Comparative analysis of drought and salt stress tolerance mechanisms in *Silene*

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Abstract

Comparative analyses of the responses to abiotic stress in related taxa with different degrees of tolerance can provide useful information to elucidate the mechanisms of stress tolerance in plants. This kind of study has been carried out in four *Silene* species, adapted to different habitats in nature, which were subjected to salt and water stress treatments under controlled . Several growth parameters, photosynthetic pigments, ions, osmolytes and non-enzymatic antioxidants levels were determined ~~after three weeks of treatment~~. Both stresses inhibited plant growth. The lowest decrease in fresh weight under salinity was observed in *S. vulgaris* while under water stress conditions in *S. sclerocarpa*. Photosynthetic pigments decreased in all species in response to NaCl except in the least affected by salinity *S. vulgaris*. Homeostasis of K^+ under saline conditions represents the main mechanism of salt tolerance in *S. vulgaris*. Proline and total soluble sugars contents increased in leaves of stressed plants but their levels did not correlate with the species´ stress tolerance and therefore do not play a key role in their cellular osmotic adjustment. Significant increase in malondialdehyde (MDA) was observed

only under water stress in *S. latifolia*, which is the species most affected by drought. Total phenolic and flavonoid variations were not statistically significant or did not correlate with the level of stress applied. According to the different evaluated parameters, the most salt tolerant species proved to be *S. vulgaris,* whereas in our experiment *S. sclerocarpa* was the most tolerant to drought. These findings are in agreement with the ecological conditions specific for these taxa in their natural habitats.

Keywords: *Silene*, salinity, water stress, monovalent ions, osmolytes, oxidative stress

Highlights

- Responses to salinity and drought in *Silene* are closely related with species' ecology.
- Degradation of photosynthetic pigments could serve as a reliable abiotic stress marker.
- The homeostasis of K^+ is the main defense mechanism against salt stress.
- Higher levels of proline do not correlate with higher stress tolerance in *Silene*.

Abbreviations

Caro – total carotenoids
Chl a – chlorophyll a
Chl b – chlorophyll b
MDA – Malondialdehyde (MDA),
Pro – Proline

Introduction

Soil salinity and drought are the most adverse environmental stress factors for agriculture, considering the damage they inflict on crop yields worldwide; they are also important because of their impact on the distribution of wild plant species in nature. Currently, more than 320 million hectares of land are affected by salinity (Munns and Tester, 2008; Rengasamy, 2010), and this area is expected to expand in the forthcoming years due to the foreseeable effects of global climate change. Climate change will also contribute to extend the surface of drought-affected areas, especially in arid and semiarid regions (ref.). The most promising strategy to increase agricultural yields and food production under the present circumstances would be the genetic improvement of the tolerance to salt and water deficit of our major crops, by classical breeding techniques and/or genetic engineering (Fita et al., 2015). To reach this goal, a deep understanding of the molecular mechanisms of abiotic stress tolerance

in plants is necessary, which explains why – apart from its academic interest – this is currently one of the most active research topics in plant biology.

The vast majority of wild plants and all major cultivated species, are highly sensitive to different abiotic stresses (Zhu, 2001; Lavor, 2013; Rejeb et al., 2015), notably to drought and salinity, although some (very few) wild taxa are adapted in nature to extremely harsh environments, such as arid (xerophytes) or saline (halophytes) habitats. It is well established that all plants, regardless of their tolerance to stress, activate the same series of basic, conserved reactions in response to abiotic stresses such as salinity or water deficit; these responses are based, for example, in the control of ion transport and ion homeostasis, the synthesis of specific compatible solutes for osmotic adjustment or the activation of antioxidant systems (Zhu, 2001; Flowers et al., 2010; Ariga et al., 2013). This fact justifies the use of salt and drought-sensitive species, such as *Arabidopsis thaliana*, as models to explore the mechanisms of response to such abiotic stresses (Sanders, 2000; Zhu, 2001; Ariga et al., 2013; Rejeb et al., 2015). Yet, the relative efficiency of these responses varies widely amongst plant species, and the contribution of a particular response to the stress tolerance of a given species or group or related taxa remains generally unknown. Therefore, no single model can provide a general view of the mechanisms of abiotic stress tolerance in plants, the elucidation of which should be based on studies performed in different species.

Silene L. is one of the largest genera of flowering plants within the Caryophyllaceae family. The genus *Silene* comprises 43 sections and about 700 species, and the natural distributions of most of them lie throughout the northern hemisphere with two main biodiversity centres: the Mediterranean region, and the south-west Asian region. However, native species can also be found in North and South America, and in Africa (Bittrich, 1990; Kilic, 2009; Fawzi et al., 2010; Rautenberg et al., 2012). This genus has been traditionally included in many genetic and ecological studies and has a number of remarkable features. Firstly, *Silene* species vary widely in terms of their breeding systems and their ecology. Secondly, several members of this mainly Holarctic genus can be easily bred and have short life cycles, and are thus convenient for both, experimental and field studies; in fact, some species continue to be widely used in the fields of ecology and evolutionary biology. The genus has also been used for over a century as a model to understand the genetics of sex determination. Other studies carried out on *Silene* species include speciation, host-pathogen interactions, biological invasions, adaptation of some populations to heavy-metal-contaminated soils, metapopulation genetics, and organelle genome evolution (Bernasconi et al., 2009; Käfer et al., 2013; Fields and Taylor, 2014; Colzi et al., 2015, Hahn and Brühl, 2016). Notably, some members of the genus hold the distinction of

harbouring the largest mitochondrial genomes ever identified (Sloan et al., 2012a,b; Rautenberg et al., 2012). Genomic resources are now becoming increasingly available in *Silene*, which makes possible to undertake genetic, quantitative genetic and molecular studies in this genus. One of the strengths of *Silene* as a model system, compared with other classical model organisms, is the availability of a large number of previous ecological studies which encompass biotic interactions with sexually transmitted fungi, pollinators and herbivores (Bernasconi et al., 2009; Hahn and Brühl, 2016; Taiti et al., 2016). Yet, even though several members of *Silene* stand out from other wild species for their resistance to abiotic stress, the mechanisms of adaptation at the physiological, biochemical and molecular levels are still poorly understood in this genus. More specifically, information on the effects of salinity and drought on the growth and development of *Silene* plants is very limited.

In this study, we have analysed the responses to salinity and water deficit of four *Silene* species adapted in nature to different habitats. Our working hypothesis is that, when comparing related taxa, the more tolerant ones will activate more efficiently those specific stress responses that are relevant for the mechanisms of tolerance. Correlation of the relative tolerance to stress of the investigated species with the level in stressed plants of biomarkers characteristic of specific response pathways, should allow distinguishing those responses that are important for tolerance, from those that are not. We have successfully used this strategy to investigate the mechanisms of tolerance to drought and salinity in other genera, such as *Limonium* (ref.), *Juncus* (refs.), *Plantago* (refs.) or *Phaseolus* (refs.).

According to the ideas above, salt and water stress treatments were applied to the selected *Silene* species, under controlled greenhouse conditions. Growth parameters were determined in control and stressed plants, to estimate their relative degree of tolerance to each of the two stress factors – and the possible correlation with the characteristics of their natural habitats. We also measured the leaf contents of some biochemical markers associated to distinct stress responses: monovalent cations, photosynthetic pigments, osmolytes and antioxidant compounds, to establish the response reactions most important for tolerance to each type of stress, drought and salinity, in *Silene*.

2. Material and Methods

2.1. Plant material

The four investigated *Silene* species were *S. vulgaris* (Moench) Garcke*, S. sclerocarpa Dufour*, *S. latifolia* Poiret, and *S. gallica* L. (Caryophyllaceae).

Silene vulgaris is an extremely variable species, occurring on sandy stands or in soils with high percent of sand throughout Europe; it is frequent in the Mediterranean region, usually growing on coastal sands and rocks. *Silene sclerocarpa* is representative of vegetation of semi-steppe shrublands in Middle Asia and the Mediterranean; the species is appropriate for xeriscaping, as it is relatively resistant to drought. *Silene latifolia* has a wide geographical range: Europe, including the whole Mediterranean region, West Asia, North Africa and North America, where it is an invasive species; it is present in foothills, mountains and subalpine areas, also in degraded zones and as a weed in cultivated land. *Silene gallica* is native to south and central Europe, it is present northwards up to Denmark, Poland and Russia, in western Asia and it can also be found in North Africa; in Australia it is currently considered as an invasive species. *S. gallica* usually grows in dry habitats, such as dry meadows, but also grows in waste lands or in arable land areas.(***)

Seeds of the aforementioned species, sampled in the'La Albufera' Natural Park, (Valencia, Spain), were provided by the 'Servicio Devesa-Albufera' of the city of Valencia, responsible for management of the Park.

2.2. Growth conditions and stress treatments

Seed germination, plant growth and stress treatments were performed under controlled greenhouse conditions: temperatures ranging between 17ºC and 23°C, a long-day photoperiod (16 h light/8 h dark) with light intensity of 130 $\mu E \cdot m^{-2} \cdot s^{-1}$, and humidity between 50–80%.

Seeds were sown in seed trays that contained a mixture of commercial peat and vermiculite (1:1). Seedlings were grown for four weeks before being transplanted into individual polyethylene pots (Ø = 11 cm) with the same substrate and placed in plastic trays (12 pots per tray). During this period, plants were regularly watered with Hoagland's nutrient solution (Hoagland and Arnon, 1950). Drought and salt stress treatments were initiated one week after the plants were transplanted. Control plants were watered twice weekly with 1.5 L Hoagland nutrient solution per tray. Salt stress treatments were performed by adding NaCl to the nutrient solution, to final concentrations of 150 or 300 mM. Water stress treatments were initiated at the same time by completely ceasing irrigation. Treatments were extended for a three-week period.

2.3. Growth parameters

At the end of the treatments, all plants were harvested and the aerial parts were weighed individually on a precision balance. Since plants of the four species differed in size, to better

compare the effects of salt stress and water deficit on growth inhibition, fresh weight measurements were expressed as percentages of the average values determined for the corresponding non-stressed control plants, taken as 100% for each species: 5.0 g (*S. vulgaris*), 6.5 g (*S. sclerocarpa*), 14.8 g (*S. latifolia*) and 6.6 g (*S. gallica*).

Part of the fresh material was weighed (fresh weight, FW) before being dried at 65 °C, until constant weight and was then weighed again (dry weight, DW). The water content percentage (WC %) was calculated as:

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

2.4. Ion content measurements

Leaf concentrations of monovalent cations, Na^+ and K^+, were determined in stressed *Silene* plants of the selected species and in the corresponding non-stressed controls. Extraction of K^+ and Na^+ was performed according to Weimberg (1987), by heating the samples (0.1 g of dried ground plant material in 25 mL of water) in a water bath for 1 hour at 95 ºC, followed by filtration through filter paper (particle retention 8-12 µm). Cations were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, USA).

2.5. Photosynthetic pigments

Photosynthetic pigments in the leaves of harvested plants were quantified by the acetone-extraction method of Lichtenthaler and Welburn (1983). About 100 mg of fresh leaf material was ground in the presence of 20 mL of ice-cold 80% acetone and shaken for 1 hour on a shaker at 4ºC, to extract, chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids (Caro). Samples were centrifuged for 15 min at 3000 x *g*; the supernatant was collected and its absorbance was measured at 663, 646, and 470 nm. The concentrations of Chl a, Chl b, and Caro were calculated according to the following equations (Lichtenthaler and Welburn 1983):

$$\text{Chl a } (\mu g\ ml^{-1}) = 12.21\ (A_{663}) - 2.81\ (A_{646}),$$

$$\text{Chl b } (\mu g\ ml^{-1}) = 20.13\ (A_{646}) - 5.03\ (A_{663}),$$

$$\text{Caro } (\mu g\ ml^{-1}) = (1000A_{470} - 3.27[\text{chl a}] - 104[\text{chl b}])/227.$$

The final values were expressed in µg g^{-1} DW.

2.6. Osmolyte quantification

Proline (Pro) content was quantified using dry leaf material according to the ninhydrin-acetic acid method of Bates et al. (1973). Pro was extracted in 3% aqueous sulphosalicylic acid and the sample was mixed with acid ninhydrin solution, incubated for 1 h at 95 ºC, cooled on

ice and then extracted with toluene. Absorbance of the supernatant was read at 520 nm using toluene as a blank. The Pro concentration was expressed as µmol g^{-1} DW.

Total soluble sugars (TSS) were measured according to Dubois et al. (1956). Dry material was ground and mixed with 3 mL of 80% methanol on a rocker shaker for 24-48 h. Sulphuric acid and 5% phenol were added and mixed before absorbance readings were taken at 490 nm. TSS contents were expressed as 'mg equivalent of glucose' per gram of DW.

2.7. Oxidative stress marker and phenolic compounds

Malondialdehyde (MDA), total flavonoids (TF) and total phenolic compounds (TPC) were determined in 80% (v/v) methanol extracts of 100 mg of dry plant material. MDA, a final product of membrane lipid peroxidation and a reliable marker of oxidative stress (Del Rio et al. 2005), was determined as described by Hodges et al. (1999). Extracts were mixed with 0.5% thiobarbituric acid (TBA), prepared in 20% TCA (or with 20% TCA without TBA for the controls), and were then incubated at 95 ºC for 20 min. After stopping the reaction on ice, the supernatant's absorbance was measured at 532 nm. The none-specific absorbance at 600 and 440 nm was subtracted and the MDA concentration was calculated with the equations described in Hodges et al. (1999).

TF were measured following the method of Zhishen et al. (1999), by reaction of the methanol extracts with $NaNO_2$ followed by $AlCl_3$ at a basic pH. Although this method is often assumed to measure ′total flavonoids′ in the sample, in fact it only detects aromatic rings bearing a catechol group, which is present in several flavonoid subclasses, such as flavonols and flavanols, but also in other phenolics, for example caffeic acid and derivatives, all with the common property of being strong antioxidants (Zhishen et al. 1999). To simplify, we refer to the $AlCl_3$-reactive compounds as 'total flavonoids' (TF) or 'antioxidant flavonoids', and express their contents in 'equivalents of catechin', used as a standard (mg eq C g^{-1} DW).

TPC were quantified as described in Blainski et al. (2013) by reaction with the Folin-Ciocalteu reagent. The extracts were mixed with the reagent and sodium carbonate and left in the dark for 90 min. Absorbance was recorded at 765 nm, and the results were expressed in equivalents of gallic acid, used as a standard (mg eq GA g^{-1} DW).

2.8. Statistical analysis

Data were analyzed using the program Statgraphics Centurion v.16. Before the analysis of variance, the Shapiro-Wilk test was used to check for the validity of normality assumption, and Levene´s test for homogeneity of variance. If the ANOVA requirements were met, the

significance of the differences among treatments was tested by a one-way ANOVA at a 95% confidence level and *post hoc* comparisons were made using the Tukey HSD test. All the means throughout the text are followed by SE.

3. Results

3.1. Salt stress

3.1.1. Growth parameters

In all four investigated *Silene* species, salt stress had a negative effect on vegetative growth, as indicated by the concentration-dependent reduction in the fresh weight of the aerial part of salt-treated plants – in relation to the corresponding controls. According to this criterion, *S. sclerocarpa* and *S. gallica* are the two species more sensitive to salt stress, showing relative FW reductions of more than 70% of the corresponding controls in the presence of 150 mM NaCl; under the same conditions, biomass accumulation was reduced by 50%, approximately, in *S. latifolia* and *S. vulgaris*; the latter appears to be the most salt-tolerant taxon, with FW reduced to about 35% of the control at the highest concentration tested, 300 mM NaCl, as compared to ~ 18 – 25% for the other three *Silene* species (Fig. 1A). Salt stress caused a slight (but statistically significant) dehydration of the aerial part of the four selected species. WC% ranged between 90% and 92% in the control plants, and decreased by 4% (in *S. vulgaris* and *S. gallica*) or by 10% (in *S. sclerocarpa* and *S. latifolia*) in the presence of high external salinity (300 mM NaCl) (Fig. 1B). Therefore, the observed reduction of fresh weight was indeed due mostly to growth inhibition, and not a mere effect of dehydration of the plants in the presence of high salt concentrations.

3.1.2. Cation contents in leaves

Leaf Na^+ contents increased in parallel to increasing external salinity, in the four selected *Silene* species, although with some quantitative differences between taxa (Fig. 2A). Yet, there is a good *negative* correlation between their relative degree of salt tolerance – as established by the growth inhibition data – and the maximum Na^+ levels accumulated in the presence of 300 mM NaCl. In the most tolerant taxon, *S. vulgaris*, Na^+ concentrations reached about 200 μmol g^{-1} DW, followed by ca. 220 μmol g^{-1} DW in *S. latifolia*; on the other hand, in *S. sclerocarpa* and *S. gallica*, which seem to be the most salt-sensitive species, Na^+ accumulated to higher values, from 260 to 275 μmol g^{-1} DW, approximately (Fig. 2A)

In *S. vulgaris*, leaf K^+ levels did not change significantly with increasing salinity, while they decreased in response to the salt treatments in the other three studied species, although no significant differences were observed between 150 and 300 mM NaCl (Fig. 2B). K^+/Na^+ ratios, calculated from the aforementioned data, decreased in the four species with increasing NaCl concentrations, with the largest relative reduction recorded for *S. latifolia* (Fig. 2C).

3.1.3. Photosynthetic pigments

Salt stress caused the degradation of photosynthetic pigments (Chl. a, Chl. b and Caro) in the leaves of all the investigated *Silene* species, except *S. vulgaris* (Fig. 3). For each of these taxa, the reduction in Chl a and Chl b was similar, ranging from 40% of the non-stressed control in *S. latifolia*, to ca. 50% in *S. sclerocarpa*, and about 60% in *S. gallica*, which is the most affected species (Fig 3, A, B). Reduction in total carotenoid levels was somewhat smaller, varying from 30% of the control in *S. latifolia*, to 55% in *S. sclerocarpa* and *S. gallica* (Fig. 3C). In all cases, average values of photosynthetic pigments decreased with increasing salt concentrations but, in general, no statistically significant differences were observed between the treatments with 150 and 300 mM NaCl (Fig. 3). The response of *S. vulgaris* to the salt treatments differed from that of the other three taxa, as the leaf contents of Chl a, Chl b and Caro measured in the controls did no change significantly in the presence of NaCl (Fig. 3).

3.1.4. Osmolytes accumulation

Proline (Pro), one of the most ubiquitous osmolytes in plants, was found to accumulate in the leaves of the salt-treated plants of all four investigated *Silene* species (Fig. 4A). In *S. Sclerocarpa*, *S. latifolia* and *S. gallica*, Pro contents were not significantly different in the controls, and increased between ~3.5-fold (in *S. latifolia*) and ~6.5-fold (in *S. gallica*) at the highest salinity level tested. Here again, *S. vulgaris* behaved somewhat differently, showing a much lower Pro concentration in the control plants, about 30% of that of the other three species; however, the relative increase of Pro contents in the presence of 300 mM NaCl (4.7-fold) was within the range observed for the other taxa (Fig. 4A). It should be pointed out that even the highest Pro concentration measured − 50 μmol g^{-1} DW in the 300 mM NaCl-treated plants of *S. gallica* − was not high enough to have a relevant contribution to osmotic adjustment under the applied salt stress conditions.

Average values of leaf total soluble sugars (TSS) also showed an increment in response to salt stress, in all four *Silene* species, although the differences with the control were not

statistically significant in *S. sclerocarpa* (Fig. 4B). As for Pro, the highest TSS content was recorded in *S. gallica* in the presence of 300 mM NaCl, reaching 67 mg eq. glucose g^{-1} DW..

3.1.5. Oxidative stress biomarker and non-enzymatic antioxidants

Leaf malondialdehyde (MDA) contents were measured in control and salt-treated plants, to estimate the level of oxidative stress. Values varied amongst the analysed species, in the range from 30 nmol g^{-1} DW (in *S. latifolia*) to 70 nmol g^{-1} DW (in *S. vulgaris*) but, for each species, they were not significantly different in stressed and non-stressed plants (Fig. 5A).

Total phenolics compounds (TPC) contents showed a salt-induced increase of about twofold over the corresponding controls in *S. vulgaris*, *S. sclerocarpa* and *S. latifolia* leaves, and of only 1.2-fold in *S gallica*. Absolute values also varied between different taxa, at each salt concentration tested, with the highest TPC contents measured in *S. latifolia* (which is the species showing the lowest leaf MAD levels) and the lowest in *S. vulgaris* (the species with highest leaf MAD contents) (Fig. 5B). Regarding total flavonoids (TF), the general pattern was similar, as *S. latifolia* and *S. gallica* contained higher concentrations than *S. vulgaris* and *S. sclerocarpa*; however, non-significant (in the latter two species) or slight (in the two former ones) increases of leaf TF were observed in the presence of salt (Fig. 5C).

3.2. Water stress

3.2.1. Growth parameters

When water stress was applied over a three-week period, growth inhibition was observed in the stressed plants of all four *Silene* species (Fig. 6A). The relative resistance to water deficit of the different taxa was different from their relative tolerance to salinity: *S. sclerocarpa* is the most drought-tolerant, with less than 40% FW reduction with respect to the corresponding control, whereas *S. latifolia* was apparently the most sensitive to water stress, with almost 70% FW reduction (Fig. 6A). The investigated species appear to be very resistant to drought-induced dehydration – under our experimental conditions – as only *S. latifolia* showed some reduction in WC% (ca. 25% of the control) in water-stressed plants; for the other three taxa, no significant changes were detected (Fig. 6B).

3.2.2. Photosynthetic pigments

Water stress had no significant effect on the levels of photosynthetic pigments, Chl a and b and Caro, in *S. sclerocarpa* or *S. gallica* (Fig. XXX). On the other hand, Chl a and Chl b

levels were reduced to a similar extent in *S. vulgaris* (by 42% - 45% of the controls) and *S. latifolia* (by 51% - 54%) (Fig. XXX). Caro contents also decreased in response to water deficit, but the differences observed between these two species were more pronounced, with reductions of 58% of the control in *S. vulgaris* and of 31% in *S. latifolia* (Fig. XXX)

3.2.3. Osmolytes accumulation

Leaf Pro contents increased significantly in response to water deficit only in *S. latifolia*, with a recorded 4.6-fold increase over the control plant concentrations (Fig. XXX). No significant changes were detected in *S. vulgaris*, *S. gallica* and *S. sclerocarpa* leaves. TSS increased in the leaves of the stressed plants of all selected *Silene* species except in *S. sclerocarpa*, especially in *S. latifolia* (almost 4-fold more than in the control plants), while in *S. vulgaris* and *S. gallica* the relative increase of TSS contents was only 1.5-fold (Fig. XXX).

3.2.4. Oxidative stress biomarker and none-enzymatic antioxidants

Leaf MDA contents did not display any significant change under water stress in the studied *Silene* species, except for *S. latifolia*, which underwent a ~2.5-fold increase in MDA levels as compared to the corresponding non-stressed plants (Fig. XXX). Control values of TPC contents were higher in *S. latifolia* and *S. gallica* than in *S. vulgaris* and *S. sclerocarpa*, but no clear pattern of variation in response to water deficit could be observed in the *Silene* species: increases of 1.5-fold and 2.0-fold over the controls were measured in *S. vulgaris* and *S. sclerocarpa*, respectively; a 25% decrease was observed in *S. gallica*, while no significant change was detected in *S. latifolia* (Fig. XXX). Regarding TF contents, they were also higher in *S. latifolia* and *S. gallica* than in *S. vulgaris* and *S. sclerocarpa* but, in contrast to leaf phenolics, no significant differences were detected in control and water-stressed plants, for any of the analysed taxa(Fig. XXX).

4. Discussion

Under salt and water stress conditions, plants activate a series of conserved responses including the control of ion transport and homeostasis, the accumulation of specific osmolytes to ensure cellular osmotic balance, or the activation of antioxidant systems to counteract oxidative stress, which is a secondary effect of these (and other) abiotic stressful conditions (Zhu, 2001; Flowers and Colmer, 2008; Nardini et al., 2014). In this paper, we present an analysis of these responses in four *Silene* species adapted to different natural habitats and therefore, presumably, with varying levels of tolerance to salinity and drought.

To the best of our knowledge, no reports have been published to date addressing a systematic study on the mechanisms of response to both, salt and water stress in *Silene*, and the knowledge on this specific topic is rather scarce (Soldaat et al., 2000; Arreola et al., 2006; Franco et al., 2008; Favre and Karrenberg, 2011). For example, in studies aimed at the commercial cultivation of *S. vulgaris* as an edible plant, Arreola et al. (2006) evaluated the application of water stress to plants in the nursery (at the seedling stage), whereas Franco et al. (2008) studied the growth rate of adult plants after transplantation to semi-arid conditions. In relation to abiotic stresses, more data are available on the response of several *Silene* species to heavy metals (Pyatt, 1999; Arnetoli et al., 2008; Taiti et al., 2016). High concentrations of metal ions in the soil usually represent a powerful selection factor for plants, and it has been shown that certain populations of *Silene armeria* (Dinelli and Lombini, 1996), *Silene paradoxa* (Arnetoli et al., 2008; Martinelli et al., 2014; Taiti et al., 2016) and *Silene vulgaris* tolerate these conditions by accumulating metal ions in roots, thus excluding them from aboveground organs (Wierzbicka and Panufnik, 1998; van Hoof et al., 2001; Ciarkowska and Hanus-Fajerska, 2008; Koszelnik-Leszek, 2012; Kaskowska and Koszelnik-Leszek, 2014).

Although abiotic stresses have a number of deleterious effects on plants, the most general and one of the first symptoms of drought or salinity s is stunted growth and reduced biomass accumulation. This is due primary to inhibition of cell elongation as a consequence of decreased turgor under conditions of osmotic stress, and can be best assessed by measuring the relative reduction of fresh weight, as compared to the non-stressed controls, in parallel with the increasing level of external stress (Manchanda and Garg, 2008; Al Hassan et al., 2016a, b). In the specific case of *Silene*, Abeli and coworkers (2015) observed that two *S. suecica* populations exposed to drought compensated for the decrease in water availability by reducing growth of shoots. Based on our experiments, and according to this criterion, *S. vulgaris* appears to be the species most tolerant to salinity, followed by *S. latifolia*, whereas *S. gallica* seems to be the most sensitive; the four species are quite resistant to salt-induced leaf dehydration, even at high external salinity, with water losses below 10% in all cases. The relative tolerance of the four taxa to water stress was different, with *S. sclerocarpa* as the most resistant species and *S. latifolia* as the most sensitive; the latter taxon was also the only one showing a significant water loss (about 25% of the control) after three weeks without watering the plants. These results, obtained under artificial greenhouse conditions, fit well with the ecological characteristics of the analysed *Silene* species. For example, the better performance of *S. vulgaris* under salt stress conditions can explain the presence of populations of this species growing at sites near the seashore, where higher contents of readily soluble salts accumulate in the soil (Cooper, 1997;

Rhind, 2015). The relative tolerance of *S. sclerocapa* to water deficit corresponds to its presence in dry habitats and is connected with limited water consumption caused by reduced stomata opening and, above all, by limited growth of aboveground organs, which is counterbalanced by extensive growth of the root system (Bunk et al., 2005). The higher sensitivity of *S. latifolia* to water stress suggests that this taxon is not adapted to soils with low moisture content and, indeed, populations of this short-lived perennial (sometimes annual) species usually grow close to cultivated and irrigated land in wild areas of the Mediterranean region (Favre and Karrenberg, 2011).

Although growth parameters are reliable and commonly used to assess the effects of stress on most plant species, they can be complemented with, or even substituted for, suitable biochemical stress markers, which include a large array of compounds that can be easily identified and quantified in plant material, using simple, sensitive and non-destructive methods (e.g. Schiop et al., 2015). Furthermore, in this and similar comparative studies, correlation of the levels of specific stress markers with the relative tolerance of the selected species can provide information on the mechanisms of tolerance.

An effective control of ion transport contributes to salt stress tolerance, and glycophytes generally cope with high soil salinity by blocking the transport of toxic Na^+ ions from underground (roots) to aboveground (stem, leaves) organs (Flowers, 1986). This seem to be also the case in *Silene*: in the present study, all plants accumulated Na^+ in the leaves in response to increasing NaCl concentrations in the pots, but the higher levels were measured in the most salt-sensitive species, *S. sclerocarpa* and *S. gallica*, while Na^+ content was the lowest in the leaves of *S. vulgaris*, the most tolerant species; this negative correlation indicates that inhibition of Na^+ transport to the leaves is indeed relevant for salt tolerance in *Silene*. Sodium accumulation is generally associated with a drop in K^+ levels, mostly due to the competition of the two cations for the same membrane transport systems (ref.). (Tester and Davenport, 2003). Maintaining relatively high cellular K^+ concentrations under salt stress conditions is another fundamental mechanism of tolerance, as described in some halophytes, e.g., in *Thellungiella halophila*, a salt-tolerant relative of the glycophyte *Arabidopsis thaliana* (Volkov et al., 2003). In our experiments, leaf K^+ levels did not change significantly in *S. vulgaris*, in response to increasing external Na^+ concentrations, which most likely also contributes to the higher tolerance of this species; the decrease of K^+ contents observed in the other three species was more pronounced in the most sensitive *S. gallica*. Interestingly, in the two taxa with intermediate tolerance, *S. sclerocarpia* and *S. latifolia*, an increase in the average K^+ values was observed in the plants treated with 300 mM NaCl, as compared to the 150 mM NaCl treatment,

441 suggesting the activation of K^+ transport to the leaves at high salinity, which would also
442 contribute to tolerance in these species; in previous studies, we have found a similar reaction to
443 high salinity in some tolerant taxa, for example in halophytes of the genera *Juncus* (Al Hassan
444 et al. Funct Plant Biol 2016) and *Plantago* (Al Hassan et al PLoS ONE 2016). Regarding the
445 water stress treatment, no significant change in the leaf levels of Na^+ or K^+ was detected in any
446 of the four investigated *Silene* species, as expected (not shown).
447 Apart from growth inhibition, degradation of photosynthetic pigments appears to be a
448 reliable salt and drought stress marker since reductions in chlorophylls (a and b) and carotenoid
449 contents have been reported to closely correlate, in many different species, with the intensity of
450 the stress applied to the plants (e.g., Sairam et al., 2002; Jaleel et al., 2009; Schiop et al., 2015).
451 In the present study, the salt-induced variation in pigments contents confirmed the relative
452 tolerance of the investigated species: no significant changes with respect to the corresponding
453 controls were observed in stressed plants of *S. vulgaris*, the most tolerant species, while the
454 highest reductions were measured in the more salt-sensitive, *S. sclerocarpa* and, especially, *S.*
455 *gallica.* Under conditions of water deficit, the qualitative patterns of variation of photosynthetic
456 pigments were analogous, in the sense that no significant changes were observed in *S.*
457 *sclerocarpa*, the most drought tolerant of the four selected *Silene* species, whereas the most
458 pronounced reduction was detected in water-stressed *S. latifolia*, the most sensitive taxon.
459 Mechanisms of abiotic stress tolerance in plants involve the synthesis and accumulation
460 of different osmolytes for osmotic adjustment, including sugars (mainly sucrose and fructose),
461 sugar alcohols (e.g., glycerol, mannitol or sorbitol) and amino acids and derivatives (such as
462 proline and glycine betaine), among others (Hasegawa et al., 2000). Proline (Pro) is one of the
463 most ubiquitous compatible solutes in plants, accumulating in many species in response to
464 different stressful conditions causing osmotic stress in the plants (Ashraf and Foolad, 2007;
465 Parida et al., 2008). In addition to its osmotic effects, Pro – and other osmolytes as well – acts
466 as an 'osmoprotectant', directly stabilising proteins and macromolecular structures under stress,
467 and as scavenger of free radicals and other deleterious 'reactive oxygen species' (ROS)
468 (Hasegawa et al., 2000; Hincha and Hageman, 2004; Kavi-Kishor et al., 2005). Numerous
469 studies have shown that, in response to the same stress treatment, tolerant species (or varieties
470 or cultivars of a given species) accumulate higher Pro levels than related taxa that are more
471 sensitive to stress (see reviews by Ashraf and Harris, 2004; Ashraf and Foolad, 2007). Yet, this
472 correlation between Pro accumulation and osmotolerance is not a general phenomenon; in many
473 other cases there is no correlation, or there is even a *negative* correlation, of Pro contents with
474 the relative degree of tolerance (Ashraf and Foolad, 2007; Chen et al., 2007; Lin and Kao, 1996;

475 Liu and Zhu, 1997). The latter seems to be the case in *Silene*; in the salt treatments, the highest
476 absolute levels of Pro (and its largest relative increase over the controls) were measured in *S.*
477 *gallica*, the less salt-resistant of the analysed species, whereas the lowest contents were
478 determined in the most tolerant, *S. vulgaris*. On the other hand, no significant differences in Pro
479 contents were found between control and drought-stressed plants, except for *S. latifolia*, which
480 happens to be the species most sensitive to water deficit. Therefore, it seems that Pro is not
481 directly involved in the mechanisms of tolerance to salt or water stress in *Silene*, but it is a
482 suitable marker of the degree of stress affecting the plants which, under the same conditions,
483 will be obviously higher in the relatively less resistant species. Similar results have been
484 obtained, for example, when comparing the responses to drought and salinity of several
485 *Phaseolus* cultivars (Al Hassan et al. Int. J. Mol. Sci. 2016; Morosan et al. The EuroBiotech
486 Journal 2017)

487 Soluble carbohydrates play also an important role as osmolytes in many plants species,
488 although their possible functions in abiotic stress tolerance mechanisms is more difficult to
489 assess than for other compatible solutes; this is mostly due to the multiple biological roles of
490 sugars, as direct products of photosynthesis, metabolites precursors, major energy sources and
491 signalling molecules (Gil et al., 2011). In the present study, average values of total soluble
492 sugars (TSS) contents increased in response to salinity or drought in the four tested *Silene*
493 species, and the differences with the corresponding controls were statistically significant in all
494 cases except in *S. sclerocarpa*. Yet these data do not allow confirming the possible participation
495 of soluble carbohydrates in stress tolerance of *Silene*, as no clear correlation could be
496 established between the observed changes and the relative tolerance of the studied species.
497 Fractionation of the extracts and identification and quantification of individual sugars will be
498 required to prove this point.

499 As mentioned above, different abiotic stresses, including drought and salinity, generally
500 cause oxidative stress as a secondary effect. One of the symptoms of oxidative damage is cell
501 membrane degradation, and MDA, which is a product of membrane lipid peroxidation, is
502 considered to be an excellent marker of oxidative stress (Del Rio et al., 2005). However, no
503 significant increase in MDA was detected in salt-treated plants, in any of the *Silene* species,
504 indicating that they were not affected by salt-induced oxidative stress. A similar situation was
505 observed in response to water stress treatments, except that MDA levels did increase
506 significantly in *S. latifolia*, the species most sensitive to drought. These results suggest that
507 possible mechanisms of tolerance based on the activation of antioxidant systems are not

relevant in this genus. In agreement with this idea, the changes observed in the levels of total phenolic compounds or antioxidant flavonoids, in response to the stress treatments, were non-significant, very small or did not did not correlate with the relative tolerance of the plants. In many other species, however, there is overwhelming evidence that these secondary metabolites participate in the mechanisms of tolerance to practically all types of abiotic stress (Gould and Lister, 2006; Di Ferdinando et al., 2014; Bautista et al., 2016) due to their strong antioxidant character and ROS scavenging activity.

In conclusion, the studies reported here demonstrate that, amongst the investigated *Silene* species, *S. vulgaris* and *S. gallica* are the most tolerant and the most sensitive, respectively, to salt stress, whereas *S. sclerocarpa* is the most resistant and *S. latifolia* the most sensitive to drought. This behaviour in response to controlled stress treatments fits well with the characteristics of the natural habitats of the different species. Tolerance to both stresses appears to be due, in part, to a strong resistance to salt- and drought-induced leaf dehydration, and is negatively correlated with the degradation of photosynthetic pigments, chlorophylls and carotenoids. The functional osmolytes participating in the mechanisms of tolerance in *Silene* have not been identified, but do not include proline, a compound that can be considered as a reliable stress biomarker in this genus. Tolerance to high salinity, specifically, seems to largely dependent on mechanisms blocking the transport of toxic Na^+ ions to the aerial part of the plants and maintaining relatively high leaf K^+ concentrations in the presence of salt.

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References

Abeli, T., Orsenigo, S., Guzzon, F., Fae, M., Balestrazzi, A., Carlsson-Garner, U., Müller, J. V., Mondoni, A., 2015. Geographical pattern in the response of the arctic-alpine *Silene suecica* (Cariophyllaceae) to the interaction between water availability and photoperiod. Ecol. Res. 30, 327–335.

Al Hassan, M., López-Gresa, M.P., Boscaiu, M., Vicente, O., 2016. Stress tolerance mechanisms in *Juncus*: responses to salinity and drought in three *Juncus* species adapted to different natural environments. Funct. Plant Biol. 43, 949–960.

542 Al Hassan, M., Pacurar, A., López-Gresa, M.P., Donat-Torres, M.P., Llinares, J,V., Boscaiu,
543 M., Vicente, O., 2016. Effects of salt stress on three ecologically distinct *Plantago*
544 species. PLoS ONE 11(8):e0160236, doi:10.1371/journal.pone.0160236.
545 Ariga, H., Katori, T., Yoshihara, R., Hase, S., Nozawa, S., Narumi, I., Iuchi, S., Kobayashi, M.,
546 Tezuka, K., Hayashi, T., Taji, T., 2013. *Arabidopsis sos1* mutant in a salt-tolerance
547 accesion revealed an importance of salt acclimation ability in plant salt tolerance. Plant
548 Sign. Beh. 8 (7) e24779, doi: 10.4161/psb.24779.
549 Arnetoli, M., Vooijs, R., Ten Bookum, W., Galardi, F., Gonelli, C., Gabrielli, R., Schat, H.,
550 Verkleij, J.A.C., 2008. Arsenate tolerance in *Silene paradoxa* does not rely on
551 phytochelatin-dependent sequestration. Environ. Pollut. 152, 585–591.
552 Arreola, J., Franco, J.A., Vicente, M.J., Martinez-Sánchez, J.J., 2006. Effect of nursery
553 irrigation regimes on vegetative growth and root development of *Silene vulgaris* after
554 transplantation into semi-arid conditions. Jour. Hort. Sci. Biotech. 81, 583–592.
555 Ashraf, M., Foolad, M.R., 2007. Roles of glycine betaine and proline in improving plant abiotic
556 stress resistance. Environ. Exp. Bot. 59, 206–216.
557 Ashraf, M., Harris, P.J.C., 2004. Potential biochemical indicators of salinity tolerance in plants.
558 Plant Sci. 166, 3–16. doi: 10.1016/j.plantsci.2003.10.024.
559 Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water
560 stress studies. Plant Soil 39, 205–207.
561 Bautista, I., Boscaiu, M., Lidón, A., Llinares, J.V., Lull, C., Donat, M.P., Mayoral, O., Vicente,
562 O., 2016. Environmentally induced changes in antioxidant phenolic compounds levels
563 in wild plants. Acta Physiol. Plant. 38, 9. doi: 10.1007/s11738-015-2025-2.
564 Bernasconi, G., Antonovics, J., Biere, A., Charlesworth, D., Delph, L.F., Filatov, D., Giraud,
565 T., Hood, M.E., Marais, G.A., McCauley, D., Pannell, J.R., Shykoff, J.A., Vyskot, B.,
566 Wolfe, L.M., Widmer, A., 2009. *Silene* as a model system in ecology and evolution.
567 Heredity 103, 5–14.
568 Blainski, A., Lopes, G.C., Palazzo de Mello, J.C., 2013. Application and analysis of the Folin
569 Ciocalteu method for the determination of the total phenolic content from *Limonium.*
570 *Brasiliense* L. Molecules 18, 6852–6865.
571 Bittrich, V., 1990. Caryophyllaceae, in: Kubitzki, K., Rowher J., Brittrich, V. (Eds.).The
572 families and genera of vascular plants: Magnoliid, Hamamelid and Caryophyllid
573 families. Vol. 2. Springer Verlag, Berlin, Germany, pp. 653.
574 Bunk, C., Sánchez Gomez, P., Hensen, I., 2005. Plant regeneration mechanisms during early
575 post-fire succession in south eastern Spain. Feddes Rep. 116, 392–404.

576 Chen, Z., Cuin, T.A., Zhou, M., Twomei, A., Naidu, B.P., Shabala, S., 2007. Compatible solute
577 accumulation and stress-mitigating effects in barley genotypes contrasting in their salt
578 tolerance. J. Exp. Bot. 58, 4245–4255. doi: 10.1093/jxb/erm284.
579 Ciarkowska, K., Hanus-Fajerska, E., 2008. Remediation of soil-free grounds contaminated by
580 zinc, lead and cadmium with the use of metallophytes. Polish J. Environm. Stud. 17,
581 707–712.
582 Colzi, I., Pignatelli, S., Giorni, E., Papini, A., Gonelli, C., 2015. Linking root traits to copper
583 exclusion mechanisms in *Silene paradoxa* L. (Caryophyllaceae). Plant Soil 390, 1–15.
584 Cooper A.,1997. Plant species coexistence in cliff habitats. J. Biogeogr. 24, 483–494.
585 Del Rio, D., Stewart, A.J., Pellegrini, N., 2005. A review of recent studies on malondialdehyde
586 as toxic molecule and biological marker of oxidative stress. Nutr. Metab. Cardiovasc.
587 Dis. 15, 316–328.
588 Di Ferdinando, M., Brunetti, C., Agati, G., Tattini, M., 2014. Multiple functions of polyphenols
589 in plants inhabiting unfavourable Mediterranean areas. Environ. Exper. Bot. 103, 107–
590 116. doi: 10.1016/j.envexpbot.2013.09.012.
591 Dinelli, E., Lombini, A., 1996. Metal distribution in plants growing on copper mine spoils in
592 nothern Apenines, Italy: the evaluation of seasonal variation. Appl. Geochem. 11, 41–
593 58.
594 Dubois, M., Gilles, K.A., Hamilton, J.K., Reberd, P.A., Smith, F., 1956. Colorimetric method
595 for determination of sugars and related substances. Anal. Chem. 28, 350–356.
596 Favre, A., Karrenberg, S., 2011. Stress tolerance in closely related species and their first-
597 generation hybrids: a case study of *Silene*. J. Ecol. 99, 1415–1423.
598 Fawzi, N.M., Fawzi, A.M., Al-Halim, A.M., 2010. Seed Morphological studies on some species
599 of *Silene* L. (Caryophyllaceae). Internat. J. Bot. 6 (3), 287–292.
600 Fields, P.D., Taylor, D.R., 2014. Determinants of genetic structure in a non-equilibrium
601 metapopulation of the plant *Silene latifolia*. PLoS ONE 9(9), e104575.
602 https://doi.org/10.1371/journal.pone.0104575.
603 Fita A, Rodríguez-Burruezo A, Boscaiu M, Prohens J, Vicente O. Breeding and domesticating
604 crops adapted to drought and salinity: a new paradigm for increasing food production.
605 Front Plant Sci. 2015; 6: 978. https://doi.org/10.3389/fpls.2015.00978
606 Flowers, T.J., Galal, H.K., Bromham, L., 2010. Evolution of halophytes: multiple origins of
607 salt tolerance in land plants. Funct. Plant Biol. 37, 604–612.
608 Flowers, T.J., Colmer, T.D., 2008. Salinity tolerance in halophytes, New Phytol. 179, 945–963.

609 Flowers, T.J., Hajibagheri, M.A., Clipson, N.J.W., 1986. Halophytes. Quart. Rev. Biol. 61,
610 313–335.
611 Franco, J.A., Arreola, J., Vicente, M.J., Martinez-Sánchez, J.J., 2008. Nursery irrigation
612 regimes affect the seedling characteristics of *Silene vulgaris* as they relate to potential
613 performance following transplanting into semi-arid conditions. Jour. Hort. Sci. Biotech.
614 83, 15–22.
615 Gil, R., Lull, C., Boscaiu, M., Bautista, I., Lidón, A., Vicente, O., 2011. Soluble carbohydrates
616 as osmolytes in several halophytes from a Mediterranean salt marsh. Not. Bot. Horti
617 Agrobo. 39, 9–17.
618 Gould, K.S., Lister, C., 2006. Flavonoid functions in plants. In Flavonoids. Chemistry,
619 Biochemistry and Applications (eds Ø.M. Andersen & K.R. Markham), pp. 397-441.
620 CRC Press, Boca Raton, FL, USA.
621 Hahn, M., Brühl, C.A., 2016. The secret pollinators: an overview of moth pollination with a
622 focus on Europe and North America. Arthropod. Plant Interact. 10, 21–28.
623 Hasegawa, P.M., Bressan, A., Zhy, J.K., Bohnert, H.J., 2000. Plant cellular and molecular
624 responses to high salinity. Ann. Rev. Plant Physiol. Plant Mol. Biol. 51, 463–499.
625 Hincha, D.K., Hagemann, M. 2004. Stabilization of model membranes during drying by
626 compatible solutes involved in the stress tolerance of plants and microorganisms.
627 Biochem. J. 383, 277–283.
628 Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil.
629 California Agricultural Experimental Station Circular No. 347, pp. 1-32. University of
630 California, Berkeley.
631 Hodges, D.M., DeLong, J.M., Forney, C.F., Prange R.K., 1999. Improving the thiobarbituric
632 acid-reactive-substances assay for estimating lipid peroxidation in plant tissues
633 containing anthocyanin and other interfering compounds. Planta 207, 604–611.
634 Houliston, G.J., Olson, M.S., 2006. Nonneutral evolution of organelle genes in *Silene vulgaris*.
635 Genetics 174, 1983–1994.
636 Jaleel, C.A., Manivannan, P., Wahid, A., Farooq, M., Al-Juburi, H. J., Somasundaram,R., 2009.
637 Drought stress in plants: A review on morphological characteristics and pigments
638 composition. Int. J. Agric. Biol. 11, 100–105.
639 Käfer, J., Talianová , M., Bigot, T., Michu, E., Guéguen, L., Widmer, A., Žlůvová, J., Glémin
640 S., Marias, G.A.B., 2013. Patterns of molecular evolution in dioecious and non-
641 dioecious *Silene*. J. Evol. Biol. 26, 335–346.

642 Kaskowska, D., Koszelnik-Leszek, A., 2014. Ecological features of spontaneous vascular flora
643 of serpentine post-mining sites in lower Silesia. Arch. Env. Prot. 40, 33–52.
644 Kavi-Kishor, P.B., Sangam, S., Amrutha, R.N., Sri-Laxmi, P., Naidu, K.R., Rao, K.R.S.S., Rao,
645 S., Reddy, K.J., Theriappan, P., Sreenivasulu, N., 2005. Regulation of proline
646 biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant
647 growth and abiotic stress tolerance. Curl. Sci. 88, 424–438.
648 Kilic, S., 2009. Anatomical and pollen characteristics in the genus *Silene* L. (Caryophyllaceae)
649 from Turkey. Bot. Res. J. 2, 34–44.
650 Koszelnik-Leszek A., 2012. Accumulation of heavy metals by *Silene vulgaris* occuring on
651 serpentine waste dump in Grochow (Lower Silesia). Ecol. Chem. Eng. 19, 411–420.
652 Lavor, D.W., 2013. Genetic engineering to improve plant performance under drought:
653 physiological evaluation of achievements, limitations, and possibilities. J. Exp. Bot. 64,
654 83–108.
655 Lichtenthaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and
656 chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591–
657 592. doi: 10.1042/bst0110591.
658 Lin, C.C., Kao C.H., 1996. Proline accumulation is associated with inhibition of rice seedling
659 root growth caused by NaCl. Plant Sci. 114, 121–128.
660 Liu, JP., Zhu, J.K., 1997. Proline accumulation and salt-stress-induced gene expression in a
661 salt-hypersensitive mutant of Arabidopsis. Plant Physiol 114, 591–596.
662 Manchanda, G., Garg, N., 2008. Salinity and its effects on the functional biology of legumes.
663 Acta Physiol. Plant. 30, 595–618.
664 Martinelli, F., Giorni, E., Colzi, I., Luti, S., Meerts P., Pazzagli L., Gonelli C., 2014. Can
665 adaptation to metalliferous environments affect plant response to biotic stress? Insight
666 from *Silene paradoxa* L. and phytoalexins. Environ. Exp. Bot., 108, 36–45.
667 Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Ann. Rev. Plant Biol. 59, 651–
668 681.
669 Nardini, A., Lo, Gullo, M.A., Trifilò, P., Salleo S., 2014. The challenge of the Mediterranean
670 climate to plant hydraulics: Responses and adaptations. Environ. Exp. Bot. 103, 68–79.
671 Parida, A.K., Dagaonkar, V.S., Phalak, M.S., Aurangabadkar, L.P., 2008. Differential
672 responses of the enzymes involved in proline biosynthesis and degradation in drought
673 tolerant and sensitive cotton genotypes during drought stress followed by recovery.
674 Plant Biotechnol. Rep. 1, 37–48.

675 Peñas, J., Cabello, J., Tendero, F.V., Mota, J.F., 2001. Comunidades vegetales rupícolas y
676 subrupícolas del sudeste ibérico (Sierra de Los Filabres). Lazaroa 22, 95–107.
677 Prentice, H.C., Andersson, S., Månsby, E., 2011. Mosaic variation in allozyme and plastid DNA
678 markers in the Europaean ranges of *Silene vulgaris* and its partially sympatric realative
679 *S. uniflora* (Caryophyllaceae). Bot. J. Linn. Soc. 166, 127–148.
680 Pyatt, F.B., 1999. Comparison of foliar and stem bioaccumulation of heavy metals by Corsican
681 pines in The Mount Olympus area of Cyprus. Ecotoxicol. Environ. Safety 42, 57–61.
682 Queiro´s F., Fidalgo, F., Santos, I., Salema, R., 2007. *In vitro* selection of salt tolerant cell lines
683 in *Solanum tuberosum* L. Biol. Plant. 51(4), 728–734.
684 Rautenberg, A., Sloan, D.B., Aldén, V., Oxelman, B., 2012. Phylogenetic relationships of
685 *Silene multinervia* and *Silene* section Conoimorpha (Caryophyllaceae). Syst. Bot. 37(1),
686 226–237.
687 Rejeb, K.B., Lefebre-De Vos, D., Le DIsquet, I., Leprince, A.S., Bordeneave, M., Maldiney,
688 R., Jdey, A., Abdelly, C., Savouré, A., 2015. Hydrogen peroxide produced by NADPH
689 oxidases increases proline accumulation during salt or mannitol stress in *Arabidopsis*
690 *thaliana*. New Phytol. doi: 10.1111/nph.13550.
691 Rengasamy, P., 2010. Soil processes affecting crop production in salt-affected soils. Funct.
692 Plant Biol. 37, 613–620.
693 Rhind, P.M., 2015. Conservation and management of coastal slope woodlands with
694 particularreference to Wales. J. Coast Conserv. 19, 861–873.
695 Rodríguez-Navarro, A. 2000. Potassium transport in fungi and plants. Biochim. Biophys. Acta
696 1469, 1–30.
697 Sairam, R.K., Rao, K.V., Srivastava, G.C., 2002. Differential response of wheat genotypes to
698 long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte
699 concentration. Plant Sci.163,1037-1046. doi: 10.1016/S0168-9452(02)00278-9.
700 Sanders, D., 2000. The salty tale of *Arabidopsis.* Curr. Biol. 10, 486–488.
701 Schiop, S.T., Al Hassan, M., Sestras, A.F., Boscaiu, M., Sestras, R.E., Vicente, O., 2015.
702 Identification of Salt Stress Biomarkers in Romanian Carpathian Populations of Picea
703 abies (L.) Karst. PLoS ONE 10(8): e0135419. doi: 10.1371/journal.pone.0135419.
704 Sloan, D.B., Alverson, A.J., Chuckalovcak, J.P., Wu, M., McCauley, D.E., Palmer, J.D.,
705 Taylor, D.R., 2012a. Rapid evolution of enormous, multichromosomal genomes in
706 flowering plant mitochondria with exceptionally high mutation ates. PLoS Biol. 10(1):
707 e1001241. doi: 10.1371/journal.pbio.1001241.

708 Sloan, D.B., Keller, S.R., Beradi, A.E., Sanderson, B.J., Karpovich, J.F., Taylor, D.R., 2012b.
709 *De novo* transcriptome assembly and polymorphism detection in the flowering plant
710 *Silene vulgaris* (Caryophyllaceae). Mol. Ecol. 12, 333–343.
711 Soldaat, L.L, Lorenz, H., Trefflich, A., 2000. The effect of drought stress on the sex ratio
712 variation of *Silene otites*. Folia Geobot. 35 (2): 203-2010.
713 Taiti, C., Giorni, E., Colzi, I., Pignatelli, S., Bazihizina, N., Buccanti, A., Luti, S., Pazzalli, L.,
714 Mancuso, S., Goneli, C., 2016. Under fungal attack on a metalifferous soil: ROS or not
715 ROS? Insights from *Silene paradoxa* L. growing under copper stress. Env. Pollut. 2010,
716 282–292.
717 Tester, M., Davenport, R., 2003. Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot.
718 91, 503–527.
719 Van Hoof, N.A.L.M., Hassinen, V.H., Hakvoort, H.W.J., Ballintijn H.K.F., Schat H., Verkleij
720 J.A.C., Ernst W.H.O., Karenlampi S.O., Trevahauta A.I., 2001. Enhanced cooper
721 tolerance in *Silene vulgaris* (Moench) Garcke populations from cooper mines is
722 associated with increased transcript levels of a 2b-type metallothionein gene. Plant
723 Physiol. 126, 1519–1526.
724 Volkov, V.,Wang, B.,Dominy, P.J., Fricke, W., Amtmann, A., 2003. *Thellungiella halophila*,
725 a salt-tolerant relative of *Arabidopsis thaliana*, possesses effective mechanisms to
726 discriminate between potassium and sodium. Plant Cell Environ. 27, 1-14.
727 doi:10.1046/j.0016-8025.2003.01116.x
728 Weimberg, R., 1987. Solute adjustments in leaves of two species of wheat at two different
729 stages of growth in response to salinity. Physiol. Plant. 70, 381–388.
730 Wierzbicka, M., Panufnik, D., 1998. The adaptation of *Silene vulgaris* to growth on a calamine
731 waste heap (S. Poland). Environ. Pollut. 101, 415–426.
732 Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in
733 mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559.
734 Zhu, J.K., 2001. Plant salt tolerance. Trends Plant Sci. 6, 66–71.
735