulation

The levels of the commonest osmolytes in plants – proline (Pro), glycine betaine (GB) and total soluble sugars (TSS) – were determined in plant leaves after the salt treatments were concluded. Pro contents showed no significant increase over the non-stressed controls in the presence of NaCl up to 400 mM, in all three *Plantago* species. Yet stronger salt stress conditions triggered the accumulation of this osmolyte in *P. crassifolia*, at levels ca. 16-fold higher than in the control plants, in the presence of 800 mM NaCl; the induction of Pro biosynthesis was even stronger in *P. coronopus* (90-fold), while no increase over the control was detected in *P. major* under the same conditions (Fig. 5A). It should be mentioned that, despite these strong relative increases in Pro contents in the most salt-tolerant species, even the highest absolute values reached, which were all below 50 µmol/g DW, are too low to contribute significantly to osmotic balance in the salt-stressed plants. The same is true for GB, which accumulated in the leaves of *Plantago* plants to maximum concentrations of around 20 µmol/g DW (Fig. 5B), although with differences in the control levels and the patterns of accumulation: in *P. crassifolia* and *P. major*, GB synthesis was induced already in the presence of 100 mM NaCl and the contents of the osmolyte did not increase significantly at higher salt concentrations; in *P. coronopus*, GB levels increased more progressively, but only at and above 400 mM NaCl (Fig. 5B). Total soluble sugars levels decreased moderately in *P. coronopus* and *P. crassifolia* with increasing NaCl concentrations, while no significant

change was detected in *P. major* (Fig. 5C). It appears, therefore, that these compounds do not play any role as osmolytes in the mechanisms of salt tolerance in the investigated *Plantago* species.

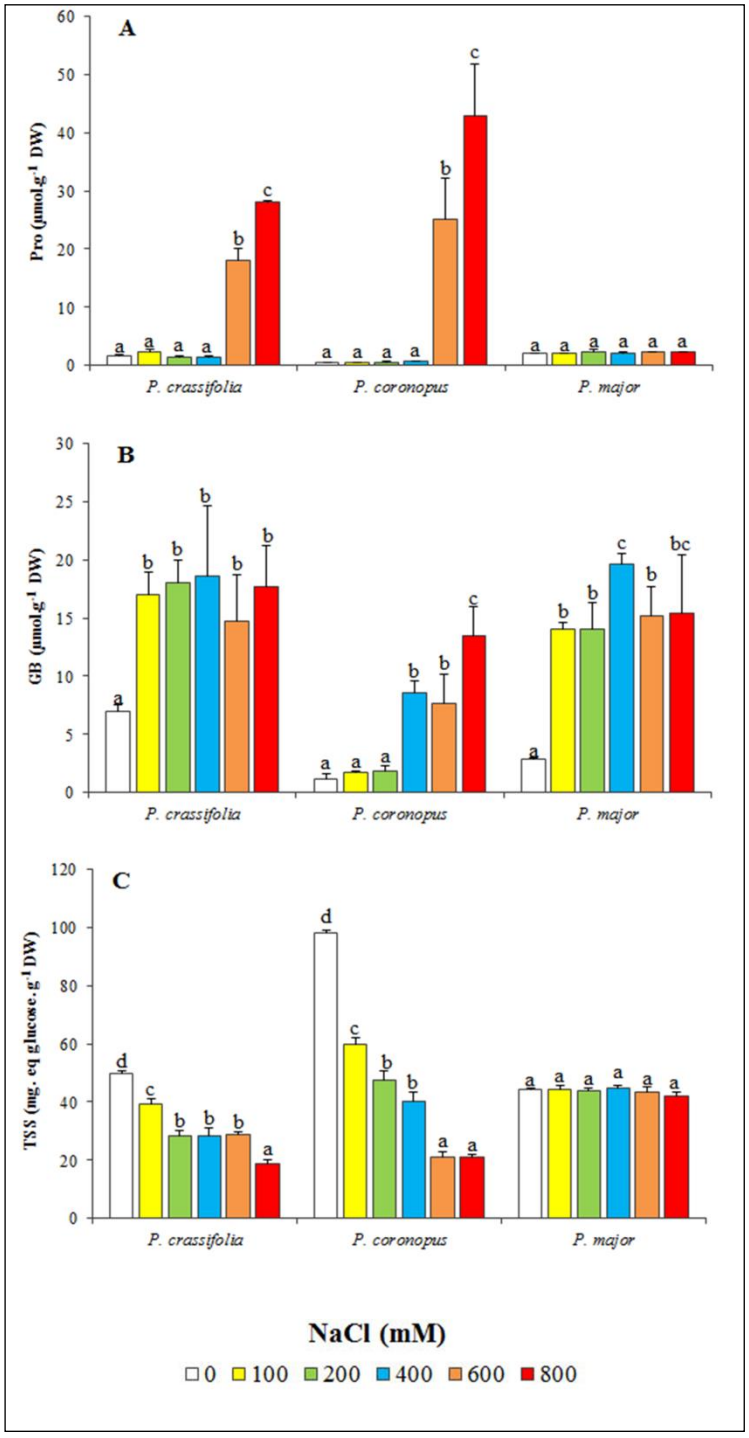

Fig. 5 Leaf contents of (A) proline (Pro), (B) glycine betaine (GB), and (C) total soluble sugars (TSS) in the selected *Plantago* species, after four weeks of treatment with the indicated NaCl concentrations (means ± SD, *n* = 5). Different lower case letters within each species indicate significant differences according to Tukey test (α = 0.05).

The spectrophotometric assay used to measure total soluble sugars does not detect polyalcohols (Ashwell, 1957) such as sorbitol (Sor), which has been identified as the major osmolyte in all *Plantago* species investigated to date (Ahmad *et al.*, 1979; Jefferies *et al.*, 1979; Lambers *et al*., 1981; Königshofer, 1983; Koyro, 2006; Flowers *et al.*, 2010; Gil *et al.*, 2011). This has been confirmed in the present study, which shows that upon treatment with NaCl, Sor levels – identified and quantified by HPLC – gradually increased in leaves of all tested *Plantago* species, in a concentration-dependent manner (Fig. 6), although some differences were observed among the three taxa. Sor contents in non-stressed *P. crassifolia* plants (ca. 500 µmol/g DW) were about half of those measured in *P. coronopus* and *P. major* controls, while the maximum level reached in the first species, in the presence of 800 mM NaCl, was higher than in the other two. Therefore, the *relative* salt-induced increase in the osmolyte concentration was highest in *P. crassifolia*: ca. 4.6-fold *vs*. 1.5-fold in *P. coronopus* and *P. major* (Fig. 6). In any case, the *absolute* Sor concentrations were about three orders of magnitude higher than those of Pro or GB, supporting the essential role of Sor in cellular osmotic adjustment under salt stress conditions in *Plantago*.

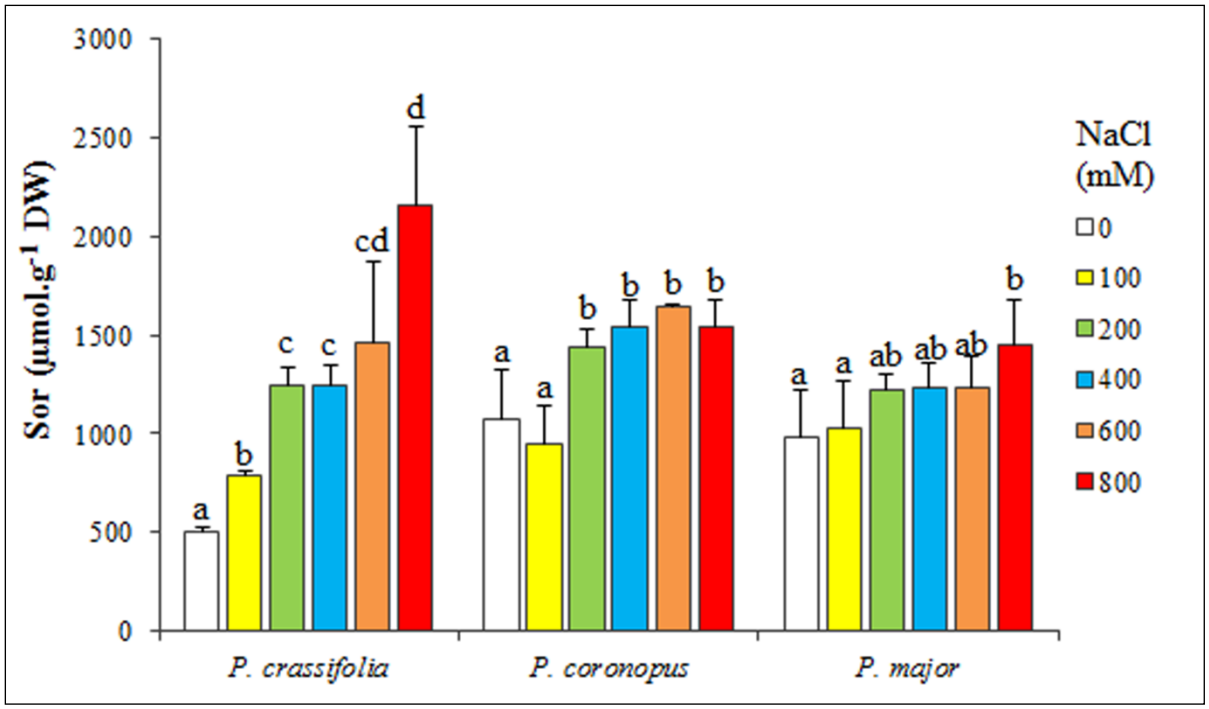

Fig. 6 Sorbitol (Sor) leaf contents after four weeks of treatement with the indicated NaCl concentrations, in the three selected *Plantago* species (means ± SD, *n* = 5). Different lower case letters indicate significant differences between treatments according to Tukey test (α = 0.05).

4.3.3.5. Effects of water stress on plant growth and biochemical stress markers

All the growth parameters, ion and osmolyte contents determined in leaves of *Plantago* plants subjected to salt stress treatments were also measured in plants kept for four weeks without irrigation. Drought also caused growth inhibition in the three species under study (Fig. 7), although their relative tolerance to water stress was not so easy to estimate. Significant reductions in the average number of leaves per plant, of ca. 50% in *P. crassifolia*, 46% in *P. major*, 20% in *P. coronopus*, were observed in water-stressed plants (Fig. 7A). However, the decrease of fresh weight in relation to the corresponding controls was much higher in *P. coronopus* (94%) or *P. major* (89%) than in *P. crassifolia* (64%) (Fig. 7B); yet this FW reduction was not only due to inhibition of plant growth under drought conditions, but also to loss of water, which was different for the different *Plantago* taxa: *P. coronopus* and *P. major* stressed plants had a much lower percentage of water in the leaves than the non-stressed controls, while water content did not change significantly in the most succulent *P.*

crassifolia (Fig. 7C). When drought-induced growth inhibition was expressed in terms of dry weight reduction, the values determined for *P. crassifolia* (ca. 40%), *P. coronopus* (63%) and *P. major* (49%) were not so different.

No changes in leaf ion levels should be expected in the plants after the water stress treatment, and this was generally true for K^+, Cl^- and the K^+/Na^+ ratio in the three *Plantago* species, as well as for Na^+ in *P. major*. Yet, in *P. crassifolia* and *P. coronopus*, Na^+ contents, which were already relatively high in the controls, increased significantly after four weeks without watering the plants (Table 2).

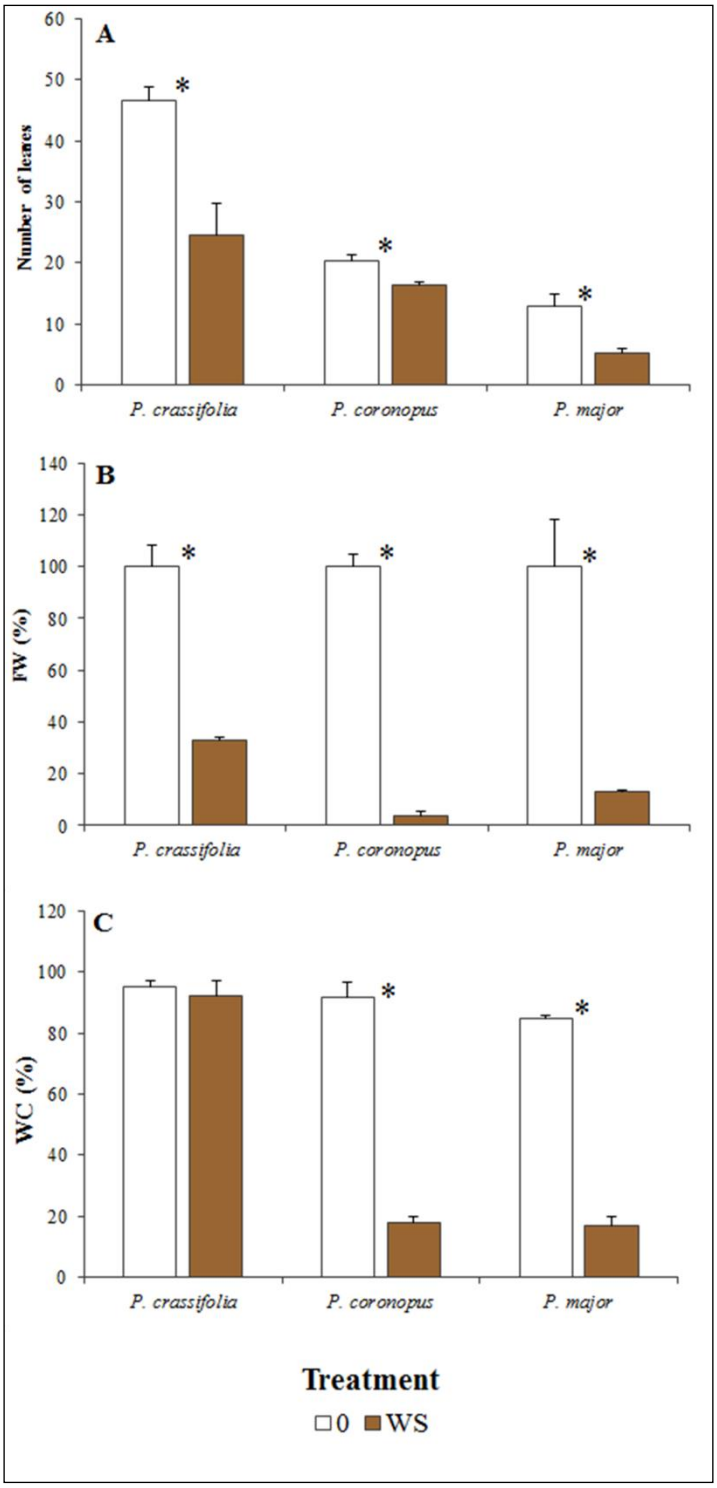

Fig. 7 Growth parameters after four weeks of water stress treatment, (A) number of leaves, (B) leaf fresh weight, values are shown as percentages of the mean FW of the control plants, considered as 100% (absolute values: 11.35 ± 1.84 g, 10.61 ± 0.86 g, and 25.83 ± 3.51 g for *P. crassifolia*, *P. coronopus* and *P. major*, respectively), and (C) leaf water content (%), in the studied *Plantago* species (means ± SD, *n* = 5). Asterisks indicate significant differences between treatments, according to Tukey test (α = 0.05).

The drought-induced accumulation of osmolytes in the three species showed patterns similar, but not identical to those observed in the presence of increasing salt concentrations

(Fig. 8). The largest increase in Pro contents (>100-fold) was observed in *P. coronopus*, followed by *P. major* (ca. 6-fold), while Pro did not accumulated in *P. crassifolia* in the drought conditions used in the experiments (Fig. 8A).

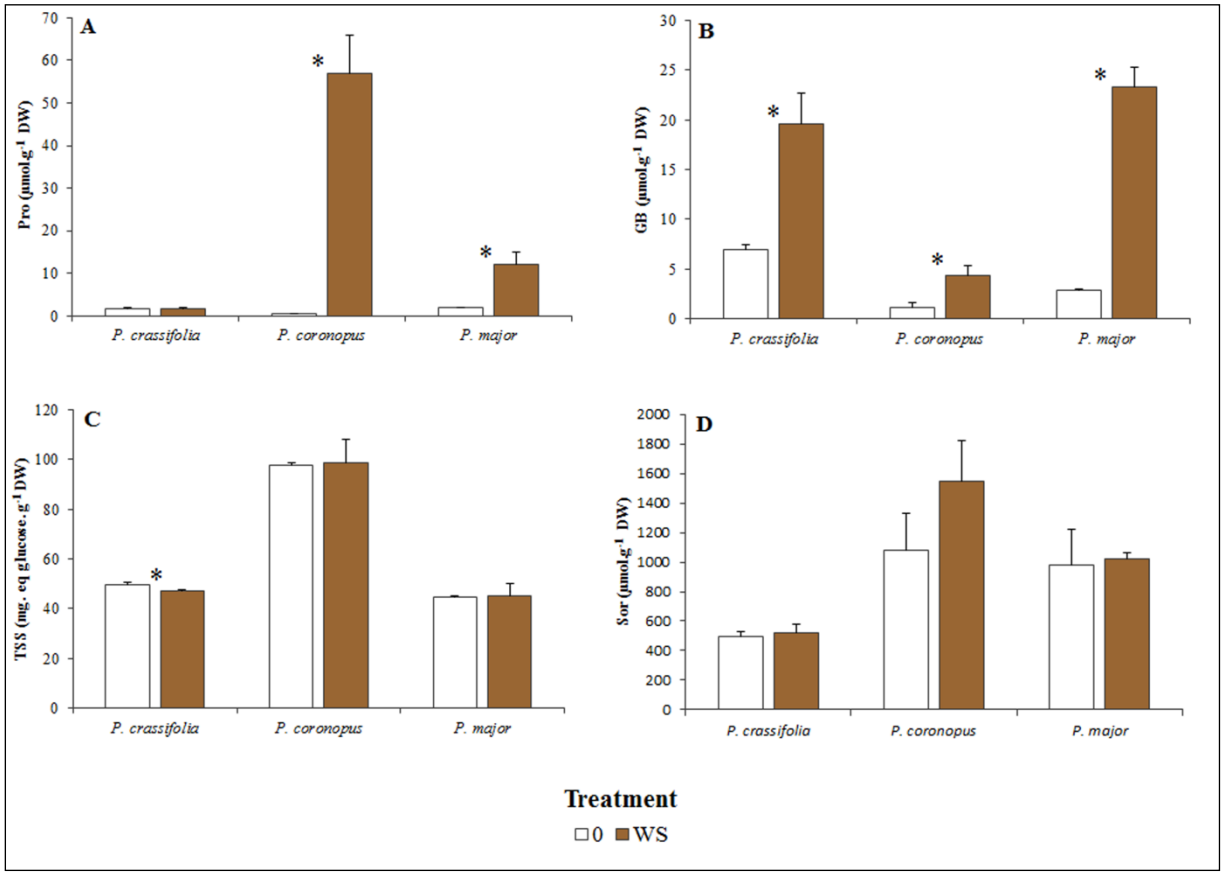

Fig. 8. Leaf contents of (A) proline (Pro), (B) glycine betaine (GB), (C) total soluble sugars (TSS), and (D) sorbitol (Sor) in the studied *Plantago* species after four weeks of water stress treatment (means ± SD. *n* = 5). Asterisks indicate significant differences between treatments, according to Tukey test (α = 0.05).

As in the case of salt-stressed plants, *P. coronopus* showed somewhat lower levels of GB than the other two species (Fig. 8B), and soluble sugars did not appear to be involved in the response of the plants to drought, since no significant differences with the controls were detected in their levels (Fig. 8C). Sorbitol contents, however, did not change as a response to water stress in *P. crassifolia* or *P. major*, and only a small increase in the mean values was detected in *P. coronopus*, but the difference with the control was not statistically significant (Fig. 8D).

4.3.4. Discussion

4.3.4.1. Effect of salt stress and drought on plant growth

Growth of all glycophytes and most halophytes is optimal in the absence of salt and is progressively reduced by increasing salt concentrations; only in some highly tolerant dicotyledonous 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; Redondo-Gómez *et al.*, 2006; 2010). Therefore, a quantitative assessment of salt-induced growth inhibition in different species should provide a reliable ranking of their relative degree of salt tolerance. This kind of analysis, applied to the three *Plantago* species included in the present study, indicated that *P. major* is more sensitive to salt than *P. crassifolia* and *P. coronopus*, the latter apparently being the most tolerant; this would roughly agree with the distribution of the three taxa in nature: *P. crassifolia* and *P. coronopus* are both halophytes, although adapted to different types of saline habitats, while *P. major* is considered as a glycophyte, never found in natural saline environments. Yet, quantitative differences in growth parameters of the three species were relatively small, and *P. major* plants actually survived for one month the treatment with very high NaCl concentrations. We have previously shown, in an independent study (Al Hassan *et al.*, 2014), that after two months exposure to NaCl up to 400 mM, *P. major* plants still produced some fertile flowers that generated viable seeds. Considering the accepted definition of halophytes as those plants able to complete their life cycle at soil salinity levels equivalent to at least 200 mM NaCl (Flowers and Colmer, 2008), *P. major* behaves as a moderate salt tolerant species, rather than as a typical glycophyte. Nevertheless, as it will be discussed below, the present

work revealed clear differences in the responses to salt stress of this species and its two more resistant congeners.

Regarding the relative drought tolerance of the three *Plantago* species, one should expect *P. coronopus* to be also the most tolerant. The responses to salinity and water stress are similar in plants and are based on practically the same mechanisms, except those related to ion transport. In the first phase of stress, plants exposed to saline conditions reduce their growth due to the osmotic component of salt stress and only later, in a second stage, ions accumulated in the leaves reach toxic levels which will produce premature senescence (Munns, 2002). In addition, *P. coronopus* is often found on sand dunes and other dry-type habitats, whereas the other two taxa grow in nature in more humid soils, either saline (*P. crassifolia*) or not (*P. major*). Contrary to this expectation, in terms of reduction of fresh or dry weight, *P. crassifolia* was found to be the most drought tolerant species, and *P. coronopus* the most sensitive; this is probably due to the fact that the latter species was the one that suffered the strongest dehydration (calculated as the loss of water content in leaves) after one month without irrigation, while no significant reduction of water content was observed in the succulent *P. crassifolia* under the same conditions. Interestingly, the three *Plantago* species appear to possess efficient mechanisms to avoid salt-induced dehydration of the leaves, even in the presence of very high concentrations (600 – 800 mM NaCl).

Soil salinity is considered a major restrictive factor for plant survival in nature, so that only halophytes are adapted to highly saline soils, conditions that are lethal for non-tolerant species. However, our results indicate that the degree of stress tolerance, *per se*, is not the only factor – perhaps not even the most important factor – for the distribution of these species in their natural environments. Other components, such as interspecies competition, may play a fundamental role (Grigore *et al.*, 2012). Many halophytes were probably refugees, outcompeted by glycophytes from non-saline areas, which remained highly competitive

under saline conditions suboptimal for non-tolerant species. The three taxa analysed here grew better in the absence of salt, but all tolerated relatively high NaCl concentrations. Yet it is likely that only *P. crassifolia* and *P. coronopus* are more competitive under saline conditions, whereas *P. major*, with a faster growth rate, may be more competitive in the absence of salt.

4.3.4.2. Effect of salt stress on ion accumulation

One basic response of glycophytes and monocotyledonous halophytes to cope with high salinity in the soil is the limitation of sodium uptake. On the contrary, dicotyledonous halophytes tend to accumulate toxic ions in the plants' aerial parts, which are maintained at low cytsolic concentrations by compartmentalisation in vacuoles. This is an advantageous mechanism to increase osmotic pressure in foliar tissues, cheaper, in terms of energy consumption, than the synthesis of organic solutes for osmotic adjustment (Raven, 1985). In many salt-tolerant plants, Na^+ concentrations are often well above 200 mM on a tissue-water basis, concentrations that completely inhibit the activity of many enzymes *in vitro* (Munns and Tester, 2008). In the present work, the highest levels of Na^+ and Cl^- in leaves were found in *P. crassifolia*, followed by *P. coronopus*, and the lowest in the less tolerant *P. major*. It has been previously reported that non-halophytic *Plantago* species kept lower foliar Na^+ levels under saline conditions than the halophyte *P. maritima* (Könnigshofer, 1983). The ability of *P. maritima* to accumulate sodium in its shoots was based on its higher capacity of Na^+ translocation from the roots, rather than on a higher uptake rate, which was similar to that found in other species of this genus (Erdei and Kuiper, 1979; Tánczós *et al.*, 1981). Differences in salt tolerance of *Plantago* species were also related to the efficiency of intracellular ion compartmentalisation mechanisms; for example, Staal *et al.* (1991) reported a considerably greater tonoplast Na^+/H^+ antiporter activity under salt stress in the halophyte

P. maritima than in the salt-sensitive *P. media*. The higher concentrations of Na^+ and Cl^- we have determined in leaves of *P. crassifolia* and *P. coronopus*, as compared to *P. major*, are also related to the anatomic structure of the former species, with succulent leaves (especially in *P. crassifolia*), and therefore with increased vacuole volume and better ion sequestration capacity. Succulence, as well as excretion of Na^+ and Cl^- by salt glands or bladders, is a basic anatomic adaptation to salinity in some dicotyledonous plants (Flowers *et al.*, 1986). Yet, the most interesting information provided by these experiments is probably that the most tolerant taxa accumulated relatively high concentrations of Na^+ (and also of Cl^-, in the case of *P. crassifolia*) in the leaves of control, non-treated plants, whereas the more salt sensitive *P. major* did not. These data clearly suggest the existence in *Plantago* of constitutive mechanisms of tolerance, based on the active uptake and transport of sodium to the leaves, even under low salinity conditions, where the cation will be used as osmoticum – in addition to the accumulation, if necessary, of organic osmolytes – in agreement with the halophytes' 'pre-adaptation to stress' hypothesis (Gong *et al.*, 2005; Sanchez *et al.*, 2008). The fact that Na^+ also seems to contribute to osmotic adjustment in water-stressed plants – a statistically significant increase of Na^+ levels was detected in *P. crassifolia* and *P. coronopus*, but not in *P. major*, one month after watering was stopped – lends support to this idea.

The accumulation of sodium in plants is generally associated with a decrease of potassium levels in the shoots. Since the two cations compete for the same binding sites, Na^+ interferes with K^+ transport into the cell by using its physiological transport systems (Flowers *et al.*, 1977; Greenway and Munns, 1980; Flowers *et al.*, 1986). Moreover, sodium uptake produces a depolarization of the plasma membrane causing the activation of outward-rectifying K^+ channels and consequently a K^+ loss (Shabala *et al.*, 2003; 2005). These processes lead to a significant reduction of K^+/Na^+ ratios, and maintenance of relatively high cellular K^+/Na^+ values appears to be a relevant mechanism of salt tolerance (Maathuis and

Amtmann, 1999). Although the three *Plantago* species somewhat followed this general trend, here again there were clear differences between *P. crassifolia* and *P. coronopus*, on the one side, and *P. major*, on the other. It is especially interesting to note the significant *increase* of K^+ levels observed in the more salt-resistant taxa at high external NaCl concentrations – under lower salinity conditions, a decrease in K^+ was detected, as expected – suggesting the activation of potassium transport to the leaves, which could partly compensate the accumulation of sodium, thus contributing to salt tolerance by avoiding a drastic reduction of K^+/Na^+ ratios. In the less tolerant *P. major*, K^+ contents in the absence of salt were higher than in the other two taxa, and did not change significantly up to ca. 400 mM NaCl; at higher salinity levels, however, a decrease of K^+ levels was detected, leading to a sharp reduction of the K^+/Na^+ ratio.

4.3.4.3. Osmolyte synthesis

Environmental stress conditions leading to cellular dehydration, including salt and water stress, trigger the cytosolic accumulation of different organic compatible solutes, or osmolytes. The contribution of osmolytes to stress tolerance is not limited to their function in osmotic adjustment, as they have multiple additional roles as osmoprotectants, directly stabilising proteins and macromolecular structures under stress conditions, as ROS scavengers or, in some cases, as signalling molecules involved in the induction of changes in gene expression patterns (Munns, 2002; Flowers and Colmer, 2008; Munns and Tester, 2008; Türkan and Demiral, 2009). In the genus *Plantago*, all available data indicate that the polyalcohol sorbitol is the major functional osmolyte (Gorham *et al*., 1981; Koyro, 2006; Flowers and Colmer, 2008; Flowers *et al*., 2010). Sorbitol accumulation in response to salt treatments has been reported in the salt-tolerant *P. maritima* (Ahmad *et al*., 1979; Jefferies *et al*., 1979; Königshofer, 1983) and *P. coronopus* (Gorham *et al*., 1981; Koyro, 2006), as well

as in the salt-sensitive *P. media* and *P. lanceolata* (Königshofer, 1983). In one of our previous studies on *P. crassifolia*, we confirmed accumulation of this compound in plants growing in a littoral salt marsh in SE Spain, and showed how seasonal changes in sorbitol levels correlated with the degree of environmental stress affecting the plants in their natural habitat (Gil *et al*., 2011).

Sorbitol accumulated in response to salt stress, in the three species studied here. Yet, the relative increases over the corresponding controls were very poor, since sorbitol concentrations were already high in the absence of salt, especially in *P. coronopus* and *P. major* (about 1 $mmolg^{-1}$ DW). Therefore, these species appear to accumulate the osmolyte in the absence of salt, so that a strong induction of its synthesis when the plants are actually affected by high salinity conditions is not necessary, and the regulation of the response to salt stress could be mostly based on changes in the intracellular localisation of sorbitol. This is obviously also in agreement with the idea of a 'pre-adaptation' or 'metabolic preparedness' to stress in tolerant species. In *P. crassifolia*, sorbitol content in the absence of salt was about 50% of that measured in *P. coronopus* or *P. major* – which could be compensated, in terms of osmotic balance, by the higher levels of monovalent ions, Na^+ and Cl^- – and the maximum absolute values measured at the highest NaCl concentration tested were somewhat higher; therefore, the relative level of salt-dependent accumulation of sorbitol was higher in this species. Regarding responses to drought, a slight increase in sorbitol levels after four weeks of water stress was detected in *P. coronopus*, the species that appears to be most affected under these conditions, in terms of growth inhibition and dehydration of the leaves, but no significant drought-induced accumulation of sorbitol was detected in the other two *Plantago* taxa.

Pro and GB are probably the commonest osmolytes in plants, used by many angiosperm species to help maintain cellular osmotic balance. In a study on 51 halophytes

sampled in their natural environment in an inland salt marsh in Turkey, Tipirdamaz *et al*. (2006) found these two osmolytes as almost omnipresent solutes. In *P. maritima* they detected even higher levels of Pro and GB than of sorbitol (which was also substantial), despite the fact, mentioned above, that sorbitol is considered the major functional osmolyte in the genus *Plantago*; this could be explained by biosynthesis of Pro and GB from O_2-dependent metabolic pathways that would be promoted in non-submerged salt marshes, while sorbitol would accumulate under more anaerobic conditions, such as those present in marine salt marshes (Tipirdamaz *et al*., 2006). Pro accumulation in salt-treated *P. maritima* plants, in both roots and shoots, has also been recently reported (Sleimi *et al*., 2015). The concomitant synthesis of different osmolytes has been previously observed in many halophytes (e.g., Briens and Larcher, 1982), although it is generally assumed that each species uses preferentially one particular compound for osmotic balance under stress conditions. For example, typical GB accumulators first accumulate this compatible solute, and only later-on, when stress is more accentuated, they start to accumulate Pro (Stewart *et al*., 1979).

A similar behaviour has been observed in the most salt-tolerant *Plantago* species included in the present study, *P. crassifolia* and *P. coronopus*, in which no significant Pro accumulation was observed at salinity levels equivalent to those present in their natural habitats, at or below 400 mM NaCl. Only at very high salt concentrations, 600 – 800 mM NaCl, large relative increases of Pro contents were detected. In the more sensitive *P. major*, no induction of Pro biosynthesis was observed, at any of the tested salt concentrations. Some of these data confirm our previous Pro measurements performed in leaves from *P. crassifolia* plants grown in the field (Gil *et al*., 2014). Accumulation of additional osmolytes under increased levels of salt stress, for example Pro in the *Plantago* salt tolerant taxa, may be a built-in mechanism which could enable halophytes to rapidly adapt to and withstand possible increases in the degree of salt stress in their natural habitats, either short-term temporary

changes in soil salinity or long-term increased salinisation, such as that that can be expected as a consequence of climate change in salt marshes of the Mediterranean basin and other arid and semiarid regions. The patterns of Pro accumulation in *Plantago* species under water stress conditions also supported the notion that this 'secondary' osmolyte only accumulates when the plants are strongly affected by stress: a very large increase in Pro contents (> 100-fold over the control) was only detected in *P. coronopus*, the species more sensitive to drought, but no increase at all was measured in the succulent *P. crassifolia*, which could very efficiently avoid dehydration and was apparently little affected by the lack of irrigation.

It should be pointed out that the maximum Pro concentrations reached under the strongest stress conditions tested are still too low – three orders of magnitude below those of sorbitol – to contribute substantially to osmotic adjustment, even assuming that it accumulated exclusively in the relatively small volume of the cytoplasm. The same could be said of GB, which was found to slightly increase in the three investigated species, as a response to both, salt and water stress, but to reach maximum absolute contents below those of Pro, and much lower than those reported for plants that are true GB accumulators (e.g., Khan *et al*., 2000; Tipirdamaz *et al*., 2006; Gil *et al*., 2014). Nevertheless, it is likely that Pro, and maybe also GB, play a significant role in the tolerance mechanisms of these species in the presence of strong stress conditions. Yet these mechanisms would not involve maintenance of cellular osmotic balance, but rather be based on the osmolytes function(s) as a low-molecular-weight chaperons, ROS scavengers and/or signalling molecules (Ashraf and Foolad, 2007; Chen and Murata 2008; Nawaz and Ashraf, 2010; Szabados and Savouré, 2010; Chen and Murata, 2011; Grigore *et al*., 2011).

4.3.5. Conclusions

The comparative analyses shown here, regarding the responses to salt stress of three *Plantago* species adapted to different natural habitats, support the notion that stress tolerance in this genus is mostly based on transport of toxic ions to the leaves, their sequestration in the vacuole, and accumulation of sorbitol in the cytoplasm for osmotic adjustment. The relative tolerance of the three taxa is partly dependent on quantitative differences in the efficiency of these processes – for example, Na^+ and Cl^- leaf contents are lowest in *P. major*, the most salt-sensitive of the investigated taxa. Yet, there also exist specific mechanisms characteristic of the more tolerant species, such as the capacity to use inorganic ions as osmotica, even under low salinity conditions: Na^+ and Cl^- in *P. crassifolia*, Na^+ in *P. coronopus*. In addition, these salt-tolerant taxa are better adapted to withstand strong increases in soil salinity: in the presence of very high NaCl concentrations, they accumulate relatively large amounts of a secondary osmolyte (Pro), and appear to activate K^+ transport to the leaves, thus avoiding a drastic reduction in K^+/Na^+ ratios. Inorganic and organic solutes are also involved in the responses to water stress in *Plantago*, but in the succulent *P. crassifolia* – the most tolerant of the selected species – the extreme resistance to dehydration, not shared by *P. coronopus* or *P. major*, seems to be the critical factor contributing to drought resistance. Despite species-specific quantitative differences, some of these response mechanisms appear to be constitutive, as monovalent cations and sorbitol leaf contents may be relatively high even in the absence of stress. Therefore, our results also support the hypothesis of a 'pre-adaptation' or 'metabolic preparedness' to stress in tolerant species of the genus *Plantago*.

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Author contributions

Analyses in plant material were performed by M.A.H. and A.P. (treatment application, growth parameters, and osmolytes quantification), J.L. (ions), and M.-P.L.G. (sorbitol measurement via HPLC). M.B. was responsible for the identification of plant material and the co-supervision of plant-related work. Soil analyses were performed by M.A.H. and A.P. Statistical analyses and preparation of figures were carried out by M.A.H. and M.B., and O.V. was responsible for the general coordination of the project, the supervision of the biochemical work and the preparation of the manuscript (with contributions from M.A.H. and M.B. for the sections referring to botanical, soil and statistical aspects).

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Tab. 1 Electric conductivity (dS/m) in 1:5 water extracts (EC1:5) of pot soil samples after salt and drought treatment of the plants. Values shown are means ± standard deviations (n = 5); different lower case letters in each column indicate statistically significant differences between treatments of the same species according to Tukey test (α = 0.05).

Treatment	*P. crassifolia*	*P. coronopus*	*P. major*
Control	0.51 ± 0.04a	0.50 ± 0.04a	0.61 ± 0.04a
Water stress	0.56 ± 0.02a	0.46 ± 0.02a	0.68 ± 0.04a
100 mM NaCl	2.21 ± 0.13b	1.97 ± 0.12b	2.16 ± 0.11b
200 mM NaCl	3.97 ± 0.28c	4.11 ± 0.28c	4.06 ± 0.2c
400 mM NaCl	7.41 ± 0.38d	6.9 ± 0.55d	7.02 ± 0.56d
600 mM NaCl	8.45 ± 0.27e	8.47 ± 0.39e	8.59 ± 0.36e
800 mM NaCl	9.56 ± 0.66f	9.44 ± 0.39f	10.12 ± 0.72f

Tab. 2 Levels of sodium (Na+), potassium (K+), chloride (Cl-), and potassium/sodium (K+/Na+) ratios in leaves of the studied Plantago species, at the end of the water stress treatment (4 weeks) (means ± SD, n = 5). Different lower case letters indicate significant differences between control and water-stressed samples, for each species, according to Tukey test (α = 0.05).

Species	Treatment	Na^+ (µmol g^{-1} DW)	K^+ (µmol g^{-1} DW)	Cl^- (µmol g^{-1} DW)	K^+/Na^+
P. crassifolia	Control	1099.07 ± 186.23a	438.62 ± 89.89a	1183.55 ± 260,56a	0.41 ± 0.14a
	Water stress	1486.44 ± 174.83b	564.54 ± 99.26a	884.73 ± 117.60a	0.38 ± 0.09a
P. coronopus	Control	1343.94 ± 93.12a	659.84 ± 79.15b	516.53 ± 98.53a	0.49 ± 0.05a
	Water stress	1636.43 ± 198.84b	395.70 ± 39.17a	554.87 ± 150.95a	0.44 ± 0.04a
P. major	Control	249.07 ± 38.42a	861.84 ± 79.90a	395.90 ± 10.58a	3.52 ± 0.66a
	Water stress	236.12 ± 23.72a	955.40 ± 52.79a	443,55 ± 60,56a	4.06 ± 0.23a

Publication IV:

Subchapter 4.4.

Growth and Reproductive Success under Saline Conditions of Three Plantago Species with Different Levels of Stress Tolerance

Reference:

Al Hassan, M., Pacurar, A., Gaspar, A., Vicente, O., Boscaiu, M. (2014). Growth and reproductive success under saline conditions of three *Plantago* species with different levels of stress tolerance. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 42(1), 180-186.

Growth and Reproductive Success under Saline Conditions of Three *Plantago* Species with Different Levels of Stress Tolerance

Abstract. Growth and responses to salt stress at reproductive stages were studied in three *Plantago* species, two of them halophytes (*P. coronopus* and *P. crassifolia*) and one glycophyte (*P. major*). Plants were treated with increasing NaCl concentrations (0, 100, 200 and 400 mM NaCl) under controlled conditions in the greenhouse. Besides fresh biomass, several reproductive parameters were analysed: number of fertile/aborted spikes per plant, length of scapes and spikes, mean number of seeds per plant and mean seed weight. Seeds of those plants were germinated in controlled conditions, after which germination vigour, germination rate and percentage, length of hypo-cotyledon, length of cotyledon, length of roots, and angle of cotyledon were measured as well. All measured parameters were found to change significantly in parallel to the increase of the salt treatment, also the more salt tolerant species (*P. major*) was found to be more affected with this treatment.

Keywords: *Plantago*; cotyledon; flowering; halophytes; glycophytes; seed germination; seedlings.

4.4.1. Introduction

Salinity is an important environmental factor that affects the distribution of plants in their natural ecosystems and is one of the major threats for agriculture. According to recent data (FAO, 2008) more than 800 million ha of land world-wide are affected by salinity. The extension of saline soils is increasing approximately 1% each year due to poor rainfall, evaporation of water from the soil, weathering of rocks, and anthropogenic alterations, such

as irrigation with saline water, or abusive and indiscriminate use of fertilizers (Flowers *et al.*, 1995).

The immense majority of terrestrial plant species and practically all cultivated ones belong to the category of glycophytes, or plants that cannot survive in conditions of saline stress. On the contrary, the halophytes include species that remain viable and complete their life cycle in the presence of salt concentrations of at least 200 mM NaCl (Flowers *et al.*, 1986). Halophytes represent a small group of plants, of only about 350 species belonging to a few genera and families, adapted to saline ecosystems under harsh environmental conditions, thus avoiding competition with glycophytes (Flowers *et al.*, 1986).

Tolerance to salt stress is extremely complex and there are numerous interactions between stress factors and molecular, biochemical and physiological processes affecting the development of the plant (Ashraf and Foolad, 2007). However, most experimental studies on mechanisms involved in salt tolerance are carried out on glycophytes, especially on model plants. The limitations of these kinds of approaches and the necessity of increasing ecophysiological research on halophytes have been recently discussed by Grigore *et al.* (2011). Mechanisms of response to stress are similar in all plants, but their efficiency largely varies among species, but only in halophytes they are efficient enough to confer salt tolerance. Salt stress induces changes in the expression of genes encoding proteins involved in numerous processes, but it is known that in halophytes such mechanisms are more efficient and specialized. Differences between glycophytes and halophytes are the result of changes in the regulation of same basic packages of genes (Zhu, 2000; 2001; Xiong and Zhu, 2002); thus such differences are only quantitative and not qualitative (Greenway and Munns, 1980; Flowers *et al.*, 1986; Zhu, 2001; Vicente *et al.*, 2004).

A very useful approach for a better comprehension of the concepts of tolerance *vs.* adaption, which often are confused, is the use of species closely related taxonomically, hence

genetically proximate, but with different degrees of stress tolerance. The genus *Plantago* with more than 200 species and a total of 483 taxa (Tutel *et al*., 2005), out of which at least 20 species considered as halophytes, is one of the most suitable for such comparative studies. Despite the relatively high number of papers dealing with one or several *Plantago* species, there are no comparative studies on responses to saline stress at a crucial stage in plants life, which is reproduction. Flowering, but mostly seed germination and seedling development represent the bottle neck in the lifecycle of plants confronted to adverse environmental conditions. Germination is strongly inhibited by high salinity even in most halophyte species and, therefore plants adapted to saline environments developed strategies to postpone germination till the rain season, when soil salinity is alleviated. A study on effects of salt stress on germination in three *Plantago* halophytes (*P. coronopus, P. crassifolia*, and *P. microrrhiza*) proved that that salinity strongly affects their seed germination, and that in all three, there is an initial dormancy period, longer at higher temperatures (Luciani *et al*., 2001). Dormancy is a common trend in halophytes, and actually seeds of numerous Mediterranean halophytes germinate in autumn when soil salinity is alleviated by abundant rainfalls that wash out salts.

Although there are numerous publications of physiological and biochemical responses on species of the genus *Plantago*, only in *P. crassifolia* flowering and quality of seeds produced by plants grown in salt stress conditions were analysed (Boscaiu *et al*., 2005).

The aim of this study was to achieve a better comprehension of the differences between mere response to stress in the glycophyte *Plantago major*, and real adaption to stress in the halophytes *P. coronopus* and *P. crassifolia*. The paper compares responses to saline stress during growth and reproductive stage in the three species, but is focused mostly on seed quality, as expression of the reproductive success of plants grown under saline

treatments. Besides growth parameters and those related to seed production and seed quality, also seedlings characteristics was thoroughly analysed by a computer programme.

4.4.2. Material and methods

Studied species

Plantago coronopus L. is a cosmopolitan annual or biennial, present in a wide range of variable habitats, mostly on disturbed lands, waste places, or littoral sand dunes. It is considered as an indicator of salinization of marginal soils, since it tolerates moderate salt stress, its growth being even slightly stimulated by low saline concentration (Koyro, 2006). On the other hand Luciani *et al*. (2005) found its seed germination as the most sensitive to salt stress among the *Plantago* species they studied. Taxonomically it belongs to section *Coronopus* (Lam &DC) Dietr. of the subgenus *Coronopus* (Lam. & DC.) Rahn, which includes 11 Mediterranean species (Rahn, 1996).

Plantago crassifolia is a perennial halophyte, common in littoral salt marshes and other saline habitats in the Mediterranean region. Previous studies on the species (Vicente *et al*., 2004; Boscaiu *et al*., 2005) proved that salt tolerance of this species is moderate: its optimal growth was in absence of salt and a drastic reduction of seed quantity and quality was registered under high saline conditions. In natural environments the species appears only in areas with moderate salinity, on salt marsh borders, but never in strongly saline areas dominated by high tolerant halophytes. From the taxonomic point of view it is closely related to previous species, belonging to the same section.

Plantago major L. is a perennial (seldom annual) glycophyte, with origins in Eurasia and North Africa but naturalised throughout the world. It is much less stress tolerant than the two previous species and requires moderate humidity. Although the species has some salt

tolerance (Weber and Hanks, 2006), it cannot be included in the category of halophytes. Taxonomically it is not so closely related to the two previous species, since it is placed in the section *Plantago* of the subgenus *Plantago*, the largest within the genus, with 130 species worldwide (Rønsted *et al*., 2003).

Plant growth and saline treatments

Seeds of the three species under study were sown in seed trays containing a mixture of commercial peat and vermiculite mixture (3:1) and placed in a greenhouse with regulated temperatures of 17-23 °C, photoperiod of 16 hours, and a humidity of 85%. Twenty days after sowing young plants were transferred to individual pots on the same substrates. After four weeks treatments with increasing salt concentrations (100, 200 and 400 mm NaCl) were applied, maintaining a control watered with distilled water. The number of plants for each treatment and species was of 3-5, and irrigation was carried out twice a week with a volume of 1.5 l of salt solutions, and distilled water for control treatment respectively, for each tray containing 12 standard 9 cm in diameter pots. After two months all plants were harvested and vegetative parts individually weighed on a precision balance. Length of the longest leaf was also measured.

Analysis of the reproductive traits of plants

At the end of treatments floral parts were separated, and for each plant the total number of spikes was registered, and length of floral scape and spike measured. Seed set was estimated by counting and weighing seeds from approximately half of the fertile spikes per treatment and species.

Quality of seeds produced by the plants submitted to salt and control treatments was tested through investigating the germination capacity of seeds. Four replicas of 25 seeds per

species and treatment were placed in standard petri dishes with cotton and filter paper moistened with 25 mL of milliQ water and sealed with parafilm to avoid evaporation and incubated in a growth chamber, under a photoperiod of 16 hours, and a temperature of 25 °C. Number of germinated seeds, considered upon radicle emergence, was counted daily for a period of two weeks. Germination capacity was expressed as final percentage of germination and germination rate as MTG (mean time of germination) according to the formula given below (Ellis and Roberts, 1980): $MGT = \Sigma D n / \Sigma n$.

Where, D are the days from the beginning of the germination test, and n is the number of seeds newly germinated on day D.

The programme Image J was applied for the seedling analyses. Length of radical, hypocotyle and of cotyledon leaves, and angle between the cotyledons were measured in 60 randomly selected seedlings per species and treatment. In addition, seedlings were weighed on the precision balance. Seedling Vigour Index (SVI) was calculated by the formula by Abdul-Baki and Anderson (1973):

SVI = Germination percentage x [Mean root length (mm) + Mean hypocotyle length (mm)]

Since plants of *P. coronopus* watered without salt did not flower over the two months treatment, seeds from initial stock were used as control for the germination assay and seedlings measurements.

Statistical analysis

Data were analysed using SYSTAT v. 16. Levene test was applied to check whether the requirements of the analysis of variance are accomplished. Germination percentages were normalized by arcsine transformation prior to analysis of variance. Significance of differences among treatments and among species was tested by applying one-way ANOVA.

When the ANOVA null hypothesis was rejected, post-hoc comparisons were performed using the Tukey test.

4.4.3. Results and Discussion

Sodium chloride treatments inhibited growth in a concentration-depended manner in the three species under study. Maximal fresh weight (FW) was registered in the most vigorous species *P. major* in control plants, but it decreased by 10 fold in plants from 400 mM NaCl treatment. Fresh weight of *P. coronopus* and *P. crassifolia* decreased as well in a concentration depended manner, but only by 4 and 5.5 folds respectively (Fig. 1). This finding is in perfect agreement with the ecological requirements of the three species, since the most affected by salt is exactly the species that in its natural environment is never confronted with salinity.

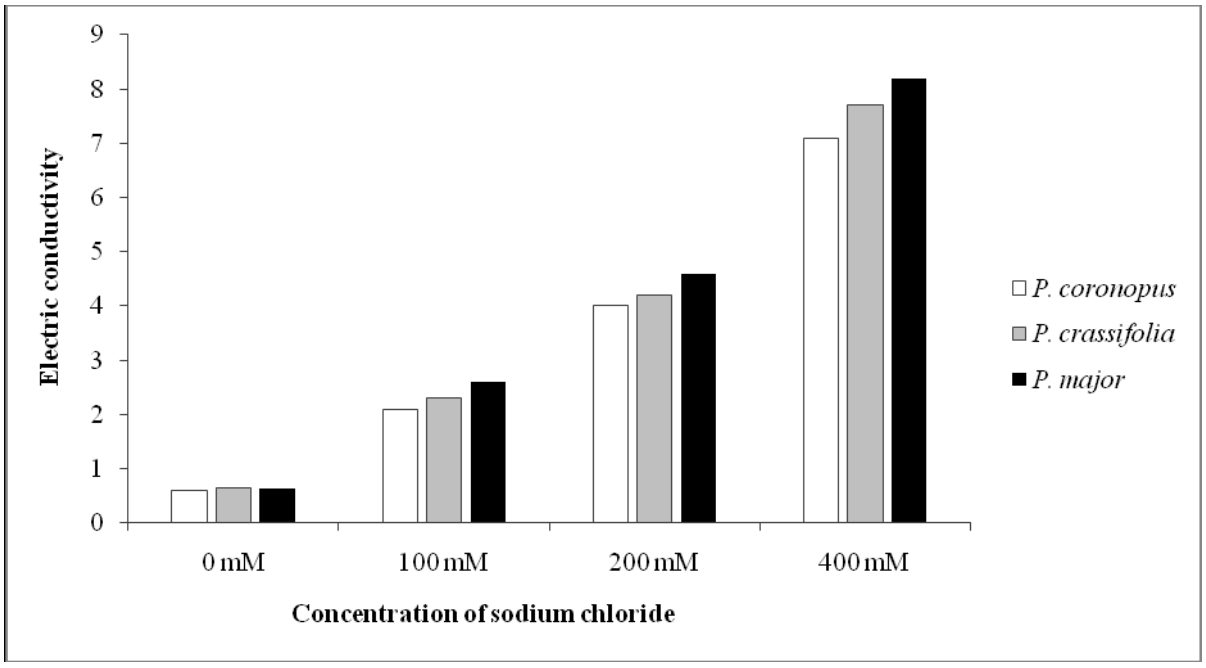

Fig. 1 Electric conductivity ($EC_{1:5}$) of the substrate after two months of salt treatments.

Another parameter used for the analysis of the effect of saline stress in is the length of the longest leaf, which generally is affected by salt. When comparing plants from control to those from the most saline treatment a 1.3-fold reduction was registered *in P. coronopus*, one of 1.8 in *P. crassifolia,* and one of 1.7 in *P. major* (Fig. 2). Although salt clearly affected the length of the longest leaf, there were no significant differences among species. Data related to the vegetative growth are similar to previous reports in *P. crassifolia* (Vicente *et al*., 2004), but no stimulation of growth by low salinity was detected in *P. coronopus*, as reported by Koyro (2006).

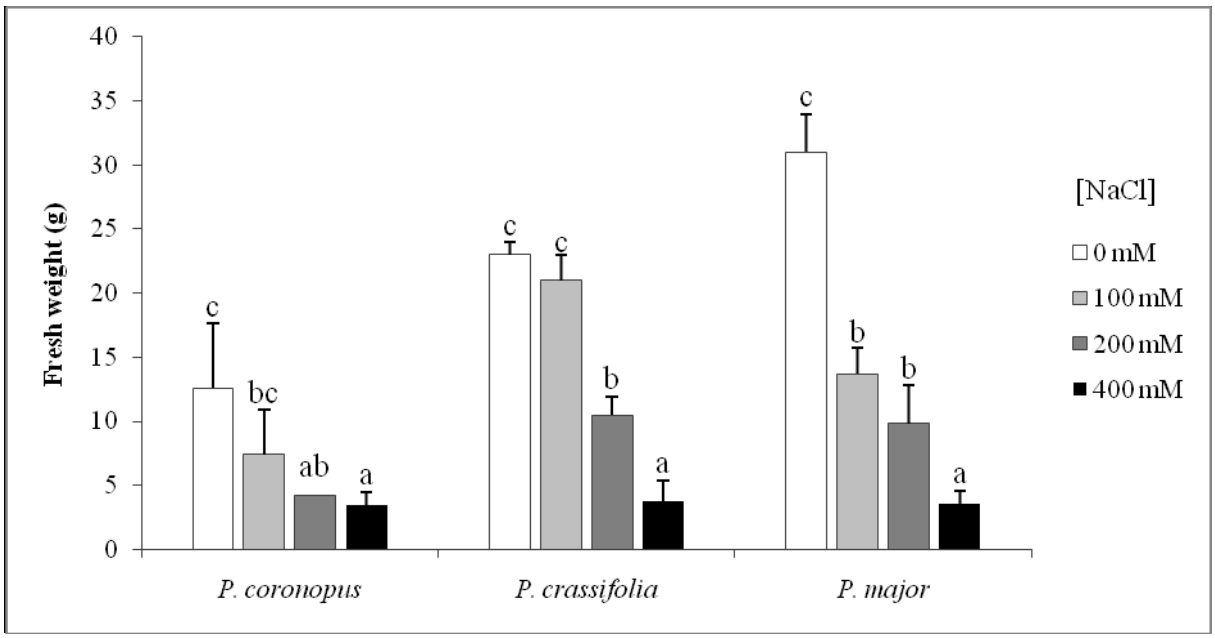

Fig. 2 Fresh weight of plants of *Plantago coronopus, P. crassifolia* and *P. major* treated for two months with the indicated NaCl concentrations. Same letters indicate homogeneous groups among treatments for each species.

Effects of salinity on floral characters were diverse among species. In *P. coronopus* only plants from treatments with 200 and 400mM NaCl produced flowers (Tab. 1). In *P. crassifolia* the mean number of spikes per plant increased in the treatments of 100 and 200 mM NaCl as reported in previous studies (Boscaiu *et al*., 2005, Grigore *et al*., 2012). At concentration of 400 mM NaCl the average number of spikes was similar to that registered in control (Tab. 1), whereas in a previous study (Boscaiu *et al*., 2005) after five months of

treatment with salty at this concentration plants did not flower at all. Salinity can induce both, inhibition or stimulation of flowering, early or delayed flowering, depending on the plant species (Grigore *et al*., 2012). The strategy of survival through next generation occurs both by early flowering (Blanvillain *et al*., 2011) and by increase of number of flowers produced as we found in *P. crassifolia* and *P. coronopus*. However, the glycophyte *P. major* responded completely different. In this species was registered a concentration dependent reduction of spike numbers, of 5.6 –fold when comparing plants in control and those from 400 mM NaCl treatment. An additional parameter, the size of the scape and spike itself was also measured and it was found to significantly decrease as the concentration of salt treatment increased in all species (Fig. 3). This finding proves that salinity may stimulate flowering, but it clearly negatively affects reproduction in the three species under study, by diminishing the quality of flowers produced under stress conditions. Perfectly correlated with the size of spikes was the number of seeds produced. Significant differences among treatments were registered, with the largest amount of seeds produced by plants from control in the three species. It is interesting to note that *P. major* produces a considerably higher amount of seeds in comparison with the other two species in all conditions. The mass of these seeds was also measured, and it was evident that seeds coming from control plants had the highest mass, that reduced with increasing salt concentrations in all species (Tab. 1).

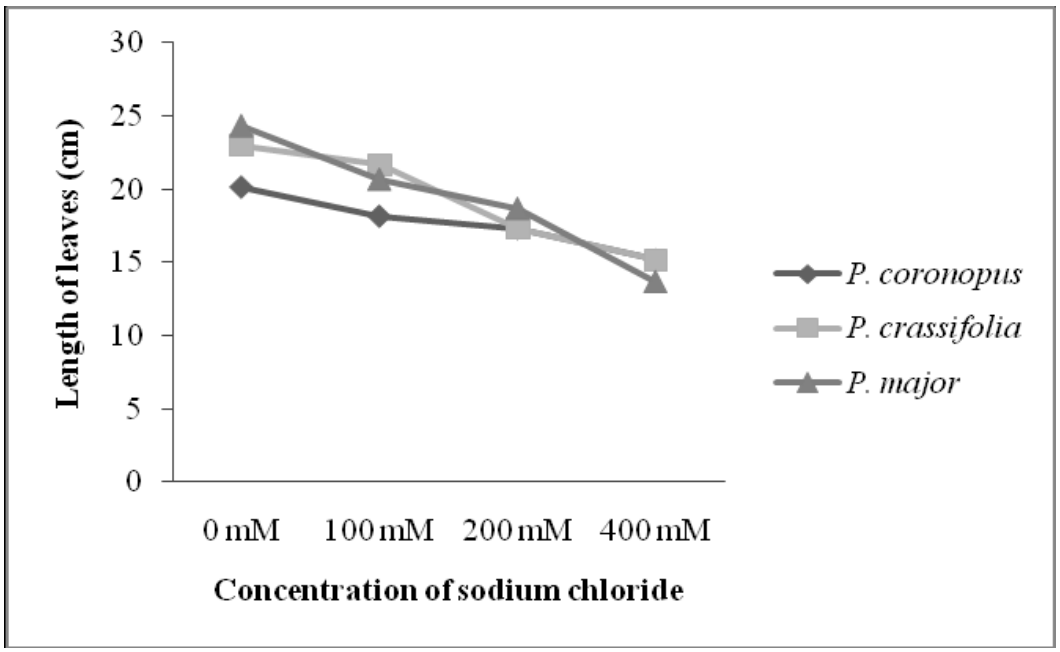

Fig. 3 Length of the longest leaf in the plants of *Plantago coronopus*, *P. crassifolia* and *P. major* treated for two months at the indicated NaCl concentrations.

Germination capacity of seeds from all treatments was also tested on filter paper moistened with distilled water, as shown in Methods. The percentage of germination, the mean rate of germination (MGT) and the seedling vigour index (SVI) are summarised in Table 1. There were no significant differences in the percentage of germination, whereas MGT slightly increased in saline treatments, but again differences were not statistically significant. On the contrary, the quality of seedlings was clearly affected by saline treatments. Significant was the loss of vigour of seedlings (SVI) which decreased 1.4 folds in *P. coronopus*, 2.5 folds in *P. crassifolia*, and 2.2 folds in *P. major*, when comparing seeds coming from control treatment and those from 400 mM NaCl treatment. The weight of seedlings followed the same patter, reducing with increasing salt concentrations (Tab. 1). In *P. crassifolia*, seedlings coming from plants watered without salts weighed 20% more than those from 400 mM treated plants; in *P. coronopus* such differences were much larger (290%) and maximal in *P. major* (320%). This finding supports the idea that the most salt

tolerant of the three species is *P. crassifolia*, which is minimally affected when compared with the other two

The length of root, hypocotyle and cotyledon and the angle between the cotyeldons was measured by the computer programme Image J. The roots length decreased 1.5-folds m in *P. coronopus*, 2.63-folds in *P. crassifolia*, and 2.3 folds in *P. major*; the hypocotyle length 1.7- folds in *P. coronopus*, 1.4-folds in *P. crassifolia*, but almost 3-folds in *P. major*; the cotyledon length two folds in *P. coronopus*, only slightly (1.22) in *P. crassifolia*, but more than three folds in *P. major*, when comparing seedlings with origin in control and 400 mM NaCl treatments (Fig. 4).

Although germination rate was high in *P. major* even for seeds with produced by plants treated with salt, their seedlings showed only poor quality, and almost half of those with origin in plants from the 200 and 400 mM NaCl treatments did not produced cotyledons, their development being blocked after radicle emergence, as it can be seen in (Fig. 5). The angle of the cotyledons (Tab. 1), increased with the increase of salt treatment mostly in *P. major*.

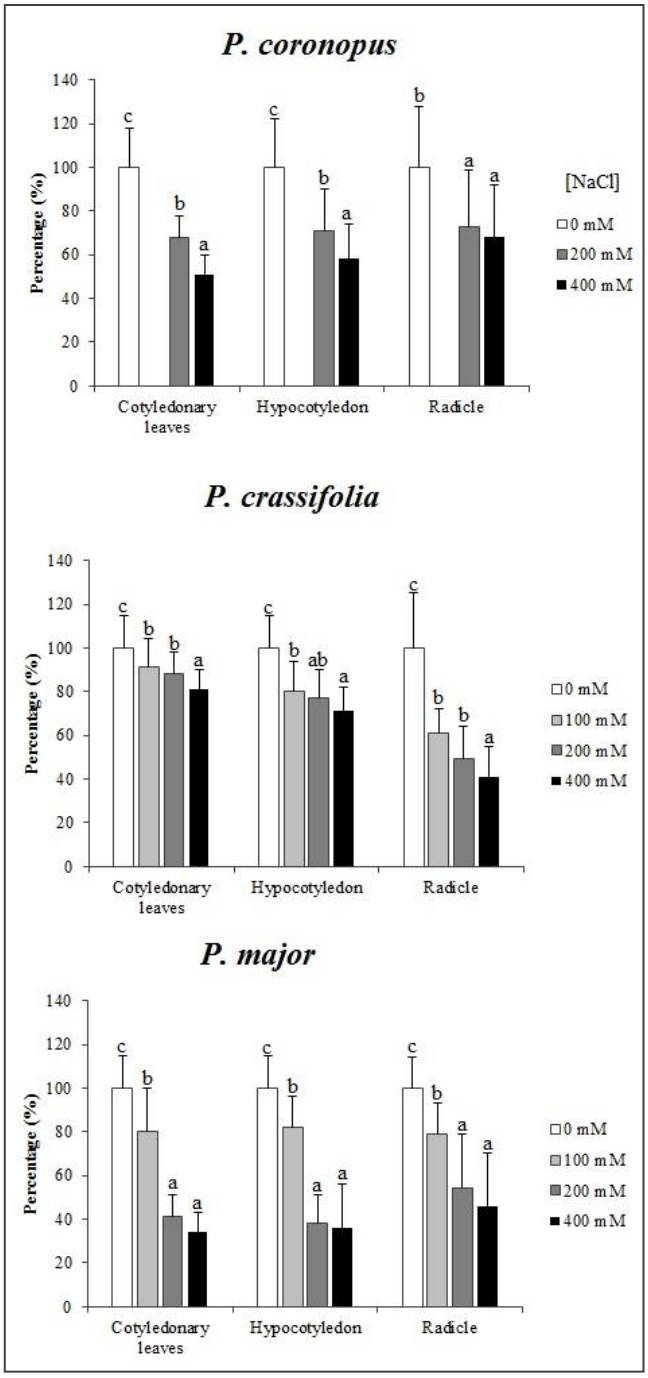

Fig. 4 Radicle, hypocotyle and cotyledon length reduction in 12 days-old seedlings obtained from seeds sampled from the plants treated for two months at the indicated NaCl concentrations. Percentages were calculated in relation to the controls' mean values which were considered 100%. Same letters indicate homogeneous groups among treatments for each species.

The angle of the cotyledon may be considered as a predictor of growth rate. Lower *et al.* (1978) reported that cucumber (*Cucumis sativus*) seedlings which displayed their cotyledons in an upward position (smaller angle) grew faster than plants which displayed

their cotyledons in a downward position (larger angle). An upward cotyledon angle was also correlated with increase in plant height, node number, and fresh weight at later growth stages.

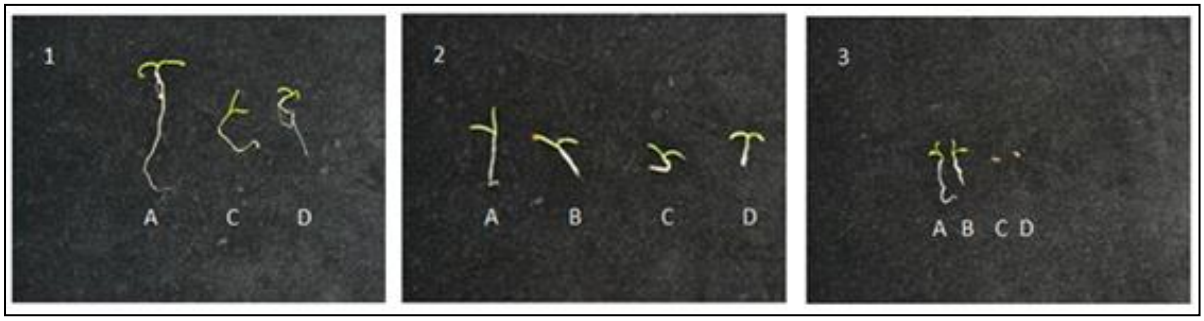

Fig. 5 Seedlings of *Plantago coronopus* (1), *P. crassifolia* (2) and *P. major* (3) obtained from the plants treated for two months with distilled water (A), 100 mM NaCl (B), 200 mM NaCl (C) or 400 mM NaCl (D)

4.4.4. Conclusions

Salinity affected vegetative and reproductive traits analysed in the three species. Plants produced under salt stress were less vigorous and their reproductive success was clearly affected by salinity, but two different patterns were detected, one corresponding to the halophytes *P. coronopus* and *P. crassifolia* and one to the glycophyte *P. major*. Fresh weight in *P. coronopus* and *P. crassifolia* reduced with increasing salinity, but this reduction was much stronger in *P. major*. Apparently salinity stimulated flowering in the halophytes, as spikes were produced only in the 200 and 400 mM NaCl treatments in *P. coronopus*, and their number increased in *P. crassifolia* in the 100 and 200 mM NaCl treatments in respect to control. The more vigorous *P. major* produced more spikes, but their number decreased according to the salt treatment. However, reproductive success was affected by salinity in the three species, since seed set and mass of seeds was reduced. Viable seeds maintained their germination capacity, as proved by high germination rates for all seeds, from all treatments, but quality of seedling obtained from seeds harvested in plants submitted to salt treatment was affected. Here again, *P. major* showed a different response: almost half of the seedlings

with origin in the plants watered with high saline concentrations (200 and 400 mM NaCl), blocked their development at very early stages. Although seedlings' weight and size decreased in saline conditions also in *P. coronopus* and *P. major*, reduction was much more accentuated in *P. m*ajor. Therefore, also this species may tolerate some salinity; it is obvious that it behaves as a glycophyte, with a very strong inhibition of growth and reproduction in condition of salt stress. When comparing the two halophytes, our findings support the idea that *P. crassifolia* is more salt tolerant than *P. coronopus*, since its responses to salinity especially at seedling level are leaser. This is also supported by ecological characteristics, only *P. crassifolia* being able to inhabit typical saline areas as salt marshes.

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Tab 1. Effects of increasing saline solutions on the traits related to reproductive success in *Plantago crassifolia*, *P. coronopus* and *P. major*. Mean ± SD values are shown. Same letters indicate homogeneous groups among treatments for each species, n.d. not determined since plants did not flower, and * seeds from the initial stock

Character	Species	0 mM NaCl	100 mM NaCl	200 mM NaCl	400 mM NaCl
Mean spike no/plant	*P. crassifolia*	3.66 ± 1.15a	4.33 ± 1.52ab	6.66 ± 1.52b	3.33 ± 0.57a
	P. coronopus	n.d.	n.d.	1.66 ± 0.57b	1.33 ± 0.57b
	P. major	7.66 ± 3.21b	5.66 ± 1.15b	4.66 ± 2.08ab	1.33 ± 0.57a
Mean seed no/spike	*P. crassifolia*	24.55± 9.02c	18.89 ± 7.52 b	11.32 ±3.18 a	7.09 ± 1.41a
	P. coronopus	n.d.	n.d.	34.30 ± 8.04b	10. 00 ± 3.08a
	P. major	28.34 ± 4.19d	23.33 ± 1.72c	17.54 ± 1.35b	8.81 ± 1.41a
Mean seed weight/plant (mg)	*P. crassifolia*	5.15 ± 2.42c	4.84 ± 1.5c	3.00 ± 0.63b	2.00 ± 0.38a
	P. coronopus	6.70 ± 1.78b	n.d.	6.70 ± 1.78b	1.62 ± 0.47a
	P. major	10.42 ± 2.94 c	8.12 ± 1.38b	7.37 ± 0.9b	3.50 ± 0.75a
Percentage of germination	*P. crassifolia*	88.71 ± 15.33c	72.83 ± 2.75b	70 ± 9.05b	48.66 ± 3.05a
	P. coronopus	n.d.	n.d.	289.5±27.57b	41±29.69a
	P. major	1369.20 ± 439b	1345.83 ± 230 b	1358.33 ± 281b	512.85 ± 291.0a
MGT (days)	*P. crassifolia*	0.49 ± 0.10c	0.49 ± 0.07c	0.41 ± 0.10b	0.39 ± 0.11a
	P. coronopus	0.34 ± 0.02c*	n.d.	0.27 ± 0.02b	0.17 ± 0.01a
	P. major	0.39 ± 0.02c	0.33 ± 0.02b	0.28 ± 0.01a	0.26 ± 0.01a
Seedling weight (mg)	*P. crassifolia*	98.40 ± 4.00b	84.00 ± 0.00a	81.00 ± 2.00a	89.00 ± 11.00a
	P. coronopus	96.00 ± 4.60a*	n.d.	99.00 ± 2.00ab	92.00 ± 0.00a
	P. major	99.00 ± 2.00a	98.00 ± 4.00a	97.00 ± 6.00a	98.00 ± 2.30a
SVI	*P. crassifolia*	4.79 ± 0.60a	4.94 ± 0.20a	4.66 ± 0.40a	5.14 ± 1.10a
	P. coronopus	2.92 ± 0.30a*	n.d.	2.76 ± 0.40a	3.02 ± 0.10a
	P. major	3.90 ± 0.20a	4.01 ± 0.10a	4.25 ± 0.14a	4.19 ± 0.20a
	P. major	6.45 ± 0.22b	6.32 ± 0.01b	5.94 ± 0.12b	5.22 ± 0.26a
Cotyledon angle (º)	*P. crassifolia*	5.07 ± 0.06c*	n.d.	3.57 ± 0.01b	1.75 ± 0.02a
	P. coronopus	3.01 ± 0.39b	2.18 ± 0.77ab	0.98 ± 0.36a	0.94 ± 0.38a
	P. major	2438.352	1176.84	1109.7	954.08

Publication V:

Subchapter 4.5.

Expression of the Vacuolar NA^+/H^+ Antiporter Gene (*NHX1*) in Three Plantago Species Differing in Salt Tolerance

Reference:

Al Hassan, M., Daniso, E., Martinelli, F., Boscaiu, M., Vicente, O. (2015). Expression of the vacuolar Na^+/H^+ antiporter gene (*NHX1*) in three *Plantago* species differing in salt tolerance (In preparation).

Expression of the Vacuolar NA^+/H^+ Antiporter Gene (*NHX1*) in Three *Plantago* Species Differing in Salt Tolerance

Abstract. Through homology we managed to successfully isolate and sequence the Na^+/H^+ antiporter gene *NHX1* of three species of the genus *Plantago*. *Plantago crassifolia* (*PcrNHX1*), *Plantago coronopus* (*PcoNHX1*), and *Plantago major* (*PmaNHX1*). Translation of the isolated and sequenced cDNAs showed that the NHX1 proteins comprised of 562, 561, and 553 amino acid residues respectively. The amino acid sequences of the *NHX1* gene in the three studied species showed more than 92% complementarity in between each other's and 68% with the previously isolated *NHX1* gene from the glycophyte *Arabidopsis thaliana* (*AtNHX1*). Change of expression of the sequenced genes and that of *AtNHX1* by the effect of salt stress was observed, after 2, 4, 8 and 24 hours of NaCl application (400 mM) at mRNA level using qRT-PCR. The studies revealed a higher and more durable expression of the gene under salt stress in the more tolerant *P. crassifolia*, while the least tolerant *P. major* had the lowest level of *NHX1* expression among the three studied species. A bioinformatics analysis was carried out to study the theoretical secondary structures of the isolated sequences, as well as generating a functional phylogenetic tree concerning a number of previously isolated NHX1 proteins in glycophytes and halophytes, that placed our three de novo sequences in the group to which most halophytes belong to.

Keywords: *NHX1; Plantago coronopus; P. crassifolia; P. major; salinity.*

4.5.1. Introduction

Plant growth and productivity are affected by a number of environmental factors, among which salinity has been determined as the most damaging and limiting (Allakhverdiev

et al., 2000). Worldwide more than 800 million ha of land is salt-affected (Rengasamy, 2006). And due to unsustainable irrigation practices about 1.6 million ha/year of irrigated lands become saline and go out of production (secondary salinization) (Tanji, 2002).

The detrimental effects of salinity on plant growth are associated with: (1) altered osmotic pressure (2) nutritional imbalance, (3) specific ion effect (ion toxicity, at high concentrations, Na^+ is toxic to plants because of its adverse effects on mineral and water uptake, enzyme activities, photosynthesis and metabolism (Niu *et al.*, 1995) or (4) a combination of all mentioned factors (Ashraf, 1994).

According to their abilities to cope with salt stress, plants have been classified into salt loving and tolerating halophytes and salt sensitive glycophytes. Halophytes have managed to withstand the deleterious effects of salt through three mechanisms: (1) avoidance (salt exclusion or the ability to exclude salts occurs through filtration at the surface of the root, or salt excretion/extrusion through salt bladders like in *Atriplex* (Mozafar and Goodwin, 1970) and *Mesembryanthemum crystallinum L.* (Agarie *et al.*, 2007) or through the cuticle like in *Tamarix* (Evert, 2006), or most importantly by Compartmentation of ions in vacuoles), (2) osmotic adjustment (the production of osmolytes to counter the osmotic pressure and oxidative stress caused by the excess of salt in the soil (Boscaiu *et al.*, 2013), (3) specificity in ions uptake and distribution (increased specificity in ion pumps for the uptake of K^+). Although these mechanisms are also present in glycophytes, they are less efficient in providing tolerance against high salt levels, and this could be due to anatomical and morphological changes during evolution (Meychik *et al.*, 2013), over expression of some genes conferring salt tolerance, or the possibility that these genes are merely constitutively expressed in halophytes, which is a matter of debate.

Na^+/H^+ antiporters are functional proteins that play vital roles in conferring salt tolerance in plants (Blumwald and Poole, 1985; Ballesteros *et al.*, 1997). Two types of

Na^+/H^+ antiporters are present in plants, *NHX1* located in the vacuole and *SOS1* located in the plasma membrane. The first functions in compartmentalizing Na^+ in vacuoles that reduces the toxicity of this ion in the cytosol and contributes to osmotic regulation (Takahashi *et al*., 2009), while the other type of Na^+/H^+ contributes in extruding Na^+ from cells (Blumwald *et al*., 2000). These proteins have been the focus of a number of studies and have been found to provide an increased tolerance to salinity when over expressed, whether the protein was originally isolated from glycophytes (Apse *et al*., 1999) or from halophytes and finally expressed in glycophytes like rice (Ohta *et al*., 2002).

To investigate whether those Na^+/H^+ antiporters coming from halophytes induce more tolerance to saline conditions than those isolated from glycophytes such as *A. thaliana*, we decided to use species from the genus *Plantago*, out of which three were chosen as the focus of our work, due to their different habitats, ecological requirements and salt tolerancy. The genus *Plantago* includes more than 200 species (Tutel *et al*., 2005), of which at least 20 species are considered halophytes, is one of the most suitable for such studies.

The three species of our choice are *P. crassifolia*, *P. coronopus*, and *P. major* that have been under the scope of our group´s work biochemically and physiologically under salt and drought stress (Boscaiu *et al*., 2005; Al Hassan *et al*., 2014; Gil *et al*., 2014). Two of the mentioned species are stress tolerant but are present in different ecological habitats, *P. crassifolia* (sandy littoral soils and salt marshes), and *P. coronopus* (disturbed lands, waste places, or littoral sand dunes). *P. major* which is less tolerant than the other two aforementioned *Plantago* species is frequent in non-saline, humid areas, and therefore considered as a glycophyte.

To understand the mechanisms of ion homeostasis in the salt-tolerant plants, we isolated *NHX1* cDNAs of the aforementioned species as well as that of the salt sensitive *A. thaliana* (*AtNHX1*), and compared their expression under salt stress via RT-qPCR, as well as

checking their theoretical secondary structures and their functional phylogenetic placement among other isolated and sequenced *NHX1*s of halophytes and glycophytes.

4.5.2. Material and Methods

4.5.2.1. Plant Material

Seeds of *P. crassifolia*, and *P. coronopus* have been acquired from the Natural Park if Albufera (Province of Valencia, Spain) and the seeds of *P. major* were bought from a commercial firm (Spicegarden, Barcelona, Spain), while seeds of *A. thaliana* ecotype Columbia-0 (Col-0), were acquired from the *"Arabidopsis* stock center", University of Nottingham, United Kingdom. Seeds (of *Plantago* species) were sterilized using hypochlorite (diluted with water) prior to germination on petri dishes for 3 weeks at 25 $^{\circ}$C with 16 hours photoperiod. Seedlings were transplanted onto a moistened mixture of peat (50%), perlite (25%) and vermiculite (25%) in 1 liter pots; the substrate was kept moist using Hoagland´s nutritive solution for another 3 weeks before the initiation of the treatments. Treatments were performed by adding 200 ml of nutritive solution supplemented with 400 mM NaCl directly on the pot, while those of control plants were irrigated with a similar volume with the nutritive solution without any additions. Plants were harvested after the start of treatments by 2, 4, 8 and 24 hours. All experiments were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 hours of light), temperature (23°C during the day and 17^{o}C at night), CO_2 level (≈ 300 ppm). Humidity ranged between 50-80% during the time course of the treatments (4 weeks).

4.5.2.2. Gene Isolation and Cloning

Fresh plant material was crushed utilizing liquid nitrogen and used for total RNA extraction using the CTAB-PVP protocol (Gambino *et al*., 2008). After quantification and checking of the RNA quality using nanodrop; Total cDNA synthesis was carried out using Maxima First Strand cDNA Synthesis Kit for RT-qPCR. Oligios designed through homology with cDNA of *P. maritima* ´s *NHX1* gene that was previously sequenced (Staal *et al*. 1991), were used to isolate the *NHX1* gene of the species understudy. The sense primer PmNHX1F0 (5´-ATGGTGTTCGACTCAGAAACTATG-3´), and the antisense primer PmNHX1R0 (5´-TCACTGCTTGGGTTCTTCAGGCCA-3´) were used to isolate the full length of the cDNA of the three *Plantago* species that are studied in this work, while sense prime AtNHX1F0 (5´-ATGTTGGATTCTCTAGTGTCG-3´) and antisense primer AtNHX1R0 (5´-TCAAGCCTTACTAAGATCAGG-3´) were used to isolate the cDNA of *NHX1* gene of *A. thaliana*.

Isolated sequences were cloned in pGEM®-T Easy Vector and were sequenced by the sequencing service in the IBMCP, UPV, Valencia, Spain. Alkaline lysis method (Brinboim and Doly, 1979) was used for minipreps, to check for the presence of insert in the pGEM®-T Easy Vector through digestion by restriction enzymes prior to sending to sequencing.

4.5.2.3. RT-qPCR Expression Assay

A fragment of β-Actin gene was used as housekeeping gene for RT-qPCR analysis using sense oligio ActF1 (5´-TGTATGTCGCTATTCAGGC-3´) and antisense oligio ActR1 (5´- AGTAACCTCTCTCTGTCAG-3´), while the sense oligio AtNHX1F1 (5´-GGAACCTTTGACTTGGGTGA-3´) and the antisense oligio AtNHX1R1 (5´-ATCAAAGCTCTGAATCGCGT-3´) were used to study the expression of gene *AtNHX1*. Expression of *PcrNHX1*, *PcoNHX1*, and *PmaNHX1* through RT-qPCR was carried out using sense primer PmNHX1F1 (5´-CAGTGGACATGTTGGCAACTTC-3´) and antisense primer

PmNHX1R1 (5´-ATGGGAACTTTTACCCTGACTTATTAG-3´). The expression study was carried out using SYBR® Green PCR Master Mix acquired from Thermo Fisher Scientific.

4.5.2.4. Bioinformatics Analysis

Protter programme was used to predict the secondary structure of the isolated proteins (Omasits *et al*. 2014). Multiple sequence alignment and generation of a phylogenetic tree were performed with ClustalΩ (Thompson *et al*., 1994) and TreeView software, respectively.

4.5.3. Results

Isolated cDNA sequences of the three *Plantago* species under study, were found to be 1686, 1683, and 1659 bp for *PcrNHX1*, *PcoNHX1*, and *PmaNX1*, respectively (sequences in details, in Appendix). Deduced amino acids sequences were aligned and showed 92% similarity in between the three aforementioned *Plantago* species, and 62% with *AtNHX1* (Fig. 1).

Fig. 1 Alignment of deduced NHX1 sequences of amino acid residues of AtNHX1, PcrNHX1, PmaNHX1, and PcoNHX1. Symbol "*", ":", and ":"; Is used to denote a residue that is similar in all four, three, and two studied species, respectively.

Prediction of secondary structures of the de novo isolated sequences of *Plantago* species showed the presence of 12 transmembrane domains (Fig. 2), which is similar to that of *AtNHX1*.

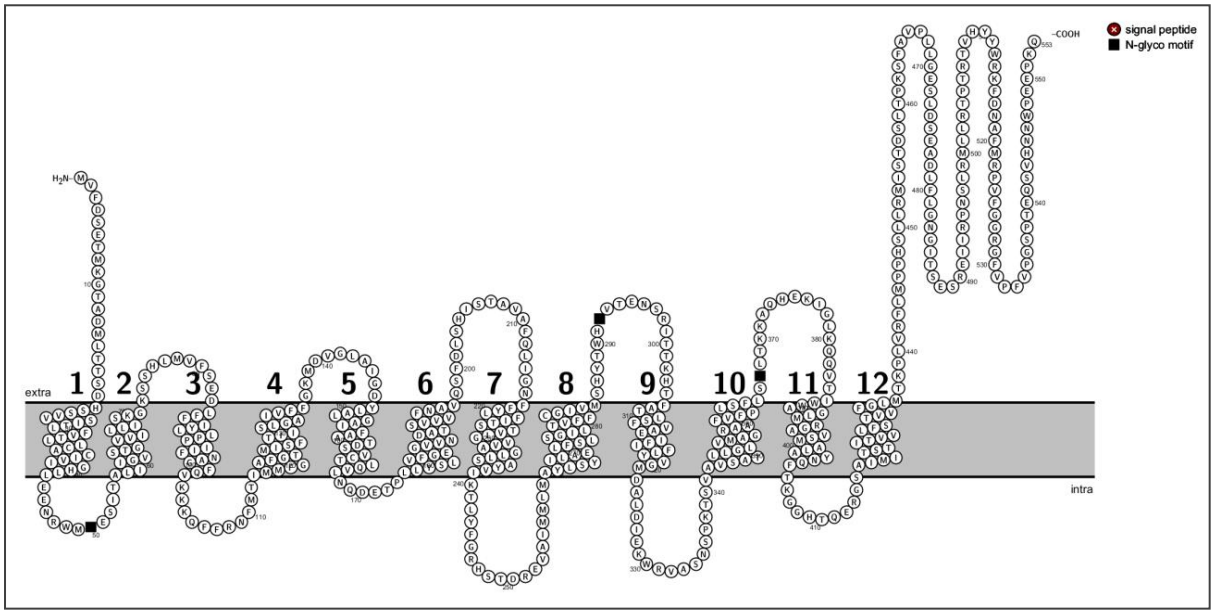

Fig. 2 Predicted secondary structure of *PmaNHX1*, with 12 transmembrane regions, that is similar to that of *AtNHX1*, *PcraNHX1* and *PcoNHX1*.

RT-qPCR analysis of the *NHX1* mRNA expression levels after the application of 400 mM NaCl after 2, 4, 8, and 24 hours, showed a higher and more durable expression in *PcrNHX1* (Fig. 3*)*, reaching 6 folds at 8 hours, and maintaining a high level of 5 folds at 24 hours. Whereas, *PcoNHX1* required more time to elevate its expression to such levels that reached 4,8 folds at 24 hours. *PmaNHX1* showed a minor overexpression by 2 folds, after 8 and 24 hours of salt application, which is higher than the levels recorded by *AtNHX1*, which nearly didn't show a significant increase in expression.

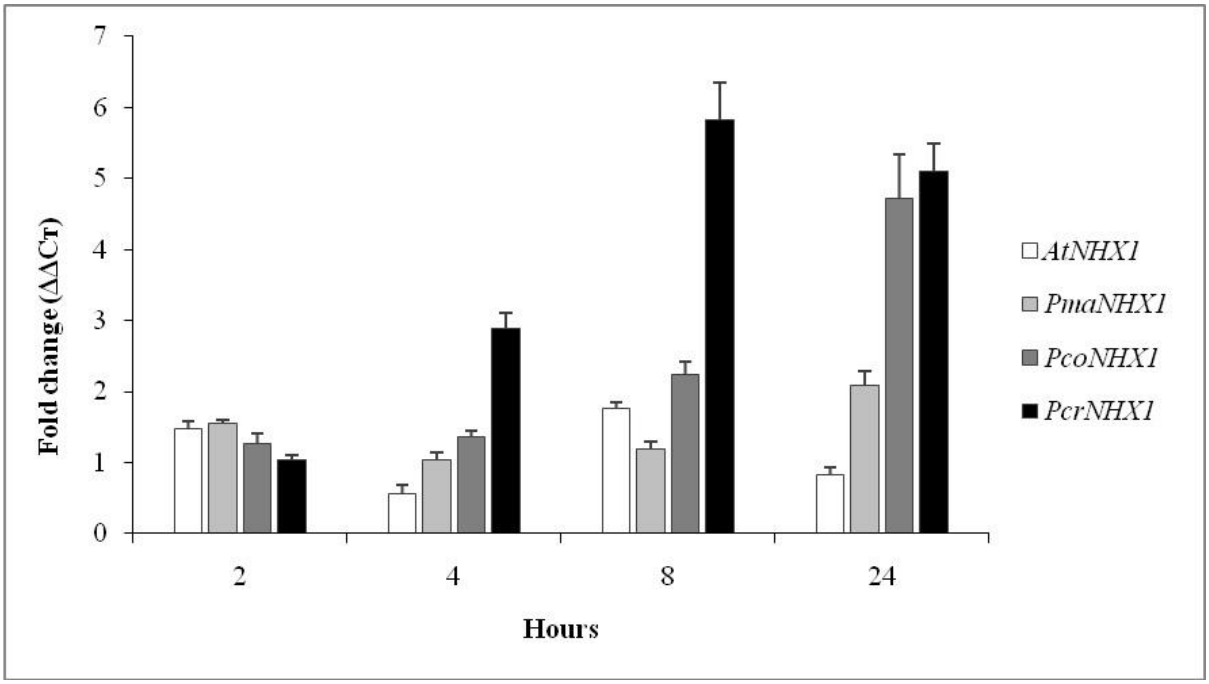

Fig. 3 Fold change ($\Delta\Delta C_T$), in the expression of *NHX1* after application of 400 mM NaCl, with respect to control plants expression levels of the three *Plantago* species under study and *A. thaliana*.

Functional phylogenetic studies showed that the *Plantago* species understudy belong to the same functional group containing most halophytes (Fig. 4), while *A. thaliana´*s NHX1 (AtNHX1) was found to belong to another, alongside crop species such as *Zea mays* and *Oryza sativa*.

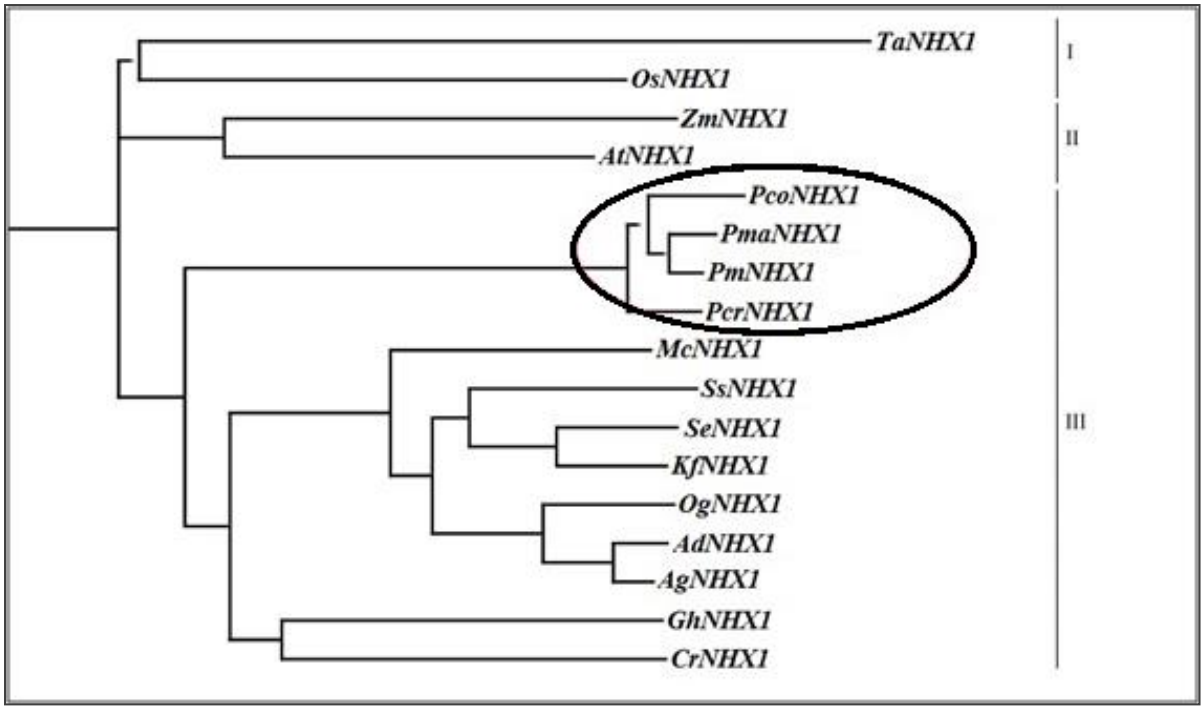

Fig. 4 Neighbor-joining phylogenetic tree of Na^+/H^+ antiporter protein sequences. TreeView was used to do multiple sequence alignment and to generate phylogenetic tree. NHX1 sequences of *Triticum aestivum* (TaNHX1), *Oryza sativa* subsp. Indica (OsNHX1), *Zea mays* (ZmNHX1), *Plantago maritima* (PmNHX1), *Mesembryanthemum crystallinum* (McNHX1), *Suaeda salsa* (SsNHX1), *Salicornia europaea* (SeNHX1), *Kalidium foliatum* (KfNHX1), *Oxybasis glauca* (OgNHX1), *Atriplex dimorphostegia* (AdNHX1), *Atriplex gmelinii* (AgNHX1), *Gossypium hirsutum* (GhNHX1), and *Citrus reticulata* (CrNHX1), were used alongside AtNHX1, PcrNHX1, PcoNHX1, and PmaNHX1.

4.5.4. Discussion

Understanding the mechanisms underlying plant salt tolerance, is essential for developing resistance in salt sensitive and affected crop species and thus crop yield (Blumwald, 2000; Munns and Tester, 2008). This made the idea of exploring how halophytes tolerate salt interesting, most importantly in part of the work to improve agricultural production (Inan *et al*., 2004; Orsini *et al*., 2010) especially in light of the recent decade's climate change and increasing human population and demand on food.

In saline soils, where Na^+ is the predominant toxic ion, excess accumulation of Na^+ in cytosol is detrimental to many metabolic and physiological processes, vital for plant growth and productivity, as it causes ion imbalance, hyper osmotic stress, and oxidative damage to plants (Hasegawa *et al*., 2000). To deal with the influx of Na^+ plants have evolved a number

of sophisticated mechanisms that includes restricted uptake/exclusion of Na^+ from cell, and compartmentalization of Na^+ into vacuoles.

Vacuolar Na^+/H^+ antiporter *NHX1* is responsible for the compartmentalization of Na^+ into vacuoles which not only provides an efficient mechanism to avert deleterious effects of Na^+ in cytoplasm, but also utilizes Na^+ in maintaining osmotic potential for driving water into the cell (Blumwald *et al*., 2000; Hasegawa *et al*., 2000).

Vacuolar compartmentalization of Na^+ is a critical process in salt adaptation, conserved in both halophytes and glycophytes. *NHX1* has been studied comparatively concerning on functional and molecular levels between halophytes and glycophytes, which were distinct and genetically unrelated (Li *et al*., 2008); A matter that we aimed to address though the work at hand, via comparative studying of the *NHX1* gene expression of *Plantago* species with different salt tolerance levels.

Through the isolation and sequencing of *NHX1* belonging to the *Plantago* species under study, we conclude that the sequence and structure is highly conserved among those species, even though there are some differences from *AtNHX1*. However, to investigate differences on functionally on a structural level, we attempted to study the *NHX1* gene from a number of halophytes and glycophytes through the alignment and generation of a phylogenetic tree. The results showed that most halophytes studied reside functionally together (even though this group does contain some glycophytes as well). However, to exclude the possibility of coincidence and evolution playing a role in this distribution, we studied another gene in a similar manner (*Rbcl*) (Fig. 5).

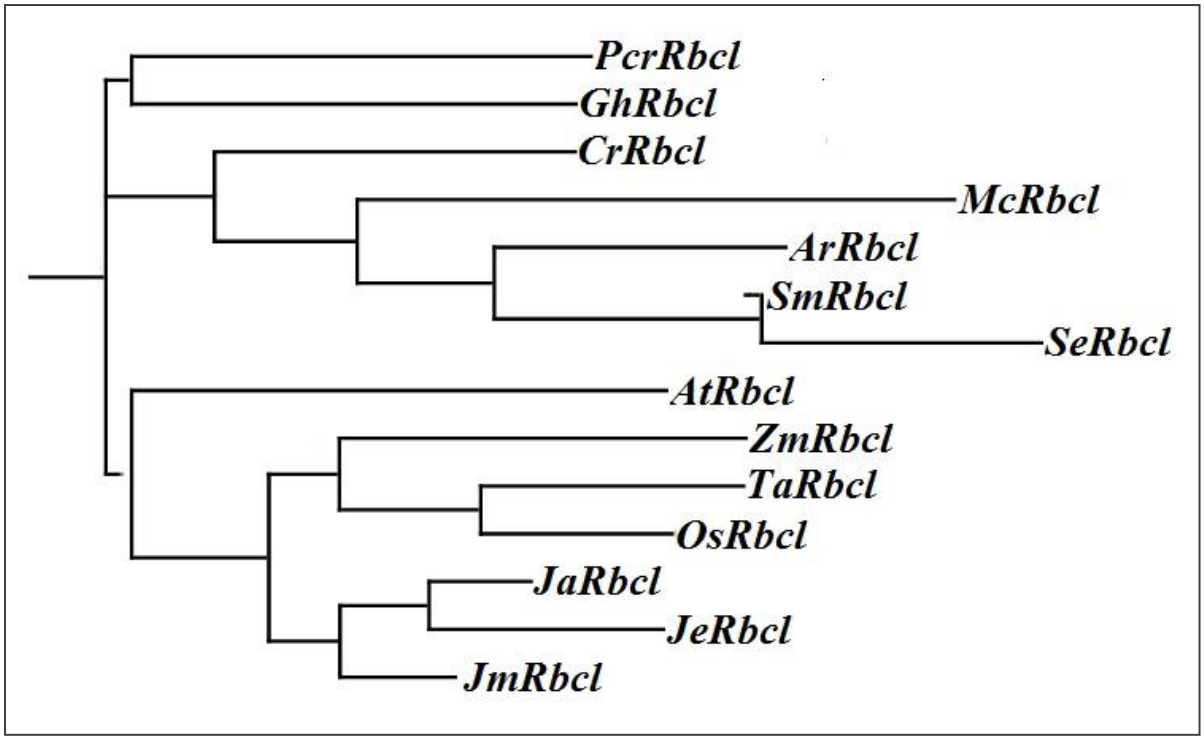

Fig. 5 Neighbor-joining phylogenetic tree of Ribulose bisphosphate carboxylase large chain (Rbcl) protein sequences. TreeView was used to do multiple sequence alignment and to generate phylogenetic tree. NHX1 sequences of *Plantago crassifolia* (PcrRbcl), *Gossypium hirsutum* (GhRbcl), *Citrus reticulata* (CrRbcl), *Mesembryanthemum crystallinum* (McRbcl), *Atriplex rosea* (ArRbcl), *Suaeda maritima* (SmRbcl), *Salicornia europaea* (SeRbcl), *Zea mays* (ZmRbcl), *Triticum aestivum* (TaRbcl), *Oryza sativa* subsp. Indica (OsRbcl), *Juncus acutus* (JaRbcl), *Juncus effuses* (JeRbcl), and *Juncus maritimus* (JmRbcl), were used alongside *Arabidopsis thaliana* (AtNHX1).

The distribution of *Rbcl* was completely different than that of *NHX1*, a fact that made us conclude that the distribution of *NHX1*, with most halophytes lumped together in a single group (with our de-novo isolated *NHX1*s from *Plantago*, included in that group), could be because of a functional distinction on the structural level that differentiates halophytic NHX1 proteins than glycophytic ones.

To investigate if this functional distinction is the only factor playing a role in the elevated function and expression (previously reported (Li *et al*., 2008)) of *NHX1* from halophytes, we studied the gene expression of *NHX1* of 3 *Plantago* species and *Arabidopsis thaliana*, after 2, 4, 8 and 24 hours of salt application. Our findings showed an increase of *NHX1* expression in all *Plantago* species, but the up regulation in expression was related with tolerance levels since *P. crassifolia* the most tolerant of the 3 studied *Plantago* species had

the highest increase in expression and the most durable, in reverse to the least tolerant studied *Plantago* studied, *P. major*. *AtNHX1* showed no significant increase within a day of salt application, even though other studies showed an increase but after a longer period (Sottosanto *et al*., 2007).

4.5.5. Conclusions

Na^+ compartmentalization is a key strategy in plant´s salt stress resistance strategy, in which *NHX1* as a Vacuolar Na^+/H^+ antiporter plays a major role. Our work showed that there are some functional differences on the structural level between halophytes and glycophytes, however it is not the only reason responsible for the difference in expression, since *PcrNHX1* showed a higher expression than *PmaNHX1*, even though both have nearly identical sequences. As such, we conclude that the main reason for higher and more efficient expression and function of *NHX1* in halophytes, maybe not due to structure or sequence of the protein itself, but rather its activation, expression and folding machinery including promotor, cis elements, transcription factors among others….

4.5.6. References

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Publication VI:

Subchapter 4.6.

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

This manuscript has been submitted to the Journal; *Functional Plant Biology*, and is currently under review.

Reference:

Al Hassan, M., Lopez-Gresa, M.P., Boscaiu, M., Vicente, O. (2015). Stress tolerance mechanisms in *Juncus*: Responses to salinity and drought in three *Juncus* species adapted to different natural environments (Under revision).

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

Abstract. Comparative studies on the responses to salt and drought stress, regarding control of ion transport and accumulation of specific osmolytes, have been carried out in three *Juncus* species adapted to different natural habitats, two halophytes (*J. maritimus* and *J. acutus*) and a salt-sensitive taxon (*J. articulatus*). This experimental approach was proven to be successful for defining several mechanisms relevant for stress tolerance in this genus. Salt tolerance in *Juncus* partly depends on the inhibition of transport of toxic ions to the aerial part of the plants: in the three selected taxa, Na^+ and Cl^- accumulated to the same extent in the roots of salt treated plants, in parallel to increasing NaCl concentrations; however, ion contents were lower in the culms and correlated with the relative salt sensitivity of the species: the lowest levels were measured in the halophytes, whereas the highest were determined in *J. articulatus*. Activation of K^+ transport at high salt concentration also seems to contribute to salt tolerance in the halophytes. Maintenance of cellular osmotic balance is mostly based on the accumulation of soluble sucrose in the three species – together with glucose and fructose in *J. articulatus*. Yet, neither the relative salt-induced increase in sugar contents, nor the absolute concentrations reached in each species, can explain the observed differences in salt tolerance. Proline contents, on the contrary, increased significantly – almost 60-fold in the presence of 400 mM NaCl, as compared to the controls – only in the salt-tolerant *J. maritimus* and *J. acutus*, but not in *J. articulatus*. Similar patterns of osmolyte accumulation were observed in response to water stress. This results support a functional role of proline in stress tolerance mechanisms in *Juncus*, probably not only contributing to osmotic adjustment, but also because of its additional 'osmoprotectant' activities.

Keywords: *Juncus*; proline; osmolyte; salt stress; drought; ion transport.

4.6.1. Introduction

Salinity, together with drought, is one of the most severe environmental stress factors which shape the distribution of plant species in nature, and is also responsible for large losses in crop production worldwide: accumulation of salts dissolved in irrigation water is leading to the progressive 'secondary' – of anthropic origin – salinisation of arable land, mainly in arid and semi-arid regions; this problem will worsen in the near future due to the forecasted effects of climate change (Boyer, 1982; Bartels and Sunkar, 2005; Watson and Byrne, 2009; IPCC, 2014; Fita *et al*., 2015). While all major crops and most wild species are relatively sensitive to salt stress, some plants – the halophytes – have evolved different mechanisms that allow them to withstand high salinity levels in their natural habitats. Halophytes have been defined as those plants specific for saline environments that can complete their life cycle in soils with salinity levels equivalent to, at least, 200 mM NaCl (Flowers *et al*., 1986; Flowers and Colmer, 2008). They constitute a small fraction of angiosperm species, only about 0.25%, and are widely distributed in different plant families and genera (Flowers *et al*., 2010).

Their academic interest and practical implications for agriculture have prompted intensive research, over the last decades, on the responses to salt stress and the mechanisms of salt tolerance in plants. There is now overwhelming evidence that plants react to increased soil salinity by activating a series of basic, conserved response pathways – which overlap with the responses to other abiotic stresses, such as drought, cold or high temperatures. These responses include the control of ion transport, compartmentalisation of toxic ions in vacuoles and accumulation of osmolytes in the cytosol to maintain cellular osmotic balance, the synthesis of 'protective' metabolites and proteins, or the activation of antioxidant systems

(Flowers *et al.*, 1986; Glenn *et al.*, 1999; Hussain *et al.*, 2008; Vinocur and Altman, 2005; Zhu, 2001; Ozgur *et al.*, 2013; Bose *et al.*, 2014; Kumari *et al.*, 2015; Volkov, 2015). These basic mechanisms are not specific for halophytes, but shared by all plants, and their activation does not necessarily lead to salt tolerance; in fact, as mentioned above, most plant species are glycophytes; that is, salt sensitive. Therefore, salt tolerance, which varies widely in different species, must depend on the relative efficiency of the aforementioned mechanisms of response (Pang *et al.*, 2010, Kumari *et al.*, 2015). Moreover, there is no single halophytic 'model species', as different salt tolerant plants use different mechanisms to efficiently cope with the deleterious effects of high soil salinity. Yet, the relative contribution of different salt stress responses to salt tolerance in a given species – or in a group of related taxa – remains largely unknown.

We believe that performing comparative studies on the responses to salt stress of genetically related taxa with different degrees of tolerance – such as congener wild species adapted to distinct habitats – will help to elucidate relevant salt tolerance mechanisms, by correlating the relative salt tolerance of the species under study with salt-induced changes in biochemical markers associated to particular response pathways.

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

Three species adapted to different natural habitats were chosen for this study. *J. maritimus* Lam. is a halophyte, common in temporarily flooded wetlands in the temperate regions of the world, including the Mediterranean basin. *J. acutus* L. is a sub-cosmopolitan species, that often coexist with *J. maritimus* but is common also on dunes, where water is the

main limiting ecologic factor; it has been reported as less salt tolerant than *J. maritimus* (Boscaiu *et al*., 2011; 2013). *J. articulatus* L. seems to be a much more sensitive species; it is frequent in the northern hemisphere and in Australia, in different humid areas such as wetlands, and also along the creeks and rivers (Albrecht, 1994). No previous study on the stress tolerance of this *Juncus* species has been carried out, but it is never found in saline habitats, (Chambers *et al*., 1995).

Regarding the taxonomic relation of the three *Juncus* species, *J. acutus* and *J. maritimus* are recognised as close taxa belonging to the same subgenus (*Juncus*), whereas *J. articulatus* was classified within the subgenus *Septati* Buchenau, section *Ozophyllum* Dumort (Fernádez-Carvajal, 1981). Molecular systematic analysis confirmed that *J. acutus* and *J. maritimus* are closely related, as inferred from DNA sequence data from the plastid *rps*16 intron, *trn*L intron, and *trn*L–F intergenic spacer (Jones *et al*., 2007). In a strict consensus tree obtained from the 555 most parsimonious trees of the combined rbcL and trnL-F DNA matrix, *J. articulatus* and *J. maritimus* were found to be more distantly related (Drábkóva *et al*., 2006).

The major aim of this work was to correlate the relative salt tolerance of the aforementioned *Juncus* species – established from their distribution in nature and by measurements of salt-induced growth inhibition under controlled experimental conditions – with specific responses based on the control of ion transport and the accumulation of different osmolytes. Since the responses to drought and salinity seem to overlap, the analysis was extended to plants subjected to water stress treatments, to check whether the same mechanisms were responsible for the relative resistance of the analysed *Juncus* species to both stresses. In line with the ideas discussed above, the results of this study should contribute to our knowledge on the general mechanisms of stress tolerance in plants and,

particularly, should help to distinguish those stress responses that are relevant for tolerance in *Juncus*, from those that are not.

4.6.2. Material and Methods

4.6.2.1. Plant material and Experimental Design

Seeds of *J. acutus* and *J. maritimus* were harvested in a salt marsh located in 'La Albufera' Natural Park (Province of Valencia, Spain), and those of *J. articulatus* in a non-saline area of the same Natural Park. All seeds were sown directly into a moistened mixture of peat (50%), perlite (25%) and vermiculite (25%) in 1 liter pots (Ø = 11 cm). During the entire course of the germination process the substrate was kept moderately moist, using Hoagland nutritive solution. Water and salt stress treatments were started 42 days after germination. Salt stress experiments were performed with three different concentrations of salt (100, 200 and 400 mM NaCl). The control plants were watered twice a week with Hoagland nutritive solution (1.5 l for each tray, containing 12 pots), and salt stressed plants with the same volume of nutritive solution containing NaCl, at the concentrations indicated above. Drought treatments were performed by completely ceasing irrigation. All experiments were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 hours of light), temperature (23^{o}C during the day and 17^{o}C at night), and a CO_2 level of ca. 300 ppm. Humidity ranged between 50-80% during the course of the treatments (eight weeks).

4.6.2.2. Soil analysis

Electrical conductivity ($EC_{1:5}$) of the substrate was measured after eight weeks of treatment. Soil samples were taken from five pots of each treatment, air-dried and then passed

through a 2-mm sieve. A soil:water (1:5) suspension was prepared in deionised water and mixed for one hour at 600 u/min, at room temperature. Electric conductivity was measured with a Crison Conductivity meter 522 and expressed in dS/m.

4.6.2.3. Plant growth parameters

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

4.6.2.4. Ion content measurements

Contents of potassium, sodium and chloride were determined in culms and roots of the plants sampled after the stress treatments. Measurements were performed according to Weimberg (1987), in aqueous extracts obtained by incubating the samples (0.15 g of dried and ground plant material in 25 ml of water) for 1 h at 95ºC in a water bath, followed by filtration through a filter paper (particle retention 8-12 µm). Sodium and potassium were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, USA) and chlorides were measured using a Merck Spectroquant Nova 60® spectrophotometer and its associated test kit (Merck, Darmstadt, Germany).

4.6.2.5. Osmolyte quantification

Proline (Pro) content was determined in fresh plant material by the ninhydrin-acetic acid method described by Bates *et al*. (1973). Pro was extracted in 3% aqueous sulfosalicylic

acid, the extract was mixed with acid ninhydrin solution, incubated for 1 h at 95ºC, cooled on ice and then extracted with two volumes of toluene. The absorbance of the organic phase was measured at 520 nm, using toluene as a blank. Pro concentration was expressed as µmol g^{-1} DW.

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

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

4.6.2.6. HPLC analysis of carbohydrates

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

4.6.2.7. Statistical analysis

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

4.6.3. Results

Effects of salt stress

4.6.3.1. Electrical conductivity of substrates

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

4.6.3.2. Growth parameters

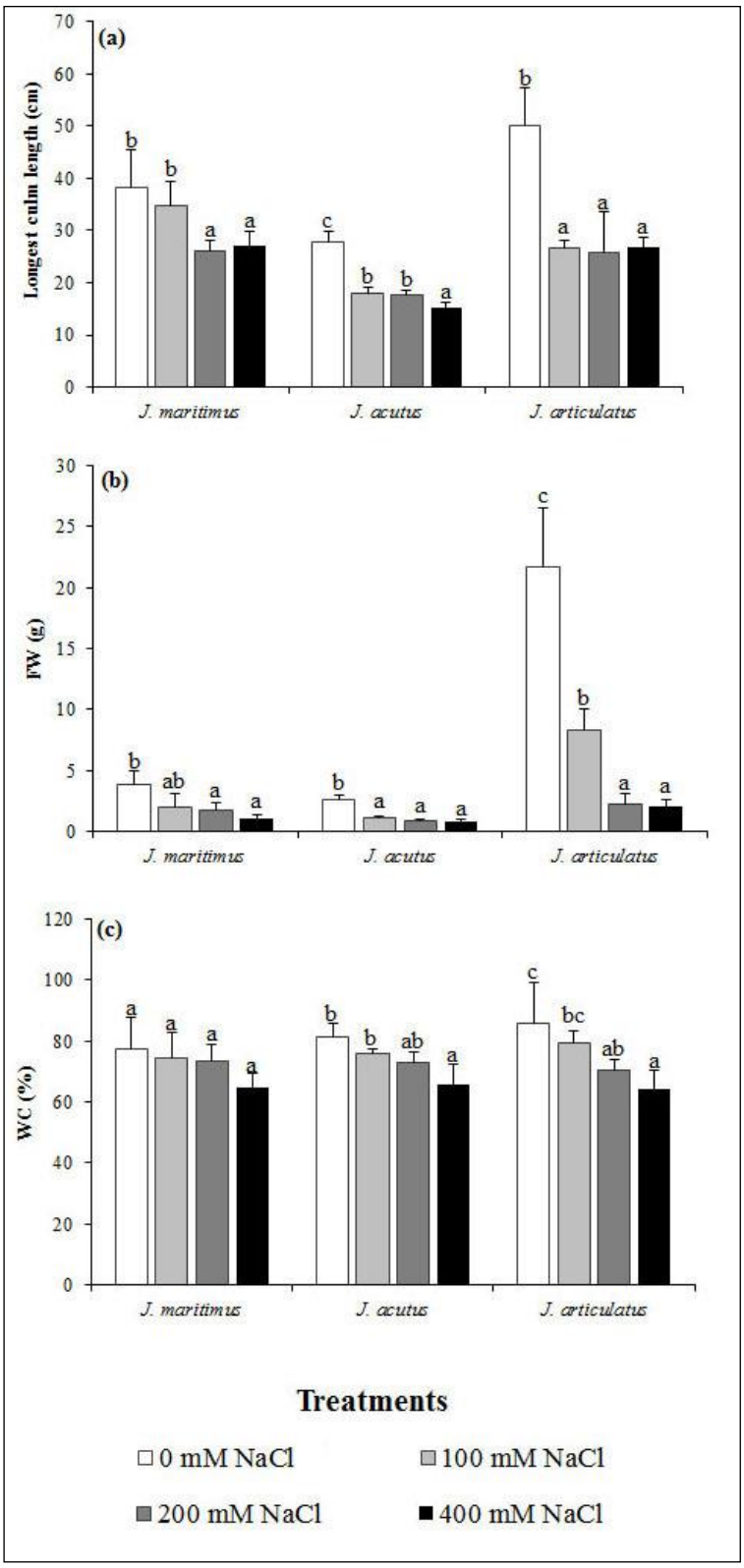

Fig. 1 Changes in (a) longest culm length (cm) (b) fresh weight (g), and (c) water content (%), in the three studied *Juncus* species, after treatment for eight weeks with the indicated NaCl concentrations (means ± SD, n = 5). Different letters indicate significant differences according to Tukey test (α = 0.05).

Salt treatments inhibited growth of *Juncus* plants, in a concentration-dependent manner, as shown by determination of several growth parameters (Fig. 1). For example, the length of the longest culm decreased in *J. articulatus* and *J. acutus* by nearly 2-fold in the presence of 400 mM NaCl, with respect to control plants. A slightly smaller decrease in culm length (about 1.5-fold) was observed in *J. maritimus* under the same conditions (Fig. 1a). Fresh weight also decreased in response to salt stress; the relative reduction of fresh weight in the 400 mM NaCl treatment was similar for *J. acutus* and *J. maritimus* (65% and 70%, respectively) but of more than 90% in *J. articulatus* (Fig. 1b), thus confirming that this species is the most sensitive to salinity of the analysed *Juncus* taxa, as indicated by its distribution in nature. Water contents decreased with increasing external salt concentrations, from about 80% in control plants to 65%, approximately, in plants treated with 400 mM NaCl, without significant differences detected in the three *Juncus* species under study (Fig. 1c). Therefore, the observed reduction of fresh weight is indeed due mostly to growth inhibition, and not simply to loss of water under salt stress conditions.

4.6.3.3. Ions contents in roots

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

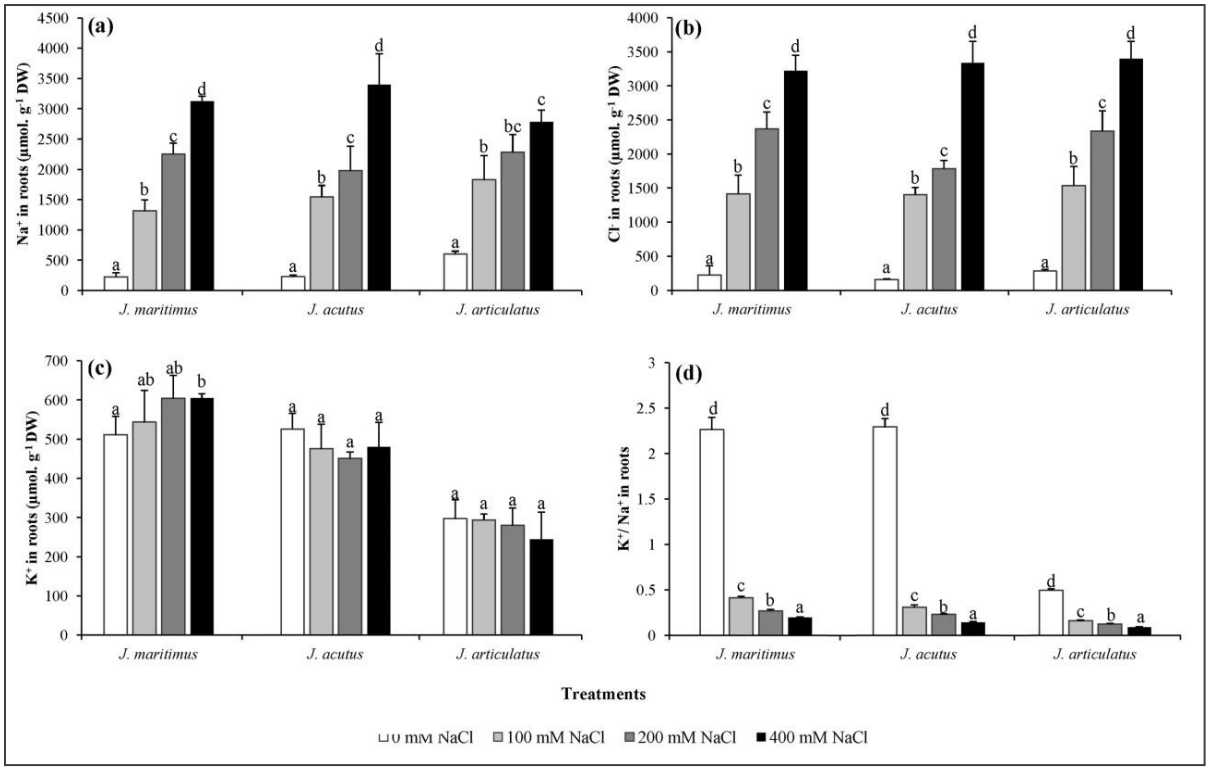

Fig. 2 Ion contents (µmol g^{-1} DW): (a) Na^+, (b) Cl^-, (c) K^+, and (d) K^+/Na^+ ratios, in the roots of the three *Juncus* species under study, after eight-week treatments with the indicated NaCl concentrations (means ± SD, n = 5). For each species, different letters above the bars indicate significant differences according to Tukey test (α = 0.05).

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

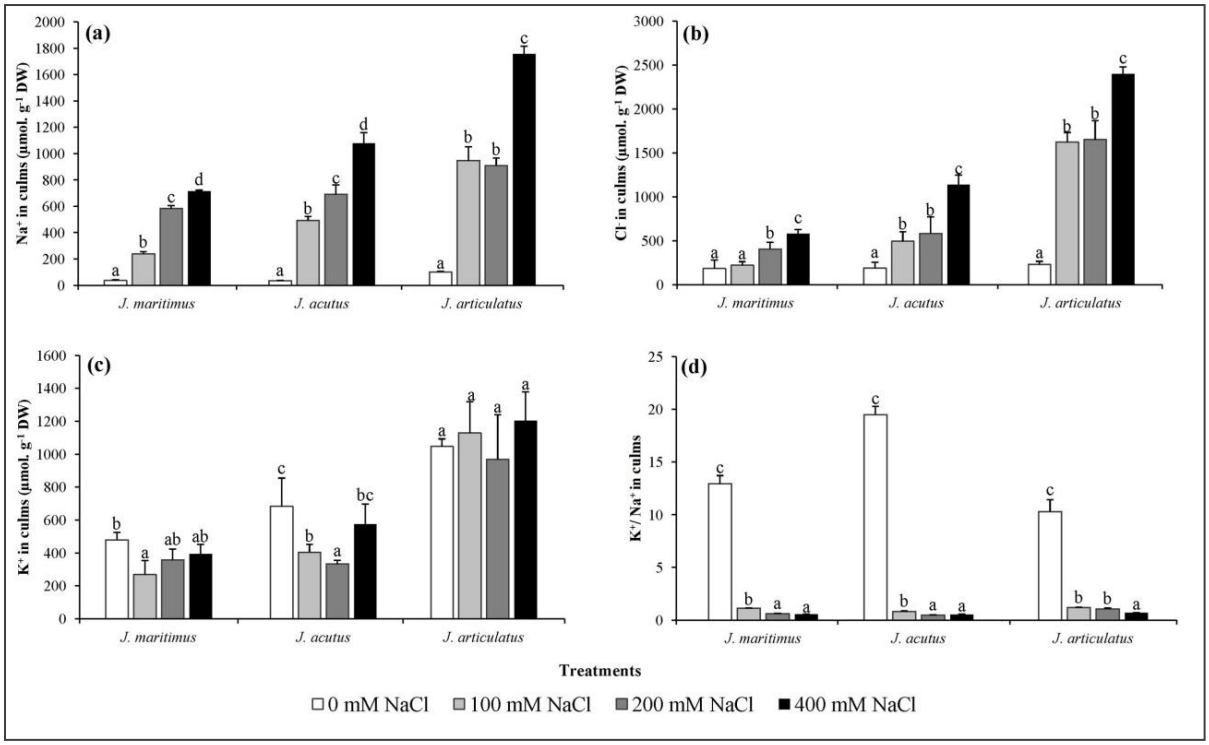

Fig. 3 Ion contents (µmol g^{-1} DW): (a) Na^+, (b) Cl^-, (c) K^+, and (d) K^+/Na^+ ratios, in the culms of the three *Juncus* species under study, after eight-week treatments with the indicated NaCl concentrations (means ± SD, n = 5). For each species, different letters above the bars indicate significant differences according to Tukey test (α = 0.05).

4.6.3.4. Ions contents in culms

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

Accumulation of K^+ in culms, in the presence of increasing NaCl concentrations, also showed different patterns depending on the relative tolerance of the species under study. In *J.*

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

4.6.3.5. Osmolyte contents

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

HPLC fractionation of the extracts revealed three major peaks of soluble carbohydrates, corresponding to glucose, fructose and sucrose (Fig. 5). All three sugars

accumulated in the aerial part of salt-treated *J. articulatus* plants, reaching similar concentrations (about 150 µmol g^{-1} DW) in the presence of 400 mM NaCl, the highest concentration tested. In the halophytes *J. acutus* and *J. maritimus* a large increase in sucrose contents – but not in those of glucose or fructose – was observed in response to the salt treatments (Fig. 5)

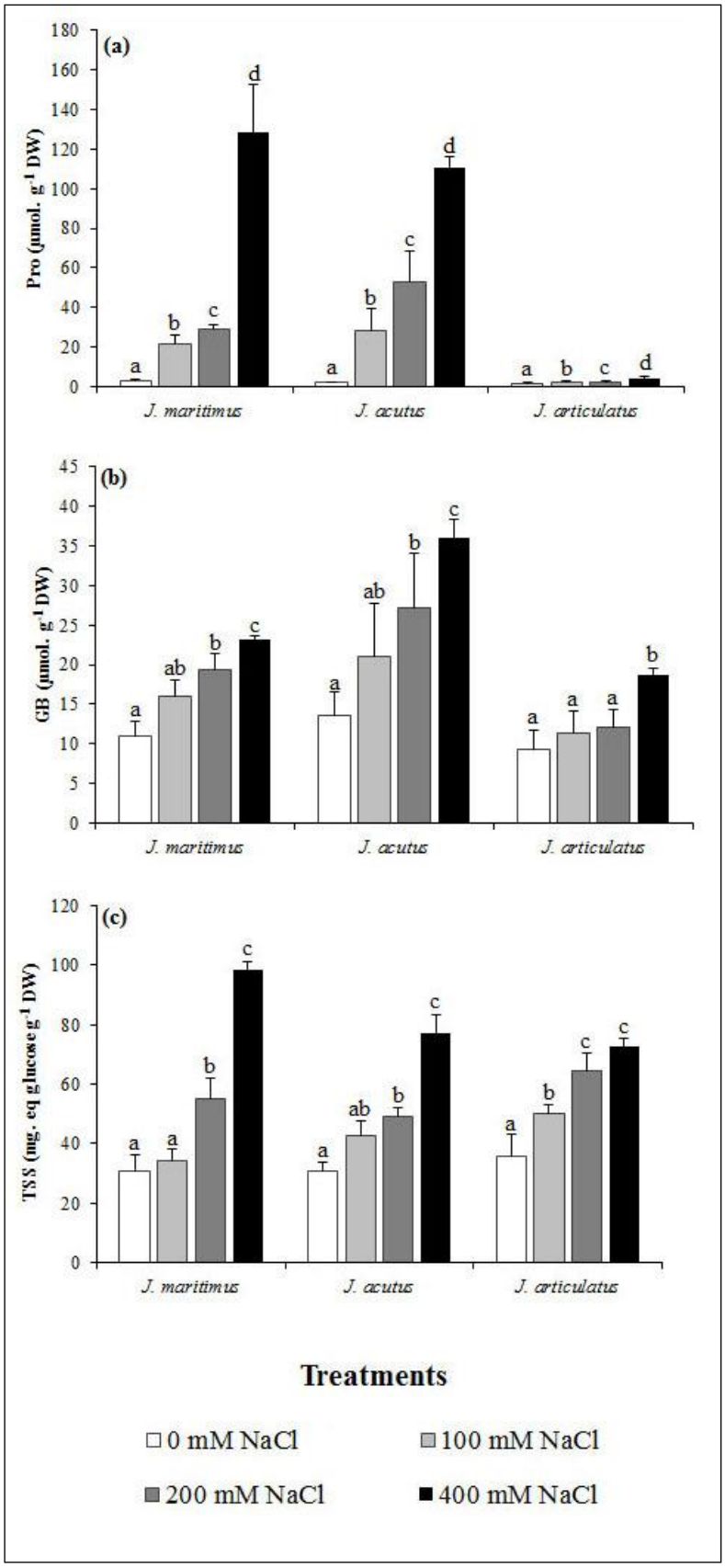

Fig. 4 Osmolyte contents: (a) proline (Pro), (b) glycine betaine (GB), and (c) total soluble sugars (TSS), in the culms of the three *Juncus* species under study, after eight-week treatments with the indicated NaCl concentrations (means ± SD, n = 5). For each species, different letters above the bars indicate significant differences according to Tukey test (α = 0.05).

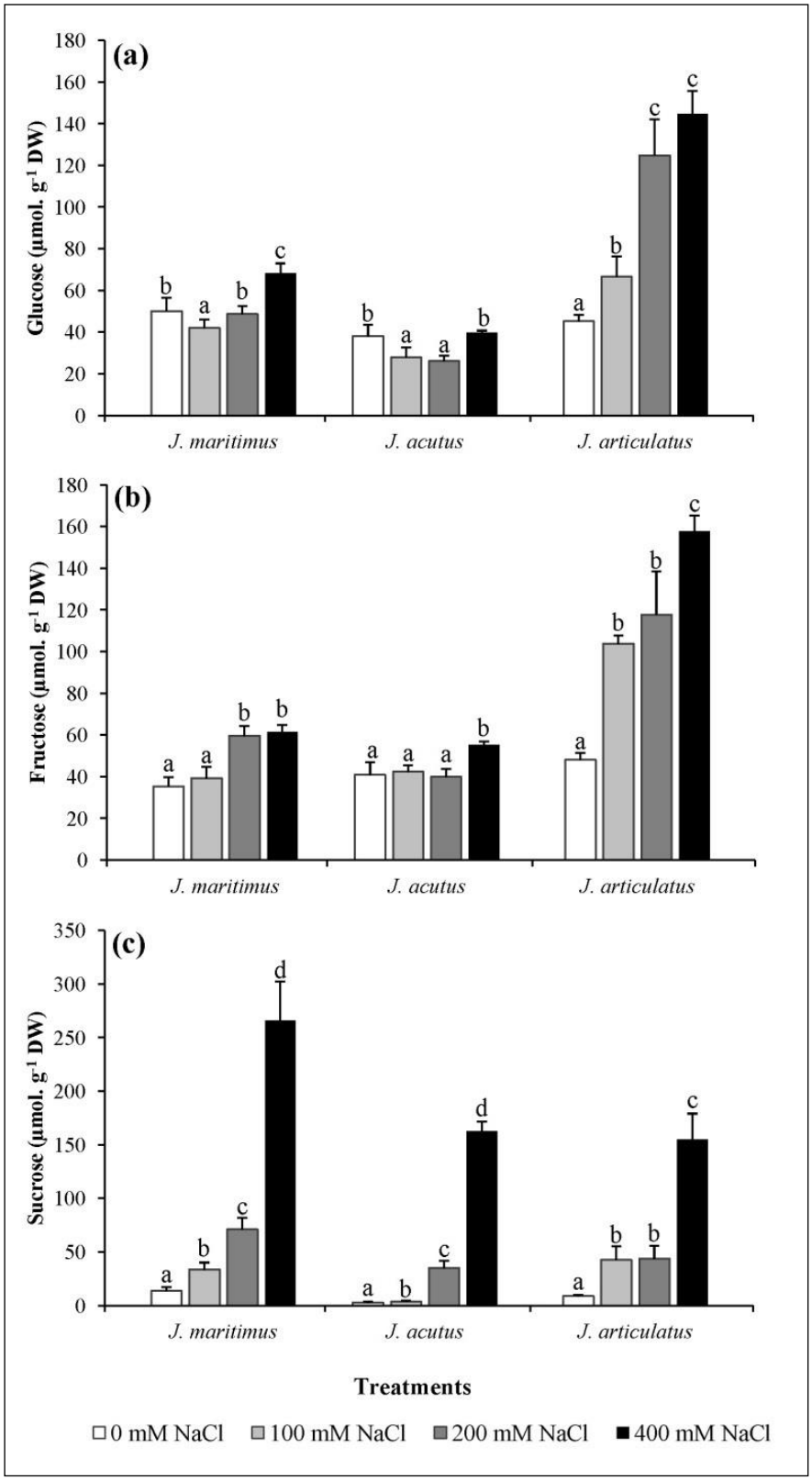

Fig. 5 Levels of soluble sugars (µmol g^{-1} DW): (a) glucose, (b) fructose, (c) sucrose, in the culms of the three *Juncus* species under study, after eight-week treatments with the indicated NaCl concentrations (means ± SD, n = 5). For each species, different letters above the bars indicate significant differences according to Tukey test (α = 0.05).

Effects of drought stress

The same parameters measured in salt-treated plants were determined as well in *Juncus* plants subjected to a water stress treatments – by completely stopping watering – for eight weeks. Drought also inhibited growth, as indicated by the reduction in the length of the longest culm of the plants and, more clearly, by a strong decrease in the fresh weight of the water-stressed plants as compared to the non-stressed controls (Table 1). According to this criterion, the glycophyte *J. articulatus* is also the taxon most sensitive to drought, showing a FW reduction of 97% after eight weeks without water (the corresponding values for *J. maritimus* and *J. acutus* were 88% and 83%, respectively). These data suggested that the effect of water stress on plant growth was stronger than that of salt stress at the highest NaCl concentration tested. However, in this case the reduction of fresh mass was partly due to loss of water, which ranged between 70% (in *J. maritimus*) and 90% (in *J. acutus*) (Table 1), values much higher than those observed in salt-treated plants (Fig. 1).

Concerning osmolyte contents under water stress conditions, the accumulation patterns of Pro, GB and TSS were similar to those observed in the presence of NaCl. Thus, drought induced a strong increase in Pro levels in the halophytes, between 50 and 70-fold higher than in the controls, reaching almost 200 µmol g^{-1} DW in the most tolerant *J. maritimus*; in *J. articulatus*, the most stress-sensitive taxon, Pro levels remained very low, with only a ca. 2-fold increase in the culms of the water-stressed plants (Fig. 6a). Water stress also induced the accumulation of GB (Fig. 6b) and TSS (Fig. 6c), but to a much lesser extent, between 2- and 3-fold over the controls, and without large differences among the three *Juncus* species.

The drought-dependent increase in the levels of soluble sugars detected in all three *Juncus* taxa was due to accumulation of sucrose, as demonstrated after the carbohydrates

were separated and quantified by HPLC. Sucrose contents strongly increased in water-stressed plants, reaching values of 160 – 180 µmol g^{-1} DW, without clear differences in the different species (Fig. 6f). Contrary to what was observed in salt-treated plants, water stress treatments did not induce the accumulation of glucose or fructose in *J. articulatus*; in fact, there was a significant reduction in the levels of these two sugars after the drought treatment. In the halophytes *J. maritimus* and *J. acutus*, either no significant changes or only small reductions in the contents of glucose and fructose were detected (Fig. 6d, e).

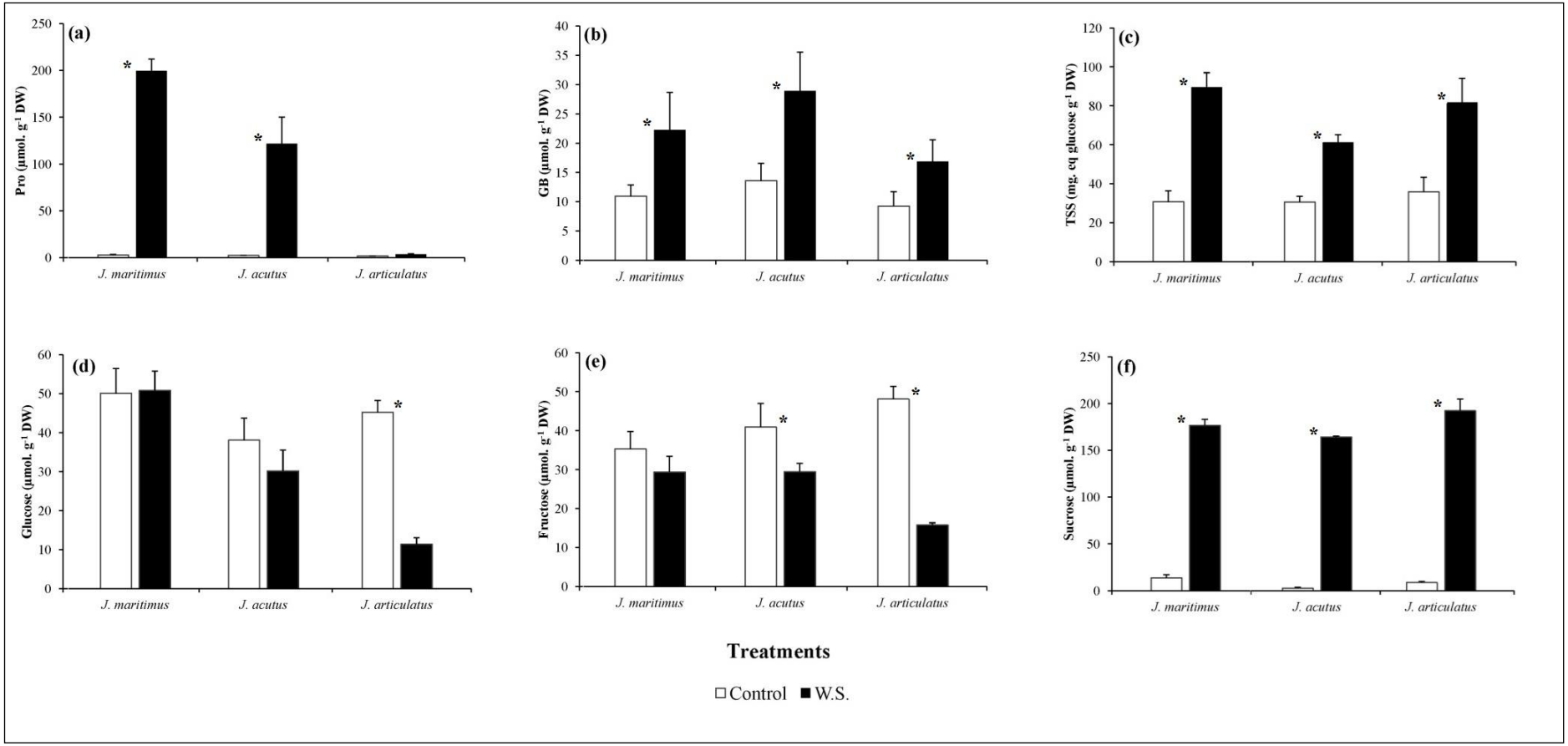

Fig. 6 Osmolyte accumulation: (a) proline (Pro), (b) glycine betaine (GB), (c) total soluble sugars (TSS), (d) glucose, (e) fructose, and (f) sucrose in the culms of the three *Juncus* species under study, after eight weeks of water stress treatments (means ± SD, n = 5). Glucose, fructose and sucrose (panels d-f) were separated, identified and quantified by HPLC. For each species, asteriks indicate significant differences between treatments according to Tukey test (α = 0.05).

4.6.4. Discussion

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

Reduction of fresh weight in parallel with increasing external salinity appears to be a reliable criterion to assess the relative salt tolerance of *Juncus* species, as previously suggested (Rozema, 1976). According to our results, *J. maritimus*, considered as a typical halophyte, is the most tolerant of the studied species, slightly more than *J. acutus*, which is also a salt-tolerant species, often reported as subhalophyte (Boscaiu *et al*., 2011; 2013). Both taxa are much more tolerant than *J. articulatus*, a species not investigated before, but that can be clearly defined as a glycophyte. Thus, the responses to salt stress under controlled artificial conditions closely correspond to the species natural distribution and their ecological optima.

In the presence of salt, the decrease in water content of the aerial part of the plants was small, and almost identical for the three species; therefore, the relative reduction of fresh weight was mostly due to growth inhibition, indicating that the *Juncus* plants possess efficient mechanisms to limit salt-induced dehydration, independently of their relative degree of salt tolerance. Water stress, on the other hand, caused a stronger dehydration of the culms, but the relative resistance of the investigated taxa to drought and salinity followed similar patterns, with *J. acutus* and *J. maritimus* showing higher tolerance than *J. articulatus.*

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

essential role in these Na^+ exclusion mechanisms (Munns and Tester, 2008; Hamamoto *et al*., 2015).

Sodium accumulation in plants is usually accompanied by a reduction in the endogenous concentrations of potassium, as both ions compete for the same membrane transporters (Niu *et al*. 1995; Rodriguez-Navarro 2000). This general reaction to salinity does not seem to take place in *Juncus*, as no significant decrease in K^+ levels was detected in the roots of any of the three taxa, or in *J. articulatus* culms. The capacity to maintain K^+ concentrations despite the progressive accumulation of toxic Na^+ ions was considered by Rozema (1976) as the basis of salt tolerance in halophytic species of this genus. Our results indicate, on the contrary, that this mechanism cannot be relevant for tolerance, as it has been observed also in the selected glycophyte, *J. articulatus*. The pattern of variation in K^+ contents in the culms of the halophytes *J. maritimus* and *J. acutus*, in response to increasing salinity, is also worth mentioning: K^+ decreases at low external NaCl concentration, as compared to the control, non-stressed plants, to increase again in the presence of higher salt concentrations. It seems, therefore, that in the salt-tolerant *Juncus* taxa accumulation of Na^+ at high levels activates transport of K^+ from the roots to the aerial part of the plants, to limit the reduction of K^+/Na^+ ratios. This mechanism most likely contributes significantly to salt tolerance in *Juncus* and, in addition, appears to be ecologically relevant. In a previous study carried out in the field, in a littoral salt marsh near the city of Valencia (Gil *et al.*, 2014), we observed that K^+ levels in culms of *J. maritimus* and *J. acutus* were higher in summer than in spring, in parallel with a higher accumulation of Na^+ (and Cl^-). In summer – normally the most stressful season in the Mediterranean climate – we determined much higher soil salinity (based on electric conductivity measurements), and Na^+ and Cl^- levels than in spring, while K^+ contents in the soil remained very low and practically constant throughout the year.

Osmolyte accumulation in the cytosol is also a general response to abiotic stress in plants, and it is generally assumed that it contributes significantly to tolerance by counteracting, at least partly, cellular dehydration caused by different stress conditions. In addition to their function in osmotic adjustment, compatible solutes may play other important roles in the mechanisms of stress tolerance, as low-molecular weight chaperones, ROS scavengers or signalling molecules (Zhu, 2001; Chen and Murata, 2002; Ashraf and Foolad, 2007; Szabados and Savouré, 2010; Smirnoff and Cumbes, 1989; Grigore *et al*., 2011; Gil *et al*., 2013). It has been reported that monocotyledonous halophytes accumulate preferentially soluble carbohydrates (sugars and polyols) for osmotic balance (Gorham *et al*., 1980; Briens and Larher, 1982). We have indeed detected a concentration-dependent increase in total soluble sugars in response to the NaCl treatments, but reaching roughly the same levels in the three *Juncus* species, irrespective of their relative salt tolerance. HPLC fractionation allowed the identification of glucose, fructose and sucrose as the major sugars present in all *Juncus* plants, as reported for *J. maritimus* and *J. acutus* grown in nature (Gil *et al*., 2011). However, the *Juncus* halophytes and the glycophyte showed different patterns of sugar accumulation: significant salt-dependent increases in glucose and fructose levels were only detected in *J. articulatus*, while sucrose contents increased in the three taxa. The high sugar concentrations measured should clearly contribute to osmotic adjustment in the presence of NaCl, thus protecting the plants against the effects of salt stress. Yet, here again, it is important to point out that there is no positive correlation between sugar contents and the relative degree of salt tolerance of the *Juncus* taxa – actually, the combined concentrations of glucose, fructose and sucrose were somewhat higher in the most salt-sensitive species, *J. articulatus*, than in the halophytes. Therefore, differences in salt tolerance within the genus *Juncus* do not seem to be due to differential accumulation of soluble carbohydrates.

Proline is not generally considered as a preferential functional osmolyte in monocotyledonous salt-tolerant plants, and the concentrations of free Pro measured in control plants – around 2 µmol g^{-1} DW – were much lower than those of sugars. In salt-treated plants, however, a large increase in Pro content was observed, up to 50 to 60-fold over the controls in the presence of the highest NaCl concentration tested (400 mM), but only in the halophytes *J. maritimus* and *J. acutus*. In the salt sensitive *J. articulatus* Pro levels increased only about 2-fold under the same conditions. This differential accumulation of this osmolyte in the culms of *Juncus* plants, depending on the relative salt tolerance of the studied species, clearly supports a functional role of Pro in the mechanisms of salt tolerance in this genus. Pro probably participates in cellular osmotic adjustment under salt stress conditions, although it reached maximum absolute levels lower than those of soluble sugars. Yet its contribution to salt tolerance mechanisms is probably mediated, to a large extent, by its additional activities as 'osmoprotectant' – low-molecular-weight chaperon and ROS scavenger (Szabados and Savouré, 2010).

4.6.5. Conclusion

Salt tolerance in *Juncus* depends to a large extent on the partial inhibition of transport of toxic ions (Na^+ and Cl^-) from the roots to the plants aerial parts and on the activation of K^+ transport at high external salt concentrations (to limit the reduction of K^+/Na^+ ratios). In addition, the accumulation to relatively high levels of Pro in the culms of the plants is also important for tolerance to both, salt and water stress, since it contributes to osmotic adjustment but also because of the 'osmoprotectant' roles of this osmolyte. The efficiency of these processes correlated positively with the relative tolerance of the investigated species, and could be distinguished from other stress responses that were activated to a similar extent

in the three *Juncus* taxa, irrespective of their relative tolerance to stress, and therefore could not be directly involved in their mechanisms of tolerance to stress.

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Tab. 1 Changes in longest culm length (cm), FW (g), and WC (%) in the 3 studied *Juncus* species with water stress (W.S.), at the end of treatments (8 weeks) (means ± SD, n = 5). Asteriks indicate significant differences between treatments, according to Tukey test (α = 0.05).

Parameter	Treatment	*J. maritimus*	*J. acutus*	*J. articulatus*
Longest culm length (cm)	Control	38.16±7.28*	27.83±2.08*	50±7.21*
	W.S.	22±2.64*	19.66±1.52*	26.66±2.51*
FW (g)	Control	3,85±1,1*	2,6±0,45*	21,69±4,83*
	W.S.	0,45±0,05*	0,43±0,01*	0,71±0,05*
WC (%)	Control	77.50±4.09*	81.22±1.42*	86.03±10.03*
	W.S.	24.01±3.71*	9.19±0.53*	15.95±2.15*

Publication VII:

Subchapter 4.7.

Differential anti-oxidative responses under salinity and drought challenges in two halophytes and one glycophyte of the genus *Juncus*

Reference:

Al Hassan, M., Cortes, J., Gaspar, A., Boscaiu, M., Vicente, O. (2015). Differential anti-oxidative responses under salinity and drought challenges in two halophytes and one glycophyte of the genus *Juncus* (In preparation).

Differential anti-oxidative responses under salinity and drought challenges in two halophytes and one glycophyte of the genus *Juncus*

Abstract. Salt stress tolerance, in halophytes and glycophytes alike, is based on conserved mechanisms, such as osmolyte accumulation, control of ion homeostasis, and synthesis of chemic and enzymatic anti-oxidants. However, quantitative and qualitative differences in those responses are responsible for halophytes´ ability to tolerate high salt concentrations a, and glycophytes´ susceptibility to stress. One useful approach in deciphering these differences is by comparison the responses to stress of closely related species with different levels of tolerance to abiotic stress. In this work we investigated the anti-oxidative defense mechanisms under salt stressin three species of the monocotyledonous genus *Juncus*, two halophytes (*J. acutus* and *J. maritimus*) and one glycophyte (*J. articulatus*). A water stress treatment by complete ceasing of watering was also included to check the three species´ responses to a different type of abiotic stress. Growth parameters, photosynthetic pigments, total phenolic compounds and flavonoids, and the activities of four anti-oxidative enzymes (CAT, GR, SOD, and APX) were quantified in culms of plants submitted for eight weeks to salt stress and drought (100 mM, 200 mM and 400 mM NaCl). The concentrations of MDA in plants submitted to stress were used as oxidative stress marker. Our findings showed that *J. articulatus* is more damaged by oxidative stress, with higher MDA levels and elevated disintegration of photosynthetic pigments in salt stressed plants, while *J. acutus* and *J. maritimus* expressed higher levels of chemical antioxidants (flavonoids and phenols), and their anti-oxidative enzymes activity showed better correlation with the applied stress.

Keywords: *Juncus*; MDA; photosynthetic pigments; anti-oxidative enzymes; salt stress; water stress.

4.7.1. Introduction

Drought and salinity are two of the most important stress factors inhibiting plant growth and yield worldwide. Most plants can not tolerate extended drought periods, or saline concentrations over 200 mM NaCl (Hasegawa *et al*., 2000), as the osmotic and ionic effects impair plant growth and cause oxidative stress through the formation of reactive oxygen species (Nor'aini *et al.*, 1997; Hernández *et al*., 2001; Foyer and Noctor, 2003).

Reactive oxygen species (ROS) typically result from the transference of one, two and three electrons, respectively, to O_2 to form superoxide ($O_2 \cdot^-$), peroxide hydrogen (H_2O_2) and hydroxyl radical (OH·) (Mittler 2002; Apel and Hirt 2004), all of which are highly cytotoxic, due to their reactivity to lipids, enzymes, proteins, and even DNA (Scandalios, 1993; Breusegem *et al.*, 2001; Quiles and Lopez, 2004).

ROS are by-products of normal cell metabolism in vital processes such as photorespiration, photosynthesis and respiration (Martinez *et al*., 2001; Mittler, 2002; Uchida *et al*., 2002). Under normal conditions the production and destruction of these reactive oxidative species are controlled and regulated by the cell. Under stress the production of ROS is increased dramatically due to loss of coordination between different metabolic pathways and it outpaces the scavenging processes, producing oxidative stress (Asada, 2006; Bose *et al*., 2013). ROS cause a major disturbance in intracellular ionic homeostasis by decline in cytosolic K^+ content followed by activation of proteases and endonucleases (Shabala, 2009; Demidchik *et al*., 2010) causing oxidative damage to proteins, lipids and DNA and thus, affect the cell membrane integrity, enzyme activities and function of photosynthetic apparatus and finally leading to cellular death (Yu *et al*., 2011; Kumari *et al*., 2015). In plants, however, ROS play a dual role, besides of their toxicity as by-products of aerobic metabolism they may be considered as signalling molecules to increase antioxidant defence mechanisms for

adapting to abiotic stress (Miller *et al*., 2008; Abogadallah, 2010; Jaspers and Kangasjärvi, 2010; Kumari *et al.*, 2015).

To avoid excessive ROS accumulation during stress and to maintain ROS at sufficient levels for signalling, plants use both chemical and enzymatic systems. The first includes the synthesis of non-enzymatic antioxidants such as flavonoids, ascorbic acid, glutathione, and b-carotenes. Among the enzymatic antioxidants most common in plants are superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), and redox regulatory enzymes such as glutathione reductase (GR), among many others (Noctor and Foyer 1998; Ozgur *et al*., 2013). Under unfavorable conditions, the biosynthesis and the activity of these antioxidants is altered (Rossel *et al*., 2002; Horling *et al*., 2003; Mittler *et al*., 2004).

Most studies on stress tolerance in plants have been conducted in model species that are not stress tolerant, especially in *Arabidopsis thaliana* and, to a much lesser extent, in some crops (Sanders, 2000; Zhu, 2000; 2001). However, the degree of tolerance to abiotic stress which can reach such species is not at all comparable to those of wild plants adapted to each particular environment. Although stress tolerance mechanisms are ubiquitous in plants, the molecular and biochemical pathways leading to improved tolerance act additively and synergistically in plants naturally adapted to stressful environments (Gossett *et al*., 1994; Hediye Sekmen *et al.*, 2007; Kumari *et al*., 2015; Srvisatva *et al.*, 2015). The mechanisms of response operating in stress tolerant taxa must be much more effective than those that function in non-tolerant models, and therefore comparative studies including stress tolerant and stress sensitive species are achieving a greater attention. Several salt and drought tolerant species of the genus *Thellungiela*, taxonomically related to *Arabidopsis*, were proposed as extremophile models for abiotic stress tolerance studies (Inan *et al*., 2004; Gong *et al*., 2005; Kant *et al*., 2006). The complete genome data on *T. parvula* and *T. salsuginea* enormously

contributed to elucidate the "full picture" of stress tolerance of dicotyledonous halophytes, but the involvement of ROS in the stress tolerance of monocotyledonous halophytes is still poorly studied (Ozgur *et al.*, 2015). Further studies on stress tolerant and stress sensitive taxonomically related species are of great interest and should be extended and diversified in monocot species.

The monocotyledonous genus *Juncus* (family *Juncaceae*) containing a wide range of halophytes and glycophytes was a suitable choice to carry out this study, especially that most comparative studies on abiotic stress responses in genetically related taxa had been performed either on different crop (glycophytes) cultivars, or between dicotyledonous glycophytes and halophytes.

Three species of the genus *Juncus* with different degrees of tolerance to drought and salt stress were selected for this comparative study. The two halophytes *J. maritimus* Lam. and *J. acutus* L. are common in littoral salt marshes on temporally flooded humid soils but have different ecological optima. *J. maritimus* is more salt tolerant than *J. acutus* (Boscaiu *et al.*, 2011; 2013) and is restricted to saline moistened soils, whereas *J. acutus* is found mostly on salt marsh borders or other areas with lower salinity. Since this species tolerates well the drought, its presence is favored on soil with sandy texture, such in small depressions among dunes and even on gypsum soils (Boira, 1995). *J. articulatus* L. is a glycophyte that occupies river banks and humid, not saline wetlands (Albrecht, 1994; Talavera *et al*., 2010).

The aim of the study was to compare the defense mechanisms against oxidative stress induced by salt and drought stress in the aforementioned three related species with different degrees of tolerance. In this work we analyzed (a) changes in growth parameters (aerial fresh weight, dry weight percentage, and photosynthetic pigments), (b) levels of MDA as oxidative stress marker and total phenolic compounds and flavonoids as non-enyzmatic antioxidants,

and (c) the activity of four antioxidant enzymes (SOD, CAT, GR, and APX). The working hypothesis is that the synthesis of chemical antioxidants and modulation of the activity of these enzymes at seedling stage may be important in the investigated species ability to tolerate salt and water stress. The relative quantitative differences registered could sustain the performance of the two halophytes *J. maritimus* and *J. acutus* in natural saline environments and the restriction of *J. articulatus* to non-saline wetlands.

4.7.2. Material and methods

4.7.2.1. Plant material and Experimental Design

Seeds of *J. acutus, J. maritimus* and *J. articulatus* were obtained from the Natural Park of La Albufera (Province of Valencia, Spain). After a short procedure of sterilization with commercial bleach solution and repeated washes with distilled water, seeds were sown on a mixture of commercial peat, perlite and vermiculate (2:1:1) and watered with Hoagland nutritive solution. After six weeks, when seedlings were robust enough, salt and drought treatments were initiated and continued for eight weeks. Following concentrations of NaCl were used for the salt treatments: 100 mM, 200 mM and 400 mM NaCl. The drought treatment was performed by ceasing irrigation completely. Control plants were watered twice a week with Hoagland nutritive solutions and salt stress plants with the same volume but with NaCl (respective to the concentration of treatment) added to the nutritive solution prior to irrigation. All experiments were conducted in a controlled environment chamber at the greenhouse, under the following conditions: long-day photoperiod (16 hours of light), temperature (23ºC during the day and 17°C at night), CO_2 level (≈ 300 ppm) and humidity 50-80%. Plant material was harvested after 8 weeks. Fresh weight (hereafter FW) of each plant was measured and part of the material was dried at 65ºC until constant weight to calculate the dry weight percentage (DW).

4.7.2.2. Quantification of photosynthetic pigments

Total carotenoids (carot), chlorophyll a (chl a), and chlorophyll b (chl b) were measured following Lichtenthaler and Welburn (1983): fresh plant material was crushed and diluted in 80% ice-cold acetone prior to being vortexed and centrifuged. The supernatant was separated and its absorbance was measured at 663 nm, 646 nm, and 470 nm. The concentration of each group of compounds was calculated according to the following equations:

Chlorophyll a (µg. ml) = 12.21 (A_{663}) - 2.81 (A_{646}),

Chlorophyll b (µg. ml) = 20.13 (A_{646}) - 5.03 (A_{663}),

Total carotenoids (µg. ml) = ($1000A_{470}$ - 3.27[chl a] - 104[chl b])/227

The values were converted into mg g^{-1} DW.

4.7.2.3. MDA and chemical antioxidants

Malondialdehyde (MDA), total phenolics compounds and flavonoids were extracted from dry plant material using 80% (v/v) methanol. Malondialdehyde (MDA) content was determined as an indication of leaf lipid peroxidation according to the method described by Hodges *et al*. (1999). Extracts were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA, and 20% TCA without TBA, and then incubated at 95°C for 20 min. After stopping the reaction, the supernatant absorbance at 532 nm was then measured. After subtracting the non-specific absorbance at 600 nm and 440 nm, MDA concentration was determined using the equations described by Hodges *et al.* (1999).

Total phenolic compounds (TPC) were quantified according to Blainski *et al.* (2013), utilizing Folin-Ciocalteu reagent. The extracts were mixed with sodium bicarbonate and

Folin-Ciocalteu reagent and left in the dark for 90 mins. Absorbance was recorded at 765 nm, and the results expressed in equivalents of gallic acid (mg. eq. GA g^{-1} DW).

The antioxidant total flavonoids (TF) were measured following the method described by Zhishen *et al.* (1999); extracts were mixed with 10% sodium nitrite, followed by 5% aluminum chloride and 1 M sodium hydroxide. The absorbance was measured at 510 nm, and the amount of antioxidant total flavonoids was expressed in equivalents of catechin (mg eq C g^{-1} DW).

4.7.2.4. Protein extraction and quantification

Crude protein extracts were prepared from fresh plant material stored and frozen at −80°C. Protein extractions were prepared following the method described by Gil *et al.* (2014). Protein concentration in the extracts was determined by the method of Bradford (1976), using bovine serum albumin as a standard and utilizing the Bio-Rad reagent.

4.7.2.5. Anti-oxidative enzyme activity assays

Catalase activity (CAT) was determined as described by Aebi (1984). Ascorbate peroxidase (APX) activity was determined by the measurement of the diminution of the absorbance of oxidized ascorbate at 290 nm (using quartz cuvettes) according to Nakano *et* Asada (1981). Glutathione reductase (GR) activity was determined according to Connell and Mullet (1986), following the oxidation of NADPH, the cofactor in the GR-catalysed reduction of oxidized glutathione (GSSG). Superoxide dismutase (SOD) activity was determined according to Beyer and Fridovich (1987) by monitoring the inhibition of nitroblue tetrazolium (NBT) photoreduction. All of the aforementioned assays were modified according to those described by Gil *et al*. (2014).

4.7.2.6. Statistical analysis

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

4.7.3. Results

Salt stress

4.7.3.1. Growth parameters

Fresh weight of plants´ aerial part showed a decrease under salt stress in the three studied species; however this reduction was more evident in *J. articulatus*, which lost nearly two thirds of its weight under 100 mM NaCl, and over 90% in 400 mM NaCl treated plants (Tab. 1A), whereas plants of *J. maritimus* and *J. acutus* submitted to the same treatment registered a reduction of 71% and 67% respectively.

Dry weight percentage (DW %), increased to similar values under salt stress in the three species, but it was noticeable that *J. articulatus* had a lower dry weight percentage in its control plants (Tab. 1B), and thus recorded a higher relative decrease of water content under salt stress in comparison to the other studied *Juncus* species.

The photosynthetic pigments measured in the leaves showed a reduction with the increase of external salt concentration more pronounced in *J. articulatus*. Chlorophyll a (Fig 1A), chlorophyll b (Fig 1B), and total carotenoids (Fig 1C) levels recorded no significant

decrease in 100 and 200 mM NaCl treated plants of *J. maritimus* and *J. acutus*, but rather a slight increase. Only under the highest salt concentration photosynthetic pigments decreased in *J. acutus*, while in *J. maritimus* were registered no significant changes in chlorophyll a and chlorophyll b levels, and even a minor increase in total carotenoids levels (Fig. 1C).

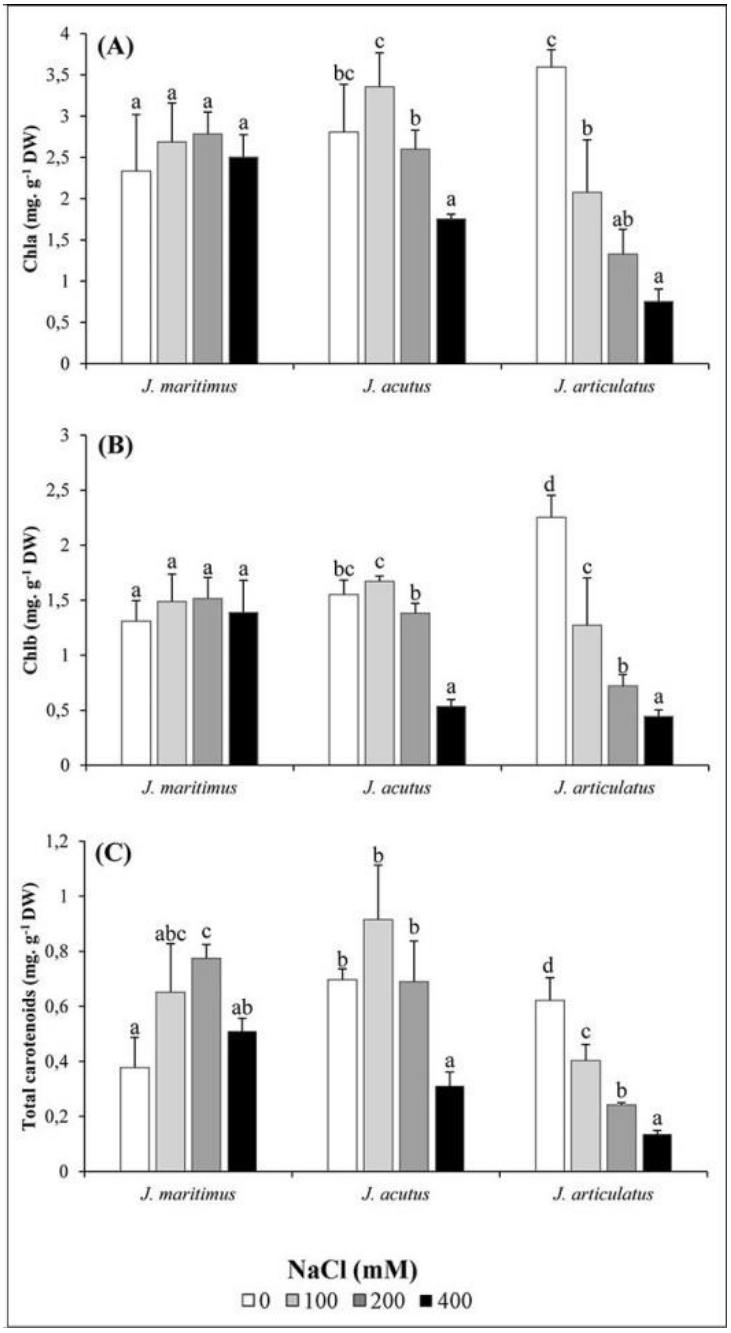

Fig. 1 Photosynthetic pigments in the leaves of the three *Juncus* species under study, after 8-week treatments with the indicated NaCl concentrations. Variations in (A) chlorophyll a, (B) chlorophyll b, and (C) total carotenoids. Means with SD (n = 5). For each species, different letters above the bars indicate significant differences in between treatments according to the Tukey test (α = 0.05).

4.7.3.2. Malondialdehyde and non-enzymatic anti-oxidants

Malondialdehyde (MDA) levels increased slightly in stressed plants of *J. maritimus* and *J. acutus*, but showed a three-fold increase in plants of *J. articulatus* from the 400 mM NaCl treatment (Fig. 2A). Total phenolic compounds and flavonoids increased in all stressed plants of *J. maritimus* and *J. acutus* at levels surpassing those of *J. articulatus*, that did not show an increase correlated with the salt concentration applied (Fig. 2B, Fig. 2C).

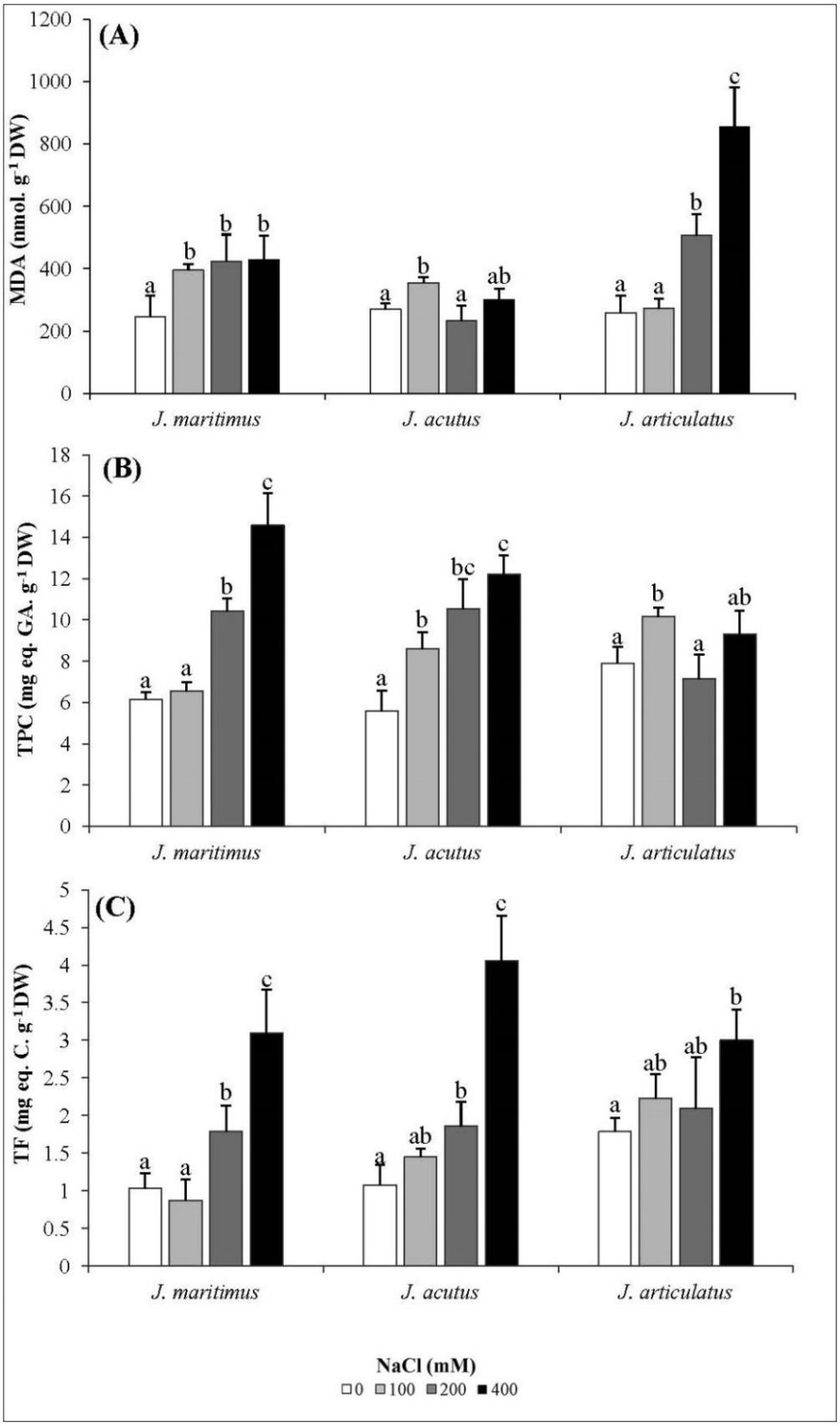

Fig. 2 Oxidative stress markers and non-enzymatic antioxidants in the leaves of the three *Juncus* species under study, after 8-weeks treatments with the indicated NaCl concentrations. Variations in (A) malondialdehyde (MDA), (B) total phenolic compounds (TPC), and (C) total flavonoids (TF). Means with SD (n = 5). For each species, different letters above the bars indicate significant differences in between treatments according to the Tukey test (α = 0.05).

4.7.3.3. Anti-oxidative enzymes activity

Activity of SOD increased in a concentration-dependent manner in *J. acutus*, reaching values in plants from 200 and 400 mM NaCl treatments almost double as those in control. Only a small increase of SOD under stress was registered in *J. maritimus*, while it fluctuated without correlation to the applied concentration in *J. articulatus* (Fig. 3A).

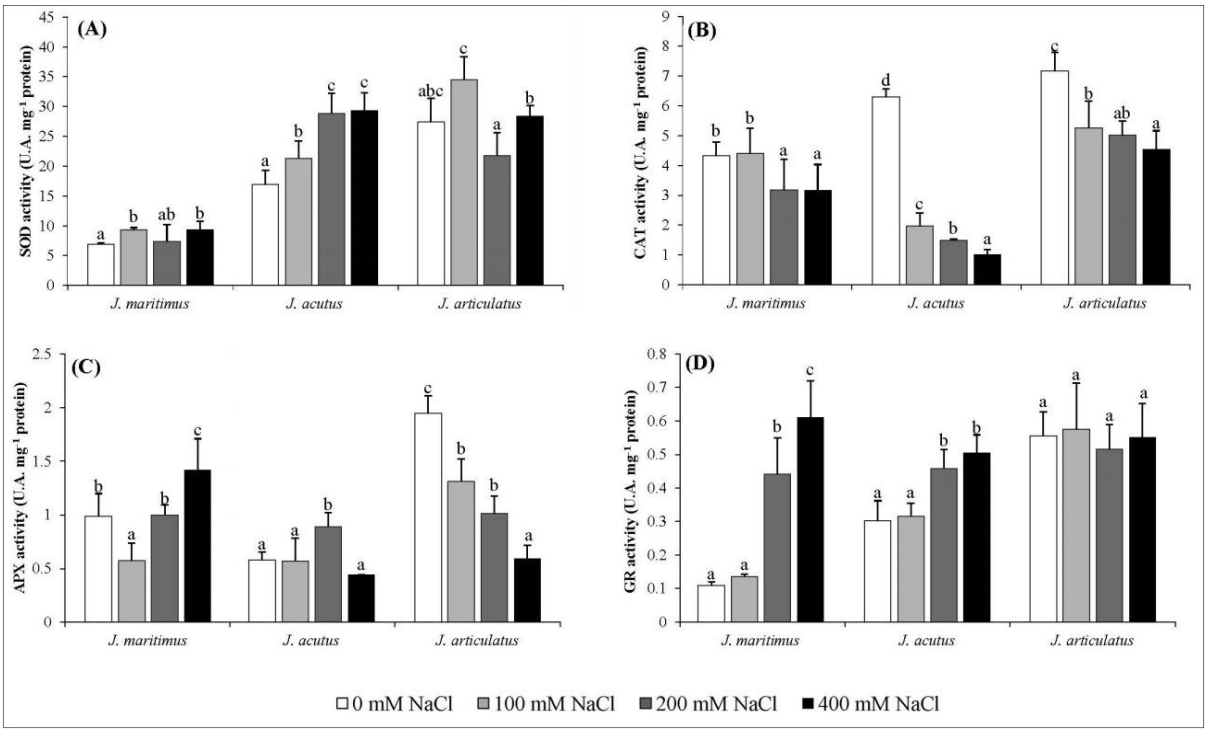

Fig. 3 Activity of antioxidant enzymes in the leaves of the three *Juncus* species under study, after 8-weeks treatments with the indicated NaCl concentrations. Variations in activities of (A) SOD, (B) CAT, (C) APX, and (D) GR. Means with SD (n = 5). For each species, different letters above the bars indicate significant differences in between treatments according to the Tukey test (α = 0.05).

CAT activity decreased with salt stress in the three studied species, showing a diminution in the plants from 400 mM NaCl treatments by nearly 50% with respect to that from control in *J. maritimus* and *J. articulatus*. This reduction was much stronger *J. acutus* plants that showed a 6 fold drop (Fig. 3B). APX activity demonstrated different variation patterns in the three species, with that of *J. maritimus* showing an increase under salt stress in contrary to that of *J. articulatus*, while that of *J. acutus* showed nearly no change (Fig. 3C). The activity of GR exhibited a surge in stressed plants of *J. maritimus* and *J. acutus* reaching

6 folds and 2 folds, respectively (Fig. 3D). *J. articulatus* activity showed no change under salt stress with respect to that recorded in control plants.

Drought stress

4.7.3.4. Growth parameters

Fresh weight of aerial parts decreased in plants undergoing drought in all three studied *Juncus* species, with the strongest relative decrease in respect to control in *J. articulatus* of 97% followed by a reduction of 88% in *J. maritimus* and 83% in *J. acutus* (Tab. 2A). Dry weight percentage (DW %), increased in water stressed plants in comparison with control plants by 3.37, 4.83, and 6 folds in *J. maritimus*, *J. acutus* and *J. articulatus*, respectively (Tab. 2B).

Photosynthetic pigments (chlorophyll a, chlorophyll b, and total carotenoids) levels decreased in the three studied *Juncus* species. The biggest diminution of pigments was found in water stressed plants of *J. articulatus*, that lost four fifth of their chl a, chl b, and total carotenoids (Tab. 3A, B, and C, respectively). The loss of photosynthetic pigments was lower in *J. maritimus* and *J. acutus*, that lost nearly half of their chl a, and chl b only, but *J. acutus* had a ratio of loss in carotenoids levels similar to that found in *J. articulatus*..

4.7.3.5. Oxidative biomarkers and non-enzymatic anti-oxidants

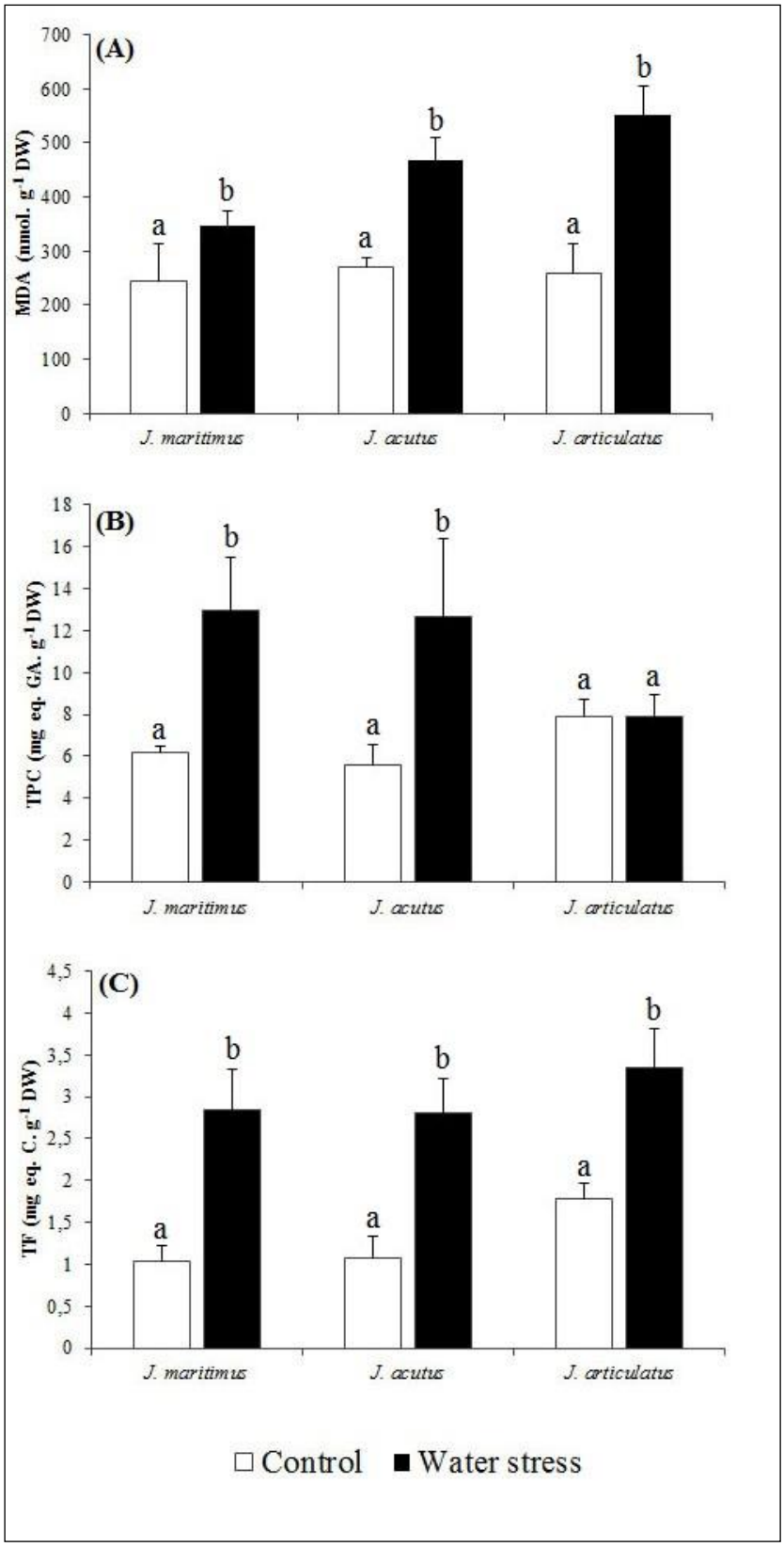

Fig. 4 Oxidative stress markers and non-enzymatic antioxidants in the leaves of the three *Juncus* species under study, after 8-weeks of water stress treatments. Variations in (A) malondialdehyde (MDA), (B) total phenolic compounds (TPC), and (C) total flavonoids (TF). Means with SD (n = 5). For each species, different letters above the bars indicate significant differences in between treatments according to the Tukey test ($\alpha = 0.05$).

MDA levels increased by nearly two folds in stressed plants of *J. articulatus* in comparison with the levels in their control plants (Fig. 4A), while this increase was lower in their counterparts of *J. acutus*, and especially in *J. maritimus*. Total phenolic compounds (TPC) levels increased significantly in stressed plants of *J. maritimus* and *J. acutus* (Fig. 4B), while those of *J. articulatus* showed no increment. Total flavonoids (TF) concentrations showed a rise in stressed plants of the three studied *Juncus* plants under study (Fig. 4C), by nearly 2 folds.

4.7.3.6. Anti-oxidative enzymes activity

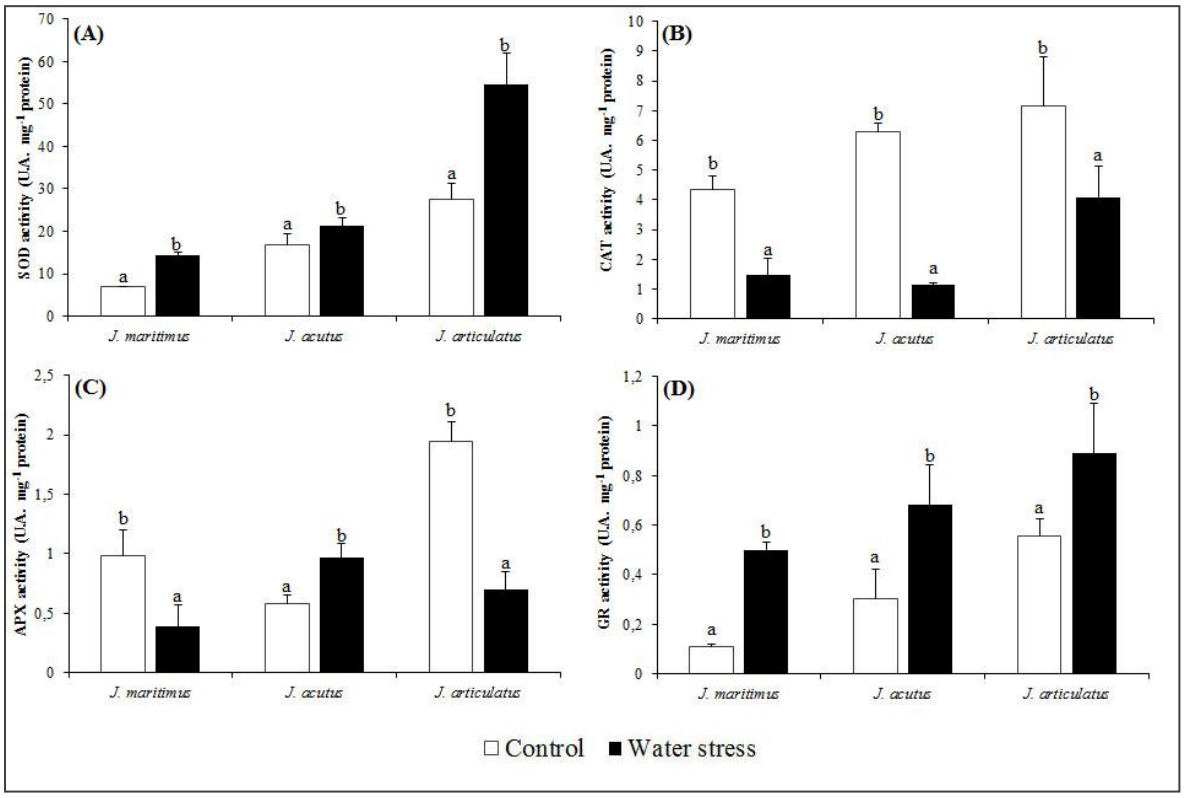

Fig. 5 Activity of antioxidant enzymes in the leaves of the three *Juncus* species under study, after 8-weeks of water stress treatments. Variations in activities of (A) SOD, (B) CAT, (C) APX, and (D) GR. Means with SD (n = 5). For each species, different letters above the bars indicate significant differences in between treatments according to the Tukey test (α = 0.05).

Anti-oxidative enzymes activities varied in plants undergoing water stress treatments in all three studied *Juncus* species. The activity of SOD showed an increase in stressed plants

of the three species in comparison with their control plants (Fig. 5A), however the activity levels in control plants of *J. articulatus* was higher than those of *J. acutus* and *J. maritimus*. The increase in activity of SOD of stressed plants in comparison with that of control plants was nearly by two folds in *J. articulatus* and *J. maritimus*, while the increase in *J. acutus* was nearly insignificant. CAT activity decreased in stressed plants in all studied taxa, with the strongest reduction recorded in *J. acutus* (by nearly six-fold), followed by that of *J. maritimus* (four-fold) and *J. articulatus* (two-fold) as it is shownin Fig. 5B. APX activity increased in water stressed plants of *J. acutus*, contrary to *J. maritimus* and *J. articulatus*, which showed a decrease in APX activity in plants under stress, more pronounced in the latter (Fig. 5C). Activity of GR increased in water stressed plants by nearly five-fold in *J. maritimus*, nearly 2.5 fold in *J. acutus* and 1.5 folds in *J. articulatus*, whose control plants had the highest activity levels (Fig. 5D).

4.7.4. Discussion

Water deficit in the soil (water stress) and high salt concentration (salt stress), generate low osmotic pressure in root cells, producing an osmotic pressure that initiates a cascade of deleterious effects that initiates with the hindering of vegetative growth (Cramer *et al*., 1994; Hummel *et al*., 2010), followed by accumulation of ROS (Apel and Hirt, 2004), nutritive imbalance (Grattan and Grieve, 1994), desiccation and even death.

Under stress, growth is inhibited since plants redirect their energy and resources from biomass generation to the activation of stress tolerance mechanisms (Zhu, 2001; Munns and Tester, 2008). Reduction of growth under stressful conditions is more pronounced in stress sensitive plants than in the stress tolerant ones (Demiral and Türkan, 2005; Hediye Sekmen *et al*., 2007), hence, monitoring a number of growth parameters, represents a suitable method to

determine the relative degree of tolerance among the investigated taxa. The degree of sensitivity of different species to certain types of abiotic stress could is related to their distribution in nature (Dunson and Travis, 1991). As expected halophytic *J. acutus* and *J. maritimus* proved to be more stress tolerant and showed a smaller reduction in measured fresh weight and water content under stress than that recorded in the glycophyte *J. articulatus*.

Another important effect of salt and drought stress on plant growth is the degradation of photosynthetic pigments (chlorophyll a and b, and total carotenoids); hindering the photosynthetic yield in affected plants and causing the failure of stress defense mechanisms on the long term due to the lack of needed energy and resources. Monitoring the decrease of photosynthetic pigments in affected plants can be used as a biomarker of stress (Schiop *et al*., 2015), which supposedly would be more pronounced in the less tolerant ones (Li *et al*., 2006; Lee *et al.*, 2007). There are numerous reports of decreased levels of total carotenoids and chlorophyll at high salinities in different plant taxa (Parida *et al*., 2004; Sai Kachout *et al*., 2013), which confirms our findings as all three species showed a decrease in their photosynthetic pigments under stress, but the strongest reduction was found in the stress sensitive *J. articulatus*.

In previous reports we analyzed osmolytes biosynthesis and ionic homeostasis in species of the genus *Juncus* (Boscaiu *et al.*, 2011; 2013; Gil *et al.*, 2013) and found that halophytes have a greater ability than the glycophyte to synthesize proline and to reduce the transport of Na^+ and Cl^- to the aerial parts of the plants (unpublished data).

This study analyses effects of the oxidative stress associated to salinity and drought. MDA is a product of membrane lipid peroxidation and a reliable marker of oxidative stress (Del Rio *et al*., 1996). Higher MDA contents should correspond to a higher degree of oxidative stress. As expected, MDA levels increased with both types of applied stresses,

however *J. articulatus* recorded higher levels, than those found in its more tolerant congeners under study. This finding reflects the severity of oxidative stress experienced by the more sensitive *J. articulatus* due to drought and salt stress.

Phenolic compounds and especially a subgroup of them, the flavonoids, are plant secondary metabolites which are important in the mechanisms of adaptation to abiotic stresses (Farah and Donangelo, 2006), among many other biological functions. Since many flavonoids and other phenolic compounds are strong antioxidants, their accumulation in plants can reduce the oxidative damage induced by different abiotic stresses, including high salinity (Hussain *et al*., 2013). Flavonoids are regarded as a secondary ROS scavenging system activated in plants under severe stress because of the depletion of primary antioxidant defence systems. Therefore the biosynthesis of antioxidant flavonoids is triggered especially under severe stress conditions, when the activities of antioxidant enzymes, considered the first line of defence against ROS, decline (Fini *et al.*, 2011).The stress related increase in levels of total flavonoids and total phenolic compounds was recorded in the studied species under both water and salt stress. However, the increase was much higher and stress related in the two studied halophytes *J. acutus* and *J. maritimus* in comparison with that measured in *J. articulatus*; a finding which shows a more efficient activation of non-enzymatic antioxidants under stress in the studied. It must be noted however, that the study of TPC and TF contents in *J. acutus* and *J. maritimus* experiencing abiotic stress in nature (Gil *et al*., 2014) showed insignificant changes, in contrast to data reported in this study. Such apparent disparity in findings could be explained by the fact that plants of *J. acutus* and *J. maritimus* studied in the wild where experiencing lower level of stress than those in the controlled conditions implemented in this study (eight consecutive weeks of water stress and salt concentrations reaching 400 mM). This study analyses effects of the oxidative stress associated to salinity and drought. This explanation is also supported by the fact that TPC and TF in the

aforementioned species did not increased at lower concentration of NaCl in plants submitted to salt treatments.

In order to limit oxidative damage under stress condition plants have developed a series of detoxification systems that break down the highly toxic ROS (Larkindale and Huang, 2004). Plants protect cell and subcellular systems from the cytotoxic effects of the active oxygen radicals using antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, and catalase (Singh Gill and Tuteja, 2010).

SOD is the most effective enzymatic antioxidant, which is ubiquitous in all aerobic organisms prone to ROS mediated oxidative stress and is considered as acting in "the first line of defense against oxidative stress in plants" (Alscher *et al.,* 2002; Larkindale and Huang, 2004) . As previously mentioned SODs remove $O_2 \cdot^-$ by catalyzing its dismutation, one $O_2 \cdot^-$ being reduced to H_2O_2 and another oxidized to O_2 (Gill and Tuteja, 2010). SOD activity has been reported to increase under water stress (Sharma and Dubey, 2005; Zlatev *et al*., 2006; Wang *et al*., 2008; Wang and Li, 2008) and salt stress (Harinasut *et al*., 2003; Kukreja *et al*., 2005; Gapinska, 2008) in a wide range of plant species, including stress tolerant ones. In glycophytes there are reports of positive correlation but also negative ones, or no correlation at all between SOD activity and the salt stress applied, whereas all reports in halophytes indicate a positive correlation (Bose *et al*., 2014). In the present study SOD activity under salt stress did correlate with the stress applied in *J. articulatus*, but a substantially increase was registered in *J. acutus*, and only a slight increase in *J. maritimus*. Under water stress the pattern of variation was different, the slightest increase being noticed in the more drought tolerant *J. acutus*, and the strongest in *J. articulatus*, which in its natural habitat is present only in humid non-saline environments. This findings match with the ecological requirements of the analyzed species, but also with previous few comparative studies which reported a higher SOD activity in the halophytes than in the glycophytes (Bose

et al., 2014 and references within). These previous comparative studies were either carried out on not so closed taxonomically related taxa, belonging to different genera (Elouzi *et al.*, 2011; Svrivastava *et al*., 2015) or when referred to congener species mostly on dicots (Mittova *et al*., 2000, 2003; Sekmen *et al*., 2007), but only scarce on monocotyledonous plants (Seckin *et al*., 2010).

Catalase (CAT) is an enzyme with the capability with the potential to directly dismutate H_2O_2 into H_2O and O_2; it was found to be indispensible for the detoxification of ROS in salt and drought stressed plants and thus was studied under such stressful conditions where some reported that its activity was up regulated in a number of glycophytic plant species (Eyidogan and Oz, 2005; Yang *et al*., 2008), while others found that this activity decreased upon the application of the aforementioned stresses (Kukreja *et al*., 2005; Pan *et al*., 2006). The same heterogeneity in its activity in relation to salinity was reported in halophytes, indicating either an increase, decrease or no changes in CAT activity, but comparative studies on related glycophyes and halophytes are still very scarce as indicated (Bose *et al*., 2014). We found here that CAT activity decreased under salt and water stress in the three *Juncus* species, but the strongest reduction was registered in *J. acutus* under both stresses. These findings confirm previous studies on *J. acutus* and *J. maritimus* performed on plants in their natural environments in a Mediterranean salt marsh, reporting a decrease of CAT activity in summer when environmental conditions were more stressful (Gil *et al*., 2014)

APX has a higher affinity for H_2O_2 than CAT, and is thought to play a vital role scavenging ROS during stress. APX activity was found to be enhanced under salt and drought stress in a number of species (Srivastava *et al*., 2005; Zlatev *et al*., 2006), including halophytes, which were reported to have higher APX activity than their glycophtyic related species (Mittova *et al*., 2000, 2003; Shalata *et al*., 2001; Sekmen *et al*., 2007). In the studied

Juncus species APX activity was found to increase in parallel with the increase of salt concentration applied in *J. maritimus* and *J. acutus,* but it reduced in *J. articulatus*. Under water stress it increased only in *J. acutus,* and decreased in both *J. articulatus* and *J. maritimus,* finding that may be relevant in explaining why *J. acutus* better stands water stress than the other two species.

GR plays an essential role in defense system against ROS by sustaining the reduced status of GSH. The activity of GR was found to increase significantly in *J. maritimus* and *J. acutus* in salt stressed plants in comparison to control ones, but not in *J. articulatus* stressed plants. These results agree with the findings of higher GR activity under salt stress in the halophytes *Plantago maritima* than in the glycophyte *P. media* published by Sekmen *et al.* (2007). However, under drought GR activity increased in the three species, mostly in *J. maritimus*, followed by *J. acutus* and to a lower level in *J. articulatus*.

4.7.5. Conclusions

The halophytes *J. acutus* and *J. maritimus* are less affected by salt and water stress than *J. articulatus,* as proved by their smaller reduction of growth and degradation foliar pigments. Their lower levels of MDA indicate that they suffer less oxidative stress under both conditions. This better tolerance is justified by their more efficient anti-oxidant mechanisms under both types of stress than in the glycophyte *J. articulatus.* Phenolics and flavonoids levels were higher in *J acutus* and *J. maritimus* and the activity of SOD, and GR increased under stress in these two species. On the contrary CAT does not seem to be important in the antioxidant defense systems of the plants of the genus *Juncus*, since its activity decreased under stress in the three species. Although some responses were similar under salt and water

stress, the glycophyte *J. articulatus* activates its enzymatic defense mechanism only under drought but not under saline conditions.

4.7.6. References

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Tab. 1 (A) Variation of aerial fresh weight (g) and (B) of dry weight percentage (DW %) in *J. maritimus*, *J. acutus*, and *J. articulatus* plants after 8 weeks of salt treatments. The values shown are means with SD (n = 5). For each species, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

(A) Treatments (mM of NaCl)	*J. maritimus*	*J. acutus*	*J. articulatus*
0	3,85 ± 1,1c	2,6 ± 0,45b	21,69 ± 7,83c
100	2,04 ± 0.4b	1,12 ± 0,11a	8,33 ± 1,71b
200	1,75 ± 0,59ab	0,88 ± 0,09a	2,26 ± 0,9a
400	1,11 ± 0,33[a]	0,86 ± 0,08a	2,01 ± 0,6a

(B) Treatments (mM of NaCl)	*J. maritimus*	*J. acutus*	*J. articulatus*
0	22,50 ± 1,19a	18,78 ± 0,33a	13,97 ± 1,63a
100	25,60 ± 1,15ab	23,91 ± 1,1b	20,65 ± 1,59ab
200	26,54 ± 2,48ab	27,06 ± 2,75b	29,40 ± 2,12bc
400	35,34 ± 0,70b	34,06 ± 1,5c	35,60 ± 3,83c

Tab. 2 (A) Variation of aerial fresh weight (g) and (B) of dry weight percentage (DW %) in *J. maritimus*, *J. acutus*, and *J. articulatus* plants after 8 weeks of drought stress treatments. The values shown are means with SD (n = 5). For each species, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

(A) Treatments	*J. maritimus*	*J. acutus*	*J. articulatus*
Control	3,85 ± 1,1b	2,6 ± 0,45b	21,69 ± 7,83b
Water stress	0,46 ± 0,06a	0,44 ± 0,1a	0,72 ± 0,16a
(B) Treatments	*J. maritimus*	*J. acutus*	*J. articulatus*
Control	22,50 ± 1,19a	18,78 ± 0,33a	13,97 ± 1,63a
Water stress	75,99 ± 11,75b	90,81 ± 5,25b	84,05 ± 11,35b

Tab. 3 Photosynthetic pigments in the leaves of the three *Juncus* species under study, after 8-week treatments with water stress. Variations in (A) chlorophyll a, (B) chlorophyll b, and (C) total carotenoids all expressed in mg. g^{-1} DW. Means with SD (n = 5). For each species, different lower-case letters indicate significant differences between treatments according to the Tukey test (α = 0.05).

(A) Treatments	*J. maritimus*	*J. acutus*	*J. articulatus*
Control	2,33±0,68b	2,8±0,57b	3,59±0,21b
Water stress	1,2±0,15a	1,65±0,13a	0,76±0,16a
(B) Treatments	*J. maritimus*	*J. acutus*	*J. articulatus*
Control	1,31±0,18b	1,55±0,13b	2,25±0,19b
Water stress	0,63±0,13a	0,85±0,15a	0,51±0,11a
(C) Treatments	*J. maritimus*	*J. acutus*	*J. articulatus*
Control	0,38±0,11a	0,7±0,04b	0,62±0,08b
Water stress	0,24±0,04a	0,15±0,01a	0,12±0,03a

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Subchapter 4.8.

Anatomical modifications under salt stress in two ecologically distinct *Juncus* species

Reference:

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Anatomical modifications under salt stress in two ecologically distinct *Juncus* species.

Abstract. The anatomic structure of roots and culms of two *Juncus* species with different degrees of salt tolerance was analysed in plants grown for two months under salt stress (NaCl treatments) and in control, non-treated plants. The aim of the study was not only to compare the anatomical structures of a halophyte *(J. acutus*) and a related glycophyte (*J. articulatus*), but mostly to assess whether salt stress induced anatomical modifications, by identifying differences between control and treated plants. Several slight differences have been indeed detected, in terms of endodermis type, development of aerenchyma and extent of sclerenchyma in perivascular sheaths. The role of Casparian endodermis was here discussed in relation to its complex implications in controlling salt influx at the root level that is an efficient mechanism involved in halophytes. Aerenchyma is a common feature found in marshy halophytes, allowing them to survive naturally under flooding conditions; however, when occurring in non-waterlogged plants, as is the case of this study, it should be regarded as a genetically, constitutive adaptation rather than an inducible one. Nevertheless, such anatomic modifications should be regarded as mere alterations due to stress – that is, as stress responses – and not as truly adaptations to salinity. In this context, the nature of these modifications – either considered as adaptations or damage indicators of salt stress – should be further reconsidered.

Keywords: aerenchyma, anatomy, endodermis, halophytes, salt stress responses sclerenchyma.

4.8.1. Introduction

Nowadays, the study of plant responses to salt stress and salt tolerance mechanisms is one of the most active research fields in plant biology, since salinity – together with drought – is the main constraint for agricultural production worldwide (Marcum, 2002; Munns, 2002, 2005; Ashraf, 2004; Bartels and Sunkar, 2005; Mittler, 2006). In a '–*omics*'-dominated era, many papers have been published reporting wide analyses of salt-induced changes, at the molecular, biochemical or physiological levels, mostly in model plant species such as *Arabidopsis thaliana* or some crops (Zhu, 2000; Koiwa *et al.*, 2006; Horie *et al.*, 2012), and lots of data have been collected that are helping to elucidate the mechanisms involved in salt tolerance. Obviously, anatomical (structural) responses to salinity of salt-tolerant plants (halophytes) have been much less studied. There are a few specific reports dealing with the anatomical features of halophytes growing in their natural saline habitats (Grigore and Toma, 2010; Grigore *et al.*, 2014 and reference therein), but data on possible structural modifications of halophytes in response to controlled salt stress treatments are very scarce.

A controversial issue regarding possible changes in structural features under salt stress refers to the way in which these modifications can be interpreted. Salinity is known to induce changes in plant anatomy and morphology. These changes are often considered to be adaptations which increase the chance of the plant to endure stress imposed by salinity, but they may also be regarded as signs of damage and disruption of the normal equilibrium of life processes (Poljakoff-Mayber, 1975; Larcher, 1995; Schulze *et al*., 2005). The anatomical and morphological features typical of halophytes are usually considered to be adaptations to salinity (Poljakoff-Mayber, 1975). Since many structural characteristics in halophytes are rather constitutive, related to their family general structural scheme and to evolution during time in relation to salinity, it is a bit problematic to regard anatomic modifications of halophytes under salt stress as an adaptation, *stricto sensu* (Grigore, 2012; Grigore *et al*., 2014). There is little experimental data to reveal whether the same features occur when

halophytes are not exposed to salinity. According to Grigore *et al*. (2014) the action of salt on halophytes, at least in histo-anatomical terms, should be rather considered as having a formative effect in an ecological and adaptive sense. This would apply for halophytes growing in nature, while – for those cultivated under controlled conditions – these changes are still open to discussion (Grigore *et al*., 2014).

Maritime marshes of the Mediterranean have been described in general terms by Rikli (1943), who stated that they usually lie behind coastal dunes but are subject to salt-water inundation. *Juncus acutus* is a halophyte that shapes well defined plant communities within Mediterranean salt marshes (Chapman, 1960). *Juncus acutus* L. subsp. *acutus* grows in interior and littoral saline meadows, while *J. articulatus* L. subsp. *articulatus* is a glycophyte that occupies wet but not saline habitats in wetlands and riversides (Talavera *et al*., 2010). Several Iberian taxa of the genus *Juncus* have been the object of morphological, anatomical, biochemical, chorological and ecological studies (Fernandez Carvajal, 1981; 1982a, b; Mateu Andres, 1991, Boscaiu *et al*., 2011; Boscaiu *et al*., 2013; Mesleard *et al*., 2015). Still, the direct effect of NaCl treatments on anatomical structures of these species has not yet been investigated.

The aim of the present study was to analyse whether high saline concentration in controlled experimental conditions, beyond the range of salinity that the two *Juncus* species normally face in their natural environments, has an effect on their anatomical structure. Those structural modifications, if they are indeed detected in salt treated plants, will be thoroughly analysed to establish whether they may be considered as truly adaptations to salinity, or they just represent a stress response; that is, a mere alteration induced by salt. This approach, by including species with different degree of tolerance to salinity, may contribute to a better understanding of the concepts of tolerance *vs*. adaptation to salt stress.

4.8.2. Materials and Methods

4.8.2.1. Plant material and experimental design

Seeds of *J. acutus* were collected from a salt marsh located in the Natural Park of La Albufera (Province of Valencia, Spain) and those of *J. articulatus* from a non-saline area in the Natural Park. The seeds were sown directly into a moistened mixture of peat (50%), perlite (25%) and vermiculite (25%) in 1 liter pots (Ø=11 cm). During the entire course of the germination process the substrate was kept moderately moist, using Hoagland nutritive solution.

Forty-two days after sowing, salt treatments were started, maintaining half of the plants as non-treated controls. The control plants were watered twice a week with Hoagland nutritive solution (1.5 l for each tray containing 12 pots), and salt-stressed plants with the same volume but with NaCl added to the nutritive solution, to a final concentration of 400 mM, prior to irrigation. Treatments were carried out over a period of two months that is sufficient to detect effects of salt stress on *Juncus*.

All experiments were conducted in a controlled environment chamber in the greenhouse, under the following conditions: long-day photoperiod (16 hours of light), temperature (23^{o}C during the day and 17^{o}C at night), CO_2 level (≈ 300 ppm). Humidity ranged between 50-80% during the time of the treatments.

4.8.2.2. Fixation, preparation of slides and microscopic studies

Plant material was harvested at the end of the treatments from different tissues (aerial tissue and roots), and placed directly in FAE (10% formaldehyde, 50% ethanol, and 5% glacial acetic acid) for fixation (Feder and O'Brien, 1968). Dehydration through successive washes of the tissue with increasing concentrations of ethanol was performed, followed by application of histo-clear and paraffin wax. The tissues were processed using a microtome

(Leica RM2025), stained with toluidine blue and analysed under a light microscope (Nikon SMZ800).

4.8.3. Results

4.8.3.1. Effect of salt on the anatomy of Juncus articulatus

At root level, in *J. articulatus* control plants (Fig. 1A and B), rhizodermis was largely exfoliated. The cortex was very thick and had a very weakly suberised exodermis. The cortical parenchyma was also very thick (8-10 cell layers), with very big meatuses between them; they had a squared, rhomboidal or rectangular shape. The endodermis had cells with Casparian strips slightly noticeable. The stele was very thin and had a parenchymatous pericycle. There were 13-15 xylem vascular bundles – only metaxylem vessels were easily noticeable – and the same number of phloem bundles, which were difficult to distinguish. Three-four large central vessels, without connection with those of xylem bundles, were also observed. The pith was thick, of the parenchymatous-cellulosic type.

In salt-treated plants (Fig. 1C and D), the rhizodermis had been entirely exfoliated. The cortex was thick and had an exodermis consisting of three suberised cell layers, partly exfoliated here and there. The cortical parenchyma contained cells slightly prolonged radially. The three internal layers had rectangular, overlapped cells. The endodermis was typically of the tertiary type, with lignified Casparian strips, in a horseshoe shape – the internal wall was very thick. The stele had a parenchymatous pericycle. There were 13-15 xylem vascular bundles, some of them in direct contact with the six large central metaxylem vessels, as well as 13-15 phloem bundles, noticeable only by the very large cells of the phloem parenchyma. The pith was sclerified and lignified; in its width, six large vessels of metaxylem were embedded.

Culms, which had been analysed at the basal level, had an elliptic outline. The epidermis of culms in control plants (Fig. 2 A and B) presented cells with the external wall thick and lignified. Here and there, small tannin and silica cells could be noticed. The cortex was very thick, of the parenchymatous-lignified type, with many cells containing silica and tannin. It consisted of 3-4 layers of external parenchyma, with very short palisade cells, 20 small vascular bundles, numerous large air-storing cavities, prolonged radially, and 1-2 layers of internal parenchyma. The stele was thick and presented a sinuous sclerenchyma ring, which reached the periphery of vascular bundles, protruding between them. There were also about 40 big and small vascular bundles, alternating with each other, all of them surrounded by a sclerenchyma sheath, which was thicker on the phloem area and in contact with sclerenchyma ring. In many vascular bundles, tracheogenesis process was still running. The pith was parenchymatous, with many cells containing tannin; a few central cells appeared to be in the process of disorganisation.

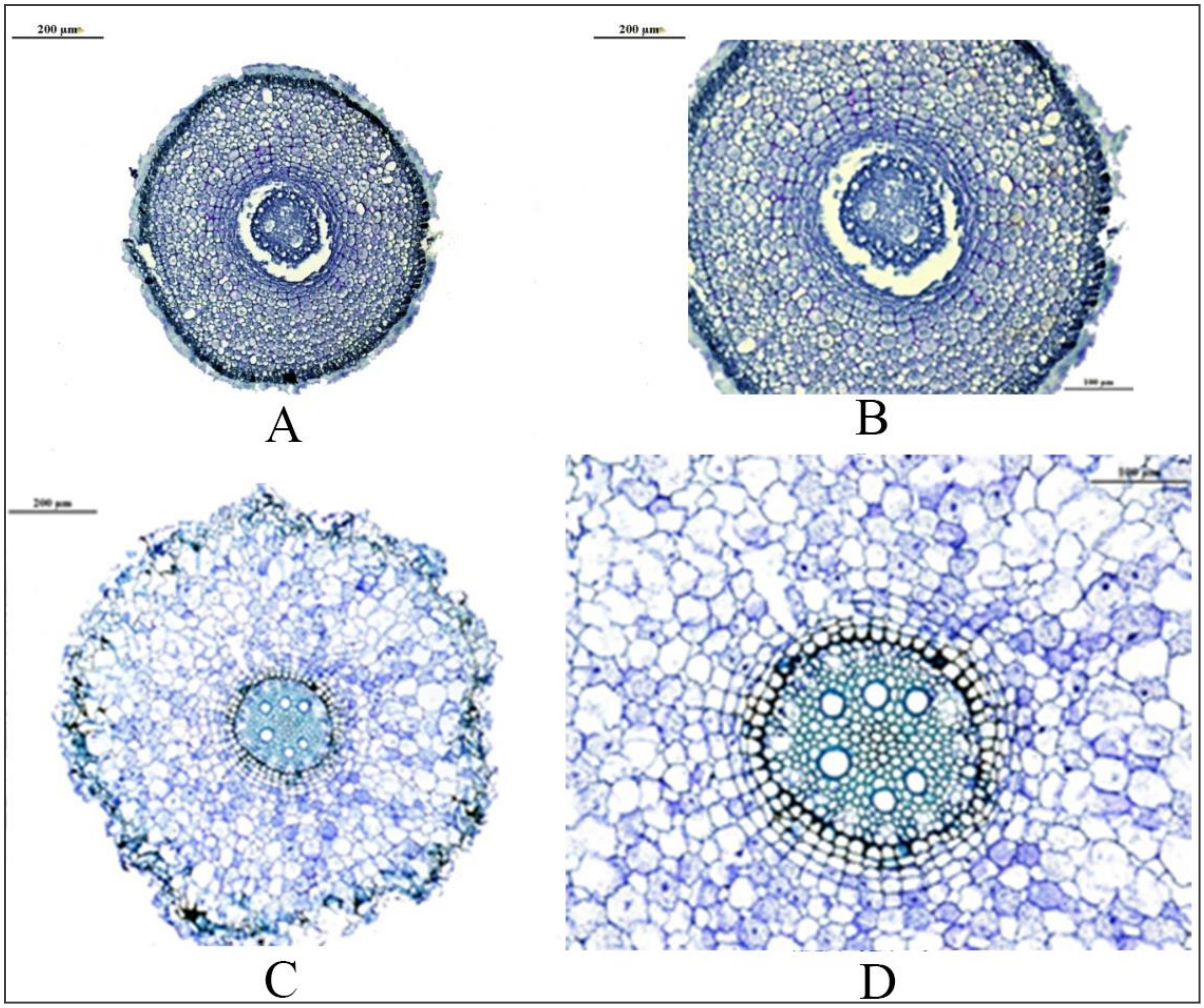

Fig. 1 Cross-sections of roots in *Juncus articulatus* control plants (A and B) and in plants treated for eight weeks with 400 mM NaCl (C and D).

In salt-treated plants (Fig. 2 C and D), the culm cross-section outline was circular. The epidermis had cells with external walls moderately thickened and lignified. The cortex was thicker than in control plants and presented 18-22 vascular bundles located in the outer area of the cortical parenchyma, as well as abundant air-storing cavities, strongly elongated radially and separated by fragments of parenchyma. The stele consisted of a peripheral sclerenchyma ring and 30-32 vascular bundles, from which 1-2 deeply protruded in the pith, thus losing the contact with the sclerenchyma ring. This and the sclerenchyma sheaths surrounding vascular bundles had cells with very thick walls. The pith was parenchymatous, compact, with cells containing tannin and amiliferous cells.

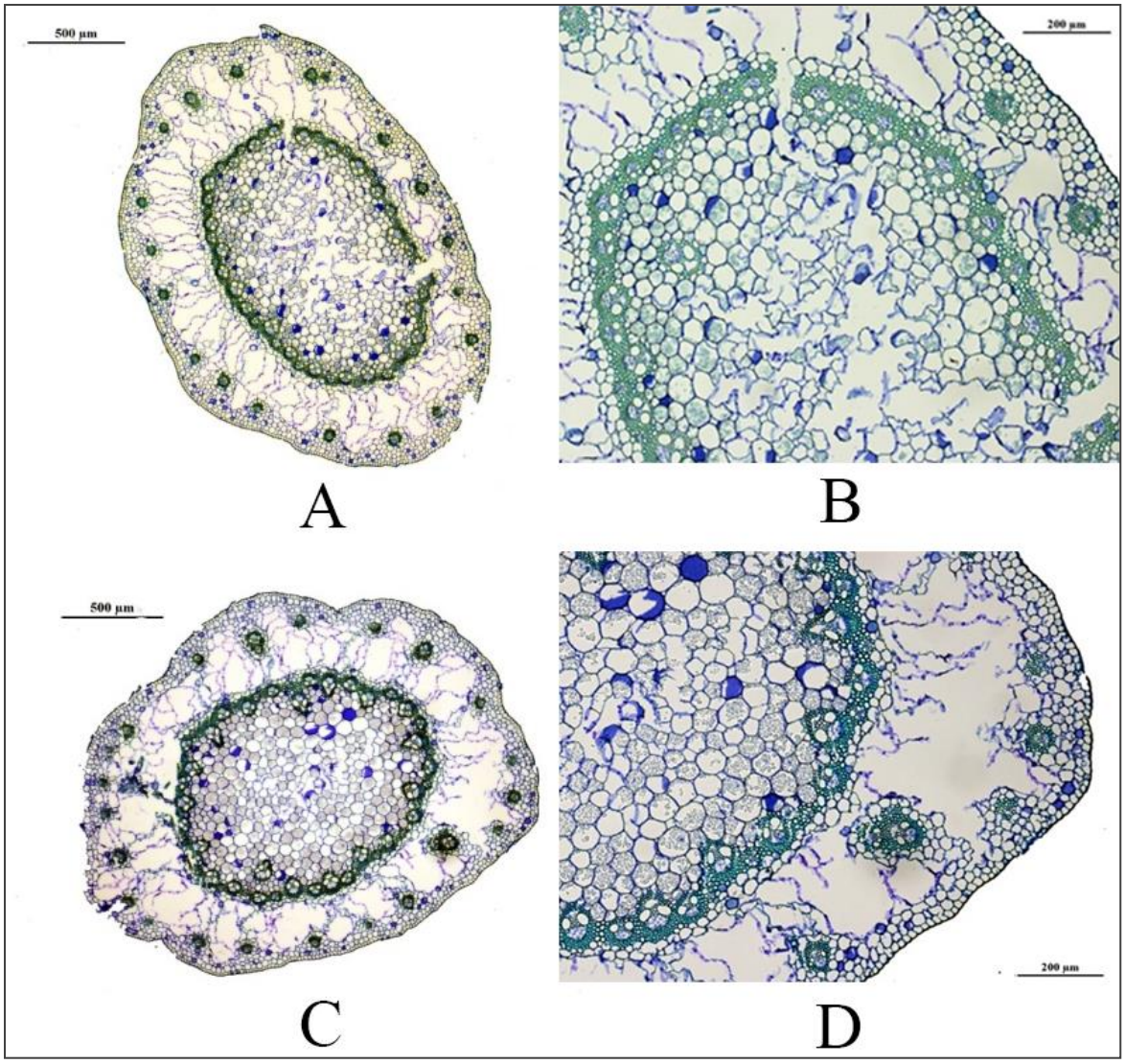

Fig. 2 Cross-sections of *Juncus articulatus* culms, in control plants (A and B) and in plants treated for eight weeks with 400 mM NaCl (C and D).

4.8.3.2. Effect of salt on the anatomy Juncus acutus

At the level of roots, which were adventitious and very thin, in *J. acutus* control plants (Fig 3 A and B) the rhizodermis had relatively short, abundant absorbing hairs. The cortex was relatively thick, including an exodermis with 2-3 layers of cells possessing thin, less suberised walls; the cortical parenchyma was thick, with many large air-storing cavities, very close to each other and separated only by fragments of disintegrated parenchymatous cells and by the stele; the endodermis had large cells, with internal and radial walls moderately thickened and lignified, which did not have yet the horseshoe shape typical for most

monocots. The stele was relatively thick, consisting of a pericycle with cells smaller than those of the endodermis, with which they alternated, and with all walls being thin. In the parenchyma, which was moderately sclerified and lignified, 7-8 large metaxylem vessels were embedded, arranged in a circle. The vascular bundles, of xylem and phloem types, were difficult to distinguish, because protoxylem vessels – in contact with the pericycle – did not differ significantly from cells of sclerified and lignified parenchyma; in addition, the few phloem elements had already slightly thickened and lignified walls. Overall, at a close look, 15-17 xylem and phloem alternating bundles could be noticed. The pith was intensely sclerified and lignified.

At the same level, in the roots of salt-treated plants (Fig. 3 C and D), the rhizodermis had few absorbing hairs. The cortex consisted of an exodermis, in the process of suberification; air-storing cavities in the cortical parenchyma were separated by radial strips of parenchyma cells; the endodermis was similar to that observed in control plants. The stele appeared thinner than in the absence of salt; in its structure, 8-10 large metaxylem vessels with very thin walls were present. Phloem bundles were not observed, but many small groups of protoxylem (whose very narrow vessels were similar to cells belonging to sclerified and lignified parenchyma) and isolated vessels in contact with pericycle could be noticed. The medullar parenchyma had cells with thin and moderately lignified walls. The stele was separated from cortical air-storing cavities by 4-5 layers of parenchyma.

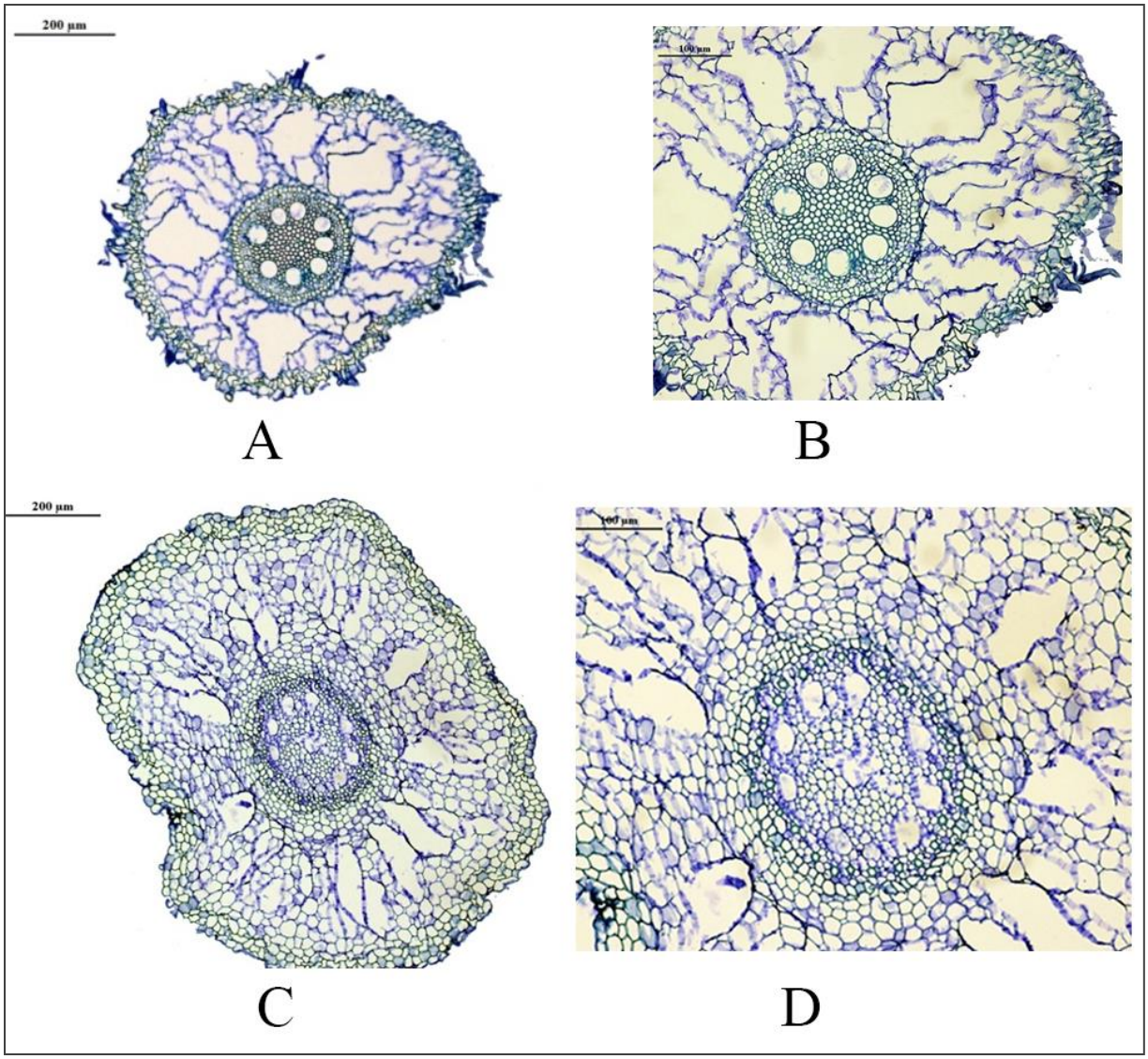

Fig. 3 Cross-sections of *Juncus acutus* roots, in control plants (A and B) and in plants treated for eight weeks with 400 mM NaCl (C and D)

Culms, analysed at the upper level, had an oval outline in cross section (Fig. 4A and B). At this level, the epidermis consisted of cells with thick and intensely lignified external walls; here and there, stomata could be observed. The cortex comprised 9-10 strands of hypodermic sclerenchyma, of different sizes and number of components, and a palisade tissue with 2-3 layers of cells, located between and underneath sclerenchyma strands, interrupted at the level of sub-stomatal cavities. No endodermis or special pericycle could be noticed. The stele consisted of fundamental parenchyma, of the meatic type. The vascular bundles, of collateral closed types, were arranged in two rings: an external one, with small

(5-6) vascular bundles and an internal one, with large (5-6) vascular bundles; all of them were surrounded by a sclerenchyma sheath, consisting of elements with moderately thickened but intensely lignified walls. The axial parenchyma was incompletely disintegrated, forming air-storing cavities of irregular outline.

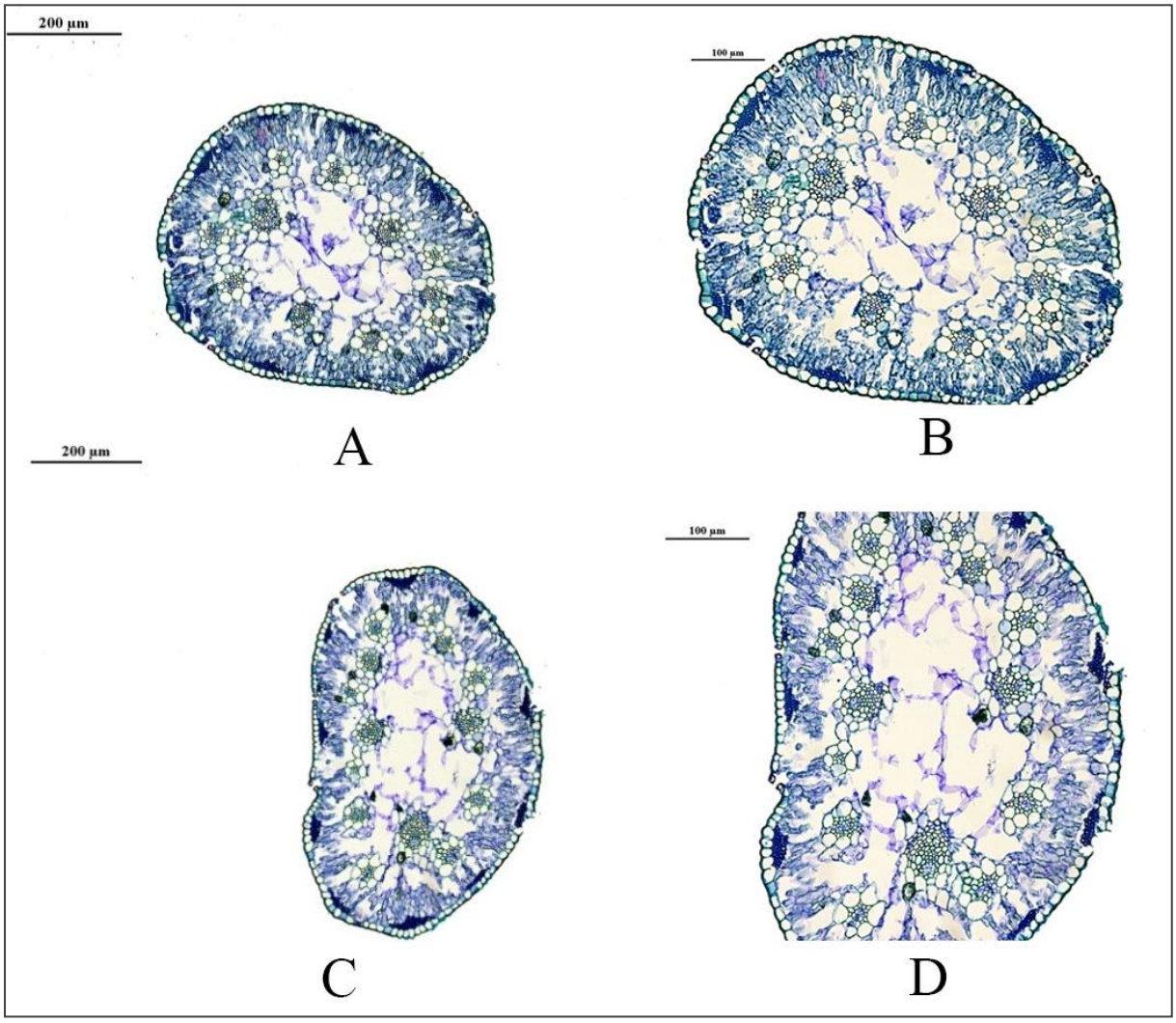

Fig. 4 Cross-sections of *Juncus acutus* culms, in control plants (A and) and in plants treated for eight weeks with 400 mM NaCl (C and D).

In salt-treated plants (Fig 4C and D), there were 10-12 hypodermic sclerenchyma strands; palisade tissue had shorter cells. Large, internal (6) and smaller, external (8) vascular bundles could be observed. Many cells from the cortical and fundamental parenchyma were partly disintegrated, thus giving the appearance of air-storing cavities.

4.8.4. Discussion

The anatomical features in both control and salt-treated plants fit to the general structural plan of *Juncus* species (Blau, 1904; Burduja and Toniuc, 1983-1984; Cutler, 1969; Napp-Zinn, 1973; 1974; 1984; Vierhapper, 1930; Grigore *et al.*, 2014). Several anatomical differences between the two species are evident, independently of the stress treatment. For instance, in *J. acutus* a well-developed aerenchyma was noticed in the root, while in *J. articulatus* it was absent. Since aerenchyma forms in both, control and salt-stressed plants, it should be assumed that this is a constitutive feature rather than a response to the salt treatment. Formation of aerenchyma must be related instead to the conditions where this species grows, in a marsh, where this is a common anatomical feature (Grigore *et al.*, 2014). In the control plants, the aerenchyma seems to be in direct contact with the central cylinder, while in treated plants it is separated from the stele by several layers of parenchyma cells. Yet, it is not clear whether this difference can be correlated with the salt treatment. The possibility that aerenchyma formation can be induced by increasing salinity has been discussed by Colmer and Flowers (2008), but there are no relevant data supporting this hypothesis. Nevertheless, there are several additional differences between control and salt-treated plants regarding their anatomical characteristics, modifications that can be assumed to be induced by salt stress. For instance, in *J. articulatus* subjected to high NaCl concentrations, the root endodermis is well developed, being of the tertiary type, with typical Casparian strips; in non-stressed control plants, on the contrary, these strips are hardly noticeable. In addition, the root exodermis of treated plants is highly suberised, while there is a weakly suberification in the controls. Grigore *et al.* (2014) emphasised that the exodermis, and especially the endodermis, may act as 'barriers' that can control and restrict the flux of

ions to the plant organs in the presence of high soil salinity; this mechanism could contribute significantly to plant survival under severe conditions, natural or experimental. Fahn (1964) and Ginzburg (1966) studied the role played by the endodermis in these mechanisms, particularly on desert halophytes. They discovered that the Casparian strips were wide and thick in the roots of these species, and suggested that the endodermis barrier appears in a highly developed form in plants of such habitats. Poljakkof-Mayber (1975) also found that the ratio between the widths of the Casparian strips and the radial wall of endodermis cells showed large variations: from 1 in hydrohalophytes to 0.9-0.8 in xerohalophytes, and from 0.6-0.5 in dune plants, to 0.33-0.27 in cultivated plants.

It is well known that Casparian strips of root endodermis contain aliphatic and aromatic suberins (Schreiber *et al*., 1999), which make the endodermis impermeable to ions and high-molecular-weight compounds, but allow the continuum of water and other low-molecular-weight solutes. Waisel (1972) showed that, in certain species, (*Suaeda monoica, Vicia faba*) Casparian strips cover almost the entire radial walls of the endodermis as compared to less than one-third covered in glycophytic dicotyledonous. Therefore, due to its particular wall differentiation and ultrastructural features, the primary endodermis is generally regarded as the main apoplastic transport barrier for the passive uptake of water with dissolved ions, from the soil solution into the xylem vessels located in the stele of the root (Robbins II *et al*., 2014). It has been stated that water and ions, which have passively moved from the soil solution to the endodermis through the cell walls of the root cortex, must penetrate the protoplast of the living endodermis cell to gain access to the central cylinder of the root (Geldner, 2013). In this way, it is assumed that root selectivity allows the separation between nutrients and harmful substances (Marschner, 1995).

The role of the Casparian strips as a barrier for solutes and ions has been suggested in several ways: the absence of diffusion of fluorescent dyes beyond the Casparian strip into the

stele (Alassimone *et al*., 2010), by the accumulation of salts at the cortical side of the Casparian strip (Alassimone *et al*., 2012), and by the drop in root pressure observed after puncturing the endodermis (Peterson *et al*., 1993; Steudle and Peterson, 1998).

In high-saline environments, the endodermis limits free apoplastic diffusion of sodium ions into the vascular flux (Robbins II *et al*., 2014). Apparently, this leads to the accumulation of sodium ions in tissues located at the periphery of endodermis (Møller *et al*., 2009).

In addition, there are some data suggesting that the exodermis might form an important barrier towards passive apoplastic diffusion in roots (Clarkson, 1991; Grigore *et al*., 2014). It is well known that hypodermal cell walls are also incrusted with lipophilic and aromatic compounds (Peterson, 1997). In addition, in response to certain environmental factors, there the formation of Casparian strips may occur in hypodermis (Enstone and Peterson, 1998).

In the stem of *J. articulatus*, analysed at the basal level, the cortex of salt-treated plants is thicker and has many and large air-storing cavities than in control plants. However, it is questionable if developed aerenchyma has an inducible-adapted value or is just a sign of tissue damage induced by salinity that may produce a disintegration of cortical cells.

4.8.5. Conclusions

The anatomical modifications found in plants subjected to salt stress do not differ significantly from the general features specific for *Juncus* species. However, the observation under salinity conditions of a tertiary endodermis in the root, and the well-developed aerenchyma in root and stem of treated plants could suggest that these are modifications induced by salt, rather than anatomic adaptations in its broader sense.

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Chapter 5:
General Discussion

The ever growing human population, projected to grow by almost 30% over the next 35 years, to reach 9.2×10^9 individuals by 2050 (Fita *et al*., 2015), leads to an increased demand on global food production, which would need to increase by nearly 60% by 2050 (Wild, 2003), if food supply is to be maintained, in comparison with amounts produced in 2005-2007 (Alexandratos and Bruinsma, 2012). Environmental abiotic stress, notably drought and salinity, are the most detrimental factors affecting crop yields world-wide. Soils affected by salt account to nearly 20% of all irrigated croplands (and nearly 50% in other estimates) (Boyer, 1982; Owens, 2001). Breeding of salt resistant crop varieties has become an essential need, to ensure our ability as a human race to feed ourselves in the coming future. Addressing this objective, will require a clear understanding of the complex mechanisms of salt stress tolerance, which we are still lacking despite intensive research during the last few decades (for recent publications, see Flowers and Colmer, 2008; Munns and Tester, 2008; Tester and Langridge, 2010; Grigore *et al*., 2011; Peleg and Blumwald, 2011; Boscaiu *et al*., 2012; Gil *et al*., 2013).

In our work, we aimed to come to a better understanding of the mechanisms contributing to drought and salt stress tolerance, many of which are found to be conserved in all plants, with distinctive differences that we tried to highlight and investigate. Considering the complexity of the mechanisms of salt tolerance, it seems worthwhile to investigate the responses to salt stress in plants with different levels of tolerance, but with similar genetic backgrounds: different species of the same genus, or different cultivars or varieties of the same crop. This approach may help to establish which of the observed responses are relevant for salt tolerance in a given species (or group of related taxa) and which are not.

We selected for our comparative studies among crops, three cultivars of the species *Phaseolus vulgaris* (cv. 'Maxidor', 'Judia de Franco', and 'The Prince'), and one cultivar of *P.coccineus* cv. 'Moonlight'), while *Solanum lycopersicum* var. cerasiforme (cherry tomato)

was used to characterize the biochemical responses of crops to drought and salt stress. For the study carried among the dicotolydenous halophytes, we selected three species of the genus *Plantago*, that are as follows in descending level of tolerance to the aforementioned stresses; *P. crassifolia*, *P. coronopus* and *P. major*. As for the monocotolydenous halophytes, three species of the genus *Juncus* were choiced out to carry out comparative studies on; two halophytes, *J. maritimus* and *J. acutus* and one glycophyte; *J. articulatus*.

The focus of our comparative studies was firstly to establish the relative degree of tolerance of the investigated crop species or cultivars, based on growth inhibition measurements; however regarding wild species, these measurements served to confirm that their distribution (zonation) in nature, is based on their relative stress tolerance potential (Rogel *et al*., 2000; Engels *et al*., 2011). Secondly, to establish the correlation of the studied taxa´s relative tolerance to stress, with the levels of particular 'physiological/biochemical stress markers' associated with conserved stress response pathways like ion transport, osmolyte accumulation, activation of antioxidant systems (enzymatic and non-enzymatic)…, in order to establish the contribution of those responses to stress tolerance.

We aimed through this work to shed light on a number of specific points, which are listed and discussed below:

5.1. Stress-induced growth inhibition

The most general effect of abiotic stress (including salt and water stress), and the easiest to quantify, is inhibition of growth, which allows plants to survive under adverse conditions by re-directing their resources (metabolic precursors and energy) from normal metabolism and growth to the activation of specific stress defense mechanisms (Zhu, 2001; Munns and Tester, 2008). Water and salt stress causes a reduction in growth rate, stem

elongation, leaf expansion and stomatal movements (Hsiao, 1973), as well as changes in a number of physiological and biochemical processes governing plant growth and productivity (Daie, 1988). However these alterations, especially the deleterious ones are more acute in stress sensitive plants than in tolerant ones (Gossett *et al*., 1993). Relative abiotic stress tolerance in plants limits their distribution in nature according to the stress-gradient hypothesis (Greiner La Peyre *et al*., 2001; Maestre *et al*., 2009) with the most tolerant being present in semiarid, arid, and saline areas, including deserts, dunes, salt marshes and other salt affected areas, where they hold a competitive advantage over less tolerant taxa (Maestre *et al*., 2009).

The monitoring of several growth parameters allowed us to establish the relative degree of resistance to water and salt stress of the investigated crop cultivars and wild species; in the latter case, it was checked whether the relative resistance to stress under controlled experimental conditions corresponded to the distribution of the plants in nature.

Among the measured parameters, the loss of fresh weight of leaves (or aerial part, depending on the species) and reduction of aerial length (length of leaves, if applicable) were essential in determining the effect of the applied stress on growth parameters of the studied taxa, and were used to define the relative tolerance to stress among the studied crop cultivars. Those parameters showed diminution in parallel with increasing salt concentrations and longer drought treatments, in all studied cultivars and species. Among the studied cultivars of *Phaseolus*, stress-induced growth inhibition (estimated from the decrease of fresh mass) was highest in *P. vulgaris cv*. 'The Prince', while *P. vulgaris cv*. 'Maxidor' recorded the smallest reduction. Therefore, *P. vulgaris cv*. 'Maxidor' is the most tolerant cultivar, while *P. vulgaris cv*. 'The Prince' was considered as the most sensitive.

Halophytes like *J. acutus* and *J. maritimus*, or *P. crassifolia* and *P. coronopus* showed relatively smaller reduction of growth under stress, in comparison with their less tolerant

congeners, *J. articulatus* and *P. major*, respectively. Those findings confirmed the expected levels of tolerance to stress according to the distribution in nature of the different species.

Therefore, the stress-gradient hypothesis of natural distribution of species in nature according to stress tolerance coincided with our findings recorded in wild species grown and treated in controlled greenhouse conditions.

5.2. Control of ion transport

One basic response of glycophytes and monocotyledonous halophytes to cope with high salinity in the soil is the limitation of sodium transport from the roots to the leaves (Flowers, 1986) a feature we detected to be highly efficient in *J. acutus* and *J. maritimus* and in *P. vulgaris cv*. 'Maxidor'. On the contrary, dicotyledonous halophytes tend to accumulate toxic ions in the plants' aerial parts, which are maintained at low cytsolic concentrations by compartmentalization in vacuoles (Hasegawa *et al*., 2000; Blumwald *et al*., 2000) such as recorded in the *Plantago* species. This is an advantageous mechanism to increase osmotic pressure in foliar tissues, cheaper, in terms of energy consumption, than the synthesis of organic solutes for osmotic adjustment (Raven, 1985).

Phaseolus plants are known to be able to exclude sodium from the shoots, even in the presence of relatively high NaCl concentrations in the soil (Seemann and Critchley, 1985) through the blocking of transport to the leaves from the roots. In our findings, it was found that the more tolerant cultivar *P. vulgaris* cv. 'Maxidor' recorded lower chloride and sodium levels in their leaves than those measured in their less tolerant studied cultivars, even though they all underwent the same salt stress treatment. In conclusion, the more tolerant *P. vulgaris* cv. 'Maxidor' blocked the transport of Na^+ and to a lesser extent Cl^- to the leaves, a finding that correlated with the levels of salt tolerance among the studied cultivars (three of *P.*

vulgaris and one of *P. coccineus*), and as such shows that this mechanism (blocking transport of ions to aerial parts) is relevant for tolerance.

Unlike *Phaseolus* (dicot glycophyte), and *Juncus* (monocot); *Plantago* (dicot halophyte) species are supposed to utilize compartmentalization of ions in vacuoles, which explains the higher concentrations of sodium and chloride ions in the more tolerant *P. crassifolia* and *P. coronopus*, that allows the exclusion of these ions from the cytosol, avoinding disruption of the functions of enzymes and also reducing ionic and osmotic imbalance inside the cell. The presence and function of vacuolar antiporters is common among all plant species, however the presence of elevated ion levels in leaves of tolerant species *P. crassifolia* and *P. coronopus*, at control levels (in the absence of external salt), suggest the existence of constitutive mechanisms of tolerance in some species of *Plantago*, based on the active uptake and transport of sodium to the leaves, even under low salinity conditions, where the cation will be used as osmoticum – in addition to the accumulation, if necessary, of organic osmolytes – in agreement with the halophytes' 'pre-adaptation to stress' hypothesis (Gong *et al*., 2005; Sanchez *et al*., 2008). The fact that Na^+ also seems to contribute to osmotic adjustment in water-stressed plants – a statistically significant increase of Na^+ levels was detected in *P. crassifolia* and *P. coronopus*, but not in *P. major*, one month after watering was stopped – lends support to this idea. The decrease in K^+ levels significantly in *P. major*, in contrary to the other 2 studied *Plantago* species; suggest that the maintenance of K^+ has some importance in conveying salt stress tolerance in *Plantago*.

In control plants, the more tolerant *J. acutus* and *J. maritimus* showed a higher K^+/Na^+ ratio in culms and roots than that of the sensitive *J. articulatus*. However, under stress, there was an increase in Na^+ and Cl^- contents in roots of salt-treated plants of the three species, reaching similar levels, but findings in the culms showed lower chloride and sodium

concentrations in the more tolerant species. Therefore, inhibition of ion transport is more efficient in the tolerant species, and as such this mechanism would be relevant for tolerance.

5.3. Osmolyte accumulation

A general response to abiotic stress is the synthesis and accumulation in the cytosol of specific compatible solutes known as osmolytes, to counteract the cellular dehydration caused by different stresses, such as salt, drought, cold or high. It is nonetheless difficult to establish whether the stress dependent increase in the concentration of a particular osmolyte has a functional role in the mechanisms of tolerance of a given species, as response doesn't neccesarly confer tolerance.

Osmolytes may significantly contribute to stress tolerance, even if they do not reach cellular concentrations high enough to have a significant osmotic effect, based on their additional roles as 'osmoprotectants': low-molecular-weight chaperons, ROS (reactive oxygen species) scavengers, or as signalling molecules involved in regulation of gene expression and metabolic processes (Szabados & Savouré, 2010; Smirnoff & Cumbes, 1989; Ashraf & Harris, 2004).

Comparative studies on the stress responses of related taxa should provide evidence in favor of the contribution to stress tolerance of specific osmolytes, if a positive correlation between osmolyte levels and the relative degree of tolerance can be found.

5.3.1. Proline

Proline is one of the most common osmolytes in plants, and is synthesized in many different stress situations, such as salinity, drought, cold, high temperature, nutritional deficiencies, heavy metals, air pollution or high UV radiation (Hare and Cress 1997, Grigore *et al*., 2011). Apart from acting in osmotic adjustment, Pro may play other roles in the mechanisms of stress tolerance, such as directly stabilizing proteins, membranes and other subcellular structures, scavenging free radicals, balancing the cell redox status under stress conditions or, as a regulatory molecule, activating the expression of genes involved in stress responses (Smirnoff and Cumbes, 1989; Verbruggen and Hermans, 2008; Szabados & Savouré, 2010).

In *Phaseolus*, the relevance of Pro accumulation for stress tolerance remains unclear, since apparently contradictory results have been obtained when comparing different varieties. Higher free Pro levels have been reported in more drought-tolerant (Ghanbari *et al*., 2013) or salt-tolerant (Cárdenas-Avila *et al*., 2006) bean cultivars than in less tolerant ones. However, there are also reports of higher Pro levels in sensitive than in more resistant cultivars, in *P. vulgaris* (Jiménez-Bremont *et al*., 2006; Rosales *et al*., 2012; Zadehbagheri *et al*., 2012), as well as in many other plant genera where no positive correlation between Pro contents and tolerance has been found (Ashraf and Foolad, 2007; Lutts *et al*., 1996; Guerrier, 1998; Chen *et al*., 2007).

The present study revealed that Pro accumulation is a common response to salt and water stress in the four analyzed cultivars, in agreement with the aforementioned published results. Yet, Pro biosynthesis cannot contribute significantly to their stress tolerance, since the levels reached in the most tolerant variety, cv. 'Maxidor', were by far lower than in the other cultivars, and *vice versa*: the highest Pro contents were measured in the most sensitive cultivar, cv. 'The Prince'. In this case, Pro should be considered as a marker of the level of

stress affecting the plants, and these results simply reflect the fact that 'Maxidor' plants were less stressed than the others.

On the other hand, in the studied *Plantago* species, it appears that Pro is accumulated only in extreme conditions (water stress and 600 mM NaCl or more) in the most tolerant of the studied species (*P. crassifolia*, and *P. coronopus*), and even though its levels are much lower than that of sorbitol (*Plantago*'s main accumulated osmolyte), it also may contribute to osmotic adjustment, and surely has an acting role in osmoprotection (ROS scavenging, low molecular chaperons), among other functions… Its differential accumulation seems to explain difference in tolerance among the studied species of this genus. The concomitant synthesis of different osmolytes has been previously observed in many halophytes (e.g., Briens and Larcher, 1982), although it is generally assumed that each species uses preferentially one particular compound for osmotic balance under stress conditions. Such behavior is reported here in the most tolerant studied *Plantago* species, *P. crassifolia* and *P. coronopus* under extreme conditions (600-800 mM NaCl and water stress). It should be pointed out that the maximum Pro concentrations reached under the strongest stress conditions tested are still too low -three orders of magnitude below those of sorbitol- to contribute substantially to osmotic adjustment, even assuming that it accumulated exclusively in the relatively small volume of the cytoplasm. Nevertheless, it is likely that Pro, plays a significant role in the tolerance mechanisms of these species in the presence of strong stress conditions. Yet these mechanisms would not involve maintenance of cellular osmotic balance, but rather be based on the osmolytes function(s) as a low-molecular-weight chaperons, ROS scavengers and/or signaling molecules (Ashraf and Foolad, 2007; Szabados and Savouré, 2010; Grigore *et al.*, 2011).

Among the studied species of *Juncus*, Pro levels increased especially under the highest salt treatment (400 mM NaCl) and in plants experiencing water stress. The recorded Pro increase was many fold higher in the tolerant *J. acutus* and *J. maritimus*, than in the stress sensitive *J. articulatus*, even though the levels were nearly equal in control plants. This finding strongly support the idea that proline biosynthesis confers salt tolerance to stressed plants, and is a not merely a response to stress, at least among the species of this genus. A significant similar increment of Pro in NaCl-treated plants was reported in other salt tolerant species of *Juncus*, such as *J. roemerianus* (Cavalieri and Huang, 1979) and *J. krausii* (Naidoo and Kift, 2006). All of which confirms the role of Pro in conferring tolerance to drought and salt stress among species of the *Juncus*. Other osmolytes were found to accumulate as well with the onset of drought and salt stress, like sucrose, fructose and glucose. These soluble carbohydrates contribute to the osmotic adjustment among other functions (sucrose in the *J. acutus* and *J. maritimus* and all three aforementioned soluble carbohydrates in *J. articulatus*), however they do not explain differences in tolerance like proline did with the studied species of *Plantago*, or among the studied species of *Juncus*.

We can conclude from our studies, the different roles that an osmolyte as Pro can undertake, ranging from a biomarker of stress in *Phaseolus*, to an osmolyte relevant for tolerance to salinity and drought under strong stress conditions, functioning as an osmoprotectant in *Plantago*, or as found in *Juncus* where it acts both as an osmoprotectant and being involved in maintenance of osmotic balance (with sugars). In conclusion, Pro is generally accumulated under stress in most plant species; however its mechanisms of action and contribution to stress tolerance vary, in a species-specific manner, depending on the concentration it reaches in the cell and the synthesis of other major osmolytes.

5.3.2. *Glycine-betaine*

Glycine betaine is another osmolyte synthesized in response to salt and water stress in many different plant groups (Ashraf & Foolad, 2007; Hanson & Scott, 1980; Rhodes & Hanson, 1993). However, a few plants use GB as a major soluble solute and accumulate it in very high levels –termed, glycine betaine accumulators- such as *Inula crithmoides, Sarcocornia fruticosa*, *Salicornia europea*, *Suaeda maritima* (Moghaieba *et al*., 2004; Gil *et al*., 2014)… none of which were among the species investigated in this work.

There are only a handful of references describing the presence of this osmolyte in *Phaseolus* (Ashraf & Iram, 2005; Ali & Abdel-Fattah, 2006), although at concentrations lower than those reported here, that nonetheless remain insignificant in comparison to the levels found in GB accumulators. In any case, the levels of GB measured in the analyzed *Phaseolus* cultivars are too low and this compound probably does not play a significant role in salt stress tolerance in this genus.

GB was found to slightly increase in the three investigated *Plantago* species, upon the application of salt and water stress; yet, it reached maximum absolute contents below those of Pro, and much lower than those reported for plants that are true GB accumulators (e.g., Khan *et al*., 2000; Tipirdamaz *et al*., 2006; Gil *et al*., 2014). Nevertheless, it is possible that GB still plays a role in the tolerance mechanisms of these species in the presence of strong stress conditions not through osmotic adjustment, but rather as an osmoprotectant (Ashraf and Foolad, 2007; Nawaz and Ashraf, 2010).

In the studied *Juncus* species, GB was found to increase by 2 to 3 fold in all cases, under both salt and water stress; however, the increase and the levels reached where insignificant compared to those reported for true glycine betaine accumulators (Kahn *et al*., 2000; Tipirdamaz *et al*., 2006). GB contents were also lower than those of other osmolytes measured by us in *Juncus*; nevertheless, a contribution of this compound to stress tolerance, based on its osmoprotectant roles, cannot be ruled out, since under stress tolerant plants may

trigger the synthesis of several osmolytes, with different functions in the mechanisms of stress tolerance.

In conclusion, glycine betaine doesn't seem to be essential in tolerance to salt and water stress among the species we studied in this work (except in water stress tolerance of *P. coccineus*), since it nearly never showed a variation that explain relative tolerance in the studied taxa. It might however contribute to some minor extent (according to its concentration) to osmotic adjustment among other functions.

5.3.3 Soluble sugars

Assessing the role of soluble sugars as compatible solutes in the mechanisms of stress tolerance is generally difficult, since their multiple functions as direct photosynthesis products, components of primary metabolism and regulatory molecules may mask their putative function as osmolytes in the mechanisms of tolerance against abiotic stress.

Several recent reports dealing with the changes of soluble carbohydrate contents in *Phaseolus* under stress conditions have been published (Sassi *et al.*, 2010; Tazuke *et al.*, 2009), but it has been also recently reported salt stress-induced sugar accumulation in the genus *Phaseolus* barely contributes to the leaf osmotic potential (Bayuelo-Jiménez *et al.*, 2012). We did not detect significant changes, correlated with the stress treatments, in the levels of total soluble sugars in the analyzed *Phaseolus* cultivars. However, after separation of the carbohydrate fraction by HPLC, strong increases in *myo*-inositol contents were observed in cv. 'Maxidor', the most tolerant cultivar of *P. vulgaris*, and to a lesser extent also in *P. coccineus*; *myo*-inositol accumulation was induced in the presence of NaCl, in a concentration-dependent manner, but mostly in water-stressed plants. Therefore, this polyalcohol appears to play a significant role in the stress tolerance mechanisms in *Phaseolus* taxa.

In the *Plantago* species studied, there was no clear correlation between the changes in total soluble sugars contents and the intensity of the stress treatments. The major osmolyte in the *Plantago* genus is the polyalcohol sorbitol, which will be explained in detail in the next subsection.

The selected *Juncus* species revealed a similar increase in the levels of soluble sugars under salt and drought stress in all three taxa. After HPLC fractionation, it could be established that glucose, fructose and sucrose were the major sugars present in the three taxa, and that the halophytes *J. maritimus* and *J. acutus* accumulated mostly sucrose in response to stress, with non-significant or very small changes in the levels of glucose or fructose in stressed plants. The glycophyte *J. articulatus* showed a different pattern: large increases in sucrose levels were observed in response to salt and water stress, as for the tolerant species; however, the levels of glucose and fructose also increased in response to increasing salt concentrations, but decreased significantly under drought stress. Therefore, despite the general overlapping of salt and water stress effects on plants, *J. articulatus* seems to activate slightly different mechanisms of response to both stresses, at least regarding the accumulation of specific osmolytes. Although sugar accumulation contributes to maintain cellular osmotic balance under stress, it is similar in the three studied species and therefore does not explain their differences in stress tolerance which are better explained by Pro accumulation and control of ion transport (much higher sodium ion levels in leaves of *J. articulatus*, even though all three species had similar Na^+ concentrations in the roots).

5.3.4. Sorbitol

Plantago taxa are known to be accumulators of sorbitol (Gorham *et a*l., 1981; Koyro, 2006; Flowers and Colmer, 2008; Flowers *et al*., 2010), unlike *Juncus* or *Phaseolus* species; and therefore, sorbitol was only determined in the species of this genus.

Sorbitol accumulated in response to water and salt stress, in the three studied *Plantago* species. However, the relative increases over the corresponding controls were very poor, since sorbitol concentrations were already high in the absence of salt, especially in *P. coronopus* and *P. major* (about 1 mmol g^{-1} DW). Therefore, these species appear to possess constitutive mechanisms of response to stress: they accumulate the osmolyte in the absence of salt, so that a strong induction of its synthesis when the plants are actually affected by high salinity conditions is not necessary, and the regulation of the response to salt stress could be mostly based on changes in the intracellular localization of sorbitol. Sorbitol functions in maintenance of osmotic balance in *Plantago* species due to its extremely high concentration, but the accumulation of sorbitol does not explain differences in tolerance of the studied species as there are no major quantitative differences in sorbitol levels among studied species. This difference of tolerance however, is explained by control of ion compartmentalization and Pro accumulation under strong stress conditions, as mentioned previously.

These finding support the hypothesis of 'pre-adaptation' or 'metabolic preparedness' to stress in tolerant species (Bose *et al*., 2013) although we prefer to talk simply of constitutive or induced stress responses.

5.4. Activation of antioxidant systems

Reactive oxygen species (ROS) in plants are constantly produced due to normal cell metabolism, in chloroplasts, mitochondria, peroxisomes and other sites of the cell as a result of vital metabolic processes such as photosynthesis and respiration (Mittler, 2002; Uchida *et al*., 2002; Martinz *et al*., 2001). However, under these normal conditions the production and destruction of these reactive oxidative species are controlled and regulated in the cell. Plants – indeed, all organisms – have antioxidant defense mechanisms that include both non-

enzymatic and enzymatic components, to minimize the effects of ROS. Under stress (such as high light, high or low temperature, salinity, drought, nutrient deficiency and pathogen attack), however, the production of ROS increases and could outpace the scavenging processes, resulting in oxidative stress.

Accumulation of non-enzymatic antioxidant compounds and the activation of antioxidant enzymes, is an essential pathway for abiotic stress tolerance (Blokhina *et al.*, 2003; Parida and Das, 2005; Parvaiz and Satyawati, 2008). The results reported in this work showed that halophytes tended to accumulate more antioxidants than their less tolerant congeners, as well as having higher anti-oxidative enzymes activity.

Antioxidant responses to stress have been studied in the selected *Juncus* species. Phenolic compounds- and especially a subgroup of them, the flavonoids- are plant secondary metabolites that seem to be important in the mechanisms of response to abiotic stresses due to their antioxidant activity (Farah and Donangelo, 2006). Under stress conditions (both salt and water stress) the halophytes *J. acutus* and *J. maritimus* showed higher concentrations of total phenolics and flavonoids than the glycophye *J. articulatus*. Moreover, the increase in specific activities of antioxidant enzymes such as Catalase (CAT), Superoxide dismutase (SOD), Glutathione reductase (GR) and Ascorbate peroxidase (APX) showed better correlations with the intensity of the applied stress in the more tolerant *Juncus* species.

5.5. Cloning and characterisation of *Plantago NHX1* genes

Na^+/H^+ antiporters are functional proteins that play vital roles in conferring salt tolerance in plants (Blumwald and Poole, 1985; Ballesteros *et al.*, 1997). Two types of Na^+/H^+ antiporters are present in plants, *NHX1* located in the tonoplast and *SOS1* located in the plasma membrane. The first functions in compartmentalizing Na^+ in vacuoles that reduces

the toxicity of this ion in the cytosol and contributes to osmotic regulation (Blumwald *et al*., 2000), while the other type of Na^+/H^+ contributes in extruding Na^+ from cells (Takahashi *et al*., 2009; Wu et al., 1996). In the selected *Plantago* species, we isolated, sequenced and studied the expression of the vacuolar Na^+/H^+ antiporter gene *NHX1*.

The three investigated *Plantago* species show different levels of salt tolerance, based on the relative efficiency of some mechanisms of response to stress, basically the control of ion transport to the leaves and the accumulation of specific osmolytes, as discussed above. In agreement with our data, differences in salt tolerance of *Plantago* species have been also related to the efficiency of intracellular ion compartmentalization mechanisms; for example, Staal *et a*l. (1991) reported a considerably greater tonoplast Na^+/H^+ antiporter activity under salt stress in the halophyte *P. maritima* than in the salt-sensitive *P. media*. The higher concentrations of Na^+ and Cl^- we have determined in leaves of *P. crassifolia* and *P. coronopus*, as compared to *P. major*, are also related to the anatomic structure of the former species, with succulent leaves (especially in *P. crassifolia*), and therefore with increased vacuole volume and better ion sequestration capacity. Succulence, as well as excretion of Na^+ and Cl^- by salt glands or bladders, is a basic anatomic adaptation to salinity in some dicotyledonous plants (Flowers et al., 1986).

Concerning the major tonoplast transporter responsible for compartmentalizing Na^+ in vacuoles: Na^+/H^+ antiporter *NHX1*, we managed to isolate the cDNA sequences of the 3 studied *Plantago* species, via RACE-pcr (Rapid amplification of cDNA ends) through the utilization of primers designed from homology. A phylogenetic tree of their deduced protein sequences and those of *NHX1* antiporters previously isolated from other species suggested the presence of some structural features characteristic of the proteins of salt tolerant taxa. The expression patterns of the *NHX1* genes in the presence of salt correlated with the relative degree of salt tolerance of the three species: in short-term treatments with a high NaCl

concentration (400 mM), no induction was detected in the least tolerant *P. major* – and in the glycophyte *A. thaliana* – and the highest and quickest induction was observed in the most tolerant *P. crassifolia*. These data support the hypothesis that the *NHX1* antiporters play a functional role in the mechanisms of salt tolerance in plants. Thus our finding are in agreement with previous published data, stating that increased efficiency of intracellular ion compartmentalization mechanisms confer higher salt tolerance in dicots.

5.6. Inducible vs. constitutive tolerance mechanisms

The constitutive resistance mechanisms to salt and drought stress are hypothetically supposed to exist in some halophytes, whereas the inducible ones are characteristic of glycophytes (Radyukina *et al*., 2007) even though they do occur in halophytes as well.

Throughout this work, we came out with a number of conclusions regarding the question of tolerance mechanisms in halophytes and glycophytes. Constitutive tolerance mechanisms were found in *Plantago* through the presence of high concentrations of sorbitol even in control conditions, however among the 3 studied species, the levels were nearly equal at control and the increase in sorbitol levels wasn't very significant (a few folds) regardless of the species' tolerance potential. Hence, the accumulation of sorbitol, even though vital for osmotic balance, didn't explain the relative tolerance levels to stress in *Plantago*. Similarly, relatively high concentrations of toxic ions were measured in the leaves of control plants of the tolerant species: *P. crassifolia* (Na^+ and Cl^-) and *P. coronopus* (only Na^+), but not in *P. major*, that we suppose are compartmentalized in vacuoles. This was further more confirmed by the study of expression of *NHX1* upon salt application, where tolerant *Plantago* species showed an induction in expression of the mentioned gene in contrary to *A. thaliana* and *P. major* as a response to short-term treatments with a high NaCl concentration (400 mM).

Meanwhile inducible tolerance mechanisms were found throughout the presented work, such as the synthesis or the up regulation of *myo*-inositol in *Phaseolus*, and proline under extreme conditions in *Plantago* and *Juncus*. Other inducible mechanisms include the upregulation of some anti-oxidant enzymes, non-enzymatic antioxidants, as well as the aforementioned ion transport.

Chapter 6:
Conclusions

We came out with a number of conclusions from the present work, some that confirm previous data, already published by us or by other groups, and some that provide novel insights on the mechanisms of abiotic stress tolerance in plants. The main conclusions are listed below:

1) The characterization of stress response mechanisms in *Solanum lycopersicum* var. cerasiforme (cherry tomatoes), confirmed the accumulation of proline in response to salt and water stress; however, we also detected an increase in the levels of glycine betaine, as a secondary osmolyte, despite numerous publications reporting its absence in tomato.

2) Partial blocking of transport of toxic ions to the leaves, alongside *myo*-inositol accumulation for osmotic adjustment, seems to be the major mechanisms conferring stress tolerance in *Phaseolus* cultivars. Proline is a reliable stress biomarker, but is not directly involved in stress tolerance in this genus.

3) Sorbitol is the main osmolyte in *Plantago* taxa, but its high levels in control plants and the patterns of accumulation in response to stress do not explain the differences in tolerance of the investigated species. On the other hand, large relative increases in proline contents under extreme stress conditions have been observed only in the stress tolerant *Plantago* species, indicating that proline accumulation is relevant for tolerance.

4) Transport of sodium and chloride ions to the leaves under salt stress, a process more efficient in the tolerant species, is also relevant for tolerance in *Plantago*. The increase in K^+ levels at high Na^+ concentrations seem to contribute as well to salt tolerance, by limiting the reduction of K^+/Na^+ ratios.

5) Compartmentalization of toxic ions in vacuoles may be improved in the most tolerant species of *Plantago* by the increased salt-induced expression of the tonoplast Na^+/H^+ antiporter gene *NHX1*.

6) Reproductive success under salt stress conditions in the studied *Plantago* species depends largely on their relative tolerance and correlates closely with its ecology and distribution in nature.

7) Salt tolerance in *Juncus* is based on the partial inhibition of transport of toxic ions from the roots to the plants aerial parts and the activation of K^+ transport at high external salt concentrations, as well as on the accumulation of relatively high levels of proline in the culms of the plants.

8) Tolerant *Juncus* species recorded more efficient variations in the stress-induced activity of antioxidant enzymes, closely correlated with their relative tolerance degree, as well as higher accumulation of chemical antioxidants than their stress sensitive congeners.

9) Some of our data indicate the presence of constitutive mechanisms of stress tolerance, such as the use of inorganic ions as osmotica even under low salinity or the accumulation of high sorbitol levels in non-stressed plants, in tolerant *Plantago* species, thus supporting the hypothesis of '*stress-anticipatory preparedness*' in halophytes.

10) Comparative studies of abiotic stress responses in genetically related taxa with different degrees of tolerance, proved to be a valuable approach to decipher general stress tolerance mechanisms in plants, especially regarding the distinction between responses that are relevant for tolerance from those that are not.

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(of Chapters 1, 3, and 5)

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Appendix

Comparative Analysis of Osmotic and Ionic Stress Effects on Seed Germination in *Tagetes* (Asteraceae) Cultivars

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Abstract. Drought and salinity are the most adverse environmental factors affecting crop productivity worldwide. The increasing scarcity of fresh, high-quality irrigation water is forcing the use of alternative water sources-such as sewage, waste water or brackish water in agriculture, in general, and also in floriculture. Seed germination is the first and the most important stage in a plant's life cycle, but it is also the most sensitive to environmental conditions, representing a bottleneck in plant development. Checking for stress tolerance at the germination stage is a reliable approach for the rapid screening of a large number of plant cultivars. The aim of the present study was to analyze the responses to osmotic and ionic stress, induced by isotonic solutions of polyethylene glycol (PEG) and NaCl, in 13 cultivars of *Tagetes patula*, *T. tenuifolia*, and *T. erecta* at the germination stage. Germination percentages and rates, and radicle, hypocotyls, and cotyledon lengths were determined after seven days of treatment. Responses to osmotic stress induced by PEG were similar in all selected cultivars, but significant differences were detected upon the salt treatments. In general, *T. erecta* responded better to salt stress than the other two species, but a large variability among cultivars within each species was observed. The most stress-tolerant cultivars of *T. patula* were 'Orion'and 'Robuszta', and those of *T. tenuifolia* were 'Luna Gold', 'Luna Rot' and 'Luna Orange'.

Identification of Salt Stress Biomarkers in Romanian Carpathian Populations of *Picea abies* (L.) Karst

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Abstract. The Norway spruce (*Picea abies*), the most important tree species in European forests, is relatively sensitive to salt and does not grow in natural saline environments. Yet many trees are actually exposed to salt stress due to the common practice of de-icing of mountain roads in winter, using large amounts of NaCl. To help develop strategies for an appropriate use of reproductive seed material on reforestation sites, ensuring better chances of seedling survival in salt-affected areas, we have studied the responses of young spruce seedlings to salt treatments. The specific aim of the work was to identify the optimal salt stress biomarkers in *Picea abies*, using as experimental material seedlings obtained by germination of seeds with origin in seven populations from the Romanian Carpathian Mountains. These responses included general, conserved reactions such as the accumulation of ions and different osmolytes in the seedlings needles, reduction in photosynthetic pigments levels, or activation of antioxidant systems. Although changes in the contents of different compounds involved in these reactions can be associated to the degree of stress affecting the plants, we propose that the (decreasing) levels of total phenolics or total carotenoids and the (increasing) levels of Na^+ or K^+ ions in *Picea abies* needles, should be considered as the most reliable and useful biomarkers for salt stress in this species. They all show very high correlation with the intensity of salt stress, independently of the genetic background of the seeds parental population, and relatively easy, quantitative assays are available to determine their concentrations, requiring simple equipment and little amount of plant material.

Transcriptome Analysis of *Phoenix canariensis* Chabaud in Response to *Rhynchophorus ferrugineus* Olivier Attacks

Authors: Giovino A, Bertolini E, Fileccia V, Al Hassan M, Labra M, Ruisi P, Martinelli F.

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Abstract. Red Palm Weevil (RPW, Rhynchophorusferrugineus Olivier) threatens most palm species worldwide. Until now, no studies have analyzed the gene regulatory networks of Phoenix canariensis (Chabaud) in response to RPW attacks. The aim of this study was to fill this knowledge gap. Providing this basic knowledge is very important to improve its management. Results: A deep transcriptome analysis was performed on fully expanded leaves of healthy non-infested trees and attacked trees at two symptom stages (middle and late infestation). A total of 54 genes were significantly regulated during middle stage. Pathway enrichment analysis showed that phenylpropanoid-related pathways were induced at this stage. More than 3300 genes were affected during late stage of attacks. Higher transcript abundances were observed for lipid fatty acid metabolism (fatty acid and glycerolipids), tryptophan metabolism, phenylpropanoid metabolism. Key RPW-modulated genes involved in innate response mediated by hormone crosstalk were observed belonging to auxin, jasmonate and salicylic acid pathways. Among transcription factors, some WRKYs were clearly induced. qRT-PCR validation confirmed the upregulation of key genes chosen as validation of transcriptomic analysis. Conclusion: A subset of these genes may be further analyzed in future studies to confirm their specificity to be induced by RPW infestations.

Elucidating grapevine responses to Stolbur phytoplasma infections, in copresence with viruses and at 'recovered' status.

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Abstract. After providing a wide picture of global transcriptomic changes of grapevine responses to "stolbur" phytoplasma, the recovery status and the molecular responses to phytoplasma and virus copresence were analyzed. NimbleGen® V. vinifera genome arrays were used. A lower transcript abundance of genes involved in photosynthesis, trehalose, phospholipids was observed in response to "stolbur" phytoplasma presence. The recovered plants showed increased transcripts involved in ATP synthesis and amino acid metabolism, secondary metabolism and biotic stress-related pathways. The recovery was associated with tetrapyrrole pathway repression. Coinfection with viruses induced genes involved in hormone categories (cytokinin, gibberellin, salicylic acid and jasmonates).