Manipulating the metabolic composition of halophytes to increase their cultural and economic value through saline cultivation

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades
Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation
von
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2017

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Tag der Promotion: 19.04.2016

Erklärung kumulative Dissertation

aus:

Gemeinsame Ordnung für die Promotion zur Doktorin der Naturwissenschaften oder zum Doktor der Naturwissenschaften (Dr. rer. nat.) an der Gottfried Wilhelm Leibniz Universität Hannover (25.03.2013),

§ 8 Dissertation

A: (3)

„[…] [2]Es ist eine ausführliche Darstellung voranzustellen, die eine kritische Einordnung der Forschungsthemen und wichtigsten Erkenntnisse aus den Publikationen in den Kontext der wissenschaftlichen Literatur zum Thema vornimmt […]"

Die voranzustellende ausführliche Darstellung ist in dieser Arbeit aufgeteilt in die Kapitel 1 und 6.

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Publikation (Kapitel 2)

Boestfleisch, C., Wagenseil, N.B., Buhmann, A.K., Seal, C.E., Wade, E.M., Muscolo, A., Papenbrock, J. (2014) Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation. *AoB PLANTS* **6**, plu046.
DOI: 10.1093/aobpla/plu046.

Die Idee für Experimente mit den älteren Pflanzen wurde von J. Papenbrock, C. Boestfleisch und A. Buhmann entwickelt. Die Idee für die Experimente mit Samen und Keimlingen stammt von C. Seal and A. Muscolo. Die Experimente mit *Lepidium latifolium* sowie die Analyse von Flavonoiden, Phenolen, Ascorbat und ORAC im selbigen Pflanzenmaterial als auch im Pflanzenmaterial von allen anderen älteren Pflanzen wurden von Christian Boestfleisch durchgeführt, ebenfalls auch das Auswerten der Daten sowie das Erstellen von Graphen und Tabellen, teilweise auch für Daten der Experimente von C. Seal and A. Muscolo. Große Teile des Manuskripts, besonders der Material und Methodenteil sowie Teile der Einleitung und Diskussion, wurden von Christian Boestfleisch verfasst.

Publikation (Kapitel 3)

Boestfleisch, C., Hornbacher, J., Rumlow, A., Papenbrock, J. (2017): Salinity Influences Single Glucosinolate Content in the Halophyte *Lepidium latifolium*, In: Sulfur Metabolism in Higher Plants - Fundamental, Environmental and Agricultural Aspects, Proceedings of the International Plant Sulfur Workshop, Vol. 3, 2017, De Kok, L., Hawkesford, M. & Schnug, E. (eds.), Springer, Dordrecht, pp. 103-114
DOI: 10.1007/978-3-319-56526-2_10

Die Idee für die Experimente wurde von J. Papenbrock und C. Boestfleisch entwickelt. Die Pflanzenanzucht und die Analyse von Flavonoiden, Phenolen, Ascorbat und ORAC wurden von C. Boestfleisch durchgeführt. Die Glucosinolate wurden von J. Hornbacher mit der Methode von A. Rumlow bestimmt. Das Auswerten der Daten sowie das Erstellen von Graphen und Tabellen wurde von C. Boestfleisch durchgeführt. Das Manuskript wurde in großen Anteilen, bis auf die Glucosinolatmethode im Material- und Methodenteil, von C. Boestfleisch geschrieben und von J. Papenbrock teilweise überarbeitet.

Publikation (Kapitel 4)

Boestfleisch, C., Papenbrock, J. (2017) Changes in secondary metabolites in the halophytic putative crop species *Crithmum maritimum* L., *Triglochin maritima* L. and *Halimione portulacoides* (L.) Aellen as reaction to mild salinity. PLoS ONE 12(4): e0176303.

Die Idee für Experimente stammt von J. Papenbrock und C. Boestfleisch. Die Experimente mit den Pflanzen sowie die Analyse von Flavonoiden, Phenolen, Ascorbat. ORAC und Prolin wurden von C. Boestfleisch durchgeführt. Die Elementanalyse wurde von A. Rumlow and J. Theilmann durchgeführt. Die Auswertung aller Daten sowie das Erstellen von Graphen und Tabellen wurde von C. Boestfleisch mit etwas Hilfe von F. Schaarschmidt unternommen. Das gesamte Manuskript wurde von C. Boestfleisch geschrieben, mit hilfreichen Hinweisen und Verbesserungen von J. Papenbrock.

Manuskript, in Vorbereitung (Kapitel 5)

Boestfleisch, C., Glasenapp, Y., Papenbrock, J. Changes in the metabolic profile of *Crithmum maritimum* under salinity stress. (in preparation)

Die Idee für die Experimente stammt von J. Papenbrock und C. Boestfleisch. Die Experimente mit den Pflanzen wurden von C. Boestfleisch durchgeführt. Die Extrakte wurden von Y. Glasenapp mit der LC-MS analysiert. Die Auswertung aller Daten sowie das Erstellen von Graphen und Tabellen wurde von C. Boestfleisch unternommen. Der Methodenteil der LC-MS wurde von Y. Glasenapp, das gesamte weitere Manuskript von C. Boestfleisch geschrieben, mit hilfreichen Hinweisen und Verbesserungen von J. Papenbrock.

Die nachfolgend aufgelisteten Publikationen sind im Rahmen der Masterarbeit entwickelt worden und während der Promotionszeit fertiggestellt worden. Sie sind jedoch nicht Gegenstand dieser Dissertationsarbeit:

- Nehring, S.; Boestfleisch, C.; Buhmann, A.; Papenbrock, J. The North American toxic fungal pathogen G3 *Claviceps purpurea* (Fries) Tulasne is established in the German Wadden Sea. *BioInvasions Rec.* **2012**, *1* (1), 5–10.

- Boestfleisch, C.; Drotleff, A. M.; Ternes, W.; Nehring, S.; Pažoutová, S.; Papenbrock, J. The invasive ergot *Claviceps purpurea* var. *spartinae* recently established in the European Wadden Sea on common cord grass is genetically homogeneous and the sclerotia contain high amounts of ergot alkaloids. *Eur. J. Plant Pathol.* **2015**, *141* (3), 445–461.

Abstract

The world population continues rising, while the agricultural land is shrinking. A significant part of the agricultural land is lost due to soil salinity. Glycophytes, which are the majority of crop plants, hardly grow on saline soil. However, these soils can be used for agriculture with salt tolerant plants, called halophytes. They have evolved mechanisms to deal with the salt stress. These include, among others, the accumulation of secondary metabolites such as antioxidants, which act as radical scavengers, and osmolytes, which prevent loss of water. Some secondary metabolites can have a positive effect on human health. Therefore, the aim of this work was the attempt to increase the concentration of secondary metabolites through salt stress in halophytes (biofortification). However, salt stress inhibits the growth, which leads to a reduction in produced biomass. Albeit, the optimum between little biomass loss and increased antioxidant concentration should be found. Halophytes showed hardly increased secondary metabolites after several weeks of salt stress, compared to prior stress conditions and compared to non-stressed plants. It is likely that halophytes have already adapted at this time to salt stress. Therefore, partly high salt stress was induced immediately in *Lepidium latifolium* (broadleaved pepperweed) and secondary metabolites were measured in hourly to weekly intervals. The secondary metabolites rose to a maximum in the period between 24 hours and one week, and then decreased slowly afterwards. After one week, the loss in biomass overcompensated the overall yield in secondary metabolites. The exception was glucoiberin, a glucosinolate. In another experiment, salt stress was directly induced at *Crithmum maritimum* (sea fennel), *Triglochin maritima* (sea arrow grass) and *Halimone portulacoides* (sea purslane), but this time only mild salt stress was used, to prevent the effect of biomass loss. The salt stress did not increase the concentration in secondary metabolites enough to compensate for the loss due to the reduced growth. Exception to this was proline, acting as an osmolyte, in *C. maritimum*. Since two individual components have been found to increase their yield, despite high losses in biomass, it was searched for further substances by mass spectrometry. Two additional substances were discovered which increase massively under salinity stress, *p*-coumaroylquinic acid isomers and another substance that could not be identified, yet.

Keywords: *Halophytes, salt stress, metabolites, antioxidants, biofortification, nutraceutical*

Kurzzusammenfassung

Die Weltbevölkerung steigt immer weiter, während gleichzeitig die landwirtschaftliche Nutzfläche immer kleiner wird. Ein erheblicher Teil davon geht durch Bodenversalzung verloren. Glycophyten, die den Großteil der Ackerpflanzen stellen, können auf versalzenem Boden kaum wachsen. Dort kann aber Landwirtschaft mit Salztoleranten pflanzen, Halophyten genannt, betrieben werden. Sie haben Mechanismen entwickelt um mit dem Salzstress umzugehen. Dazu zählt unter anderem die Anreicherung von Sekundärmetaboliten wie Antioxidantien, welche als Sauerstoffradikalfänger agieren, sowie Osmolyten, die einen Wasserverlust verhindern. Einige Sekundärmetabolite können sich auch positiv auf die Gesundheit des Menschen auswirken. Daher war das Ziel dieser Arbeit der Versuch, die Konzentration der Sekundärmetabolite durch Salzstress in Halophyten zu erhöhen (Biofortifikation). Salzstress hemmt jedoch das Wachstum, wodurch weniger Biomasse gebildet wird. Daher sollte auch das Optimum zwischen möglichst wenig Biomasseverlust und erhöhter Antioxidantien Konzentration gefunden werden. Es zeigte sich, dass einige Halophyten nach mehreren Wochen Salzstress kaum erhöhte Sekundärmetabolitwerte gegenüber dem Ausgangswert und gegenüber nicht gestressten Pflanzen aufwiesen. Es ist anzunehmen, dass sich die Halophyten in dieser Zeit schon an den Salzstress gewöhnt haben. Daher wurde *Lepidium latifolium* (Pfefferkraut) direkt unter teilweise hohen Salzstress gesetzt und stündlich bis hin zu wöchentlich, die Sekundärmetabolite gemessen. Diese stiegen auf ein Maximum im Zeitraum zwischen 24 h und einer Woche und gingen dann langsam wieder zurück. Nach einer Woche waren die Einbußen in der Biomasse so groß, dass die Gesamtausbeute in Sekundärmetaboliten geringer war. Die Ausnahme hier war Glucoiberin, ein Senfölglykosid. In einem weiteren Experiment wurden *Crithmum maritimum* (Meerfenchel), *Triglochin maritima* (Stranddreizack) und *Halimone portulacoides* (Portulak-Keilmelde) ebenfalls direktem, jedoch geringerem Salzstress ausgesetzt, um den Effekt der Biomasseverluste durch hohen Salzstress zu verhindern. Der Salzstress erhöhte die Konzentration in Sekundärmetaboliten aber nicht stark genug, um den Verlust durch das geringere Wachstum auszugleichen. Ausnahme hierbei war das als Osmolyt wirkende Prolin bei *C. maritimum*. Da schon zwei Einzelkomponenten gefunden wurden, deren Ausbeute sich trotz hohen Biomasseverlustes steigern ließ, wurde mittels Massenspektrometrie nach weiteren Substanzen gesucht. Es wurden zwei weitere Substanzen gefunden, deren Konzentration unter Salzstress anstieg: *p*-Cumarsäure Chinasäure Ester und deren Isomere, sowie eine weitere, die sich aber noch nicht identifizieren ließ.

Schlüsselwörter: *Halophyten, Salzstress, Metabolite, Antioxidantien, Biofortifikation, Nutraceutical*

Content

Chapter 1

General introduction

The emerging problem of food insecurity

The world's population reached 7.3 billion in the year 2015 and continues growing, albeit slower than in the recent past. A further increase of the world population to 9.7 billion by 2050 and 11.2 billion by 2100 is projected (United Nations 2015). An additional production of another billion tonnes of cereals, plus 200 million tonnes of livestock products every year, is necessary to feed a population of just 9 billion (FAO 2011b). Nearly 800 million people are still undernourished, most of them in Southern Asia and Sub-Saharan Africa. Furthermore, in these areas, a lack of or inadequate intake of micronutrients, like vitamin A, results in different types of malnutrition (FAO 2015). Unfortunately, the area of agricultural land decreases slowly and climate change enhances the risks of natural hazards, as drought and flooding, and their intensity and frequency (FAO 2011a; FAO *et al.* 2015). Consequently, crops suffer from drought and salt stress, which already cause mayor problems (Boyer 1982; Athar and Ashraf 2009). To prevent an upcoming global food crisis, new ways of crop production have to be established. This study shows solutions to deal with the problems caused by salt, but first explains the reasons and influence of salt on crops.

Salt causes worldwide problems in agriculture

Before describing further effects of salt, a definition has to be made. Sodium chloride (NaCl) is the salt everybody knows from the kitchen to enhance the flavour of his food. But there are other elements which can form ionic bonds, producing salts, which have to be taken into consideration. Typical seawater contains about 35 g salts per litre, but NaCl makes just 85.7% of the ions present in seawater, the rest consisting of Mg^{2+}, SO_4^{2-}, Ca^{2+}, K^+, Sr^{2+} Br^-, F^-, and B (Millero *et al.* 2008). Assuming NaCl would make up 100% of the salts in seawater, 35 $g*l^{-1}$ would be equal to 598.9 mM NaCl, but there are actually only about 513 mM NaCl present in seawater. That makes experiments of either seawater use or NaCl use difficult to compare and there are experiments using seawater and experiments using NaCl in this study. But another possibility for the measurement of the salinity is the electrical conductivity, measuring the ionic content of the solution. But the SI unit S (siemens) is difficult to handle and compare in

real life. Therefore, in this study Practical Salinity Units (PSU) which is calculated from the electrical conductivity, is used. A solution containing 35 PSU is equal 35 g $*l^{-1}$ or to 3.5%. There is a difference conducting experiments with plants if (artificial) seawater is used or just NaCl, from the ionic point of view, but not from the osmotic point of view. Salinity of seawater is divided into three steps: low salinity (1-7 PSU), a transition zone (8-25 PSU) and high salinity (30 PSU and above) (Koyro and Lieth 1998).

Soil salinity is a severe problem for agricultural crops, but before starting with the reasons one has to know the dimensions. Figure 1 shows a graphical overview. More than 8 million km^2, 6% of the world´s land mass (without Antarctica) is covered with different types of salt-affected soils, which is more than the size of Australia (Pessarakli and Szabolcs 2010). Additionally, 11% of the irrigated agricultural area is affected by some level of salinity, according to the FAO (2011b). That is 0.34 million km^2 and occurs in very highly developed countries as well as low developed countries. The United States, China, India and Pakistan make up 0.21 million km^2 (60%) of that area. Another 0.6-0.8 million km^2 are affected by waterlogging and consequently by salinization (FAO 2011b). Irrigated agricultural land is about one fifth of arable land, but accounts 42 % of all cereal production worldwide (FAO 2011b).
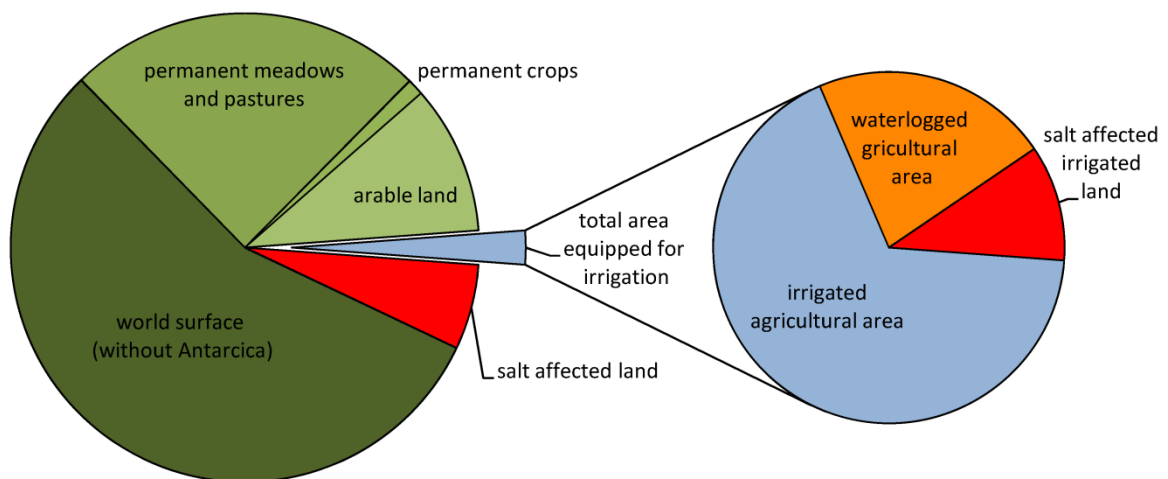
Figure 1. Salt affected land worldwide. From the world land area, 135.7 M km² (without Antarctica), 8 M km² of that area are salt affected (Pessarakli and Szabolcs 2010). 49.2 M km² of the world land is used for agriculture (The World Bank 2013), which is divided in permanent meadows and pastures, permanent crops and arable land of which 3.2 M km² are irrigated (FAO 2011a). From the irrigated area 0.34 M km² are salt affected and 0.6-0.8 M km² are affected by waterlogging (FAO 2011b).

Soil salinization happens almost exclusively in arid or semi-arid areas, where evapotranspiration is greater than precipitation (Breckle 2009). There are two types of salinization processes of soils. The primary, or natural salinization occurs over a long-time period. Weathering of saline minerals or parent rocks releases soluble soils. Furthermore, salt from the oceans is transported via air and accumulates on land. Secondary salinization of soils is a man-made problem caused by irrigating with salty water, deforestation, or overgrazing and insufficient drainage (Richards 1954; Athar and Ashraf 2009; Munns 2009). The latter one cause groundwater levels to rise, carrying salts from deeper levels to the root zone. Saline soils are defined as soils which electrical conductivity is greater than 4 ds/m which is 2.2 g NaCl $*l^{-1}$ or 37.7 mM NaCl (Richards 1954). There are other definitions of saline soils and classifications as sodic soils, for an overview see (Shahid and Rahman 2010). The problem is that soil salinity of 4 ds/m decreases the yield of most agricultural crops, for example rice (*Oryza sativa*), beans (*Phaseolus vulgaris*), carrots (*Daucus carota*) and onions (*Allium cepa*) (Hillel 2000).

The crops mentioned above are glycophytes, which happen to be the majority of crop plants worldwide, but their growth is inhibited by salinity. Cheeseman (2015) defined glycophytes as plant species that "evolved by adaptation under natural selective pressures in ecosystems with low soil sodium levels and which maintains low sodium levels in its aboveground tissues, especially in its leaves." This adaption may have been useful to prevent herbivore thread, but glycophytes do not thrive in saline soil. There was some effort and attempts to cultivate glycophytes under salinity with conventional breeding, which, despite some success, was disappointing (Epstein *et al.* 1980; Flowers, Galal and Bromham 2010). Furthermore, the transfer of single genes to improve crop yields in saline soils was very modest (Panta *et al.* 2014).

Halophytes as alternative crop plants on saline soils

Instead of using glycophytes as agricultural crop plants, other species can be used which thrive in saline soil: Halophytes. They have adapted to survive in saline soil. "They are plant species which complete their life cycle in 200 mM NaCl or more" (Flowers, Hajibagheri and

Clipson 1986). Even though some glycophytes are more salt-tolerant than others, they are not as tolerant as halophytes.

Figure 2 shows a simplified graph of the growth of halophytes and glycophytes under the influence of salt. Salt sensitive crops (glycophytes) can tolerate just a tiny amount of salt and their yield decreases fast, if their threshold is exceeded. At low salt concentrations, salt-tolerant crops thrive up to a threshold, which is around the start of the transition zone, and beyond the growth is steadily reduced. Barley (*Hordeum vulgare*), cotton (*Gossypium hirsutum*), rye (*Secale cereale*), and asparagus (*Asparagus officinalis*) are plants classified as salt-tolerant and are capable of producing a decent yield in saline soils (Hillel 2000). However, increasing salinity would reduce the yield steadily, because they are still glycophytes. The growth of halophytes is increased at low salinity, but high salinity decreases the yield slowly. They have an optimum growth at a transition zone between low and high salinity.
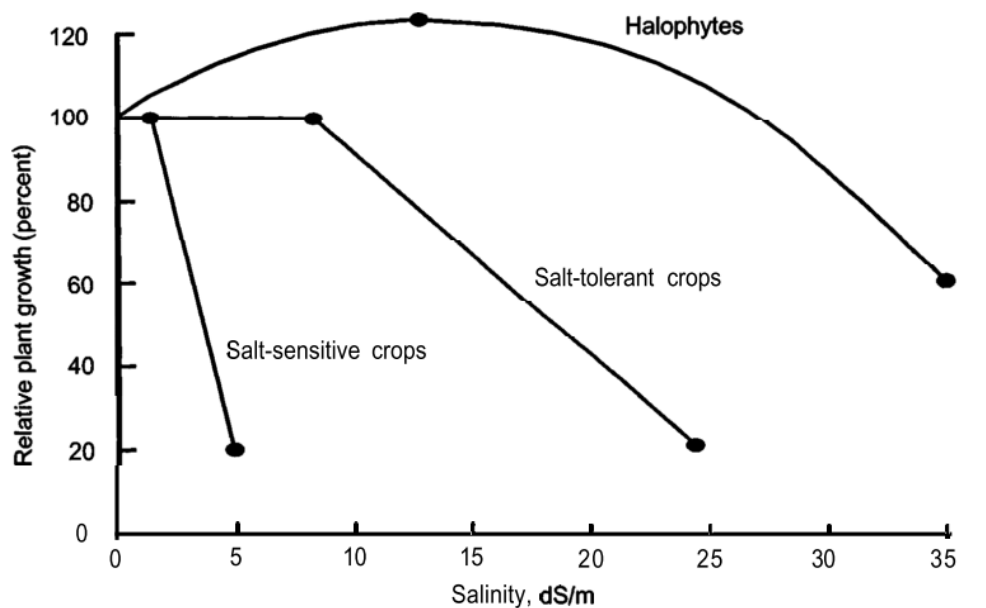
Figure 2. Response of halophytes, salt-tolerant crops (glycophytes) and salt-sensitive crops (glycophytes) towards salinity. The salinity of 5, 10, 15, 25 and 35 dS/m is about 2.8, 5.5, 8.5, 16.5 and 23.9 g/l respectively. Taken from (Hillel 2000)

There are some halophyte species which are in use as crop plants already, like quinoa (*Chenopodium quinoa*), sea fennel (*Crithmum maritimum*), sea aster (*Tripolium pannonicum*), *Salicornia* and *Sarcocornia* species and many more (Ventura and Sagi 2013; Panta *et al.* 2014; Ventura *et al.* 2014). Common species like wild rocket (*Diplotaxis tenuifolia*) and old culinary herbs like Common Scurvygrass (*Cochlearia officinalis*), turn out to be salt-tolerant, adding more to the list of halophyte crops. For some, agricultural practices and marketing need to be

examined (Lieth, Zeilinger and Papenbrock 2002; de Vos *et al.* 2013). There are also other fields of application for halophytes besides crop plants. They are suitable for forage and feed for animals, oilseed and protein crops, energy crops (biofuels and biogas), phytoremediation (desalination and phytoremediation of polluted soils) and treatment of nutrient-rich saline (waste)water (from aquaculture). Furthermore, their components are valuable for pharmaceutical applications and commercial products like cosmetics, functional food and nutraceuticals (Buhmann and Papenbrock 2013; Panta *et al.* 2014; Buhmann and Waller 2015; Turcios, Weichgrebe and Papenbrock 2016)

Reasons for the salt tolerance of halophytes

Halophytes have acquired mechanisms to cope with the salt in the environment. Salt intrusion causes osmotic and ionic stress for the plants cells, so they must re-establish ionic and osmotic homeostasis (Zhu 2001). Plants have to adjust osmotically to maintain turgor pressure, they must contain more solutes inside than the outside media (Flowers, Munns and Colmer 2015). Therefore, in many halophytes species, the ions Na^+ and Cl^- are accumulated in the vacuole (intracellular compartmentation). This is different for halophyte species as some accumulate either Na^+ or Cl^- (Koyro *et al.* 2010). To adjust the osmotic potential of the cytoplasm, compatible solutes (or osmolytes) are produced. Figure 3 shows the main distribution of ions and possible osmolytes in a cell adapted to salinity. Furthermore, halophytes have a higher ability to exclude salts from the roots and developed salt glands for the excretion of ions (Flowers and Colmer 2008; Munns and Tester 2008). The compartmentation prevents the ion concentration becoming toxic for enzymes in the cytoplasm, but this process is energy consuming and currently it is unknown whether toxic concentrations that inhibit metabolism are ever reached (Flowers *et al.* 2015).
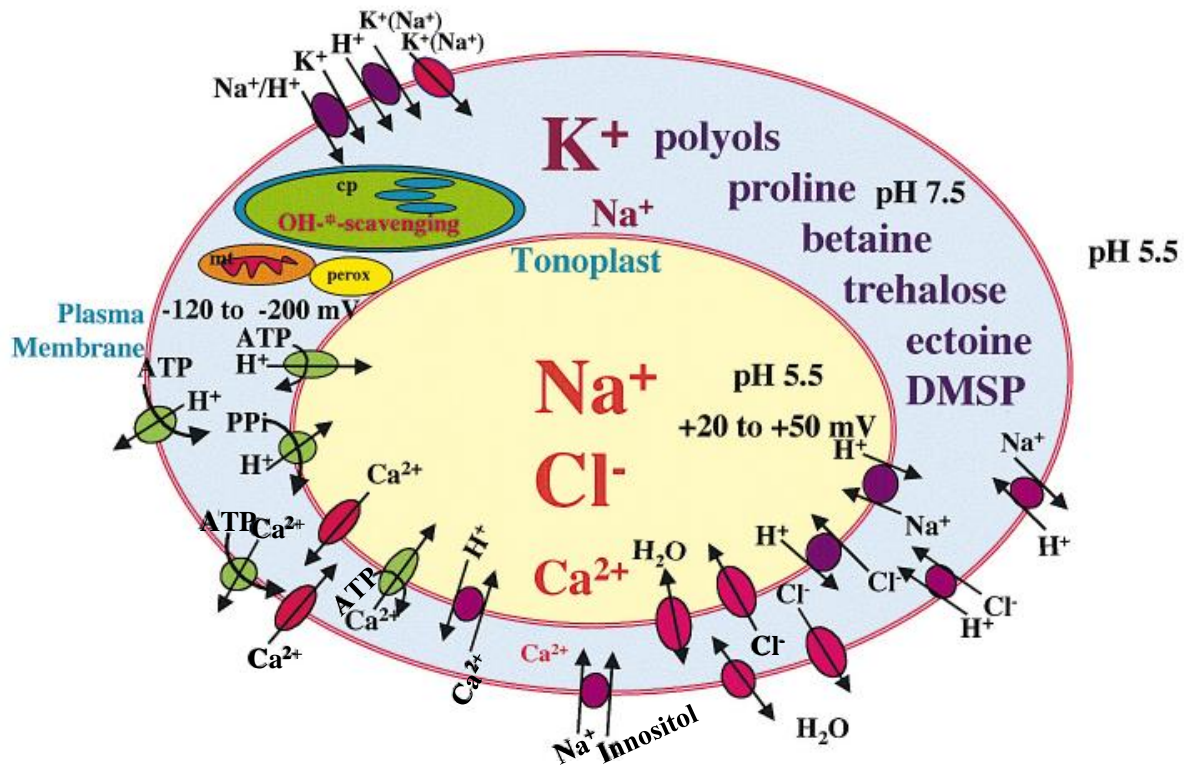
Figure 3. A cell, after salt adaptation, re-established osmotic and ionic homeostasis. Ions are compartmented mainly to the vacuole and osmolytes (polyols, proline, betaine, trehalose, ectoine and dimethylsulfoniopropionate (DMSP)) are compartmented in the cytoplasm. Na^+ and Cl^- transport proteins responsible for homeostasis, channels, and electrochemical potentials across the plasma membrane and tonoplast are indicated. Organelles (chloroplast (cp), mitochondrion (mt), and peroxisome (perox)) are included. Taken from (Hasegawa *et al.* 2000).

Before homeostasis is established or if the stress is severe, other reactions take place. Figure 4 shows the reactions of water shortage under drought, but this osmotic stress is similar to salt stress, as mentioned above. Low water potential causes stomata to close, leading to a decrease in CO_2 uptake. Reduction equivalents like $NADPH+H^+$, which are normally used for the CO_2-fixation via Calvin cycle, increase. Consequently, the $NADP^+$ concentration decreases and therefore, potential electron acceptors for the electron transport chain decrease as well. This leads to an over-reduction of the photosynthetic electron transport chain and induces the generation of reactive oxygen species through the Mehler-reaction. The antioxidative system is triggered producing enzymatic and nonenzymatic antioxidative substances (Ben Amor *et al.* 2005; Selmar and Kleinwächter 2013). Whereas enzymatic antioxidants like the superoxide dismutase (SOD) or the ascorbate peroxidase (APX) are enhanced by reactive oxygen species (ROS), the oversupply of $NADPH+H^+$ in contrast to $NADP^+$, might lead to an increased production of highly reduced compounds, like (poly)phenols, alkaloids or isoprenoids (Selmar and Kleinwächter 2013).
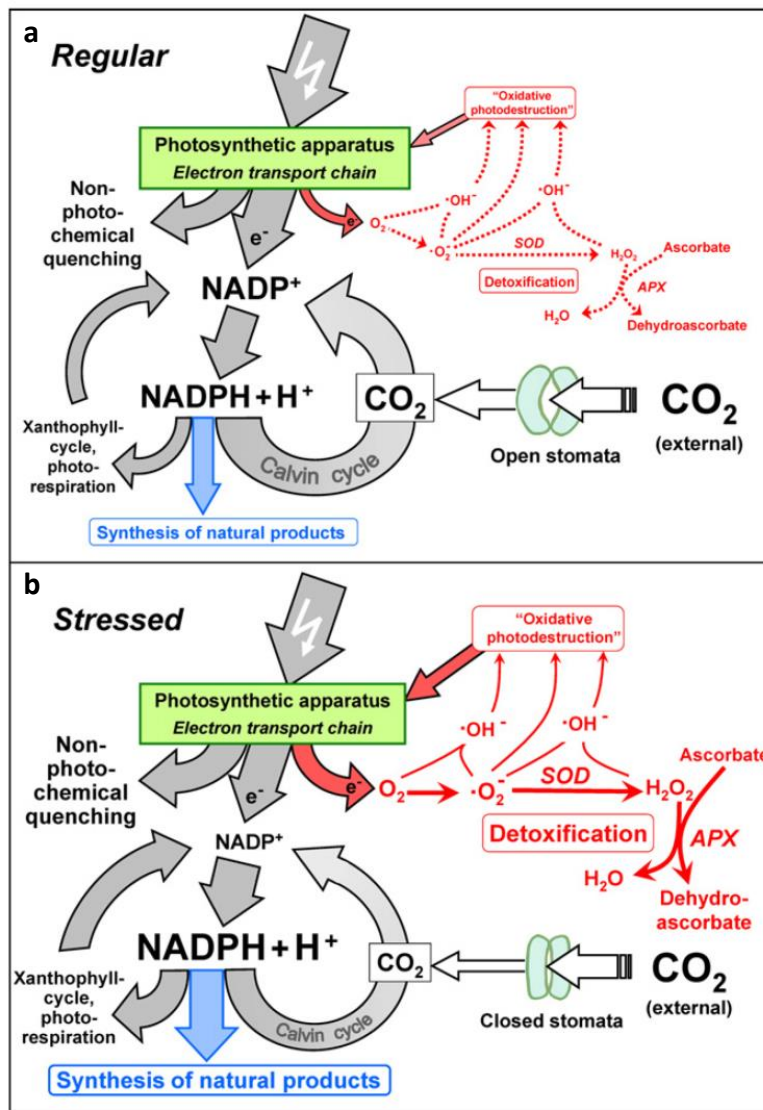
Figure 4. Overview of regular (a) and stressed (b) energy and metabolic pathways in plants. Under regular (a) conditions the energy from the photosynthetic apparatus is transferred to available $NADP^+$ or emitted by non-photochemical quenching. Therefore, only a tiny amount of ROS are produced. Under stress conditions (b) closed stomata lead to low internal CO_2 concentration, which lowers the $NADPH+H^+$ consumption in the Calvin cycle, therefore minimizing the available $NADP^+$. The surplus of energy generated by the photosynthetic apparatus can neither be reduced by non-photochemical quenching nor the xantophyll cycle or photorespiration, generating ROS by the Mehler reaction. To prevent oxidative damage on the photosynthetic apparatus or other proteins or metabolites, the ROS have to be detoxified, for example by the superoxide dismutase (SOD) and ascorbate peroxidase (APX). taken from (Selmar and Kleinwächter 2013).

Benefits of salinity stress on halophytes

Salt stress can have a beneficial effect because it may increase the non-enzymatic antioxidant concentration. There are not many studies about the positive effect of salinity on the non-enzymatic antioxidants (Ozgur *et al.* 2013; Bose, Rodrigo-Moreno and Shabala 2014). And some studies showed unclear results of antioxidants according to salt in halophytes (Ksouri *et al.* 2007; Alhdad *et al.* 2013). The factor time is not considered thoroughly, as many studies have just one observation time for their analysis of antioxidants.

The antioxidants which might be present in plants are the (poly)phenols with over 8000 different structures, divided into the major groups of flavonoids and non-flavonoid

polyphenols. Furthermore, there are alkaloids, thiols like glutathione, the vitamins C (ascorbic acid) and E (tocotrienol and tocopherol), carotenoids, uric acid and bilirubin. But even some osmolytes like proline have an antioxidative effect (Ou *et al.* 2002; Gill and Tuteja 2010; Rosa, Alvarez-Parrilla and Gonzalez-Aguilar 2010; Seal *et al.* 2010; Del Rio *et al.* 2013). As the group of antioxidants is diverse, some methods were developed to analyze the total antioxidative capacity, like the oxygen radical absorbance capacity (ORAC), however no method can analyze the combined antioxidative capacity, as either lipophilic or hydrophilic antioxidants can be analyzed (Wu *et al.* 2004). Furthermore, the ORAC mainly analyzes the chain breaking antioxidants. Chain breaking antioxidants include (poly)phenols, vitamin C and E, uric acid and bilirubin. They donate their hydrogen atom to ROS, becoming stable radical themselves. This prevents a further chain reaction or slows it down. Preventive antioxidants are superoxide dismutase, catalase, peroxidase, β-carotene, lycopene and bilirubin. They intercept oxidizing species before damage can occur (Ou, Hampsch-Woodill and Prior 2001; Ou *et al.* 2002; Buettner and Schafer 2002).

It is assumed, that this protective effect of the non-enzymatic antioxidants is still present when the antioxidants are consumed with the plant material and there are many studies indicating that antioxidants have a promising effect on human health (Crozier et al. 2009; López-Berenguer et al. 2009; Del Rio et al. 2013; Fiedor and Burda 2014; Rodriguez-Mateos et al. 2014). Therefore, halophytes can serve not only as an additional food resource; they can also be described as nutraceutical. As Kalra (2003) stated: "When functional food aids in the prevention and/or treatment of disease(s) and/or disorder(s) other than anemia, it is called a nutraceutical." Therefore, they may help to minimize threatening undernourishment or malnutrition. The process of enhancing the necessary daily micronutrients directly in the crops eaten every day is called biofortification (Hirschi 2009). Albeit halophytes are not part of the daily diet everywhere, they are in some regions, and will become more relevant in time (Kaur *et al.* 2013).

Disadvantages of salinity stress on halophytes

However, apart from the positive effect of increasing the antioxidant concentration in Halophytes, which would improve their application for pharmaceutical and commercial products, there is a negative effect. Growth is reduced as the result from the closed stomata

and therefore the inadequate photosynthesis, but the direct inhibition of cell division and expansion might be more crucial (Zhu 2001). Furthermore, the energetic cost for ion transport and the synthesis of compatible solutes and ROS damage decrease growth (Flowers *et al.* 2015).
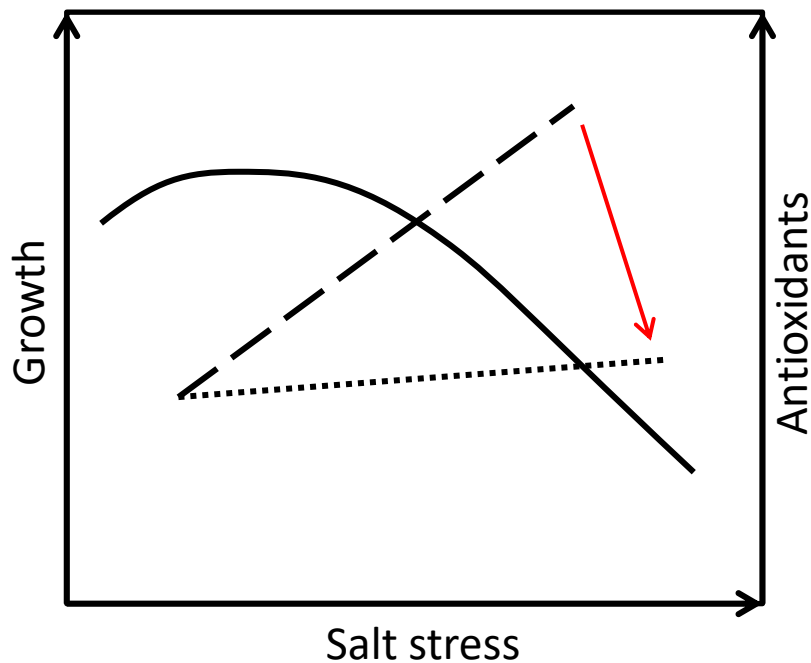
Figure 5. Contrary reactions in Halophytes under salinity stress. Solid line (——) growth; dashed line (---) antioxidant concentration short after the stress; dotted line (····) antioxidant concentration after a long exposure to salt stress; arrow indicating the time (own drawing).

These opposing reactions are delineated in Figure 5. Low salinity increases growth in halophytes but severe salt stress decreases the growth. Contrary to this phenomenon is the concentration of antioxidants in halophytes, it increases with elevated salinity. Over time the concentration of antioxidants may decrease as halophytes adapt to salt stress. Therefore, it is not clear under which salinity and at which time point the maximal yield (antioxidative concentration x growth) is achieved.

Aims of this Study

- Establishing methods for a fast and high throughput antioxidant determination in halophytes.
- Testing the possibility of a biofortification for halophyte species
  - Evaluation of changes in the metabolite concentration and composition through
    - different salt stress conditions ranging from low to high salinity stress
    - different incubation times ranging from hours to weeks
  - Finding the point in time for the maximum yield of antioxidants induced by salinity
- Evaluating different brackish water raised halophyte crop species in terms of
  - status as possible nutraceuticals
  - comparison to other crops and nutraceuticals

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Chapter 2

Boestfleisch, C., Wagenseil, N.B., Buhmann, A.K., Seal, C.E., Wade, E.M., Muscolo, A., Papenbrock, J. (2014) Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation. *AoB PLANTS* **6**, plu046.
DOI: 10.1093/aobpla/plu046.

Research Article

SPECIAL ISSUE: Physiology and Ecology of Halophytes—Plants Living in Salt-Rich Environments

Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation

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Abstract. Halophytes, salt-tolerant plants, are a source of valuable secondary metabolites with potential economic value. The steady-state pools of many stress-related metabolites are already enhanced in halophytes when compared with glycophytes, but growth under conditions away from the optimum can induce stress and consequently result in changes to secondary metabolites such as antioxidants. However, direct evidence for increasing the concentration of valuable secondary metabolites as a consequence of altering the salinity of the growing environment still remains equivocal. To address this, we analysed a range of metabolites with antioxidant capacity (including total phenols, flavonoids, ascorbate, reduced/oxidized glutathione and reactive oxygen species scavenging enzymes) in seedlings and plants from different families (Amaranthaceae, Brassicaceae, Plantaginaceae and Rhizophoraceae) and habitats grown under different salt concentrations. We show that it is possible to manipulate the antioxidant capacity of plants and seedlings by altering the saline growing environment, the length of time under saline cultivation and the developmental stage. Among the species studied, the halophytes *Tripolium pannonicum*, *Plantago coronopus*, *Lepidium latifolium* and *Salicornia europaea* demonstrated the most potential as functional foods or nutraceuticals.

Keywords: Halophytes; nutraceuticals; secondary metabolites; stress tolerance; yield.

Introduction

The existence of spatio-temporal gradients of soil salinity has traditionally been considered one of the most important physical factors in the plant zonation of salt marshes (Chapman 1974). Zonation is caused largely by the coupling of differences in germination ecology to spatio-temporal variations in soil salinity (Egan and Ungar 2000). Salt marsh habitats are characterized by diurnal,

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monthly and/or seasonal dynamics where the duration of submergence, tidal scour, waterlogging and especially salinity vary (Ungar 1991). These fluctuations require high physiological plasticity, resulting in strong phenotypic and biochemical variations between individual plants, populations and species (Pigliucci *et al.* 2006; Flowers and Colmer 2008; Richards *et al.* 2010).

Biochemical perturbations in plants exposed to salinity are associated with both primary and secondary metabolisms (Král'ová *et al.* 2012) through a complex metabolic network (Krasensky and Jonak 2012; Obata and Fernie 2012), which includes an increase in intracellular levels of reactive oxygen species (ROS). Although ROS have positive roles in the stress-response pathway, for example in signalling (Kranner *et al.* 2010), an imbalance between ROS synthesis and scavenging may cause severe damage to protein structures, inhibit the activity of enzymes of important metabolic pathways and result in the oxidation of macromolecules including lipids and DNA.

Changes in the metabolic profile during exposure to salinity are dependent on the genus, species, cultivar and developmental stage of the plants (Bernstein *et al.* 2010; Atkinson and Urwin 2012). Fraire-Velázquez and Balderas-Hernández (2013) showed that the exposure of several glycophytic genera, such as *Arabidopsis*, *Lotus* and *Medicago*, to salt resulted in similar changes to 48 metabolites, with increasing concentrations of amino acids (particularly proline), sugars and phenols and with decreasing concentrations of organic acids. Conversely, halophytes do not present such large changes in metabolite profiles and/or gene expression after exposure to salt (Taji *et al.* 2004; Sanchez *et al.* 2008; Kosová *et al.* 2013), suggesting a constitutive salt tolerance. Nevertheless, environmental conditions that are sub- or supra-optimal are likely to be stressful and thus there is potential to manipulate metabolic changes through the growing environment.

Recent studies have shown the potential of halophytes as a source of valuable secondary metabolites with likely economic value (Ksouri *et al.* 2012; Buhmann and Papenbrock 2013). However, direct evidence of increases in the concentration of valuable secondary metabolites and the antioxidant capacity as a consequence of altering the salinity of the growing environment still remain equivocal. Since halophytes differ widely in their degree of salt tolerance and natural growth rate (Flowers *et al.* 1977; Ungar 1991), this study was designed to assess and compare the effect of NaCl salinity on the antioxidant capacity of halophytic species from different genera and habitats. To obtain a broad overview of their potential for functional products, several metabolites were analysed in mature plants (total phenolics, ascorbic acid (AA), total flavonoids and total antioxidant capacity), all attributed

as antioxidants with use in fields such as pharmacognosy, functional foods and nutraceuticals (Arts and Hollman 2005; Scalbert *et al.* 2005; Gallie 2013). In seedlings, the antioxidant glutathione (GSH) and other low-molecular weight (LMW) thiols, and the activities of the antioxidant scavenging enzymes glutathione reductase, ascorbate peroxidase (APX), peroxidase (POX) and catalase (CAT) were evaluated. The aim was to maximize the antioxidant capacity by altering the saline growing environment and identify which of the study species have potential for cultivation as functional foods or nutraceuticals.

Methods

Plant material

Species were selected for study based on their habitat and taxonomy: the pioneer salt marsh plants, *Salicornia europaea*, *S. dolichostachya*, *Tripolium pannonicum* and *Suaeda maritima* (all Amaranthaceae); from the low-mid marsh, *Atriplex portulacoides* (Amaranthaceae); from the mid-upper marsh, *Atriplex halimus* (Amaranthaceae); from gravelly salty soils in dry habitats, *Plantago coronopus* (Plantaginaceae); from gravelly salty soils in wet habitats, *Lepidium latifolium* (Brassicaceae); and the woody mangrove species, *Bruguiera cylindrica* (Rhizophoraceae).

Seeds and propagules

Atriplex portulacoides, *T. pannonicum* and *S. dolichostachya* seeds were collected from the North Sea coast (53°29′13″N; 8°03′16″E). *Salicornia europaea* was collected in Poland by Agnieska Piernik, Toruń. Seeds of *P. coronopus* were purchased from Jelitto Staudensamen GmbH (Schwarmstedt, Germany). One plant each of *L. latifolium* and *A. halimus* was ordered from Rühlemann's Kräuter & Duftpflanzen (Horstedt, Germany). Seeds were produced from the *L. latifolium* plant and cuttings were obtained from the *A. halimus* plant. Mature seeds of *S. maritima* were collected at Cuckmere Haven, Seaford, East Sussex in 2010.

Germination tests

Seeds of *S. europaea* and *T. pannonicum* were selected for size homogeneity and surface sterilized for 20 min in 2.0 % (v/v) sodium hypochlorite, rinsed and soaked in distilled water for 1 h. The entire sterilization procedure including soaking took 1 h and did not affect the germination process (Ruiz-Carrasco *et al.* 2011; Burrieza *et al.* 2012). Germination tests were carried out under different concentrations of NaCl (0 mM NaCl/0 PSU, 150 mM NaCl/8.8 PSU, 300 mM NaCl/17.5 PSU and 600 mM NaCl/35.1 PSU). Seeds were placed on filter paper in 9-cm Petri dishes containing 3 mL of each solution. The Petri

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dishes were sealed with Parafilm to prevent evaporation and kept in a growth chamber (Green line WRS 96–85, KW, Scientific Equipment, Italy) under conditions indicated in Table 1 with a relative humidity of 70 %.

Seeds of *S. maritima* were cleaned and stored at 15 % relative humidity and 15 °C until 1 month prior to the start of experiments when seeds were transferred to 5 °C to break their dormancy (Wetson *et al.* 2008). Seeds were sown onto two layers of 90-mm seed test paper (Fisher, UK) in 9-cm Petri dishes containing 7.5 mL of 0 mM NaCl/0 PSU, 100 mM NaCl/5.8 PSU, 200 mM NaCl/11.7 PSU, 300 mM NaCl/17.5 PSU and 400 mM NaCl/23.4 PSU. Dishes were wrapped in plastic bags and incubated at 25 °C (12 h photoperiod). Germination was defined as radicle emergence of at least 2 mm.

Growth and cultivation of mature plants

Seeds of *L. latifolium* were germinated on propagation soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany), and after 2 weeks transplanted to sand (0–2 mm grain size, Hornbach, Hannover, Germany), watered with modified Hoagland solution (Epstein 1972) and finally transferred to aerated containers with 13.5 L solution containing 3.57 mM $NaNO_3$, 316 μM $H_2NaPO_4 \times H_2O$ and 23.5 μM Fe-EDDHA (5.7 %) (Duchefa, Haarlem, Netherlands). After 1 week, the sea salt mixture was added to obtain 0 mM $Na^+ + Cl^-$/0 PSU, 220 mM $Na^+ + Cl^-$/15 PSU, 331 mM $Na^+ + Cl^-$/22.5 PSU and 442 mM $Na^+ + Cl^-$/30 PSU (Seequasal GmbH, Münster, Germany). The concentrations of the main ions were in the same range as average compositions of sea salts given in Atkinson and Bingman (1997) and are summarized in the **Supporting Information**. Both units, mM and PSU—practical salinity units, 1 PSU = 1 g sea salt kg^{-1} water—are given because many scientists in applied research use PSU. In our study, PSU was converted to mM $Na^+ + Cl^-$ not considering the other ions of the seawater, and mM NaCl was directly converted to PSU.

Seeds of *T. pannonicum, S. dolichostachya, P. coronopus, L. latifolium, A. portulacoides* were sown on propagation soil (Einheitserde). After reaching a size of ~3 cm, the seedlings were transferred to sand (Hornbach). These seedlings and cuttings of *A. halimus* were cultivated on sand until the plants could be transferred to containers used for hydroponic culture (see Table 1 for respective time periods). After a stepwise addition of 0.5 % NaCl to Hoagland solution every second day, the plants were placed in artificial seawater (Seequasal GmbH) of 220 mM $Na^+ + Cl^-$/15 PSU.

In another experiment, 4-week-old *T. pannonicum* plants, grown as described before, were transferred to containers for hydroponic culture. After a stepwise addition of 0.5 % NaCl to Hoagland solution every second

day, the plants were placed in artificial seawater (Seequasal GmbH) of 220 mM $Na^+ + Cl^-$/15 PSU, 331 mM $Na^+ + Cl^-$/22.5 PSU and 442 mM $Na^+ + Cl^-$/30 PSU.

Bruguiera cylindrica propagules, originally from Indonesia, were grown in the greenhouse for ~3 years. Plants were transferred to hydroponic basins with 110 mM $Na^+ + Cl^-$/7.5 PSU. After an acclimation time, the salinity was raised slowly and the plants were grown at salinity levels of 220 mM $Na^+ + Cl^-$/15 PSU and 442 mM $Na^+ + Cl^-$/30 PSU.

Mature plants were grown under greenhouse conditions as summarized in Table 1: sodium vapour lamps (SON-T Agro 400, Philips, Amsterdam, Netherlands) served as an additional light source, providing the minimal light flux density shown in the table. For *S. dolichostachya* day length was elongated by applying artificial light early in the morning (from 4:00 to 8:00 h) and at night (from 18:00 to 22:00 h) to prevent early flowering of the plants. Seven-week old plants were harvested at 0, 2, 4, 8 and 24 h after addition of salt.

Determination of total flavonoids, total phenols and oxygen radical absorbance capacity (mature plants)

Unless otherwise stated, frozen ground leaf material (50 mg) of different plant species was incubated (10 min) in ice-cold methanol (800 μL, 80 %) with shaking every 2 min. After centrifugation (5 min, 15 000 g) the supernatant was collected and the pellet was re-extracted (three times) with ice-cold methanol (400 μL, 80 %) to produce 2 mL of extract. Extracts were used as detailed below. All reactions were performed at room temperature (RT, between 21 and 23 °C) unless stated otherwise.

Total phenols. Based on the method of Dudonné *et al.* (2009), 100 μL of water was pipetted into the wells of a 96-well microplate. Triplicates of 10 μL sample, blank (80 % methanol) or gallic acid standard (5–250 μg mL^{-1}) were added, and finally 10 μL of Folin–Ciocalteu reagent. After incubation for 8 min and addition of 100 μL of 7 % sodium carbonate, the plate was incubated for 100 min and measured at 765 nm. Total phenols were calculated from a standard curve.

Total flavonoids. The method was based on Dewanto *et al.* (2002) and conducted as follows: 150 μL of water was added to each well of a clear 96-well microplate, together with 25 μL of sample or catechin hydrate standard (10–400 μg mL^{-1}) or 80 % methanol as blank (in triplicate), 10 μL of 3.75 % $NaNO_3$ followed by an incubation of 6 min and an addition of 15 μL of 10 % $AlCl_3$. After 5 min incubation, 50 μL of 1 M NaOH was added and the total flavonoids were calculated from a standard curve based on the absorption at 510 nm.

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Table 1. Growing conditions for the plants used in the different experiments. *[see Supporting Information].

Species	Tissue	Time from sowing to salt acclimation (weeks)	Acclimation time to salt (weeks)	Time under salt treatment (weeks)	Photoperiod with additional light (h)/quantum fluence rate (μmol m^{-2} s^{-1})	Temperature (°C)	Data presented in
L. latifolium	Shoots	7	0	0.14	14/350	22	Fig. 1/Tables S2 and 3*
T. pannonicum	Shoots	3	1	5	14/350	22	Fig. 2/Table S4*
P. coronopus	Shoots	2	1	5	14/350	22	Fig. 3/Table S4
T. pannonicum	Shoots	4	1	5	14/350	22	Fig. 3/Table S4
L. latifolium	Shoots	4	1	5	14/350	22	Fig. 3/Table S4
A. portulacoides	Shoots	6	1	5	14/350	22	Fig. 3/Table S4
A. halimus	Shoots	7 (cuttings)	1	5	14/350	22	Fig. 3/Table S4
S. dolicostachya	Shoots	5	1	5	18/350	22	Fig. 3/Table S4
B. cylindrica	Leaves	ca. 163	6	8	14/350	22	Fig. 4/Table S4
S. maritima	Seedlings	0	0	<2	8/7	25	Fig. 5
S. europaea	Seedlings	0	0	1	16/1055	25	Table 2
T. pannonicum	Seedlings	0	0	1	16/1055	25	Table 3

Oxygen radical absorbance capacity. The oxygen radical absorbance capacity (ORAC) assay was based on Huang *et al.* (2002) and Gillespie *et al.* (2007) with modifications. A black 96-well microplate was kept on ice, 120 μL of 112 nM fluorescein in 75 mM phosphate buffer (pH 7.4) was pipetted into each well, followed by 20 μL of standards, sample or blanks. 6-Hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standard (0.25–50 μM) was diluted in the identical phosphate buffer. Samples were diluted between 1 : 40 and 1 : 150 with phosphate buffer to be within the range of the standard curve. The plate was incubated for 15 min at 37 °C and the fluorescence 485/520 was measured at time point 0. Eighty microlitres of 62 mM 2,2′-azobis(2-amidino-propane) dihydrochloride were added to each well and the fluorescence was measured every minute for 80 min. The difference in absorbance at time point 0 and after 80 min was calculated and quantified using a standard curve.

Ascorbic acid. The determination of AA, dehydroascorbic acid (DHA) and total ascorbic acid (TAA) was based on published protocols (Kampfenkel *et al.* 1995; Stevens *et al.* 2006; Gillespie and Ainsworth 2007). Frozen ground leaf material (50 mg) was weighed two times shaken with 500 μL of ice-cold TCA (6 %) and suspended on ice for 15 min until centrifugation (18 400 g, 20 min) and stored on ice before use. Cold phosphate buffer (10 μL, 75 mM, pH 7.0) and 10 μL of blank, standard (1–0.0625 mM) or sample were added to a 96-well microplate. For reduction of DHA, dithiothreitol (10 μL, 10 mM) was added to every second sample for TAA determination. After 10 min, 10 μL of *N*-ethylmaleimide (0.5 %) was added to the same second sample and 20 μL of water into the other wells. After 2 min incubation, 100 μL reaction mixture (two parts of 10 % TCA, one part of FeCl$_3$, two and a half part of 43 % H$_3$PO$_4$ and two parts of 4 % α-α'-bipyridyl solved in 70 % ethanol) was added. After 30 min incubation at 37 °C, the absorption was read at 523 nm. The difference between the measured TAA and AA is the calculated DHA.

Total antioxidant capacity (seedlings). The total antioxidant capacity was evaluated in the methanol extracts from the reduction of Mo (VI) to Mo (V) and subsequent formation of green phosphate/Mo (V) complex at acidic pH (Prieto *et al.* 1999). The extract (0.3 mL) was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the absorbance was measured at 695 nm against a blank (methanol) after cooling to RT. A calibration curve was prepared by mixing AA (31.25, 62.5, 125, 250, 500 and 1000 μg mL^{-1}) with methanol and the

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results are expressed as mg AA equivalents g^{-1} dry matter (DM).

Total phenols (seedlings). The total amount of phenols was determined using the Folin–Ciocalteu reagent (McDonald *et al.* 2001). Each plant extract (0.5 mL) was mixed with the Folin–Ciocalteu reagent (5 mL, 1 : 10 diluted with distilled water) and aqueous Na_2CO_3 (4 mL, 1 M). After 15 min the total phenols were determined photometrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200 and 250 mg L^{-1} solutions of tannic acid in methanol : water (50 : 50, v/v). Total phenol values are expressed in terms of tannic acid equivalent (mg g^{-1} DM).

Proline (seedlings). The amount of proline was assayed according to Bates *et al.* (1973). Plant tissue (500 mg) was homogenized in 3 % aqueous sulfosalicylic acid (10 mL) and filtered through Whatman No. 42 filter paper. Two millilitres of acid ninhydrin (1.25 g ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid) and 2 mL of glacial acetic acid were heated for 1 h at 100 °C. The reaction mixture was extracted with 4 mL of toluene and mixed vigorously for 15–20 s and the absorbance of the toluene layer measured spectrophotometrically at 520 nm using toluene as the blank.

Chlorophyll and carotenoids. Frozen ground leaf material (50 mg) was extracted with ice-cold 80 % acetone (400 µL) over 10 min (with mixing every 2 min) before centrifugation at 14 000 *g* for 5 min. The supernatant was collected and stored on ice. The pellet was re-extracted three times with 200 µL ice-cold 80 % acetone and centrifuged as described. All supernatants were pooled and absorption was measured at 750.0, 663.2, 646.8 and 470.0 nm using a spectrophotometer (Uvikon XS, Biotech instruments, Germany), and total chlorophyll and carotenoid contents were calculated according to Lichtenthaler (1987).

LMW thiols (seedlings)

For the determination of GSH, cysteine, cysteine–glycine and γ-glutamate-cysteine (reduced, oxidized and total), the plant material was freeze dried before grinding into a fine powder with a pestle and mortar using liquid nitrogen. Five replicates of 10–20 mg ground seeds were extracted in 1 mL of 0.1 M HCl and centrifuged at 15 000 *g* for 40 min at 4 °C, where the pellet was re-suspended in a further 1 mL of 0.1 M HCl, centrifuged as before and the two supernatants combined. The supernatant was used in the procedure as detailed by Seal *et al.* (2010) to determine the concentration of LMW thiols.

Enzyme assays

Extracts for enzyme assays (seedlings). Fresh seed material (0.5 g) was ground and then homogenized in 0.1 M phosphate buffer solution (pH 7.0) containing 100 mg soluble polyvinylpolypyrrolidone and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 15 000 *g* for 15 min at 4 °C and the resulting supernatants filtered through two layers of muslin cloth and used for enzyme activity assays. All enzyme activities were measured at 25 °C in a UV–visible light spectrophotometer (UV-1800 CE, Shimadzu, Japan) and expressed as enzyme units (U) per milligram soluble protein where one unit of enzyme was defined as the amount of enzyme necessary to decompose 1 µmol of substrate per min at 25 °C.

Guaiacol peroxidase. Peroxidase (EC 1.11.1.7) activity was measured following the change in absorbance at 430 nm during the incubation of the extracts at 25 °C with 10 mM guaiacol, 1 mM H_2O_2 in 0.1 M potassium phosphate buffer at pH 7.0 in a total volume of 3 mL. The molar extinction coefficient was 2.55 $mM^{-1}\,cm^{-1}$ (Putter 1974).

Ascorbate peroxidase. Ascorbate peroxidase (EC 1.11.1.11) activity was assayed according to Nakano and Asada (1981). The reaction mixture (1.5 mL) contained 50 mM phosphate buffer (pH 6.0), 0.1 μM EDTA, 0.5 mM ascorbate, 1.0 mM H_2O_2 and 50 μL enzyme extract. The reaction was started by the addition of H_2O_2 and ascorbate oxidation measured at 290 nm for 1 min. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 $mM^{-1}\,cm^{-1}$) and the results were expressed in μmol H_2O_2 min^{-1} mg^{-1} protein taking into consideration that 2 mol of ascorbate are required for the reduction of 1 mol H_2O_2 (McKersie and Leshem 1994).

Catalase. Catalase (EC 1.11.1.6) activity was measured according to the method of Beers and Sizer (1952), with minor modifications. The reaction mixture (1.5 mL) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 μM EDTA, 20 mM H_2O_2 and 50 μL enzyme extract. The reaction was started by the addition of the extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient (36 $mM^{-1}\,cm^{-1}$) and the results were expressed as μmol H_2O_2 min^{-1} mg^{-1} protein.

Glutathione reductase. Glutathione reductase (EC 1.6.4.2) activity was determined by measuring the rate of $NADPH_2$ oxidation as the decrease in absorbance at 340 nm $(\varepsilon = 6.2\ mM^{-1}\,cm^{-1})$. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.0),

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20 mM GSSG, 2 mM NADPH$_2$, 350 μL H$_2$O and 50 μL enzyme extract.

Statistical analysis

Mature plants. Four plants (shoots including leaves) were used at each data point in triplicate for ORAC, total phenols and total flavonoids, and in duplicate for AA. Values were tested for significance with a two-way analysis of variance (ANOVA) and Holm-Sidak post-hoc analysis using SigmaPlot 12.5 (Systat Software, Inc.). The grouping factors were salinity and time; interactions were calculated for ORAC, TAA, phenols and flavonoid values.

Three plants (shoots including leaves) were pooled as one sample, and three (one, as starting value for the species comparison) pooled samples made one data point except for *B. cylindrica* where eight to nine leaves were analysed individually. The analysis was repeated three times in triplicate measurements for ORAC, total phenols and total flavonoids, and in duplicate for AA. Carotenoids and chlorophyll were measured three times. Results were tested for significance by one-way ANOVA and *B. cylindrica* by *t*-test in SigmaPlot 12.5 (Systat Software, Inc.).

Seedlings. For experiments with *S. maritima*, five replicates of 120 seeds were used. Reduced GSH and glutathione disulfide (GSSH) values were tested for significance with one-way ANOVA ($P > 0.05$). Germination values were arcsine transformed before tested with one-way ANOVA and post-hoc analysis of LSD ($P < 0.05$).

For experiments with *S. europaea* and *T. pannonicum* seedlings, five replicates of 50 seeds were used. All data were analysed by one-way ANOVA with the salt concentration as the grouping factor. The response variables for these ANOVAs were seed germination, total antioxidant activity, total phenol and enzyme activities. Since salt concentration had four levels, on all significant ANOVAs we performed the Tukey multiple comparison test to compare all pairs of means. The germination percentage data were previously subjected to arcsine transformation and were reported in tables as untransformed values. All data collected were statistically analysed using SYSTAT 8.0 software (SPSS, Inc.).

Results

Antioxidant capacity of plants at different salinities

Lepidium latifolium. The aim was to analyse the effect of different salt concentrations on the antioxidant capacity of different halophytic species. Oxygen radical absorbance capacity values of 7-week-old *L. latifolium* plants in the control and at 220 mM Na$^+$ + Cl$^-$/15 PSU did not change significantly during the time course of the experiment (Fig. 1A, **[see Supporting Information]**). However, the higher salt concentrations (331 mM Na$^+$ + Cl$^-$/22.5 PSU and 442 mM Na$^+$ + Cl$^-$/30 PSU) led to significant increases in the ORAC values (Holm-Sidak method, $P < 0.05$). After 24 h, the values were approximately double of that at the beginning of the experiment. In parallel with changes in the ORAC values, the concentration of DHA and TAA increased significantly at higher salinities (Fig. 1B, $P < 0.05$). Also an increase of total phenols was observed in control plants (up to 24 h) and at 220 mM Na$^+$ + Cl$^-$/15 PSU (up to 8 h) (Fig. 1C) but fluctuated at higher salt concentrations. The flavonoid concentrations were almost constant in the control and at 220 mM Na$^+$ + Cl$^-$/15 PSU but increased at the higher at 331 mM Na$^+$ + Cl$^-$/22.5 PSU and 442 mM Na$^+$ + Cl$^-$/30 PSU (Fig. 1D). The grouping factors were salinity and time. For TAA and flavonoid values, there are significant interactions between salinity and time, whereas for phenol and ORAC values there are no significant interactions between time and salinity. For comparison the values are also expressed on a dry matter basis **[see Supporting Information]**. In summary, in *L. latifolium* increasing salt concentrations induced a higher antioxidative capacity based on TAA, total phenols and/or flavonoids.

Tripolium pannonicum. The antioxidant capacity of 9-week-old salt-adapted *T. pannonicum* plants did not show any significant differences from the control (4-week-old plants) at different salinities (Fig. 2A). The amounts of AA, DHA and TAA increased slightly with increasing salinity but this was not significant (Fig. 2B). The concentrations of total phenols and total flavonoids increased when the plants were grown in the presence of salt, although different salt concentrations in the medium did not increase the concentrations significantly (Fig. 2C and D). The carotenoid (Fig. 2E) and chlorophyll (Fig. 2F) concentrations increased with increasing salinity until 331 mM Na$^+$ + Cl$^-$/22.5 PSU, after which by 442 mM Na$^+$ + Cl$^-$/30 PSU the values were at their lowest. Taken together in salt-adapted mature *T. pannonicum*, increasing salt concentrations did not induce a higher antioxidative capacity although there was a slight increase in potential antioxidants, such as phenols, flavonoids and carotenoids.

Atriplex halimus, A. portulacoides, L. latifolium, P. coronopus, S. dolichostachya, T. pannonicum. In a comparison of different halophytic species, the antioxidant capacities were determined among young, non-treated plants and 7-week-old and 11-week-old plants grown at 220 mM Na$^+$ + Cl$^-$/15 PSU (Fig. 3). The ORAC showed significant differences among the salt-tolerant species (Fig. 3A). With the exception of *P. coronopus*, the ORAC

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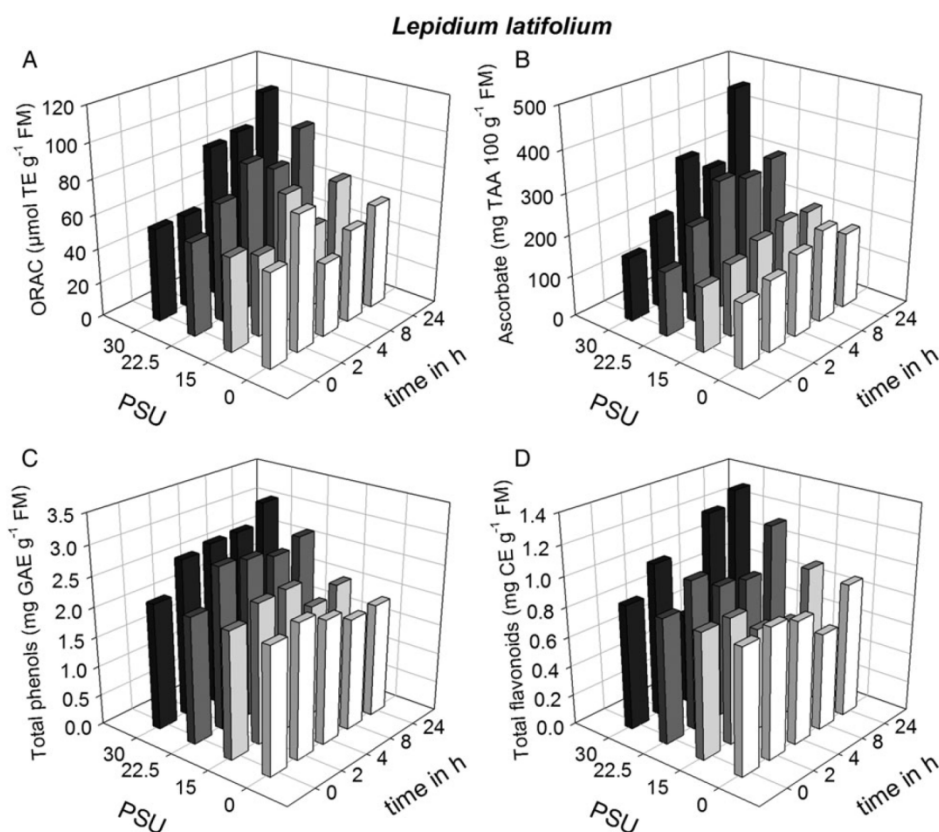
Figure 1. Six-week-old *L. latifolium* plants were transferred to basins; after 1 week the salinity was increased directly to 220 mM Na$^+$ + Cl$^-$ / 15 PSU, 331 mM Na$^+$ + Cl$^-$ /22.5 PSU and 442 mM Na$^+$ + Cl$^-$ /30 PSU. Samples were taken at time point 0 just before increasing salinity and 2, 4, 8, 24 h thereafter. Means are plotted against PSU and time. (A) ORAC values, (B) ascorbate, (C) total phenols and (D) total flavonoids. CE, catechin equivalents; GAE, gallic acid equivalents; TAA, total ascorbic acid; TE, trolox equivalents. For statistical analysis see **Supporting Information**.

values were always lower in the older, salt-treated plants. The amount of DHA and therefore the TAA amounts rose with increasing salinity (Fig. 3B).Younger and unstressed halophytes contained higher concentrations of TAA except *A. halimus* which showed a large increase in DHA. The higher ORAC values in *P. coronopus* were based on higher contents of total phenols and flavonoids whereas in the other plant species investigated the levels were lower in older, salt-treated plants (Fig. 3C and D). On the one hand, younger plants have higher concentrations of antioxidant compounds, on the other hand are much smaller and have less biomass.

Bruguiera cylindrica. The ORAC values were not influenced by doubling the salt concentration in the growth medium in *B. cylindrica* trees (Fig. 4A). However, AA, DHA and TAA as well as total phenols and flavonoid concentrations were increased at higher salt concentrations

(Fig. 4B–D) indicating a huge source of antioxidant compounds.

Antioxidant capacity of seedlings at different salinities

Suaeda maritima. The germination of *S. maritima* was highest in the absence of NaCl (58 %) and decreased with increasing NaCl concentration to 24 % at 400 mM NaCl/23.4 PSU (Fig. 5A and B). Although there were small changes in the concentration of GSH (Fig. 5A) and LMW thiols (Fig. 5B) with increasing NaCl treatment, these were not significant ($P > 0.05$). No significant differences in the concentration of GSH, GSSG or total glutathione were seen with increasing NaCl. The ratio of GSH:GSSG was similar at all salt treatments, with a slightly higher proportion of reduced than oxidized glutathione (ratio GSH:GSSG between 1.02 and 1.42), except at 300 mM NaCl/17.5 PSU where oxidized GSSG was

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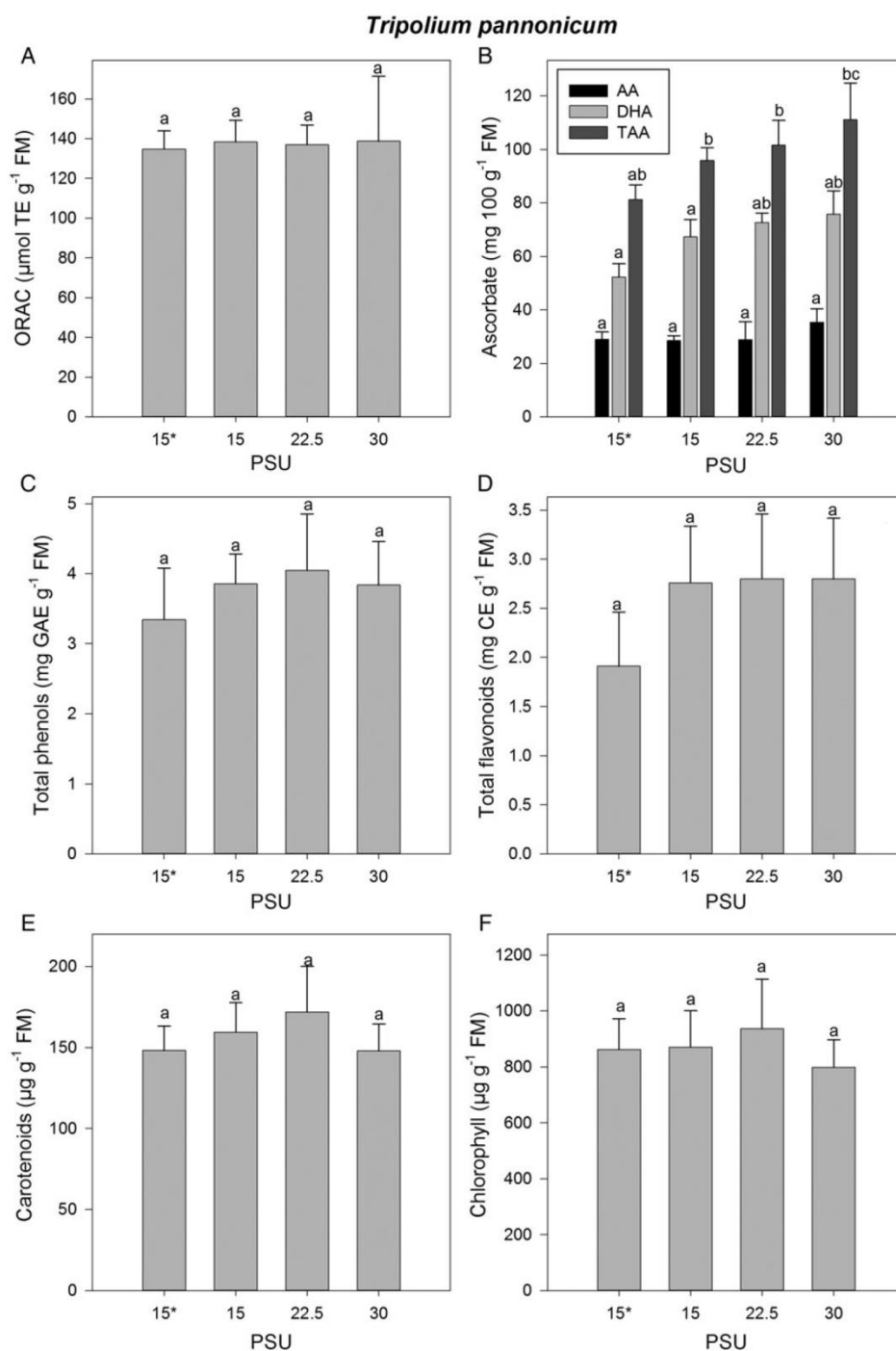
Figure 2. *Tripolium pannonicum* plants were acclimated to a salinity of 220 mM Na$^+$ + Cl$^-$/15 PSU for 1 week and thereafter transferred to basins of 220 mM Na$^+$ + Cl$^-$/15 PSU, 331 mM Na$^+$ + Cl$^-$/22.5 PSU and 442 mM Na$^+$ + Cl$^-$/30 PSU. Plants taken after the salt acclimation are indicated with an asterisk. Each sample was analysed for (A) ORAC values, (B) AA, DHA and TAA, (C) total phenols, (D) total flavonoids, (E) carotenoid and (F) chlorophyll contents. For abbreviations see legend to Fig. 1. Different letters indicate significant differences.

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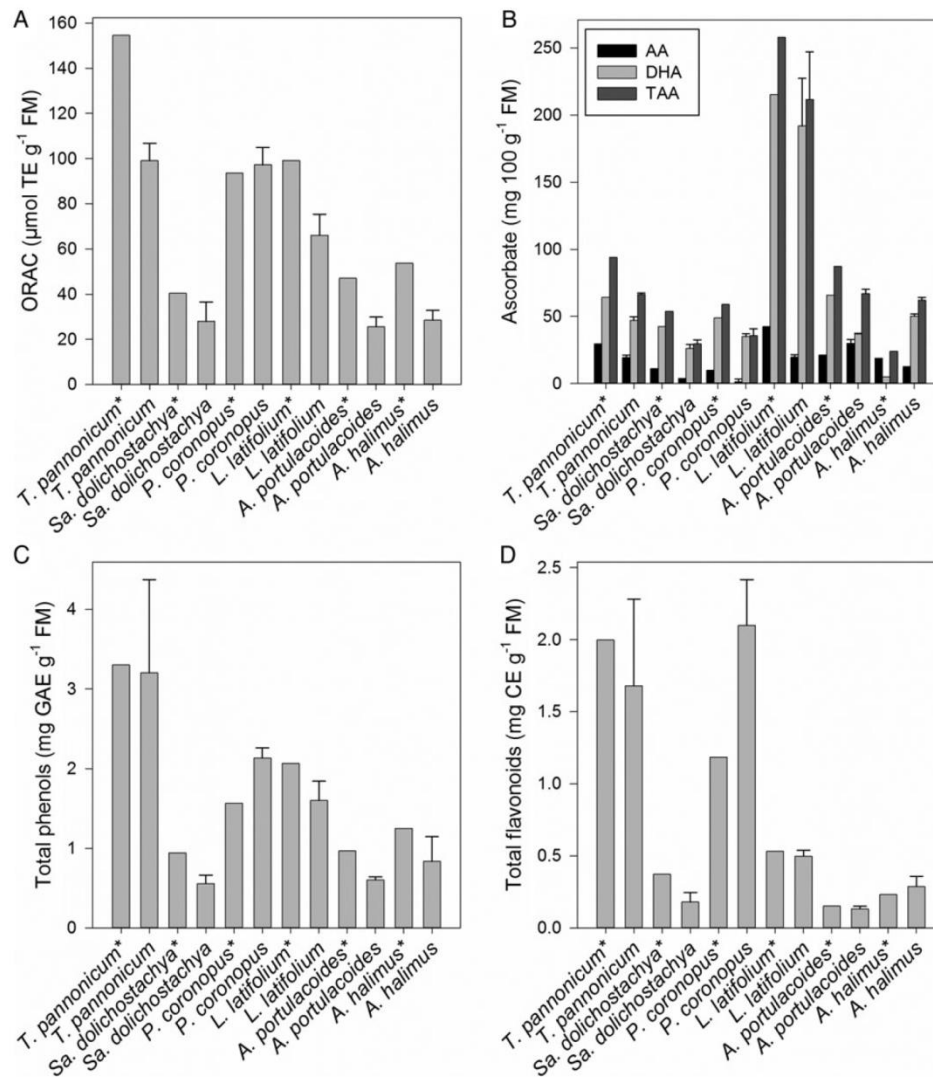
Figure 3. Different halophyte species, *T. pannonicum*, *S. dolichostachya*, *P. coronopus*, *L. latifolium*, *A. portulacoides* and *A. halimus*, were grown on sand for up to 6 weeks, acclimated for 1 week to a salinity of 220 mM $Na^+ + Cl^-$/15 PSU and transferred to basins containing 220 mM $Na^+ + Cl^-$/15 PSU. One sample was taken at the beginning of the experiment (marked with an asterisk). After 5 weeks samples were taken again. Each sample was analysed for (A) ORAC values, (B) AA, DHA and total ascorbic acid (TAA), (C) total phenols and (D) total flavonoids. For abbreviations see legend to Fig. 1. Different letters indicate significant differences.

dominant (ratio GSH : GSSG 0.56). The ratio of reduced : oxidized thiols was also similar at all salt treatments, with a slightly higher proportion of reduced than oxidized thiols (ratio LMW thiols reduced : oxidized between 1.03 and 1.35), except at 300 mM NaCl/17.5 PSU where oxidized thiols were dominant (ratio LMW thiols reduced : oxidized 0.60).

Salicornia europaea. The germination of *S. europaea* did not change with increasing salinity from 0 to 300 mM

NaCl/17.5 PSU; however, an increase in the total antioxidant capacity, APX, CAT, GR, proline and phenols was observed (Table 2). At the highest salinity (600 mM NaCl/35.1 PSU), a significant decrease in germination (from 85 to 50 %) in comparison with the other treatments was observed while all other metabolites measured, except proline, increased to their highest levels (Table 2).

Tripolium pannonicum. Seeds of *T. pannonicum* germinated in the presence of 150 mM NaCl/8.8 PSU to the same

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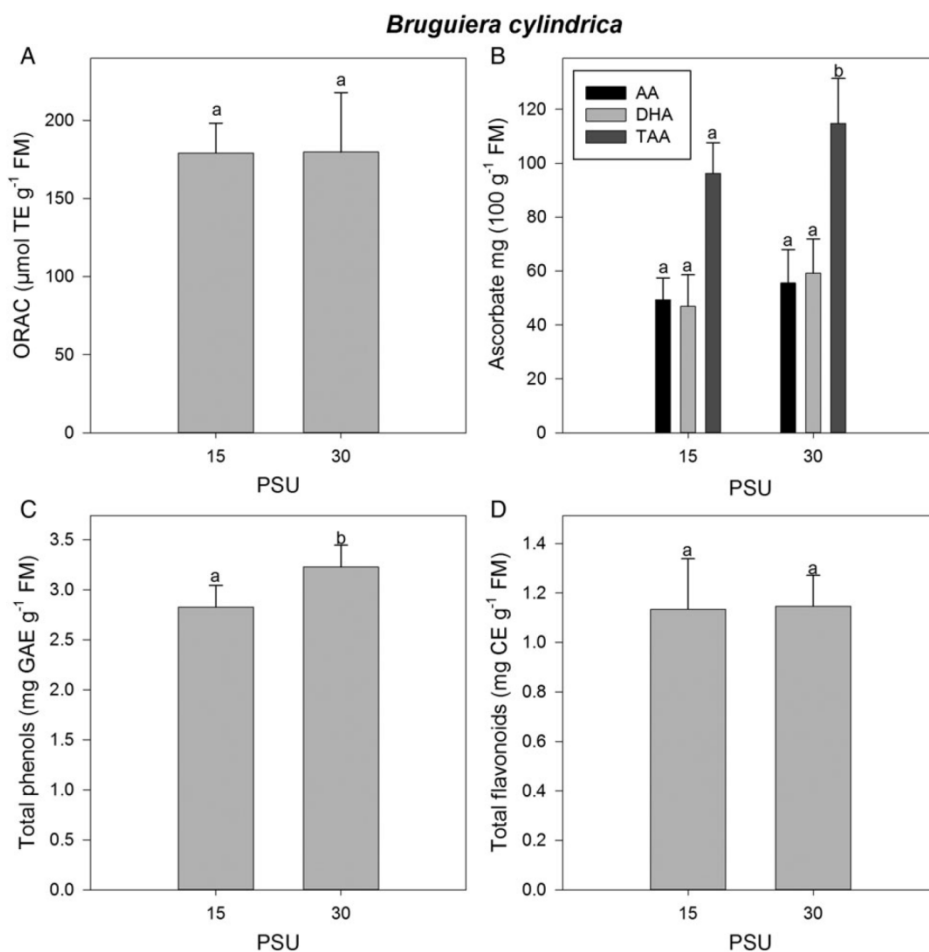
Figure 4. *Bruguiera cylindrica* plants were transferred to basins with 110 mM Na$^+$ + Cl$^-$/7.5 PSU. After an acclimation time of 7 weeks the salinity was raised slowly over 6 weeks and the plants were grown 8 weeks at salinity levels of 220 mM Na$^+$ + Cl$^-$/15 PSU and 442 mM Na$^+$ + Cl$^-$/30 PSU. Leaf material was harvested. Each sample was analysed for (A) ORAC values, (B) AA, DHA and TAA, (C) total phenols and (D) total flavonoids. For abbreviations see legend to Fig. 1. Different letters indicate significant differences.

extent as at 0 mM NaCl but increases in CAT, GR and phenols were apparent (Table 3). At 300 mM NaCl/17.5 PSU germination decreased to 50 % together with a significant increase in total antioxidant capacity, POX, APX, CAT, GR, proline and phenols (Table 3). No germination was observed at the highest salinity (600 mM Na Cl/35.1 PSU).

Discussion

Antioxidants such as phenolic compounds, carotenoids, AA and flavonoids are ubiquitous in plants and are essential to the human diet (Arrigoni and De Tullio 2002; Balasundaram *et al.* 2006; Pham-Huy *et al.* 2008; Parvaiz *et al.* 2009; Ksouri *et al.* 2012; Fiedor and Burda 2014). The association of a raised antioxidant capacity with salt

tolerance has been demonstrated in a number of salt-tolerant glycophytes and true halophytes (Broetto *et al.* 2002; Agarwal and Pandey 2004; Ben Amor *et al.* 2005; Alhdad *et al.* 2013). When salinity was applied over 24 h to plants of the annual halophyte *L. latifolium*, an increase in total antioxidant capacity in addition to phenols, ascorbate and flavonoids was observed, demonstrating that manipulation of the antioxidant capacity is possible through salinity treatment. Furthermore, the TAA contents of *L. latifolium* were higher than in the other halophyte species studied (Fig. 3B) and higher than the reported values in glycophytes within this genus such as *Lepidium sativum* (garden cress; 60 mg 100 g^{-1} FM, http://ndb.nal.usda.gov/ndb/search/list, accessed 29 March 2014) and cabbage (90–150 mg 100 g^{-1} FM), and species outside the genus such as the lemon fruit (*Citrus × limon*;

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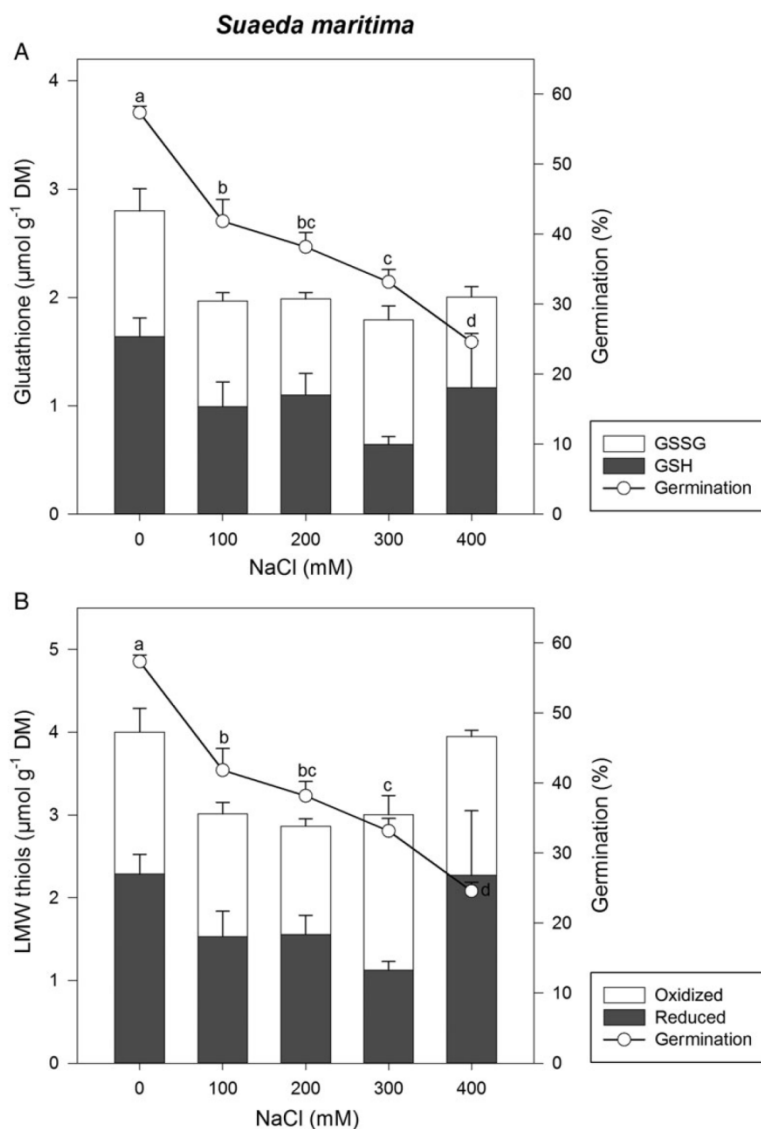
Figure 5. (A) Reduced GSH (grey bars) and GSSG (white bars) in germinated seeds of *S. maritima*. Line plot indicates viability of seeds as determined by germination test. Germination values are shown as mean ± SE. Different letters indicate significant differences of the mean. (B) Reduced concentration of the LMW thiols glutathione + cysteine + cysteinyl–glycine + γ-glutamyl-cysteinyl (grey bars) and their oxidized forms (white bars) in germinated seeds of *S. maritima*. No significant differences in the concentration of LMW thiols (reduced, oxidized and total) were seen with increasing NaCl. Line plot indicates viability of seeds as determined by germination test. Germination values are shown as mean values ± SE. Different letters indicate significant differences of the mean.

53 mg 100 g^{-1} FM, http://ndb.nal.usda.gov/ndb/search/list, accessed 29 March 2014). Therefore, the treatment of *L. latifolium* with high salt concentration for 24 h could significantly increase the ascorbate contents and could be used as a promising cultivating technique.

In contrast to the 24 h treatment of *L. latifolium*, mature plants of *T. pannonicum* did not accumulate secondary compounds when exposed to 15–30 PSU salinity for 4 weeks (Fig. 2). For many of the mature plant species studied, and in seedlings of *S. maritima*, the antioxidant capacity remained similar after weeks of salt exposure (Figs. 3–5). This could indicate that the levels of salinity were near optimal and therefore not high enough to induce a stress response or reflect a period where the

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Table 2. Total antioxidant capacity, proline content and activities of guaiacol peroxidase, CAT, APX and glutathione reductase enzymes in 7-day-old seedlings of *S. europaea* germinated under different NaCl treatments. Values are means ± SE ($n = 4$). Different letters in the same column indicate significant differences $P \leq 0.01$. AAE, ascorbic acid equivalents.

NaCl treatments	Germination percentage (%)	Total antioxidant capacity (AAE g^{-1} DM)	POX (U mg^{-1} prot)	APX (U mg^{-1} prot)	CAT (U mg^{-1} prot)	GR (U mg^{-1} prot)	Proline (mg g^{-1} DM)	Phenols (mg tannic acid g^{-1} DM)
0 mM NaCl/0 PSU	85[a]	32.74 ± 1.11[c]	0.26 ± 0.1[b]	1.41 ± 0.5[c]	25 ± 2.0[c]	0.05 ± 0.03[d]	0.33 ± 0.03[b]	237.9 ± 5.0[c]
150 mM NaCl/8.8 PSU	85[a]	44.51 ± 3.76[b]	0.29 ± 0.2[b]	1.74 ± 0.4[c]	24 ± 2.1[c]	0.11 ± 0.02[c]	0.59 ± 0.02[a]	445.9 ± 4.0[b]
300 mM NaCl/17.5 PSU	85[a]	47.93 ± 3.11[b]	0.56 ± 0.3[b]	2.52 ± 0.2[b]	29 ± 2.5[b]	0.16 ± 0.02[b]	0.62 ± 0.03[a]	437.9 ± 4.8[b]
600 mM NaCl/35.1 PSU	50[b]	58.96 ± 4.21[a]	1.1 ± 0.2[a]	3.16 ± 0.3[a]	37 ± 3.1[a]	0.20 ± 0.01[a]	0.61 ± 0.02[a]	675.3 ± 5.5[a]

Table 3. Total antioxidant capacity, proline content and activities of guaiacol peroxidase, CAT, APX and glutathione reductase enzymes in 7-day-old seedlings of *T. pannonicum* germinated under different NaCl treatments. Values are means ± SE ($n = 4$). Different letters in the same column indicate significant differences $P \leq 0.01$. AAE, ascorbic acid equivalents.

NaCl treatments	Germination percentage (%)	Total antioxidant capacity (AAE g^{-1} DM)	POX (U mg^{-1} prot)	APX (U mg^{-1} prot)	CAT (U mg^{-1} prot)	GR (U mg^{-1} prot)	Proline (mg g^{-1} DM)	Phenols (mg tannic acid g^{-1} DM)
0 mM NaCl/0 PSU	91 ± 2.1[a]	18.15 ± 1.61[b]	0.29 ± 0.21[b]	1.11 ± 0.4[b]	20 ± 2.2[c]	0.12 ± 0.02[c]	0.41 ± 0.14[b]	237.9 ± 5.0[c]
150 mM NaCl/8.8 PSU	88 ± 3.3[a]	23.33 ± 3.76[b]	0.67 ± 0.33[b]	2.04 ± 0.6[b]	31 ± 3.5[b]	0.27 ± 0.01[b]	0.60 ± 0.19[b]	345.9 ± 6.0[b]
300 mM NaCl/17.5 PSU	50[b]	39.44 ± 3.11[a]	1.68 ± 0.41[a]	3.99 ± 0.4[a]	53 ± 4.1[a]	0.43 ± 0.01[a]	1.09 ± 0.21[a]	437.9 ± 8.0[a]
600 mM NaCl/35.1 PSU	0	0	0	0	0	0	0	0

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antioxidant mechanisms have adjusted or 'resisted' (*sensu* Kranner *et al.* 2010) to the increasing stress. For other species, the antioxidant capacity decreased after 5 weeks salt exposure (Fig. 3), suggesting that increased exposure time to salinity causes failure in these protective mechanisms, which in turn leads to a loss of vigour and eventually death of the organism (Kranner *et al.* 2010; Kranner and Seal 2013). According to the triphasic stress concept of Kranner *et al.* (2010), the activation of protection and repair machinery, which includes the antioxidant defence system, occurs soon after stress is perceived at the cellular level (e.g. by changes in osmotic potential or ionic homoeostasis) and could explain the more noticeable increase in antioxidant capacity under the shorter 24-h salinity exposure period in *L. latifolium.*

Whereas 4- and 5-week-old plants of *T. pannonicum* had little change in total antioxidant, 7-day-old seedlings did (Table 3) in addition to seedlings of *S. europaea.* Cultivated for 1 week, increasing salinity was associated with a higher antioxidant capacity, particularly with respect to proline and total phenols, and the ROS-scavenging enzyme activities. Proline is an osmoprotectant and a low-molecular-weight chaperone, and can reduce the inhibitory effects of ions on enzyme activity, increase the thermal stability of enzymes and prevent the dissociation of enzyme complexes such as the oxygen-evolving complex of PSII (Hasegawa *et al.* 2000). Furthermore, the enzymes required for pathway extensions that lead to these osmolytes are often induced following salt and drought stresses (Hasegawa *et al.* 2000; Hong Bo *et al.* 2006). Many comparative studies using salt-tolerant and sensitive genotypes have correlated higher salt tolerance to an increase in the activity of antioxidant enzymes (Crowe *et al.* 1984; Ashraf 1994; Ashraf and Fatima 1995; Garcia *et al.* 1997; Hounsa *et al.* 1998). Overexpression of some antioxidant enzymes has been reported to improve salt tolerance (Singer and Lindquist 1998; Goddijn and van Dun 1999).

Other environmental factors such as light intensity should be considered. The mangrove *B. cylindrica* did contain higher phenols after salinity exposure but not flavonoid contents despite the literature reporting that mangroves use phenols and tannins as a UV protection screen (Moorthy and Kathiresan 1997). It is likely that higher light intensities in parallel with higher salt concentrations are necessary to raise the antioxidant capacity in this mangrove species. *Tripolium pannonicum* showed a maximum of carotenoid and chlorophyll contents at 331 mM NaCl/22.5 PSU. If high concentrations of photosynthetic pigments are taken as an indication for high photosynthetic activity and consequently for increased growth and biomass production, higher salinities were favourable in comparison to lower salt

concentration. However, in the case of *T. pannonicum* a lower yield in biomass production was correlated with increasing salinity. The gain in fresh weight biomass per plant during 5 weeks was 51.4 ± 12.8 g at 220 mM $Na^+ + Cl^-$/15 PSU, 27.5 ± 5.6 g at 331 mM $Na^+ + Cl^-$/22.5 PSU and 14.6 ± 2.3 g at 442 mM $Na^+ + Cl^-$/30 PSU.

The manipulation of cultivation conditions to increase antioxidant capacity is only desirable if plant growth does not decline. For example, treatment of *L. latifolium* with 15 PSU salinity (equivalent to 220 mM $Na^+ + Cl^-$) resulted in the highest biomass production among the treatments (data not shown). Previously, plants of *S. maritima* with higher antioxidant capacity induced during saline flooding were associated with a biomass yield penalty in plants grown in both the natural environment and glasshouse (Alhdad *et al.* 2013). Furthermore, an increase in phenols and flavonoids was observed in *Nitraria retusa* and *A. halimus* with increasing salinity but this was associated with decreased growth at concentrations of NaCl between 400 mM/23.4 PSU and 800 mM/46.8 PSU (Boughalleb and Denden 2011). A balance is therefore needed so that the antioxidant capacity is enhanced from salinity treatment and growth is maintained. The risk of cultivating halophytes under saline conditions too distant from the optimum environment is that energy resources may be directed into additional energy-demanding stress-response mechanisms, such as ion transport (Flowers and Colmer 2008).

Conclusion

This study has directly shown that it is possible to manipulate the antioxidant capacity of plants and seedlings by altering the saline growing environment. The length of time cultivated under saline conditions and the developmental stage may influence the metabolite levels. Seedlings of the obligate halophyte *S. europaea* are good candidates for antioxidant manipulation particularly as these plants are already established in the market as desirable edible crops. Mature plants of *L. latifolium* exposed to short-term salinity may also be a good candidate, especially as a source for AA. Among the species studied, the total antioxidant capacity in the halophytes *T. pannonicum*, *P. coronopus* and *L. latifolium* cultivated under moderate salt stress is higher than fruits and vegetables generally sold in the market (Ninfali *et al.* 2005), demonstrating the potential of these halophytes as functional foods or nutraceuticals.

Sources of Funding

Contributions by the Authors

C.B., N.B.W., A.K.B. and E.M.W. conducted the experimental work and analysed the data. C.E.S., A.M. and J.P. analysed the data and wrote the manuscript.

Conflicts of Interest Statement

None declared.

Acknowledgements

Supporting Information

The following Supporting Information is available in the online version of this article –

Table S1. Mean molarity values from eight commercially available sea salts calculated for three different salinity concentrations. Data from Atkinson and Bingman (1997).

Table S2. Mean values and SD ($n = 4$) for *L. latifolium* fresh matter (FM). See Fig. 1 for further details. Different letters at the same time point indicate significant differences ($P \leq 0.05$). All pairwise multiple comparison procedures (Holm-Sidak method) were applied. AA, ascorbic acid; CE, catechin equivalents; DHA, dehydroascorbic acid; GAE, gallic acid equivalents; TAA, total ascorbic acid; TE, trolox equivalents.

Table S3. Mean values and SD ($n = 4$) for *L. latifolium* dry matter (DM). See Fig. 1 for further details. Different letters at the same time point indicate significant differences ($P \leq 0.05$). All pairwise multiple comparison procedure (Holm-Sidak method) was applied. AA, ascorbic acid; CE, catechin equivalents; DHA, dehydroascorbic acid; GAE, gallic acid equivalents; TAA, total ascorbic acid; TE, trolox equivalents.

Table S4. Glutathione and cysteine concentrations of different species corresponding to different salinities. The asterisk marks the start values of younger plants [*1]4 weeks, [*2]up to 7 weeks old. These plants were harvested just after the adaptation to 220 mM Na^+ + Cl^-/15 PSU.

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Supporting Information

File 1. Mean molarity values from eight commercially available sea salts calculated for three different salinity concentrations. Data from Atkinson and Bingman (1997).

Major cations (mM)	15 PSU	22.5 PSU	30 PSU
Na^+	199.9	299.9	399.9
K^+	4.184	6.276	8.368
Mg^{2+}	22.13	33.19	44.25
Ca^{2+}	4.184	6.276	8.368
Sr^+	0.053	0.080	0.106
Major anions (mM)			
Cl^-	226.4	339.6	452.8
SO_4^{2-}	10.71	16.07	21.43
Nutrients (µM)			
PO_4-P	0.219	0.328	0.437
NO_3-N	2.002	3.003	4.004
NH_4-N	2.890	4.335	5.780

PSU	mM Na+ + Cl-	Time point	ORAC (μmol TE g⁻¹ FM)	AA (mg 100 g⁻¹ FM)	DHA (mg 100 g⁻¹ FM)	TAA (mg 100 g⁻¹ FM)	Total phenols (mg GAE g⁻¹ FM)	Total flavonoids (mg CE g⁻¹ FM)
0	0	0	54.44 ±31.24[a]	36.53 ±5.06[a]	120.7 ±22.33[a]	157.2 ±20.92[a]	2.129 ±0.506[a]	0.843 ±0.220[a]
0	0	2	77.86 ±16.66[a]	39.05 ±7.81[a]	134.1 ±20.06[a]	173.2 ±27.05[a]	2.259 ±0.459[a]	0.877 ±0.164[a]
0	0	4	42.66 ±8.29[a]	39.35 ±3.65[a]	160.8 ±44.72[a]	200.1 ±45.34[a]	2.085 ±0.149[a]	0.827 ±0.048[a]
0	0	8	53.92 ±18.35[a]	38.71 ±2.41[a]	186.0 ±23.10[a]	224.7 ±24.47[ac]	1.876 ±0.243[a]	0.654 ±0.243[a]
0	0	24	60.78 ±24.44[a]	26.23 ±4.30[a]	158.4 ±18.83[a]	184.6 ±21.46[a]	1.908 ±0.338[a]	0.900 ±0.106[a]
15	220	0	54.44 ±31.24[a]	36.53 ±5.06[a]	120.7 ±22.33[a]	157.2 ±20.92[a]	2.129 ±0.506[a]	0.843 ±0.220[a]
15	220	2	47.31 ±21.12[a]	43.13 ±11.39[a]	133.2 ±36.84[a]	176.3 ±25.84[a]	2.351 ±0.354[a]	0.847 ±0.120[a]
15	220	4	74.41 ±30.20[a]	45.56 ±13.12[a]	154.7 ±50.77[a]	200.3 ±45.81[a]	2.371 ±0.617[a]	0.687 ±0.222[a]
15	220	8	48.25 ±17.36[a]	35.03 ±8.73[a]	179.7 ±23.66[a]	214.7 ±22.45[ad]	1.888 ±0.087[a]	0.641 ±0.235[a]
15	220	24	67.73 ±20.99[a]	30.37 ±3.97[a]	177.3 ±7.08[a]	207.7 ±10.39 [a]	2.053 ±0.084[a]	0.929 ±0.042[a]
22.5	331	0	54.44 ±31.24[a]	36.53 ±5.06[a]	120.7 ±22.33[a]	157.2 ±20.92[a]	2.129 ±0.506[a]	0.843 ±0.220[a]
22.5	331	2	68.95 ±21.13[a]	52.11 ±5.78[a]	181.1 ±51.45[a]	233.3 ±53.61[a]	2.733 ±0.191[a]	1.004 ±0.051[a]
22.5	331	4	84.54 ±21.88[a]	53.21 ±4.98[a]	258.4 ±26.99[b]	311.6 ±22.94[b]	2.661 ±0.129[a]	0.891 ±0.357[a]
22.5	331	8	75.15 ±22.41[a]	31.98 ±2.04[a]	258.4 ±20.51[b]	290.4 ±21.70[bc]	2.533 ±0.199[b]	0.857 ±0.397[a]
22.5	331	24	92.28 ±24.94[a]	49.14 ±21.36[b]	262.1 ±42.09[b]	311.2 ±58.49[b]	2.686 ±0.229[b]	1.147 ±0.066[ab]
30	442	0	54.44 ±31.24[a]	36.53 ±5.06[a]	120.7 ±22.33[a]	157.2 ±20.92[a]	2.129 ±0.506[a]	0.843 ±0.220[a]
30	442	2	54.58 ±24.62[a]	50.09 ±5.25[a]	169.4 ±40.09[a]	219.5 ±38.75[a]	2.656 ±0.208[a]	1.034 ±0.055[a]
30	442	4	87.48 ±17.04[a]	51.40 ±5.41[a]	284.3 ±27.02[b]	335.7 ±28.81[b]	2.739 ±0.246[b]	0.611 ±0.070[a]
30	442	8	90.25 ±30.59[a]	39.81 ±10.21[a]	247.8 ±20.69[b]	287.6 ±29.13[bc]	2.757 ±0.357[b]	1.231 ±0.160[b]
30	442	24	107.57 ±29.20[a]	121.44 ±25.62[c]	335.9 ±82.73[c]	457.3 ±87.25[c]	3.091 ±0.350[b]	1.321 ±0.132[b]

File 3. Mean values and SD (n=4) for *L. latifolium* dry matter (DM). See Figure 1 for further details. Different letters at the same time point indicate significant differences (P≤0.05). All Pairwise Multiple Comparison Procedure (Holm Sidak Method) was applied. Abbreviations: Ascorbic acid, AA; catechin equivalents, CE; dehydroascorbic acid, DHA; gallic acid equivalents, GAE; total ascorbic acid, TAA; trolox equivalents, TE.

PSU	mM Na$^+$ + Cl$^-$	Time point	ORAC (µmol TE g^{-1} DM)	AA (mg 100 g^{-1} DM)	DHA (mg 100 g^{-1} DM)	TAA (mg 100 g^{-1} DM)	Total phenols (mg GAE g^{-1} DM)	Total flavonoids (mg CE g^{-1} DM)
0	0	0	419.3 ±250.8[a]	282.9 ±59.9[a]	944 ±281.1[a]	1227 ±312.4[a]	16.55 ±4.882[a]	6.534 ±1.999[a]
0	0	2	587.9 ±118.6[a]	294 ±51.0[a]	1010 ±88.2[a]	1304 ±133.7[a]	16.92 ±2.046[a]	6.566 ±0.474[a]
0	0	4	305.5 ±71.8[a]	280.2 ±31.1[a]	1136 ±288.1[a]	1416 ±285.3[a]	14.81 ±0.564[a]	5.876 ±0.223[a]
0	0	8	385.2 ±126.8[a]	277.7 ±21.8[a]	1327 ±77.8[a]	1604 ±76.5[a]	13.37 ±0.490[a]	4.742 ±1.882[a]
0	0	24	463.9 ±161.1[a]	204.3 ±32.2[a]	1229 ±29.2[a]	1434 ±52.1[a]	14.76 ±1.395[a]	7.001 ±0.551[a]
15	220	0	419.3 ±250.8[a]	282.9 ±59.9[a]	944 ±281.1[a]	1227 ±312.4[a]	16.55 ±4.882[a]	6.534 ±1.999[a]
15	220	2	334.3 ±170.0[a]	302.8 ±100.0[a]	916 ±231.2[a]	1219 ±141.2[a]	16.36 ±2.860[a]	5.878 ±0.856[a]
15	220	4	482 ±166.4[a]	293.2 ±55.2[a]	1023 ±364.6[a]	1317 ±332.4[a]	15.3 ±2.558[a]	4.485 ±1.429[ab]
15	220	8	340.8 ±115.7[a]	248.1 ±55.4[a]	1281 ±177.0[a]	1529 ±149.7[a]	13.44 ±0.277[a]	4.569 ±1.695[a]
15	220	24	470.8 ±124.6[a]	215.4 ±39.2[a]	1252 ±109.4[a]	1468 ±147.3 [a]	14.47 ±0.504[a]	6.551 ±0.398[a]
22.5	331	0	419.3 ±250.8[a]	282.9 ±59.9[a]	944 ±281.1[a]	1227 ±312.4[a]	16.55 ±4.882[a]	6.534 ±1.999[a]
22.5	331	2	393.5 ±131.2[a]	295 ±36.2[a]	1024 ±294.5[a]	1319 ±308.8[a]	15.48 ±1.387[a]	5.681 ±0.328[a]
22.5	331	4	511.6 ±117.8[a]	330.5 ±74.4[a]	1577 ±148.2[b]	1908 ±197.6[b]	16.33 ±1.870[a]	5.499 ±2.482[ab]
22.5	331	8	456.3 ±128.8[a]	195.7 ±18.4[a]	1575 ±52.0[a]	1771 ±67.7[a]	15.44 ±0.387[a]	5.228 ±2.418[a]
22.5	331	24	561 ±125.0[a]	304 ±143.5[a]	1610 ±277.3[ab]	1914 ±398.5[b]	16.44 ±0.288[a]	7.058 ±0.701[a]
30	442	0	419.3 ±250.8[a]	282.9 ±59.9[a]	944 ±281.1[a]	1227 ±312.4[a]	16.55 ±4.882[a]	6.534 ±1.999[a]
30	442	2	307 ±137.0[a]	282.6 ±32.0[a]	956 ±233.4[a]	1239 ±230.1[a]	14.96 ±1.044[a]	5.825 ±0.291[a]
30	442	4	432.4 ±80.6[a]	254.2 ±23.0[a]	1406 ±123.4[ab]	1660 ±126.3[ab]	13.55 ±1.060[a]	3.022 ±0.307[b]
30	442	8	481.9 ±148.2[a]	212.6 ±45.9[a]	1332 ±61.2[a]	1544 ±86.9[a]	14.79 ±1.236[a]	6.601 ±0.558[a]
30	442	24	571 ±249.0[a]	654.6 ±307.2[b]	1693 ±199.3[b]	2348 ±483.3[c]	15.96 ±3.127[a]	6.815 ±1.202[a]

File 4. Glutathione and cysteine concentrations of different species corresponding to different salinities. The asterisk marks the start values of younger plants *[1] 4 weeks, *[2] up to 7 weeks old. These plants were harvested just after the adaptation to 220 mM Na^+ + Cl^-/15 PSU.

Species	PSU	mM Na^+ + Cl^-	Glutathione (nmol g^{-1} FM)	Cysteine (nmol g^{-1} FM)
T. pannonicum*[1]	15*[1]	220*[1]	29.5±11.38	1.40±0.29
T. pannonicum	15	220	55.6±19.11	1.23±0.13
T. pannonicum	22.5	331	58.8±17.19	1.26±0.14
T. pannonicum	30	442	80.4±29.62	1.16±0.07
T. pannonicum*[2]	15*[2]	220*[2]	18.5	1.44
T. pannonicum	15	220	43.8±9.16	1.79±0.64
S. dolichostachya*[2]	15*[2]	220*[2]	48.5	2.06
S. dolichostachya	15	220	42.2±18.47	1.72±0.27
P. coronopus*[2]	15*[2]	220*[2]	28.5	1.64
P. coronopus	15	220	23.1±2.97	0.76±0.15
L. latifolium*[2]	15*[2]	220*[2]	14.0	2.50
L. latifolium	15	220	169.1±52.37	7.63±0.92
A. portulacoides*[2]	15*[2]	220*[2]	208.1	16.96
A. portulacoides	15	220	126.5±4.93	15.85±2.93
A. halimus*[2]	15*[2]	220*[2]	278.2	24.45
A. halimus	15	220	155.9±12.91	13.14±2.39
B. cylindrica	15	220	122.5±36.80	4.28±2.16
B. cylindrica	30	331	142.1±47.43	3.73±1.26

Chapter 3

Salinity Influences Single Glucosinolate Content in the Halophyte *Lepidium latifolium*

Christian Boestfleisch, Johann Hornbacher, Annekathrin Rumlow, and Jutta Papenbrock

Abstract   The influence of salinity on the biosynthesis of secondary metabolites with a focus on single glucosinolates (GSLs) was investigated in *Lepidium latifolium* L., which is a plant species rich in antioxidants. Mature plants were subjected to 0, 15, 22.5, and 35 Practical Salinity Units (PSU) for 1–4 weeks. While phenols, flavonoids, and the oxygen radical absorbance capacity (ORAC) increased with increasing salinity, the ascorbate concentration did not follow a specific pattern. The concentration of single GSLs was influenced by salinity in different ways: While the concentration of aliphatic GSLs like glucoiberin and sinigrin increased, the concentration of aromatic GSLs such as glucobrassicin decreased under salinity stress. Salinity increased the total GSL concentration significantly with sinigrin being the major contributing GSL. The exact molecular role of the different GSLs in abiotic stress defense needs further analysis.

The halophyte *Lepidium latifolium* L. belongs to the Brassicaceae family, known for their high abundance of glucosinolates (GSLs). The role of GSLs and their break-down products under biotic stress, especially their defense function against herbivores, insects and pathogens, is well known: upon tissue damage myrosinase hydrolyzes GSLs, releasing thiocyanates, isothiocyanates and nitriles (Agrawal and Kurashige 2003; Hopkins et al. 2009; Manici et al. 1997; Rask et al. 2000; Tierens et al. 2001). Stress caused by abiotic factors like drought has different effects on GSL composition and content. In several studies (Mewis et al. 2012; Radovich et al. 2005; Schreiner et al. 2009; Tong et al. 2014) either an increase of aliphatic GSLs under drought stress or a decrease, no effect or a less pronounced increase of aromatic including (indolic) GSLs in *Brassica* species and in *Arabidopsis thaliana* L. was shown. Other studies of GSLs in drought-stressed *Brassica* species showed a reduction or insignificant changes in the GSL content (Khan et al. 2010; Robbins et al. 2005). In both studies the content of indolic GSLs was predominant.

Only a few studies exist on the effect of salinity on GSLs. In two *Brassica napus* cultivars the total GSL concentration increased under salinity (Qasim et al. 2003).

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Brassica oleracea showed an increased total GSL content under the influence of salt (López-Berenguer et al. 2008, 2009). In these studies measurements of total GSL contents were performed, while other studies also analyzed single GSLs. In radish sprouts, for example, one aliphatic GSL was predominant and its concentration increased by increased salinity (Yuan et al. 2010). Zaghdoud et al. (2012) found that the total GSL content was not altered by salinity in a *B. oleracea* cultivar, while another cultivar showed a decrease in the total GSL content. This cultivar showed a decrease in indolic GSLs but an increase in aliphatic GSLs. Bloem et al. (2014) revealed no significant changes in contents of the aromatic GSL glucotropaeolin in salt stressed *Tropaeolum majus*. All studies previously mentioned applied low salinity to glycophytes, while in this study high salinities to a halophyte species were applied.

Several antioxidants are altered in their accumulation by salinity, often enhanced under stress conditions, like the non-enzymatic metabolites ascorbate, glutathione, carotenoids, tocopherols, and phenolics. These antioxidants serve, besides the enzymatic antioxidants, to scavenge or detoxify reactive oxygen species (ROS) induced by stresses like salinity (Noctor and Foyer 1998; Sharma et al. 2010).

In this study the salt-tolerant species *Lepidium latifolium* was investigated as we were interested in the GSL concentration in relation to salinity and to identify the time point when the GSL content was highest after beginning of the salt treatment. Sometimes classified as a halophyte, *L. latifolium* is native to southern Europe and Asia (Kaur et al. 2013; Zhao et al. 2010). Its tolerance to salt allows it to grow along the coastline but it is also found in the cold Himalayan region (Gupta et al. 2013; Kaur et al. 2013). It has been identified as an invasive species in North America (Francis and Warwick 2007). Fortunately, the GSL spectrum of *L. latifolium* was analyzed previously indicating eight GSLs with sinigrin being the dominant GSL (Kaur et al. 2013). The use as a vegetable and the medicinal utilization were also previously described (Kaur et al. 2013; Navarro et al. 1994). The influence of salinity on biomass, oxygen radical absorbance capacity (ORAC), ascorbate, phenolics and flavonoids, and GSL spectrum and contents of *L. latifolium* influenced by salt were analyzed in this study. The concentration of aromatic GSLs decreased or showed no clear reaction, whereas indolic GSLs decreased and aliphatic GSLs increased or were indifferent towards salinity stress. All other metabolites except ascorbate that showed varying responses were positively affected by salinity. Thus ORAC increased accordingly. The duration of stress application had an influence on the concentration of some of the metabolites analyzed.

Lepidium latifolium seeds (Rühlemann's Kräuter und Duftpflanzen, Horstedt, Germany) were germinated on propagation soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany). After a period of 5 weeks, plants were transplanted to sand of 0–2 mm grain size (Hornbach, Hannover, Germany). During the nursing time, the plants were watered with modified Hoagland solution (Epstein 1972). Mature plants were grown under greenhouse conditions at around 22 °C. Sodium vapor lamps (SON-T Agro 400, Philips, Amsterdam, Netherlands) served as an additional light source, providing a photoperiod of 14 h light and a quantum fluence rate of 350 $\mu mol\ m^{-2}\ s^{-2}$. Finally, the plants were transferred to aerated containers

with 13.5 L solution containing 3.57 mM $NaNO_3$, 316 mM $H_2NaPO_4 \times H_2O$ and 23.5 mM Fe-EDDHA (5.7%) (Duchefa, Haarlem, Netherlands). After 1 week of acclimatization to the hydroponic culture, the sea salt mixture (Seequasal GmbH, Münster, Germany) was added stepwise by an increase of 0.75 PSU every day to the desired concentrations of 0, 15, 22.5, and 30 PSU. Four plants of each salinity treatment were harvested at the time the cultivation solutions reached their final concentration (0 weeks) and 1, 2, 3 and 4 weeks after induction. Whole plants (shoot including leaves) were frozen in liquid nitrogen and stored at $-80\ ^{\circ}$C for further analysis. The metabolite extraction and the determination of total phenols, total flavonoids, oxygen radical absorbance capacity (ORAC) and ascorbic acid were performed as described by Boestfleisch et al. (2014). For the determination of GSLs, frozen, ground leaf material was freeze-dried. One milliliter of 80% methanol was added to 10 mg dried plant material. The sample was placed on a shaker until homogenization and then centrifuged for 5 min at 13,000 g. The pellet was re-extracted in the same way and the supernatants were combined. The supernatant was loaded onto a column (QIAGEN GmbH, Hilden, Germany) containing 2 ml of a 5% (w/v) suspension of DEAE Sephadex A25 (Sigma-Aldrich, Taufkirchen, Germany) in 0.5 M acetic acid (pH 5). The column was then flushed with 10 ml of HPLC-grade H_2O and 4 ml of 0.02 M acetic acid (pH 5). For desulfating the GSLs overnight at room temperature, 50 µl of sulfatase (Sigma-Aldrich) solution (Thies 1979) was added to 450 µl 0.02 M acetic acid (pH 5) and loaded onto the column as well. Desulfated GSLs were eluted 3 times with 2 ml HPLC-H_2O. Samples were dried in a vacuum centrifuge overnight and resolved in 300 ml HPLC-H_2O. Analysis was performed with HPLC system (Knauer, Berlin, Germany) equipped with an Ultra AQ C-18 column (150 x 4.6 mm, 5 µm particle size) (Restek GmbH, Bad Homburg, Germany). For measuring the samples, a volume of 50 µl was injected. A water (solvent A)-acetonitrile (solvent B) gradient at a flow rate of 1 ml min^{-1} at room temperature was used. Following gradient was applied: 100% A (6 min), 100–70% A (27 min), 70–40% A (0.1 min), 4.9 min 40% A, 40–100% A (0.1 min), and 19.9 min 100% A. Eluents were monitored at a wavelength of 229 nm. Identification of desulfated GSLs was achieved by comparing the retention time with commercially available GSLs (PhytoLab GmbH and Co. KG, Vestenbergsgreuth, Germany) that were treated the same way as the samples. By means of standard curves of these references, desulfated GSLs were quantified. Integration of peaks and elaboration of data were performed using ChromGate Client/Server Version 3.3.1 (Knauer, Berlin, Germany). GSLs were calculated as the mean of four biological replicates with the standard deviation of these four biological replicates. The total amount of GSLs was calculated as the sum of all individual GSLs. To asses precision and reproducibility of GLS analysis, four technical replicates were prepared by measuring GSL contents in each plant sample four times. The standard deviation relative to the individual GSL content was calculated in these replicates. Values were tested for significance ($p = 0.05$) with an analysis of variance (ANOVA) using R (version 3.2.2), displaying significant differences between metabolites corresponding to PSU values at different harvest times.

At the starting point there were no significant differences in biomass production of *L. latifolium* between the different salinities, but after 1 week there was a significant difference between plants grown at 22.5 and 30 PSU and plants grown at 15 PSU, which produced the highest biomass (Fig. 1a). The difference in biomass at the different salinities increased towards week 2 where the highest increase in biomass was observed in plants grown at 0 PSU. This effect was significantly higher than in plants grown at 22.5 and 30 PSU. Plant growth decreased with increasing salinity at this point of experimentation. After 3 and 4 weeks the increase in biomass reached a maximum for plants grown at 15 PSU, followed by 0 PSU. Plants grown at 30 PSU showed the lowest increase in biomass. The ORAC of *L. latifolium* grown at salt stress (30 PSU) was highest at all sampling dates compared to the other levels of salinity (Fig. 1b). The ORAC maximum was determined at week 0 in plants grown at 30 PSU and decreased slowly towards the 4th week. At week 0 and week 1, ORAC values in plants grown at 30 PSU were significantly higher compared to plants grown at 15 and 0 PSU. This difference decreased, but ORAC values in plants grown at 30 PSU were still significantly higher compared to plants grown at 15 PSU. In the 3rd week plants grown at 30 PSU had again a significantly higher concentration compared to lower levels of salinity. In the 4th week there was no significant difference in the ORAC. The total phenol concentration of plants grown at 30 PSU was the highest during the time of the experiment (Fig. 1c). It was significantly higher than the concentration of plants grown at 0 PSU at all sampling dates with the exception of week 3. In most cases plants grown at 0 PSU had the lowest total phenol concentration followed by 15 PSU. Higher salinity concentrations yielded higher total phenol concentrations. This effect was significant over time except for the 3rd week when plants grown at 0 and 30 PSU had higher total phenol concentrations than plants grown at 15 and 22.5 PSU.

Plants grown at higher salinities of 30 and 22.5 PSU produced higher total flavonoid values than plants grown at 0 and 15 PSU (Fig. 1d). There was a significant difference between plants grown at 30 and 0 PSU at all sampling dates. The differences in total flavonoid values between plants grown in high and low salinities were greatest at the start of experimentation and after 1 week, and became smaller after 3 and 4 weeks.

Small differences in the ascorbate concentration were detected at the beginning of the experiment but plants grown at 0 PSU had a lower concentration compared to plants grown at other salinities (Fig. 1e). In the 1st week plants grown at 0 and 15 PSU had insignificantly lower ascorbate values than plants grown at 22.5 and 30 PSU. Plants grown at 15 and 30 PSU had lower ascorbate values compared to plants grown at 0 and 22.5 PSU in the 2nd week. In the 3rd week there was a significant decline in the ascorbate concentration from plants grown at 0 to plants grown at 30 PSU. In the 4th week plants grown at 0 PSU showed significantly lower values than plants grown at all other salinities. The highest ascorbate concentration in this week was detected in plants grown at 22.5 PSU followed by 15 PSU. The ascorbate content in plants grown at these salinities were significantly higher compared to plants grown at 0 PSU at this point of time.
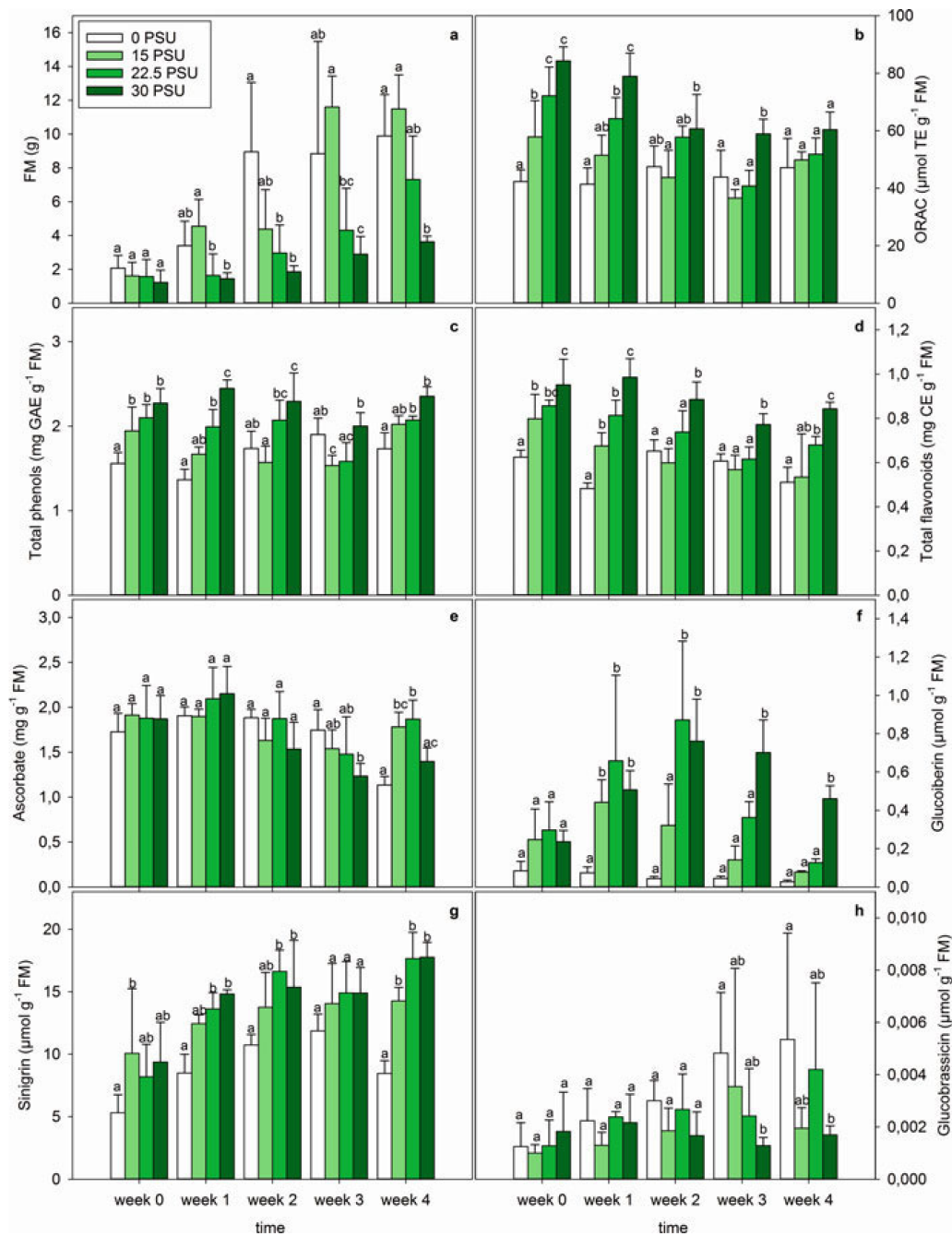
Fig. 1 Biomass production (**a**), oxygen radical absorbance capacity (ORAC, **b**) and contents of total phenols (**c**), total flavonoids (**d**), ascorbate (**e**), glucoiberin (**f**), sinigrin (**g**), glucobrassicin (**h**) of *L. latifolium* plants (n = 4). Six-week-old plants were placed into aerated containers and after an acclimatization time of 1 week the salinity was increased by 0.75 PSU every day to the concentrations of 0 PSU, 15 PSU, 22.5 PSU and 30 PSU. Fresh material was harvested upon reaching the targeted salinities (week 0) followed by a weekly interval. Different letters indicate significant differences (p = 0.05) between different PSU values within one point of time

Salinity caused diverging effects on individual GSLs. There are three ways how the GSL concentration was influenced by salinity. Figure 1f shows the first way, an increase in the concentration of glucoiberin. At the beginning of the experiment there was a trend for a higher glucoiberin concentration in plants grown at 22.5 PSU, but this effect was not significant. This trend continued in the 1st week when plants grown at all salinities had a significantly higher concentration compared to plants grown at 0 PSU. In the 2nd week plants grown at 22.5 and 30 PSU had a significantly higher glucoiberin concentration than plants grown at 0 PSU. One week later the significant maximum in glucoiberin concentration was found in plants grown at 0 PSU. This trend continued in the 4th week while all concentrations decreased.

The temporal pattern of the mean sinigrin concentration (Fig. 1g) was similar to the pattern of glucoiberin. A significant difference in the sinigrin concentration in plants grown at 0 and 15 PSU could be detected in week 0. However, after 1 week a trend was visible in such way that plants grown at higher salinities showed higher sinigrin concentrations. There was a significant difference in the sinigrin concentration between plants grown at 30 PSU and plants grown at 0 PSU in week 1 and 4. In week 2, plants grown at 22.5 PSU reflected a higher and significant (towards 0 PSU) sinigrin concentration. In week 3, plants grown at 22.5 and 30 PSU showed both high sinigrin concentrations, which differed not significantly from that of the 0 PSU treatment.

The second way how GSLs were influenced by salt stress can shown exemplary for glucobrassicin (Fig. 1h). There was no significant difference at the beginning of the experiment until the 2nd week, but a trend was observed after 2 weeks. Plants grown at lower salinities accumulated more glucobrassicin, except for plants grown at 15 PSU. After 3 weeks, there was a significant decrease in the glucobrassicin concentration in plants grown at 0 to 30 PSU, which continued towards the 4th week, but plants grown at 15 PSU had the second lowest concentration.

GSL concentrations of other GSLs are shown in Table 1. For the sake of completeness, the values of Fig. 1f–h are also presented in Table 1. The concentration of gluconapin increased with higher salinity towards the 2nd week, and showed the opposite pattern in the 4th week. Salinity decreased the concentration of glucobrassicin and gluconasturtiin. The decline in the GSL concentration from low to high salinity started around the 1st week with gluconasturtiin, but was more distinctive for glucobrassicin. The concentrations of glucocheirolin, glucoraphanin, and glucotropaeolin did not show a clear pattern for a de- or an increase under salinity stress, which represents the third way GSLs were influenced by salt stress. Within week 1 and 2 the maximum of glucotropaeolin was reached in plants grown at 30 PSU but changed to 0 PSU in week 3 and 4. The highest glucocheirolin concentration accumulated in plants grown at 15 PSU in week 2 and 3 and at 22.5 PSU in week 4. With respect to the relatively high standard deviation it is important to keep in mind that each single determination was done with individual plants.

The measurement of GSL contents in technical replicates resulted in standard deviations not higher than 20% with a mean of 8% relative to the individual GSL content (data not shown). In contrast, relative standard deviations of biological

Table 1 Mean GSL concentration (n = 4) in nmol g^{-1} FM for glucocheirolin, glucoraphanin, gluconapin, glucobrassicin, gluconasturtiin and glucoiberin; in µmol g^{-1} FM for sinigrin, glucotropaeolin and the sum of GSL

Time in weeks	PSU	Gluco-cheirolin	Glucoiberin	Sinigrin	Gluco-raphanin	Gluconapin	Gluco-tropaeolin	Gluco-brassicin	Gluco-nasturtiin	Total GSL amount
0	0	0.65 ± 0.47 a	84.1 ± 49.9 a	5.31 ± 1.45 a	15.9 ± 3.50 a	1.45 ± 1.58 a	0.39 ± 0.17 a	1.25 ± 0.91 a	31.5 ± 19.7 a	5.80 ± 1.68 a
	15	0.93 ± 0.81 a	247 ± 160 a	10.1 ± 5.18 b	34.4 ± 18.7 a	2.23 ± 2.23 a	1.14 ± 0.45 a	1.00 ± 0.31 a	72.5 ± 50.3 a	11.6 ± 5.83 b
	22.5	0.70 ± 0.57 a	297 ± 148 a	8.19 ± 2.61 ab	19.8 ± 9.76 a	3.22 ± 3.03 a	0.99 ± 0.34 a	1.28 ± 0.99 a	69.0 ± 50.2 a	9.60 ± 3.14 ab
	30	0.72 ± 0.36 a	236 ± 58.9 a	9.37 ± 3.18 ab	31.6 ± 9.14 a	6.12 ± 7.46 a	1.18 ± 0.24 a	1.83 ± 1.51 a	69.2 ± 29.9 a	10.9 ± 3.49 b
1	0	1.19 ± 0.73 a	73.9 ± 30.8 a	8.50 ± 1.49 a	21.4 ± 12.5 a	8.87 ± 12.5 a	0.99 ± 0.58 a	2.24 ± 1.23 a	227 ± 183 a	9.80 ± 2.23 a
	15	1.02 ± 0.47 a	442 ± 118 b	12.4 ± 0.70 ab	21.6 ± 3.96 a	3.64 ± 4.79 a	0.89 ± 0.22 a	1.30 ± 0.49 a	48.7 ± 13.0 a	13.8 ± 0.97 ab
	22.5	1.17 ± 0.41 a	658 ± 447 b	13.6 ± 1.31 b	34.7 ± 4.88 ab	9.41 ± 5.91 a	1.39 ± 0.30 ab	2.38 ± 0.20 a	94.8 ± 33.7 a	15.8 ± 1.21 b
	30	1.12 ± 0.48 a	507 ± 100 b	14.8 ± 0.33 b	41.2 ± 3.53 b	16.1 ± 13.0 a	2.48 ± 0.97 b	2.16 ± 1.08 a	83.9 ± 12.1 a	18.0 ± 0.95 b
2	0	1.15 ± 0.22 a	43.5 ± 11.0 a	10.7 ± 0.84 a	37.9 ± 6.13 a	3.01 ± 0.56 a	0.83 ± 0.25 a	3.00 ± 0.77 a	207 ± 41.1 a	11.9 ± 0.89 a
	15	1.23 ± 0.40 a	321 ± 216 a	13.8 ± 2.78 ab	27.1 ± 11.5 a	5.81 ± 3.37 ab	0.80 ± 0.35 a	1.86 ± 0.87 a	133 ± 117 a	15.1 ± 2.51 ab
	22.5	0.87 ± 0.56 a	871 ± 412 b	16.6 ± 1.70 b	29.9 ± 9.12 a	6.21 ± 5.49 ab	1.04 ± 0.38 a	2.67 ± 1.35 a	55.3 ± 17.6 a	18.6 ± 1.64 b
	30	0.51 ± 0.29 a	761 ± 219 b	15.4 ± 3.74 b	31.6 ± 2.51 a	17.2 ± 12.4 b	1.33 ± 0.26 a	1.67 ± 0.91 a	73.2 ± 19.8 a	17.6 ± 4.01 b

(continued)

Table 1 (continued)

Time in weeks	PSU	Gluco-cheirolin	Glucoiberin	Sinigrin	Gluco-raphanin	Gluconapin	Gluco-tropaeolin	Gluco-brassicin	Gluco-nasturtiin	Total GSL amount
3	0	1.01 ± 0.36 a	44.9 ± 12.2 a	11.9 ± 1.34 a	46.8 ± 11.5 a	3.64 ± 1.94 a	1.58 ± 1.51 a	4.82 ± 2.32 a	431 ± 284 a	14.0 ± 1.84 a
	15	1.13 ± 0.70 a	141 ± 73.4 a	14.1 ± 3.21 a	24.9 ± 12.2 b	8.56 ± 11.3 a	0.91 ± 0.84 b	3.54 ± 4.53 ab	279 ± 391 ab	15.4 ± 2.23 a
	22.5	0.85 ± 0.41 a	363 ± 82.4 a	14.9 ± 2.51 a	21.0 ± 8.20 b	6.8 ± 10.4 a	1.08 ± 0.47 b	2.42 ± 1.81 ab	72.3 ± 29.7 b	16.4 ± 2.41 a
	30	0.46 ± 0.24 a	701 ± 170 b	14.9 ± 2.05 a	27.4 ± 8.42 b	3.81 ± 3.61 a	1.01 ± 0.32 b	1.28 ± 0.31 b	53.3 ± 4.73 b	16.7 ± 2.15 a
4	0	0.63 ± 0.29 a	27.1 ± 8.95 a	8.46 ± 1.02 a	23.7 ± 3.98 a	7.63 ± 3.93 a	1.50 ± 0.86 a	5.35 ± 4.07 a	247 ± 116 a	10.3 ± 1.32 a
	15	0.90 ± 0.81 a	78.1 ± 6.46 a	14.3 ± 1.09 b	21.2 ± 9.85 a	4.14 ± 3.52 a	0.32 ± 0.06 a	1.95 ± 0.78 ab	159 ± 5.97 a	14.8 ± 1.14 b
	22.5	1.30 ± 0.89 a	127 ± 19.1 a	17.6 ± 2.10 b	39.8 ± 19.9 a	4.80 ± 5.81 a	0.47 ± 0.16 a	4.20 ± 3.31 ab	176 ± 83.4 a	18.5 ± 2.31 b
	30	0.68 ± 0.60 a	461 ± 67.4 b	17.8 ± 1.18 b	31.0 ± 10.0 a	1.52 ± 0.92 a	0.77 ± 0.17 a	1.70 ± 0.35 b	101 ± 13.5 a	19.1 ± 1.28 b
Time		NS	***	***	NS	.	*	*	*	***
PSU		NS	***	***	NS	NS	*	*	**	***
Time x PSU		NS	***	NS	**	NS	**	NS	NS	NS

Analysis of variance for the influence of time and salinity

NS, *, **, *** non-significant or significant at P ≤ 0.01, 0.05, 0.01, or 0.001, respectively

replicates showed values up to 139% with a mean of 38% (data not shown). Because of the low relative standard deviations of technical replicates compared to the ones of biological replicates, the fluctuations in GSL contents emerging from technical procedures were neglected and the standard deviation of GSL contents in biological replicates was used to calculate significant differences between the treatments.

Lepidium latifolium had an optimal growth at 15 PSU in this experiment, as the gain of biomass was the highest at this salinity condition. This was expected as this plant species is a halophyte. Nevertheless, the salt was added in a short time (4 days) and the plant was still able to survive salinity of 22.5 and 30 PSU. The concentrations of the antioxidants were significantly increased by salinity stress. The ORAC increased at the beginning of the experiment and remained on this level for a week. Total phenols and as part of them the flavonoids were affected strongest 1 week after the start of the experiment. The range of concentrations of the mentioned antioxidants was getting smaller towards the end of the experiment closing the difference between high and low salinity. The ascorbate concentration showed a different pattern in comparison to the other antioxidants. There was no significant difference towards the 3rd week of the experiment. Ascorbate concentration quickly changed in *L. latifolium* within 24 h after the beginning of the salinity treatment (Boestfleisch et al. 2014) and returned to a steady state within 4 days.

If the yield of the antioxidants is calculated (multiplying the antioxidant concentration with the biomass produced), there is only one result for all antioxidants: the increase of biomass exceeded the increase in antioxidants (data not shown).

At the beginning of the experiment only changes in the sinigrin content proved to be significant, but 1 week after the induction of salinity stress the content of four out of eight GSLs reacted significantly to salinity. The maximum of glucoiberin shifted along the timescale with increasing salinity from 15 PSU around week 1 to 30 PSU between week 2 and 3 (Fig. 1e and Table 1). Sinigrin contents showed a larger difference at week 4 compared to the beginning, and gluconapin and glucotropaeolin showed an up- and down-regulation, whereas this was more distinctive in the latter one.

GSLs seemed to react partly different than antioxidants. While ORAC, phenols and flavonoids were positively and highly significant intercorrelated ($r > 0.8$; $p < 0.001$), and all of them showed a positive significant though weak correlation with ascorbate ($r = 0.27–0.38$; $p < 0.05$), GSLs showed ten positive and two negative correlations (Pearson correlation, data not shown). The negative ones were between glucoiberin and glucobrassicin and between glucoiberin and gluconasturtiin. There were some low correlations of mainly aliphatic GSLs with ORAC (glucoiberin: $r = 0.36$; $p < 0.001$, glucotropaeolin: $r = 0.32$; $p < 0.005$ and gluconasturtiin: $r = -0.28$; $p < 0.05$), phenol (glucoiberin, sinigrin, glucoraphanin and glucotropaeolin: $r = 0.31–0.43$; $p < 0.05$), flavonoid (glucoiberin, sinigrin, glucoraphanin and glucotropaeolin, $r = 0.23–0.49$; $p < 0.05$) and ascorbate values (glucocheirolin and glucoraphanin $r = 0.28–0.3$; $p < 0.05$). From the temporal patterns of the GSLs and the correlations between them, they can be classified into

three different groups: an up regulation (aliphatic GSLs), a down regulation (aromatic including indolic GSLs) and an intermediate reaction (aromatic excluding indolic GSLs) under salinity. This up and down, or the different regulation, was previously shown for salinity (Yuan et al. 2010; Zaghdoud et al. 2012) and drought treatments (Mewis et al. 2012; Radovich et al. 2005; Schreiner et al. 2009; Tong et al. 2014). Jensen et al. (1996) gave an explanation for these observations under salinity and drought stress. They showed that GSL synthesis increased when the leaf water potential was less than -1.4 MPa for extended periods. In our study, the VWC (volumetric water content) decreased from 85% to 77% at 30 PSU and from 92 to 83% at 0 PSU (data not shown). However, only aliphatic GSLs had a significant correlation with the VWC. The decrease of glucobrassicin with increasing salinity might also be the reason for its ease of oxidation, as it has a high antioxidant capacity (Cabello-Hurtado et al. 2012). These authors demonstrated a relatively high ORAC value for glucobrassicin, much higher than ascorbic acid. However, in our study the most abundant GSL was sinigrin, which has according to Cabello-Hurtado et al. (2012) a 6–7 times lower ORAC compared to ascorbic acid. Therefore, the total GSL content did not contribute much to the ORAC resulting in a low correlation ($r = 0.33$; $p < 0.01$). Furthermore many of the studies had only one point of time for the measurement of GSLs, but we could show that GSL contents in salt-stressed plants changed over time.

AS already mentioned sinigrin was the most abundant GSL in *L. latifolium*. Therefore, an increase in salinity elevated the total GSL yield, which would be beneficial for herbivore protection under abiotic stress conditions (Agrawal and Kurashige 2003; Hopkins et al. 2009), in addition to the enhanced growth and antioxidant production at 15 PSU. It was suggested that transient allocation and redistribution of some GSLs indicate a role in signaling mechanisms under abiotic stress conditions to induce fast physiological adaptation to unfavorable conditions (del Carmen Martínez-Ballesta et al. 2013). However, the determination of the exact functions of GSLs in reaction to abiotic stress needs further investigation.

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Chapter 4

Boestfleisch, C. & Papenbrock, J. (2017): Changes in secondary metabolites in the halophytic putative crop species *Crithmum maritimum*, *Triglochin maritima* and *Halimione portulacoides* as reaction to mild salt stress, PLoS ONE 12(4): e0176303

DOI: 10.1371/journal.pone.0176303

RESEARCH ARTICLE

Changes in secondary metabolites in the halophytic putative crop species *Crithmum maritimum* L., *Triglochin maritima* L. and *Halimione portulacoides* (L.) Aellen as reaction to mild salinity

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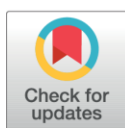
Abstract

It is assumed that salinity enhances the concentration of valuable metabolites in halophytes. The objective was to find a salt concentration and a point in time at which the yield for the valuable metabolites was maximal. Therefore, three different halophyte species were grown under different salinities and harvested over a period from shortly after stress induction up to three weeks. Various reaction patterns were found in the metabolite composition of the analyzed plant material. *Halimione portulacoides* showed a "short term response", indicated by an increase in all metabolites analyzed after a few hours, whereas *Crithmum maritimum* showed a "long term response" through accumulation of proline starting after days. *Triglochin maritima* did not change in metabolite concentration, but like the other plant species the biomass was reduced by salinity. Generally, a higher production in secondary metabolites by higher salinity was outbalanced by a reduction in biomass production. Concentrations of analyzed antioxidants showed a similar reaction and correlated with each other.

Introduction

Soil salinity is, besides drought, a major abiotic stress for plants and crops worldwide [1]. Eleven percent of the irrigated area of land which is 34 Mha is affected by salinity and another 60–80 Mha may be affected to some extent due to waterlogging [2]. With an increasing world population, increased harvest and more agricultural land are needed. Otherwise, land which is not suitable for crop production due to salinity may be used by halophytes to produce food and fodder [3,4]. Salinity causes hyperosmotic and hyperionic stress in plants, and osmolytes are produced for protection against hyperosmotic stress [5]. For the protection against oxidative stress, also caused by salinity, antioxidants are produced [6]. The antioxidants can be divided into two groups, antioxidant enzyme systems and non-enzymatic compounds [7]. Part of the non-enzymatic compounds, the secondary plant metabolites include the (poly)phenols consisting of flavonoids and non-flavonoid polyphenols and are present in many vegetables

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and fruits [8,9]. These antioxidants, among others, are beneficial for human health if consumed [10]. For example, a high intake of (poly)phenols of berries reduces the risk of a cardiovascular disease [11]. Many substances can act as antioxidants whereas the following can be present in plants: vitamin C (ascorbic acid), vitamin E (tocotrienol and tocopherol), carotenoids, (poly)phenols divided in flavonoids and non-flavonoid polyphenols, alkaloids, thiols like glutathione, uric acid and bilirubin [12–14].

As the sum of the abundance and action of many antioxidants, the oxygen radical absorbance capacity (ORAC) can be identified. This determines mainly chain-breaking antioxidants such as (poly)phenols, vitamin C, vitamin E, uric acid and bilirubin [12]. There are two types of ORAC, the hydrophilic ORAC and the lipophilic ORAC. The lipophilic ORAC, determining for example vitamin E and carotenoids, contributes just 5% or often less to the total antioxidant capacity in fruits and vegetables [15,16]. Therefore, often only the hydrophilic ORAC is determined, as in this study.

The three species investigated in this study are already in use for the human diet. *Crithmum maritimum* is consumed by humans since a long time and it is not considered to be toxic if consumed in greater amounts [17,18]. It belongs to the Apiaceae and is a facultative halophyte [19–21]. *Triglochin maritima*, a member of the Juncaginaceae, is edible [22] and an obligate halophyte [23]. *Halimione portulacoides* belongs to the Amaranthaceae and is considered an obligate halophyte [24,25]. It can be consumed raw or cooked [26].

Salinity stress reduces the biomass in vegetable crops, but also increases the secondary metabolite concentration [27,28]. It was previously shown that a manipulation of the antioxidant capacity is possible in certain halophytes [29]. However, the observation time from stress induction to harvest was limited to continuous observation of 24 h (seedlings) or only one harvest after 1, 5 or 8 weeks, for different species, respectively.

The aim of this study was to find the optimal time when the increase of metabolite concentration is still higher than the drawback of reduced growth through salinity. Therefore, plants were harvested after stress induction in the first hours with increasing intervals up to three weeks. So it was possible to determine the exact point of time when antioxidative compounds were produced. It was argued that stress conditions chosen resulting in severe growth reduction are often too harsh. Furthermore experimentally applied stress should not lead to growth reduction of plants [30]. To better understand the mechanisms of salinity, "mild stress conditions" ranging from 0 Practical Salinity Units (PSU is a value derived from conductivity, and is equal to ppt) up to 15 PSU were chosen in this study. Mild stress should not limit the growth of plants significantly compared to severe stress. However, the induced mild stress did not show the anticipated results. There was no point in time at which the concentration of the antioxidants analyzed was higher compared to the gain of biomass. To sum up, the yield of antioxidants was not higher at increased salinity. In *C. maritimum* the osmoprotectant proline had a higher concentration and total amount at a salinity of 15 PSU, despite a lower biomass compared to 0 PSU.

Materials and methods

Plant material and growth conditions

Seeds of *C. maritimum*, *T. maritima*, and *H. portulacoides* obtained from Rühlemann's (Horstedt, Germany) were germinated on propagation soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany), and after 3 to 6 weeks, depending on the season and growth of the species, transplanted to sand (0–2 mm grain size, Hornbach, Hannover, Germany), watered with modified Hoagland solution [31], and finally after 4 to 6 weeks transferred to aerated containers with 13.5 L solution containing the nutrients indicated in S1 Table. After one week, NaCl was added

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Table 1. Times for the different nursing steps of the plant species used in the experiments and their origin are shown.

Species	Sawing time to hydroponic culture in weeks	Hydroponic acclimatisation time in weeks	Salinity induction time in h
C. maritimum	12	2	0, 2, 4, 8, 24, 48, 96, 168, 336, 504
T. maritima	9	1	0, 2, 4, 8, 24, 48, 96, 168, 336, 504
H. portulacoides	7	1	0, 2, 8, 24, 96, 336

to obtain 0 PSU, 5 PSU, 10 PSU and 15 PSU. For detailed information on culture conditions see Table 1.

Mature plants were grown under greenhouse conditions with a minimum temperature of 16.8˚C at night-time and maximum of 27.8˚C at day-time. The average temperature was 19.3˚C. Sodium vapour lamps (SON-T Agro 400, Philips, Amsterdam, Netherlands) served as an additional light source, providing the minimal light flux density (350 µmol m^{-2} s^{-1} for 14 h). Plants were harvested at 0, 2, 4, 8, 24, 48 (2 d), 96 (4 d), 168 (1 week), 336 (2 weeks) and 504 h (3 weeks) after addition of salt, whereas six individuals were harvested at point of time 0 before the addition of salt and 4 individuals per PSU condition were harvested at each other point of time. The complete shoot was taken and individuals were analyzed separately.

Metabolite extraction

Methods for metabolite extraction were described previously in [29].

Total phenols were measured with Folin-Ciocalteu reagent. Gallic acid was used as standard. The protocol was modified for microtiter plates [32].

Total flavonoids were measured based on a published protocol and modified for mitrotitre plates [33]. After the addition of NaNO$_3$, AlCl$_3$ and NaOH flavonoids form a coloured complex which was measured at 510 nm. Catechin hydrate was used as standard.

The oxygen radical absorbance capacity (ORAC) assay is based on published protocols [34,35] with modifications [29]. In addition, a mathematical compensation was conducted for the thermal gradient within the plate, which leads to more precise results. The capacity of the sample to prevent fluorescein from being damaged by radicals from 2,2′-azobis(2-amidino-propane) dihydrochloride was measured and compared to the capacity of 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard.

For the determination of AA, dehydroascorbic acid (DHA) and total ascorbic acid (TAA) published protocols [36–38] were modified as described previously [29]. Furthermore small modifications have been made: Metaphosphoric acid (MPA) instead of trichloroacetic acid (TCA) for the extraction and FeCl$_3$ of a higher quality were used (sublimed grade, ≥99.9% trace metals basis 701122 Sigma-Aldrich) because the prior use of TCA and a lower quality of FeCl$_3$ led to a lower determination rate of AA due to degradation to DHA.

For the determination of proline according to the protocol [39], 50 mg of frozen ground plant material were mixed with 1 ml of 40% ethanol and slowly shaken overnight on an overhead shaker at 4˚C. Samples were centrifuged for 5 min at 18,400 *g*. Then 125 µl of supernatant were mixed with 250 µl of reaction mix. The reaction mix consisted of ninhydrin 1% (w/v) in glacial acetic acid 60% (v/v) and ethanol 20% (v/v). The samples were incubated in a water bath at 95˚C for 20 min. After cooling to room temperature, 100 µl were transferred into a microtiter plate and read at 520 nm. The results were compared to proline standards ranging from 0.1 to 2 mM.

Elemental analysis of plant material

Freshly frozen plant material of three individual plants was dried at 80˚C for 24 h. Dried plant material was analyzed separately by inductively coupled plasma-optical emission spectroscopy

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(ICP-OES) based on a published protocol [40]. Concentrations of elements were calculated referring to fresh matter (FM).

Statistical analysis and evaluation of the data

At each data point four plants (shoots including leaves) were analyzed in triplicate for ORAC, total flavonoids, total phenols, proline and in duplicate for AA. Metabolites were calculated as the mean of four individuals (n = 4). Salinity might have a positive effect on the concentration of some metabolites, but also triggers a lower biomass production. To see whether and when the increase of the concentration outruns the loss of biomass, the total amount (yield) of each metabolite for each point of time was calculated (metabolite concentration multiplied by biomass).

Values were tested for significance with an analysis of variance (ANOVA) using R (version 3.2.2). Significant differences among PSU values were calculated within points of time and among points of time within PSU values. Principal components were calculated in R (version 3.2.2). Pearson correlation was calculated in SigmaPlot (version 12.5).

Results

The main aim of this study was to find suitable conditions to increase the metabolite concentration while maintaining a high biomass production and an optimal stress induction time period.

Metabolic changes in *C. maritimum*

The mean biomass of harvested *C. maritimum* plants is shown in Fig 1A. In the beginning the average biomass was 1.16 g per plant. During the first week there were random changes and the fresh matter increased slightly up to around 2 g. The maximum mean biomass per plant was 5.45 g after 504 h at plants grown at 5 PSU. This is significantly different to the mean biomass of plants grown at 15 PSU at the same point of time.

The time pattern for ORAC, total phenols and, as a part of them, total flavonoids of *C. maritimum* are significantly correlated with each other (see Table 2 for further information). Therefore, only one metabolite profile is shown and the small differences in between are listed in the text (for the complete data set see Supporting information).

The ORAC values for *C. maritimum* are given in Fig 1B. They started at around 80 μmol TE g^{-1} FM with indistinct changes between time and salinities. There was a general, not significant, increase to the maximum of 101 μmol TE g^{-1} FM in plants grown at 15 PSU in the first week. Afterwards, there was a drop towards values measured in the third week within all plants at all salinities, reaching values of 64 μmol TE g^{-1} FM in plants grown at 0 PSU and 85 μmol TE g^{-1} FM in plants grown at 15 PSU. From 96 h onwards plants grown at 15 PSU had a significantly higher ORAC than plants grown at 0 PSU. There were small differences of total flavonoid concentrations (S1A Fig) in comparison to the ORAC values revealing slightly different trends. There was no increase in the flavonoid concentration in the first 96 h, but the flavonoid concentration at 186 h with plants grown at 10 and 15 PSU stands out more clearly compared to the ORAC values. The total phenol concentration (S1B Fig) had a more distinct slope than the ORAC values up to the maximum at 168 h. Both shared a significant difference at 168 h in between concentrations of plants grown at 0 and 15 PSU.

The amount of DHA was nearly undetectable and the amounts of TAA and AA were nearly the same. Therefore, just TAA values for *C. maritimum* are displayed in Fig 1C. The mean TAA concentration increased 2 and 4 h after induction, and increased with fluctuations to a first maximum at 10 PSU after 48 h, followed by a drop down at 96 h. A second rise with a

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Fig 1. *Crithmum maritimum* plants were set in containers, after an acclimatization time of 2 weeks, 14-week-old plants were exposed to salinity ranging in 5 PSU steps from 0 till 15 PSU. Fresh material (n = 4) was harvested at the indicated time. The mean biomass production (A), ORAC value (B), total ascorbate concentration (C) and proline concentration (D) for each salinity is plotted against the time. For better visibility the time is not true to scale. Different capital letters above the standard deviation indicate significant differences ($p < 0.05$) between points of time among a PSU value. Different lower letters indicate significant differences ($p < 0.05$) within one point of time between different PSU values.

Table 2. Analysis of variance of the parameters measured at the different species.

Species	Factor	FM	ORAC	Phenols	Flavonoids	TAA	Proline
C. maritimum	time	***	***	***	***	***	***
	PSU	** (ns log)	***	***	***	ns	***
	time*PSU	* (ns log)	*	.	ns	ns	***
T. maritima	time	***	***	***	ns	***	***
	PSU	** (***log)	ns	.	*	ns	***
	time*PSU	* (**log)	ns	*	*	**	***
H. portulacoides	time	***	***	***	***	***	***
	PSU	ns	***	***	***	***	***
	time*PSU	ns	***	***	**	***	(*log)

The asterisks indicates the significance levels * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001. n.s. not significant. ANOVA for fresh mass (FM) values was calculated in log.

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second maximum followed at 336 h after induction. Again there was a decrease in TAA concentration in plants at 504 h. It appeared that at 2 and 4 h after stress induction the plants set to higher salt concentrations had increased TAA concentrations, but this was not significant.

There was no significant change in proline concentration of *C. maritimum* in the first hours after salt induction. But from 24 h onwards a significant increase was observed, the proline concentration rose in the plants set to higher salinity (Fig 1D). For plants grown at 15 PSU this trend continued: the proline concentration increased steadily to a plateau at 336 and 504 h. Plants treated with water containing 10 PSU showed a similar significant increase in proline concentration and a plateau at 336 and 504 h but accumulated only about half the amount of plants grown at 15 PSU. Plants set to 5 PSU were only affected slightly and showed only a small increase in proline concentration. The significantly higher maximum was already reached at 168 h after induction, followed by a later decrease in the values. The proline concentration of plants grown at 0 PSU did not change significantly at all.

Metabolic changes in *T. maritima*

In Fig 2A the average fresh biomass in g of *T. maritima* over time is shown. At the beginning of the experiment there were indistinct changes, but in the later stages, after 336 and 504 h, a correlation between PSU and biomass was observed. The highest mean biomass was harvested after 504 h at plants grown at 5 PSU which was significantly different to plants grown at 15 PSU at the same time and significantly different to the starting value.

ORAC values of *T. maritima* are shown in Fig 2B. Starting at 33 μmol TE g^{-1} FM, they decreased over time to 23 up to 27 μmol TE g^{-1} FM in the end. There was some variance in between salinities and points of time, but higher salinities tended to induce higher ORAC values at 168 h and 504 h, however, this was not significant. The temporal patterns of flavonoid and phenol values of *T. maritima* appeared similar compared to the temporal pattern of the ORAC values (S2A and S2B Fig). But both flavonoid and phenol decreased in concentration with increasing salinity 2 h after the induction of salinity which was significant for phenol contents. This decrease in metabolite concentration with increasing salinity was only observed at the beginning of the experiment followed by non-significant changes between time and salinities. At 96 and 168 h the plants grown at higher salinities produced higher phenol and flavonoid concentrations. There was a significant difference for phenol concentration at 96 h between plants grown at 0 and 15 PSU. While the range of total phenols remained, with minor exceptions, at the same level over time and only decreased at 336 and 504 h, the span of total flavonoids remained, with minor exceptions, at the same level.

The mean TAA values of *T. maritima* are displayed in Fig 2C. The TAA concentration rose 2 h after beginning of the salinity treatment and was significantly higher for plants grown at 0 PSU compared to plants grown at 15 PSU. Just 4 h after induction this constellation changed and plants grown at 10 and 15 PSU had significantly higher values compared to plants grown at 0 and 5 PSU. Afterwards there was a decline without significant changes in the concentration till 48 h for all salinities. The values remained with fluctuations at the same level for the rest of the experiment, except for 336 h where TAA decreased significantly, except for plants grown at 0 PSU.

In *T. maritima* the proline mean concentrations did not change significantly within the first 8 h (Fig 2D). At 24 h plants grown at 10 PSU had significantly higher proline concentrations than the other salinities. In the later stages, the proline concentrations of plants grown at 0 and 5 PSU decreased while plants grown at 10 PSU did not change significantly. Plants grown at 15 PSU increased at 336 and 504 h, significantly higher, compared to the starting point and other salinities. This led to a significant increase in the proline concentration of
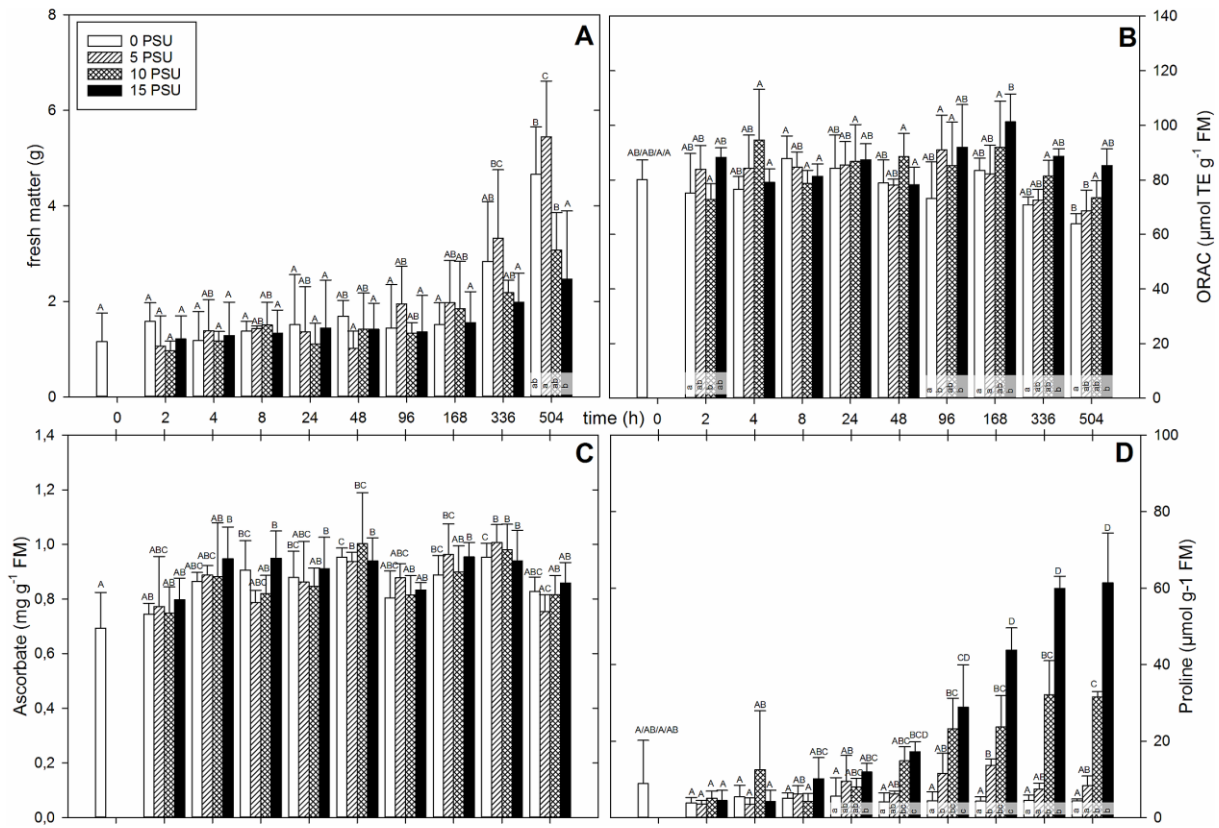
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Fig 2. *Triglochin maritima* plants were set in containers, after an acclimatization time of one week, ten week old plants were exposed to salinity ranging in 5 PSU steps from 0 till 15 PSU. Fresh material (n = 4) was harvested at the indicated time. The mean biomass production (A), ORAC value (B), total ascorbate concentration (C) and proline concentration (D) for each salinity is plotted against the time. For better visibility the time is true to scale. Different capital letters above the standard deviation indicate significant differences ($p < 0.05$) between points of time among a PSU value. Different lower letters indicate significant differences ($p < 0.05$) within one point of time between different PSU values.

plants grown in higher salinities compared to plants grown in lower salinities at 96 h, 168 h and 504 h and partly at 336 h.

Metabolic changes *in H. portulacoides*

The mean fresh biomass of *H. portulacoides* increased slowly during the first four days but increased massively towards the second week (Fig 3A). The highest biomass production was observed in the end of the experiment in plants grown at 5 PSU. There was a significant difference in comparison to the starting value for each salinity, but there was no significant difference in comparison to the last point of time.

The ORAC profile *H. portulacoides* is shown in Fig 3B. Eight hours after the induction of salinity stress the ORAC value for plants grown at 10 PSU increased significantly from about 28 up to 45 µmol TE g^{-1} FM. All other ORAC values from plants grown at other salinities at this point of time were not significantly different from the starting point and compared to each other. At 24 h all salinity levels decreased in concentration, but 10 PSU was still significantly

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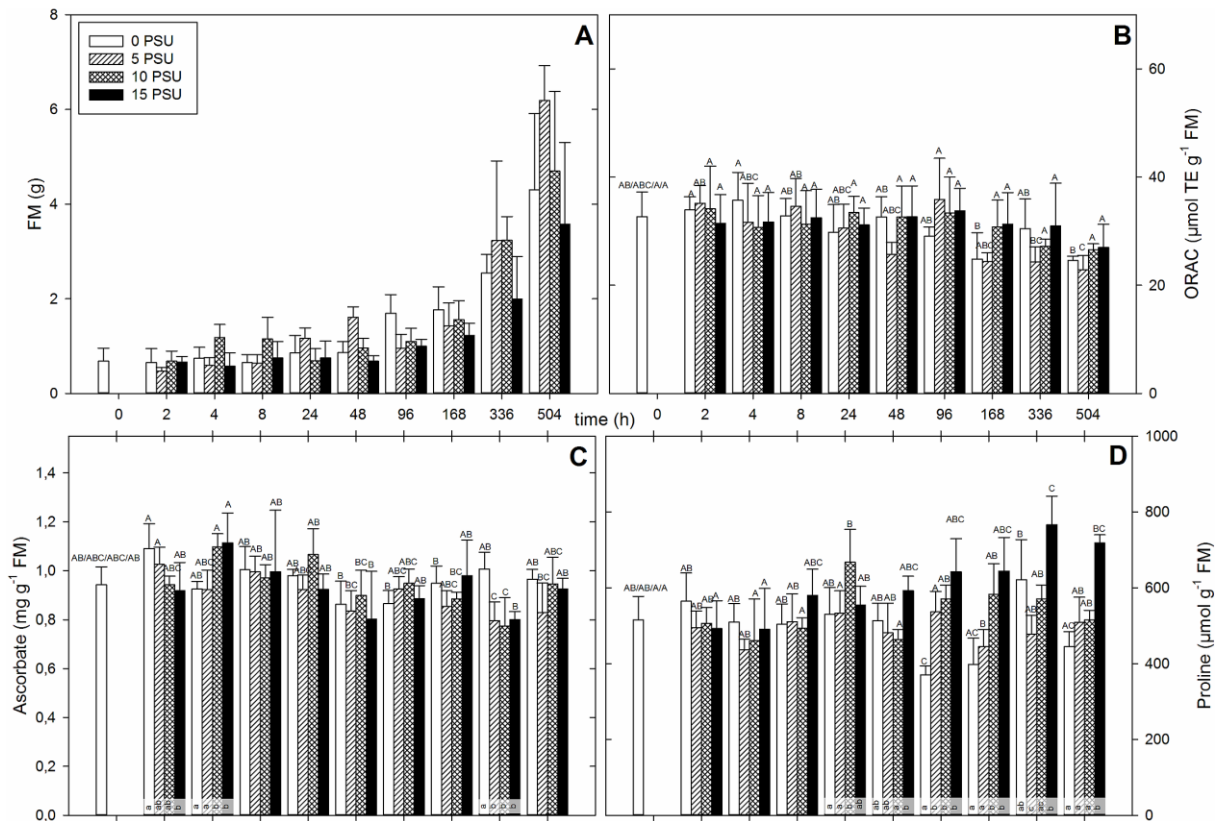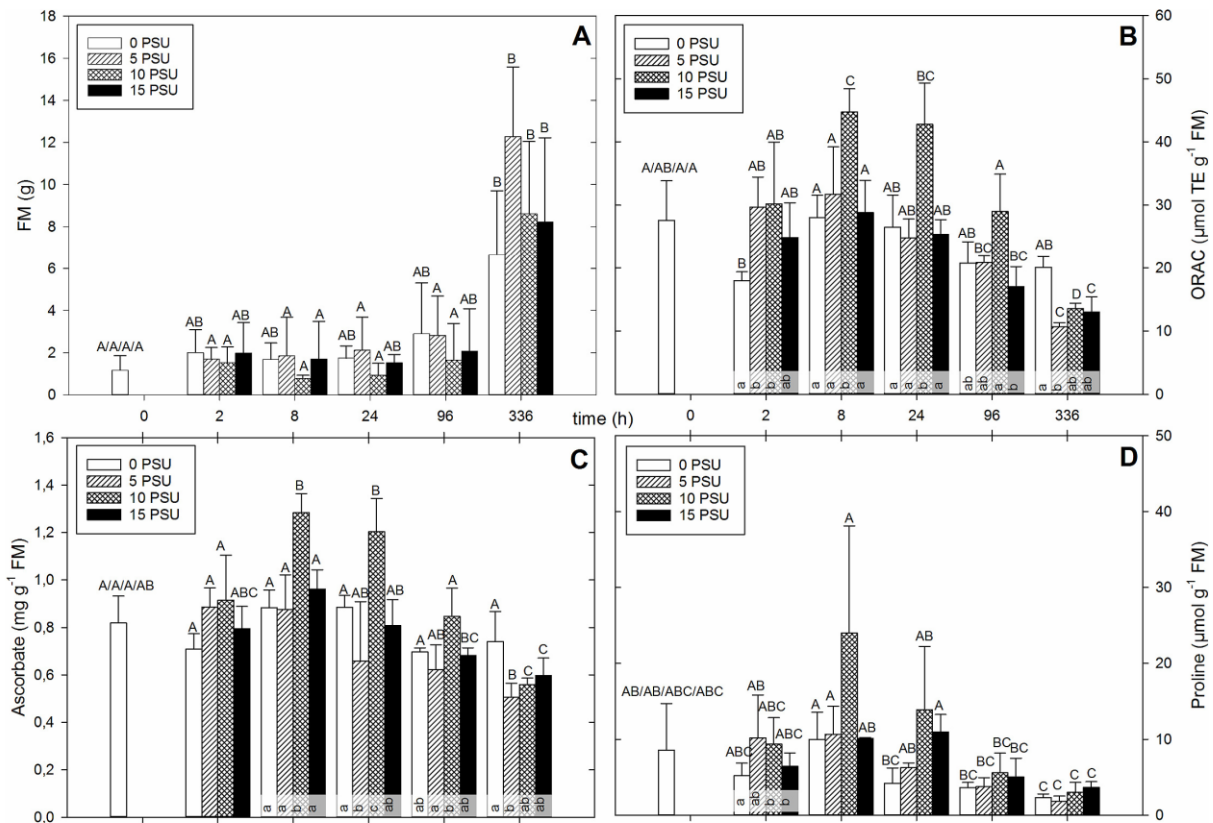**Fig 3. *Halimione portulacoides* plants were set in containers, after an acclimatization time of one week, eight week old plants were exposed to salinity ranging in 5 PSU steps from 0 till 15 PSU.** Fresh material (n = 4) was harvested at the indicated time. The mean biomass production (A), ORAC value (B), total ascorbate concentration (C) and proline concentration (D) for each salinity is plotted against the time. For better visibility the time is not true to scale. Different capital letters above the standard deviation indicate significant differences ($p < 0.05$) between points of time among a PSU value. Different lower letters indicate significant differences ($p < 0.05$) within one point of time between different PSU values.

higher than the other salinities. This continued at 96 h but was not significant. At 336 h all ORAC values were significant below the starting level except the values for 0 PSU which had a significantly higher concentration compared to the values of the other salinities. The temporal pattern for total flavonoids of *H. portulacoides* is given in S3A Fig. The pattern resembles the one of ORAC (Fig 3B) with only little differences. The starting value for the total flavonoid concentrations was higher, so there was no significant difference between that value and the maximum after 8 h at 10 PSU, however, it was still significantly higher compared to the other salinities at the same point of time. At 96 and 336 h there were no significant differences of ORAC values between plants grown at different salinities. The mean total phenol concentration was similar to the ORAC pattern (S3B Fig). After 2 h the phenol concentration of plants grown at 10 PSU was significantly higher than plants grown at 0 PSU but not significantly higher than the starting value. At 8, 24 and 96 h the total phenol concentration was significantly higher in plants grown at 10 PSU compared to the other salinities.

The mean TAA concentration over time for *H. portulacoides* is shown in Fig 3C. From the starting value, the concentration rose significantly to a peak at 8 h in plants grown at 10 PSU,

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while plants at the other salinities did not produce significantly higher concentrations of TAA. At the next point of time of 24 h there was again another significantly higher value in plants grown at 10 PSU, compared to the other salinities. All concentrations declined stepwise at 96 h and 336 h after salinity induction. At 336 h, plants at all salinities except 0 PSU produced values which were significantly lower compared to the starting value.

The temporal pattern of proline for *H. portulacoides* is displayed in Fig 3D. After a short time of salt induction (8 h) the amount of proline in salt treated plants (10 PSU) rose higher compared to plants at the other salinities. At 24 h after induction, the proline concentration for salt-exposed plants (10 and 15 PSU) was still higher, but not significant, than the lower stressed and unstressed plants (5 and 0 PSU), but not higher than the starting value. After 96 h the proline concentration decreased to a minimum at 336 h, which was significantly lower, compared to the starting value, for plants grown at 0 and 5 PSU.

Studies on the dependence of the metabolite concentration from each other

As mentioned earlier some metabolite profiles appeared to be very similar to each other within one species. To visualize the proximity and relation, the Principle component analysis (PCA) was performed and results are displayed in Fig 4A–4C. In Fig 4A the PCA for *C. maritimum* is displayed. It is clearly visible, that ORAC, total flavonoid and most of the total phenol values were influenced in the same way in the direction of PC1, whereas proline and TAA values were influenced in different ways, with TAA almost moving along PC2 and proline in the opposite way between PC1 and PC2. A similar result for the PCA of *T. maritima* is shown in Fig 4B. The total phenol and the ORAC values were influenced almost in the same way along PC1. TAA values and flavonoids were influenced similarly but shifted in opposite ways more influenced by PC2. Proline values were affected by PC2 and shifted in almost a different direction compared to TAA. Fig 4C shows the PCA of *H. portulacoides* and it is obvious that all metabolites, except proline, were affected in a similar way. A clear grouping for time or salinities could not be observed.

To investigate if there is a significant correlation between the different metabolites, the Pearson correlation was calculated (S2 Table). It strengthens the results of the PCA: ORAC, total phenols, and total flavonoids were significantly linearly correlated within *C. maritimum*, *T. maritima* and *H. portulacoides*. There was always a significantly high correlation between
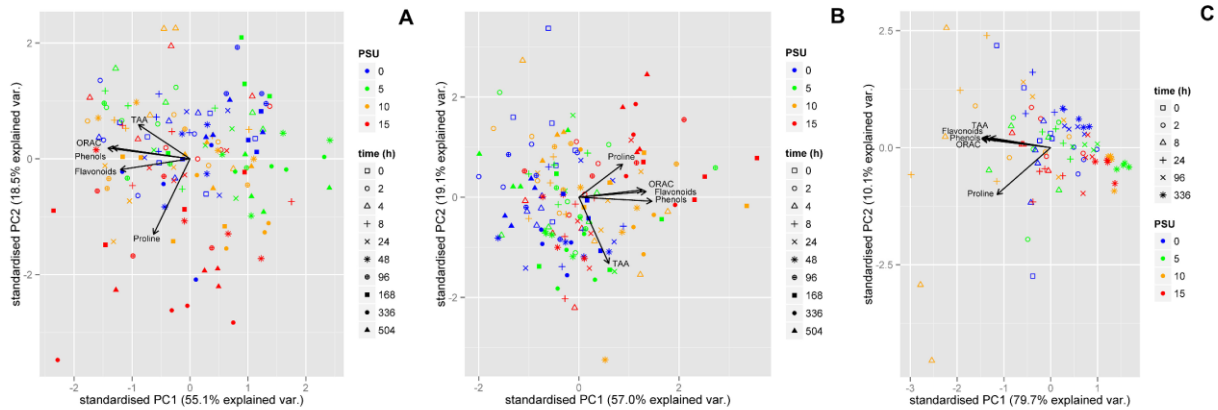
Fig 4. Principal components for individual measurements of ORAC, phenol, flavonoid and proline concentration in *C. maritimum* (A), *T. maritima* (B) and *H. portulacoides*. The different salinities are indicated by different colours and the different points of time by different symbols.

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ORAC and phenols, whereas in *C. maritimum* flavonoids had a higher correlation than phenols towards ORAC. Flavonoids had still a high correlation to ORAC in *T. maritima* and *H. portulacoides*, in the latter TAA had a higher correlation to ORAC than flavonoids. TAA also had a high correlation to phenols and flavonoids and even proline in *H. portulacoides*. The correlation of TAA to the other metabolites was generally lower in *T. maritima*, and in *C. maritimum* only TAA correlated slightly with phenols. Proline concentration correlated moderately with all other metabolites in *H. portulacoides* while in *C. maritimum* the correlation was lower and in *T maritima* the correlation was even lower.

The total yield of each metabolite was calculated. The yield of antioxidants was in general highest at the highest biomass production namely at 5 PSU (data not shown). There were some exceptions, for example the gain of proline (Table 3). At 336 h the total gain of proline in *H. portulacoides* was highest at the 15 PSU. *Crithmum maritimum* had a significantly higher gain of proline at 48, 96, 168, 336 and 504 h at 15 PSU compared to 0 PSU.

Elemental composition

To understand the physiological basis for the differences in the metabolite contents among the plant species, especially of the osmolyte proline, the elemental composition of the halophytes was measured at selected points of time (Table 4). As expected, there was an increase in the Na

Table 3. Amount of proline.

Species	PSU	Time (h)	Proline (μmol g^{-1} FM)	
H. portulacoides	0	336	14.89	±6.52 a
	5	336	21.12	±4.26 a
	10	336	27.96	±20.92 a
	15	336	30.96	±19.74 a
C. maritimum	0	48	7.08	±4.48 a
	5	48	6.42	±2.78 a
	10	48	20.32	±9.92 ab
	15	48	24.63	±10.91 b
	0	96	5.68	±2.92 a
	5	96	20.99	±8.82 b
	10	96	30.91	±11.93 b
	15	96	37.04	±16.90 b
	0	168	27.70	±14.37 a
	5	168	6.90	±3.68 b
	10	168	40.33	±18.69 b
	15	168	70.37	±40.00 b
	0	336	14.04	±9.86 a
	5	336	23.78	±7.43 ab
	10	336	69.82	±18.54 bc
	15	336	118.82	±36.68 c
	0	504	20.24	±3.11 a
	5	504	46.37	±19.45 ab
	10	504	96.79	±24.43 b
	15	504	150.23	±87.96 b

The average amount of proline for selected species is calculated and given for points of time, where the yield is higher at high salinity. Different letters in each point of time indicate significant differences ($p < 0.05$).

https://doi.org/10.1371/journal.pone.0176303.t003

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Table 4. Elemental composition of the plant material at selected points of time.

Species	Time (h)	PSU	Elemental content in the plant material			
			Na+ (mg g-1 FM)	K+ (mg g-1 FM)	Ca2+ (mg g-1 FM)	K+/Na+
C. maritimum	0	0	1.23 ± 0.68	5.65 ± 0.37	4.37 ± 0.22	5.81
	24	0	1.11 ± 0.26	5.85 ± 0.84	4.33 ± 0.30	5.50
		5	1.30 ± 0.14	6.23 ± 2.18	4.69 ± 0.47	4.86
		10	2.55 ± 0.47	4.92 ± 0.43	4.92 ± 0.53	2.00
		15	2.03 ± 0.14	5.2 ± 0.30	5.02 ± 0.21	2.57
	96	0	0.89 ± 0.24	4.71 ± 0.52	3.64 ± 0.49	5.57
		5	1.92 ± 0.38	4.8 ± 0.03	3.77 ± 0.16	2.54
		10	2.86 ± 0.86	4.83 ± 1.05	4.12 ± 0.71	1.86
		15	3.54 ± 1.06	4.43 ± 0.87	4.09 ± 0.24	1.40
	336	0	0.88 ± 0.15	4.39 ± 0.56	3.26 ± 0.15	5.12
		5	2.91 ± 0.44	4.27 ± 0.83	2.91 ± 0.30	1.46
		10	3.46 ± 0.40	3.77 ± 0.73	3.17 ± 0.44	1.11
		15	4.97 ± 0.29	4.19 ± 1.38	3.72 ± 0.32	0.84
T. maritima	0	0	3.55 ± 0.54	4.43 ± 1.03	2.16 ± 0.29	1.24
	96	0	2.59 ± 0.33	6.91 ± 2.63	2.14 ± 0.17	2.67
		5	6.8 ± 1.69	5.74 ± 1.99	2.1 ± 0.36	0.87
		10	6.96 ± 0.73	5.2 ± 1.34	2.15 ± 0.17	0.76
		15	6.92 ± 0.87	5.9 ± 1.48	2.75 ± 0.68	0.84
	336	0	2.53 ± 0.21	9.63 ± 1.33	3.45 ± 0.52	3.84
		5	6.89 ± 0.26	4.21 ± 1.54	1.77 ± 0.23	0.62
		10	9.05 ± 0.42	3.91 ± 0.4	1.43 ± 0.08	0.43
		15	10.78 ± 0.85	4 ± 0.85	1.4 ± 0.05	0.37
H. portulacoides	0	0	4.9 ± 0.76	6.67 ± 1.77	2.24 ± 0.27	1.42
	96	0	5.25 ± 2.23	7.43 ± 4.09	2.35 ± 0.66	1.76
		5	8.66 ± 0.74	5.27 ± 2.25	1.69 ± 0.14	0.62
		10	7.96 ± 0.69	5.2 ± 1.79	1.73 ± 0.51	0.64
		15	9.65 ± 0.63	6.45 ± 0.76	1.53 ± 0.27	0.67
	336	0	5.13 ± 1.6	7.74 ± 1.22	1.89 ± 0.22	1.58
		5	8.72 ± 0.81	6.37 ± 0.45	0.85 ± 0.08	0.74
		10	9.76 ± 0.54	6.36 ± 2.01	0.74 ± 0.09	0.65
		15	10.94 ± 0.66	4.07 ± 0.59	0.72 ± 0.15	0.37

Mean (± S.D.) are given (n = 3). For a better comparison the Na+ in the hydroponic solution is calculated: 0, 5, 10 and 15 PSU equals 0, 1.97, 3.93 and 5.90 mg g-1 FM.

https://doi.org/10.1371/journal.pone.0176303.t004

concentration over time correlating with increasing salinity for each species. Contents of K increased over time in *T. maritima* and *H. portulacoides* at 0 PSU and decreased at 5, 10 and 15 PSU. In *C. maritimum* K contents decreased over time at all conditions. Contents of Ca decreased slowly in all species under salinity. Within the two facultative halophytes the difference of the Ca concentration between the salinity and plants grown at 0 PSU was greater than in the obligate halophyte *C. maritimum*. The K/Na ratio decreased in saline conditions for all species, whereas it was almost stable in *C. maritimum* and *H. portulacoides* at 0 PSU. In *T. maritima* the K/Na ratio increased at 0 PSU.

Discussion

Many studies agree that there is a health-promoting effect from antioxidants of various plant sources [8,9,41]. It was also shown that salinity stress causes an increase in the concentration

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of antioxidants [6,42]. Thus, mild salt-stress might produce healthier vegetables containing high amounts of antioxidants.

Crithmum maritimum showed a significant increase in ORAC, total phenols and flavonoid concentration at 168 h after salinity induction comparing the contents at 0 and 15 PSU. This was also the point of time when the plants accumulated the highest concentrations of these antioxidants. Therefore, this would be the best point of time to harvest. This consideration leaves out the concentration of proline and TAA, because proline is of low economic value and the TAA values were not significantly different between salinities. *Triglochin maritima* showed an increased antioxidant production with increased salinity, but at different points of time as the maxima in antioxidant concentrations vary. The maximum ORAC value was measured after 96 h in plants gown at 5 PSU and the maximum flavonoid concentration at 336 h in plants grown at 15 PSU, however, there was no significant difference in comparison to plants grown at 0 PSU. The only significant maximum was determined at 96 h in the phenol concentration in plants grown at 15 PSU. TAA concentrations accumulated to a significant maximum at 4 h in plants grown at 15 PSU; therefore, there was no optimal point of time for a harvest. In the case of *H. portulacoides* it is very conclusive: the best point of time for a harvest would be 8 h after salinity induction for plants grown at 10 PSU. At this point of time there were significant differences in ORAC, total phenol, total flavonoid and TAA concentrations.

However, taking the biomass for the total antioxidant yield into consideration, the best point of time for a harvest shifts. Within one week, biomass production was not severely inhibited by salinity, however, there was a trend to a decreased biomass production in plants grown at 10 and 15 PSU. This trend continues at 336 h after salinity induction and an actual increase of biomass production of plants grown at 5 PSU became obvious. At 504 h after salinity induction there was a significant difference in the gain of biomass of *C. maritimum* and *T. maritima* between the maximum, in plants grown at 5 PSU, and the lowest gain of biomass, in plants grown at 15 PSU. A higher gain of biomass, yields a higher amount of secondary metabolites. A high antioxidant activity and a high biomass production under the same condition did not coincide, furthermore, the lower biomass production compensated the increased antioxidant concentration.

Drought stress causes a complex pattern of reactions in plants regulated by the activity of many genes [43]. Often transcriptome dynamics are in consistence enzymatic and metabolic changes [44]. The modified enzyme patterns result in an increase of antioxidants influencing, for example, stomata closing to prevent further loss of water but also preventing further uptake of CO_2 [28]. Therefore, reduction equivalents like $NADPH+H^+$ are increased, as they are not consumed for CO_2-fixation in the Calvin cycle. Thus, metabolic processes are directed to produce highly reduced secondary metabolites, such as isoprenoids, (poly)phenols or alkaloids. But the decrease of biomass under drought stress revises the yield in many cases [28]. The mechanisms of stomatal closure due to water deficiency are comparable under salinity and drought stress. However, the closed stomata preventing further CO_2-fixation, affect the growth of the plant. To work around the problem of biomass reduction, mild salinity conditions were chosen. But mild salinity obviously does not increase the yield of secondary plant products in halophytes to a high extent to overcome the reduction in biomass.

The yield of proline, which acts as an osmolyte, was different compared to the antioxidants. There was a significant increase in proline concentration with increasing salinity for all three species. This increase of the total yield was sufficient to compensate for the loss of biomass production, at least for *C. maritimum* and *H. portulacoides*. In *H. portulacoides* the total proline yield was the highest after 336 h at 15 PSU, however, this was not significant. In *C. maritimum* the proline yield was significantly higher at 15 PSU from 48 h onwards (Table 3). The search for halophytes containing valuable osmolytes increased by salinity with a high probability

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might be a promising future approach. There are already some pharmaceutical and medicinal applications for osmolytes, for example protection against carcinogenic oxidative stress, helping in vaccine stabilisation and treating the dry eye syndrome, for the complete list see the review [45].

A correlation between salinity stress and an increase in proline in halophyte species and therefore the use as a stress marker was postulated previously [46–48]. Also in glycophytes a proline up-regulation was shown, but under drought stress [49]. There are several other osmolytes present in halophytes [50], which means that both *H. portulacoides* and *T. maritima* might use other types of osmolytes for protection. In *H. portulacoides* high concentrations of glycine betaine were found in plants harvested in a salt marsh [51], as well as methylated onium compounds and sugars [45]. *Triglochin maritima* does not seem to significantly increase any specific osmolyte concentration [52]. Many osmolytes like glycine betaine and sugars were found in a moderate concentration compared to other monocotyledonous salt marsh species. Proline, pipecolate, fructose, maltose, and sucrose were also found in *T. maritima* [45]. The ten times higher proline concentration, in comparison to the other plant species investigated, which was already present at non-saline conditions, seems to be sufficient for osmotic balance. Furthermore, it was shown that the synthesis of osmolytes might be constitutive in some species as the concentration of osmolytes does not increase with increasing salinity in all cases [45].

On average, the nutritional value of the halophytes is high compared to other raw vegetables. The ORAC value of *C. maritimum* was around 80 μmol TE g^{-1} FM which is comparable to red pepper [12]. The ORAC values of *T. maritima* and *H. portulacoides* were around 20–40 μmol TE g^{-1} FM. These values are comparable to raw asparagus or raw broccoli [15]. The total phenol concentration of *C. maritimum* is around 3 mg GAE g^{-1} FM, comparable to red [52] or green pepper and broccoli [15]. *Triglochin maritima* and *H. portulacoides* have total phenol values which are comparable with asparagus, carrots, yellow onions [15] or spinach and broccoli [53]. It is important to mention that the values of the reference sources vary, but this is most likely due to the collection time, season, variety and other reasons.

It is notable that the Na^+ concentration of *C. maritimum* under salinity was always lower compared to the concentration of the surrounding culturing solution. On the one hand, this might indicate that the plant acts as an excluder to keep Na^+ out. On the other hand, this might result in an osmotic pressure, and to prevent this, the proline concentration is enhanced. The Na^+ concentrations of *T. maritima* and *H. portulacoides* are higher than in the surrounding media. *Triglochin maritima* is described as leaf-succulent halophyte acting as an excretive halophyte with glandular cells [54,55]. *Halimione portulacoides* has epidermal bladders to remove the salt, but it also accumulates a high concentration in the leaves to maintain an osmotic balance [56], as it was also confirmed by our results (Table 3). Whether the salt was transported to glandular cells or epidermal bladders cannot be discriminated as the whole plant material was analyzed.

The similar trends with respect to time in the biosynthesis of compounds with high ORAC, total phenols and total flavonoids have multiple reasons. As mentioned earlier, flavonoids are one of the two groups of (poly)phenols [8,9]. When the phenols were measured, the flavonoids were automatically measured as well, contributing half or two thirds to the amount of phenols, leading to the similar courses of these metabolites. As the ORAC value represents the sum of the most abundant antioxidants available in the plant there is a similarity towards phenols and therefore also flavonoids. This can be seen in *C. maritimum* having high flavonoid values in comparison to phenols, therefore, both values have a high correlation. The high phenol concentration contributes more to the ORAC value than the TAA concentration leading to a high correlation between ORAC and phenols (and therefore flavonoids), but a low correlation with

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ORAC and TAA. *Halimione portulacoides* has a relatively high TAA concentration in relation to phenols. Therefore, TAA is contributing more to the ORAC resulting in a higher correlation between them. A positive correlation between phenolics and antioxidant activity, degree of correlation varying upon analyzed material, was reported before [57,58]. Furthermore a correlation between ORAC, total phenolic and TAA content was found in soybean leaves [35]. We could also observe a correlation between all measured metabolites, partly weaker and partly stronger, depending on the plant species. Another study postulated that the correlation between AA and ORAC is high, when the AA concentration is high [59]. They analyzed guava fruits with an even or higher AA concentration than (poly)phenol concentration. In analyzed *Vaccinium* species with a high phenol concentration and an around 50 times lower AA concentration the AA only contributed 5% or less to the ORAC [60].

The size of the plants has to be taken into account. Some smaller plants showed higher concentration in secondary compounds and vice versa. This natural variety influences the ORAC and phenol values in *C. maritimum* and *T. maritima*, furthermore the TAA in the latter and all values in *H. portulacoides*. This leads to a fluctuation in all metabolite patterns also in the control plants grown at 0 PSU. A correlation between the secondary metabolite camptothecin and tissue age was found, and a correlation between the phlorotannin content and size of kelp, summarizing that secondary metabolites are synthesized at the cost of growth [61,62].

Conclusion

In our study mild stress promoted content of secondary metabolites partly after a few hours, partly after several days. But in all cases the biomass reduction in plants grown in mild stress exceeded the increase in metabolite concentration in mildly stressed plants resulting in an equal or even lower yield of metabolites per cultivated area. Therefore, the total amount of secondary metabolites per harvest was lower in the stressed plants, except for proline. There might be other individual metabolites affected in the same way. So far, total (poly)phenol concentrations were taken into consideration, but analyzing individual valuable phenolic compounds is a promising future approach because single components might react to salinity. Salinity-affected soils occur all over the world. These lands are not suitable for conventional agriculture, but for saline agriculture. Species that can be grown in these environments are halophytes. It was shown that it is possible to manipulate their antioxidant concentration and enhance their value. As salt affected areas are a general problem already, halophytes can be grown on saline areas where glycophytic crops do not survive. Alternatively, halophytes could be cultivated in hydroponic greenhouse conditions by using brackish water to enhance their nutritional value.

Supporting information

S1 Fig. *Crithmum maritimum* plants were set in containers, after an acclimatization time of 2 weeks, 14-week-old plants were exposed to salinity ranging in 5 PSU steps from 0 till 15 PSU. Fresh material was harvested at the indicated time. The mean flavonoid (A) and phenol concentration (B) out of four plants for each salinity is plotted against the time. For better visibility the time is not scaled natural. Different capital letters above the standard deviation indicate significant differences ($p < 0.05$) between points of time among a PSU value. Different lower letters indicate significant differences ($p < 0.05$) within one point of time between different PSU values.
(TIF)

S2 Fig. *Triglochin maritima* plants were set in containers, after an acclimatization time of 2 weeks, 14-week-old plants were exposed to salinity ranging in 5 PSU steps from 0 till 15

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PSU. Fresh material was harvested at the indicated time. The mean flavonoid (A) and phenol concentration (B) out of four plants for each salinity is plotted against the time. For better visibility the time is not scaled natural. Different capital letters above the standard deviation indicate significant differences ($p < 0.05$) between points of time among a PSU value. Different lower letters indicate significant differences ($p < 0.05$) within one point of time between different PSU values.
(TIF)

S3 Fig. *Halimione portulacoides* **plants were set in containers, after an acclimatization time of 2 weeks, 14-week-old plants were exposed to salinity ranging in 5 PSU steps from 0 till 15 PSU.** Fresh material was harvested at the indicated time. The mean flavonoid (A) and phenol concentration (B) out of four plants for each salinity is plotted against the time. For better visibility the time is not scaled natural. Different capital letters above the standard deviation indicate significant differences ($p < 0.05$) between points of time among a PSU value. Different lower letters indicate significant differences within one point of time between different PSU values.
(TIF)

S1 Table. Composition of the nutrient solution.
(DOCX)

S2 Table. Pearson correlation of four assays for each species. The number indicates the correlation coefficient, the asterisk indicates the significance levels $* \leq 0.05$; $** \leq 0.01$; $*** \leq 0.001$. n.s. not significant; TAA, total ascorbic acid.
(DOCX)

Acknowledgments

Author Contributions

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Supporting Information (figures and tables)
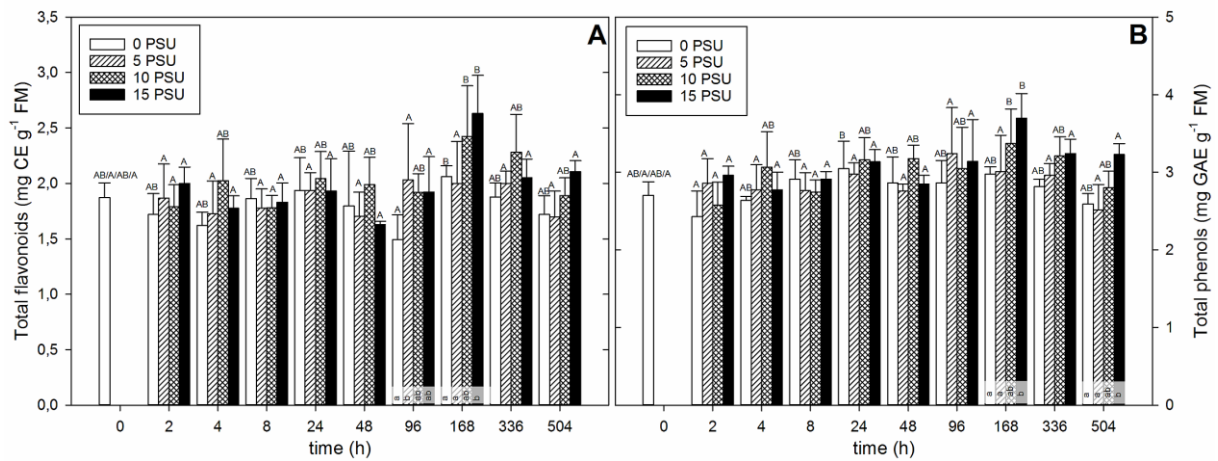
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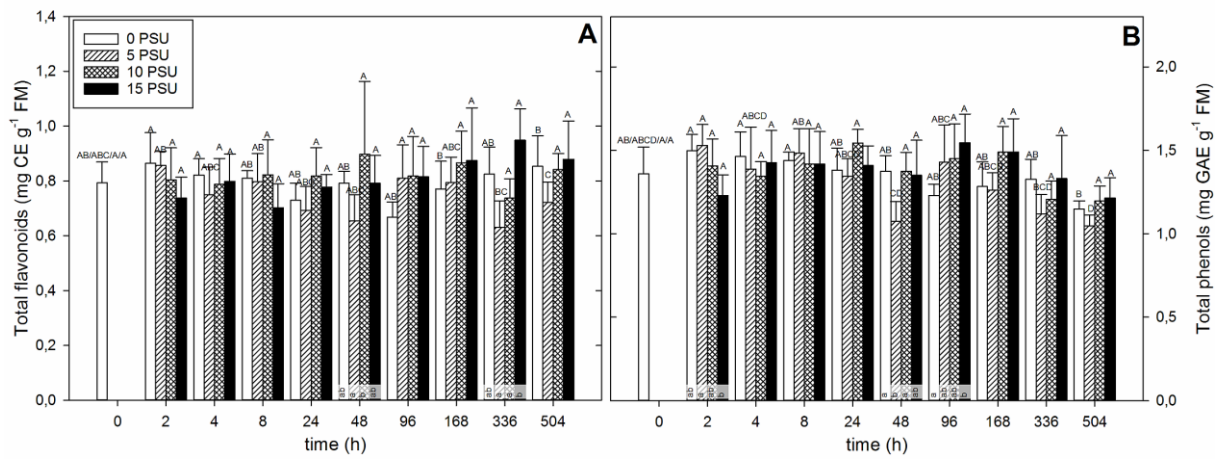
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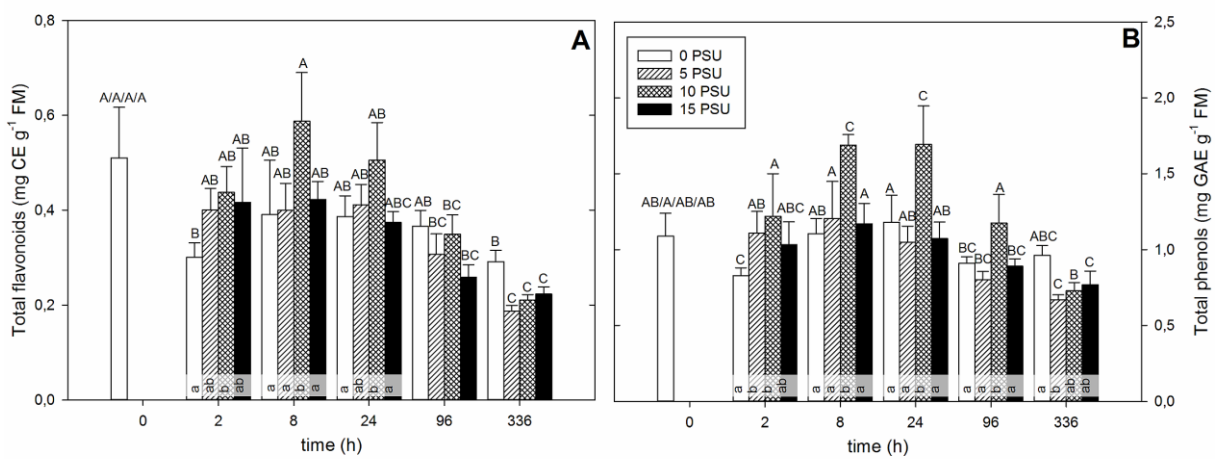
S3 Fig.

S1 Table.

Chemical compound	mg*l^{-1}	µmol*l^{-1}
KNO_3	606.60	6000
$Ca(NO_3)_2$ x $4H_2O$	944.64	4000
$NH_4H_2PO_4$	230.16	2000
$MgSO_4$ x $7H_2O$	246.48	1000
KCl	3.73	50
H_3BO_3	1.55	25
$MnSO_4$ x H_2O	0.34	2.0
$ZnSO_4$ x $7H_2O$	0.58	2.0
$CuSO_4$ x $5H_2O$	0.12	0.5
$MoNa_2O_4$ x $2H_2O$	0.12	0.5
$C_{10}H_{16}FeN_2NaO_8$	0.60	10

S2 Table.

Species	Metabolites	Flavonoids	Phenols	TAA	Proline
C. maritimum	ORAC	0.632 ***	0.781 ***	0.16 n.s.	0.297 ***
	flavonoids		0.818 ***	0.227 n.s.	0.407 ***
	phenols			0.378 ***	0.449 ***
	TAA				0.062 n.s
T. maritima	ORAC	0.577 ***	0.86 ***	0.344 ***	0.206 *
	flavonoids		0.626 ***	0.295 ***	0.296 ***
	phenols			0.475 ***	0.262 **
	TAA				0.113 n.s
H. portulacoides	ORAC	0.775 ***	0.966 ***	0.874 ***	0.643 ***
	flavonoids		0.755 ***	0.694 ***	0.552 ***
	phenols			0.902 ***	0.632 ***
	TAA				0.598 ***

Chapter 5

Boestfleisch, C., Glasenapp, Y., Papenbrock, J. Changes in the metabolic profile of *Crithmum maritimum* under salinity stress. (in preparation)

Changes in the metabolic profile of *Crithmum maritimum* under salinity stress

Boestfleisch, C., Glasenapp, Y., Papenbrock, J. (in preparation)

Abstract

Crithmum maritimum L. is a halophyte of broad scientific interest, as it harbours many promising substances of economic value. The aim was to find a suitable and fast technique for the identification of individual metabolites, which should change in *C. maritimum* under the influence of mild salt stress. Grown under salinity stress, the amount of valuable phenolic acids and osmolytes increased, albeit not uniformly. Putative *p*-coumaroylquinic acid isomers (5-*p*-coumaroylquinic acid, 4-*p*-coumaroylquinic acid and 3-*p*-coumaroylquinic acid) increased outstandingly, compared to the other (poly)phenols. Furthermore, proline and a compound, which is not identified yet, increased exceptionally. However, only prolonged salinity stress, from 48 h onwards had a visible impact on the amount of the on the polyphenols and osmolytes named above. This could indicate that individual phenols are regulated differently and might have additional functions. It is demonstrated how an overview of the changes in the metabolite profile via LC-MS was obtained.

Introduction

Halophytes are plants that have adapted to live in saline areas (Flowers and Colmer 2008). The cracks and cleaves close to the seashore affected by the sea spray at the rocky coastline of the Mediterranean Sea and European Atlantic coast is the main habitat of *C. maritimum* L., a halophyte which has a long tradition used as a food or medicinal plant (Franke 1982; Pateira *et al.* 1999). But recently promising components were also discovered, as it was shown that essential oils present in *C. maritimum* showed antioxidant and antibacterial activity (Jallali *et al.* 2014). It belongs to the family of Apiaceae and contains a number of antioxidative polyphenols and flavonoids (Atia *et al.* 2011; Siracusa *et al.* 2011). These antioxidants are generally enhanced at abiotic stress like salinity (Sharma, Jha and Dubey 2010). *Crithmum maritimum* showed indeed enhanced activity of antioxidative enzymes under salinity (Ben Amor *et al.* 2005). It was also shown that the concentrations of phenols change during the season (Meot-Duros and Magné 2009) and during application of salt stress (Boestfleisch and

Papenbrock 2017), but the influence of salt on single metabolic components in *C. maritimum* was not shown yet. The aim of this study was to see whether salinity has a strong influence on single compounds and to demonstrate how it is possible to identify putative compounds by a literature screening.

Material and methods

Plant material

The plants were raised under the conditions described in Boestfleisch and Papenbrock (2017). A methanol extraction was performed, also described therein. One individual plant sample (complete shoot material), at point of time 0 (before the addition of salt) and points of time 2, 4, 8, 24, 48, 96, 168, 336, and 504 h after salt induction, from plants grown at a salinity of 15 Practical Salinity Units (PSU) and 0 PSU for comparison, was analyzed for individual components via LC-MS.

LC-MS analysis

The analysis was performed with an LC-MS system. The HPLC system (Shimadzu, Darmstadt, Germany) consisted of a controller (CBM-20A), two pumps (LC-20AD), a column oven (CTO-20AC) and a photo diode array detector (SPD-M20A). The sample separation was performed on a Vertex Plus column (250 x 4 mm, 5 µm particle size, packing material ProntoSIL 120-5 C18-H) with precolumn (Knauer, Berlin, Germany).

Prior to analysis the samples were diluted tenfold in 80% methanol (LC-MS grade) and a volume of 10 µl was injected. The column oven temperature was set to 30°C. As eluents, water (A) and methanol (B), both containing 2 mM ammonium acetate were used with a flow rate of 0.8 ml min^{-1}. The following gradient was applied: 10% B at the start, changing linear to 90% B in 35 min, 2 min of 90% B, switch to 10% B in 1 min and subsequent equilibration at 10% B for 2 min. UV/Vis spectra from 190-800 nm were recorded. For identification, components were injected into an AB Sciex Triple TOF mass spectrometer (AB Sciex TripleTOF 4600, Canby, USA) following HPLC separation. Negative electrospray ionisation (ESI) was used at a nebulizer temperature of 600°C and an ion spray voltage floating of -4500 V. Mass spectra in the range of 100-800 Da were measured in the TOF range, in addition MS/MS spectra from 50-800 Da at a collision energy of -30 were recorded.

Standards for the identification and quantification were prepared the same way. Peaks were compared for retention time and fragmentation pattern. A standard curve of three individual measurements of proline standards ranging from 1 to 100 mM was used for quantification of the proline peaks by MultiQuant™ software (AB Sciex, Darmstadt, Germany).

Data evaluation and peak identification

To facilitate the identification process of unknown compounds a classical literature search was conducted and a list with possible substances and their molecular formula that might be present in *C. maritimum* was created in Excel® 2007 (Microsoft Corporation, Redmond, USA). This list was copied into MasterView™ (AB Sciex) that calculated an expected mass from the molecular formula. This mass was compared to the mass of the peaks present in the samples and thereby the peaks were assigned. Furthermore, isotopic distribution of each mass was analyzed and fragmentation pattern was compared to ChemSpider, wherever possible, for further reliability. The parameters of the peaks from the putative compounds, like shape and retention time, besides mass, were transferred to a quantification method in MultiQuant™. Thereby, all peaks for all substances were extracted, however, just the peak area. Only in the case of proline the area was calculated to a concentration based on the standard curve. A comparison of the unknown substances with an own library of known phenolic acids was conducted using LibraryView™ (AB Sciex).

Results

The metabolic composition of the hydrophilic extract from *C. maritimum* is shown in the chromatogram in Figure 1. The mayor metabolites were quinic acid and esters of quinic acid with caffeic acid (1-caffeoylquinic acid, 3-caffeoylquinic acid, also known as chlororogenic acid, 5-caffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid) with ferulic acid (5-feruloylquinic acid, 3-feruloylquinic acid, 4-feruloylquinic acid) and with *p*-coumaric acid (5-*p*-coumaroylquinic acid, 4-*p*-coumaroylquinic acid and 3-*p*-coumaroylquinic acid. Furthermore, sugars (sucrose and trehalose) and sugar alcohols (sorbitol, mannitol) were found. Malic acid and diosmin were also two further metabolites that were present in notable amounts. All metabolites found in *C. maritimum*, including the ones in smaller amounts which are not visible in Figure 1, are shown in Table 1.
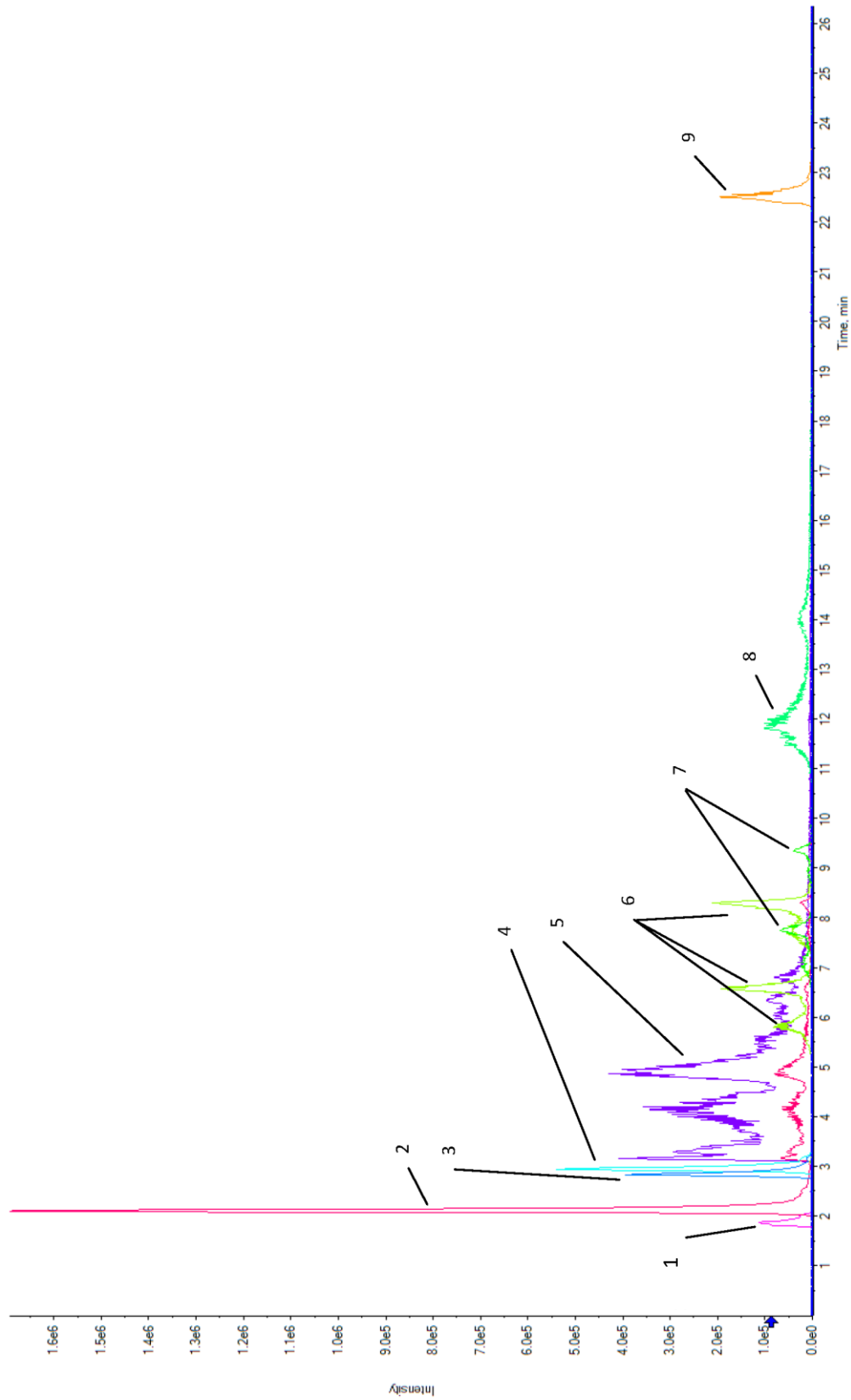
Figure 1. The chromatogramm of the main putative compounds present in *C. maritimum* methanolic extract Only mayor metabolites are indicated. The corrugated appearance of peaks indicates isomeric forms, which are listed in brackets. 1: malic acid, 2: quinic acid, 3: (sorbitol, mannitol), 4: (trehalose, sucrose), 5: (1-caffeoylquinic acid, 3-caffeoylquinic acid (chlororogenic acid), 5-caffeoylquinic acid) 6: (5-*p*-coumaroylquinic acid, 4-*p*-coumaroylquinic acid, 3-*p*-coumaroylquinic acid), 7: (5-feruloylquinic acid, 3-feruloylquinic acid, 4-feruloylquinic acid), 8: (3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid), 9: diosmin.

Table 1 Peak size of the component expressed as the ratio at each time point and salinity (n=1) compared towards the start (0 h and 0 PSU) of the experiment. Mean values from all points in time for 0 PSU and 15 PSU are compared to each other, change is given in percent. rt (retention time); DMSP (3-dimethylsufonio-propionate); *putative formula, n.y.i. (not yet identified), n.d. (not determined). Compound name and formula are taken from [1](Meot-Duros and Magné 2009), [2](Siracusa et al. 2011), [3](Atia et al. 2011), [4](Hasegawa et al. 2000) and [5](Cunsolo et al. 1993).

Component	Formula	Mass in Da	rt	0 h		2 h		4 h		8 h		24 h		48 h		96 h		168 h		336 h		504 h		mean change at salt (in %)
			PSU	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	
caffeoylquinic acid isomers[1]	C16H18O9	354.10	4.88	1.00	1.46	1.61	1.54	1.58	1.14	1.37	1.24	1.53	1.49	1.53	1.03	1.08	1.56	2.04	1.53	1.97	1.75	1.74		11
hesperidin[3]	C28H34O15	610.19	22.52	1.00	1.11	1.62	1.47	1.49	1.32	1.46	1.28	0.98	1.24	1.04	n.d.	0.76	0.74	0.94	1.31	1.04	1.41	0.99	1.44	16
p-coumaroylquinic acid isomers[2]	C16H18O8	338.10	8.28	1.00	1.30	2.65	3.09	1.38	1.81	2.13	1.42	1.45	1.10	1.46	2.00	0.78	1.23	1.25	3.24	1.34	5.13	1.33	3.99	38
feruloylquinic acid isomers[2]	C17H20O9	368.11	7.76	1.00	1.69	1.46	1.53	1.51	1.25	0.78	1.14	1.10	1.33	1.26	1.15	0.72	1.16	0.98	1.93	0.91	1.47	1.06	1.19	19
dicaffeoylquinic acid isomers[2]	C25H24O12	516.13	11.8	1.00	1.43	1.77	1.27	1.66	0.99	1.23	1.10	1.33	1.10	1.65	1.31	0.66	0.76	1.06	1.73	1.12	2.04	1.19	1.59	22
quinic acid[1]	C7H12O6	192.06	2.1	1.00	1.18	1.41	1.47	1.29	1.14	1.49	1.40	1.68	1.40	1.35	2.02	1.11	1.07	1.37	1.82	1.19	1.66	0.82	1.34	20
diosmin[3]	C28H32O15	608.17	22.52	1.00	1.16	1.59	1.49	1.55	1.26	1.40	1.27	1.21	1.27	1.27	n.d.	1.17	1.05	1.34	1.34	1.69	1.24	1.76		14
sucrose / trehalose[1]	C12H22O11	342.12	2.94	1.00	1.94	2.95	4.49	4.60	3.83	4.13	3.41	4.96	3.19	5.72	2.46	2.44	3.70	5.58	4.45	4.72	3.92	3.32		15
malic acid[1]	C4H6O5	134.02	1.86	1.00	1.14	0.83	1.12	1.06	1.18	1.26	1.31	1.22	1.30	1.20	1.03	1.19	1.10	1.07	1.08	0.94	0.98	0.59		-9
glucose[1]	C6H12O6	180.06	2.76	1.00	1.57	2.19	3.12	2.53	2.15	2.05	2.40	2.18	3.50	2.02	1.37	2.49	2.31	2.86	2.45	2.98	1.86			-1
d-ononitol[4]	C7H14O6	194.08	2.1	1.00	1.27	1.46	1.38	1.23	0.98	1.19	1.19	1.53	1.13	1.69	0.90	0.92	1.18	1.51	1.03	1.57	0.76	1.06		19
sorbitol / mannitol[4]	C6H14O6	182.08	2.84	1.00	0.79	0.71	1.14	0.99	0.73	1.15	0.62	0.54	0.80	1.17	0.52	0.88	0.60	0.83	0.75	1.17	0.65	1.03		22
glycine betaine[4]	C5H11NO2	117.08	2.12	1.00	0.28	0.09	0.22	0.33	0.13	0.48	0.07	0.20	0.11	0.19	0.24	0.13	0.19	0.21	0.22	0.19	0.22			31
DMSP[4]	C5H10O2S	134.04	1.86	1.00	1.07	0.87	1.15	1.07	1.06	1.24	1.22	1.32	1.37	1.30	1.34	1.63	1.41	1.26	1.24	1.11	1.17	0.75		-4
proline[4]	C5H9NO2	115.06	3.2	1.00	0.30	0.01	0.11	0.35	0.10	0.35	0.06	0.37	0.05	0.69	0.08	1.13	0.21	2.46	0.24	2.50	0.14	2.46		88
falcarinol[5]	C17H24O	244.18	22	1.00	0.82	0.68	0.66	0.64	0.50	0.57	0.38	0.62	0.35	n.d.	0.34	0.23	0.35	0.33	0.55	0.45	0.44	0.57		4
A (n.y.i.)	*C12H23O11Cl	377.09	2.95	1.00	3.52	4.54	5.86	5.78	4.56	3.99	4.57	5.96	4.37	6.64	5.80	5.42	6.46	7.80	6.86	7.03	6.54	6.04		9
B (n.y.i.)	*C14H24O12	383.12	2.11	1.00	1.32	1.85	1.49	1.67	1.17	1.55	1.67	2.58	1.57	1.30	1.07	1.50	2.19	1.44	2.30	0.82	1.31			22
C (n.y.i.)		401.13	2.93	1.00	1.61	2.30	3.78	3.42	4.52	4.05	3.41	4.67	5.11	2.30	2.41	3.74	5.38	4.48	4.66	3.79	3.06			19
D (n.y.i.)		170.83	2.06	1.00	0.84	1.09	0.86	0.97	0.61	0.54	0.40	0.35	0.32	0.76	0.70	0.79	1.53	0.66	1.28	0.78	1.17			33

All isomeric forms of the same mass were added up for the evaluation of the peak size. The peak area of each point in time was compared to the start of the experiment and the ratio of change is given for each metabolite, salinity and point in time. Most of the metabolites increase over time. Exceptions are sorbitol/mannitol, glycine betaine, proline, falcarionol and the unknown metabolite D (n.y.i.). Albeit, in these cases, the values for the salt-stressed plants were higher compared to the ones of the non-stressed plant. In all metabolites analyzed in this study, the salt stressed plants had higher values than the unstressed plants which could be especially well observed in the phenols, but also in the osmolytes. Proline with an increase of 88%, *p*-coumaroylquinic acid isomers with an increase of 38% and D (n.y.i.) with an increase of 33% were the most dominant ones. There were only three metabolites that decreased in concentration through salinity stress, which are malic acid, glucose, and DMSP with -9%, -1% and -4%, respectively. However, in these values, the mean was calculated for the sum of all points in time, for the corresponding salinity. The mean values of the two salt concentrations were compared as a percentage change. However, this calculation did not consider the changes of the components in time. If the changes in time are taken into account, the difference between 0 PSU and 15 PSU will be more apparent. Figure 2 is a more detailed presentation of the changes in peak area for selected metabolites in time.
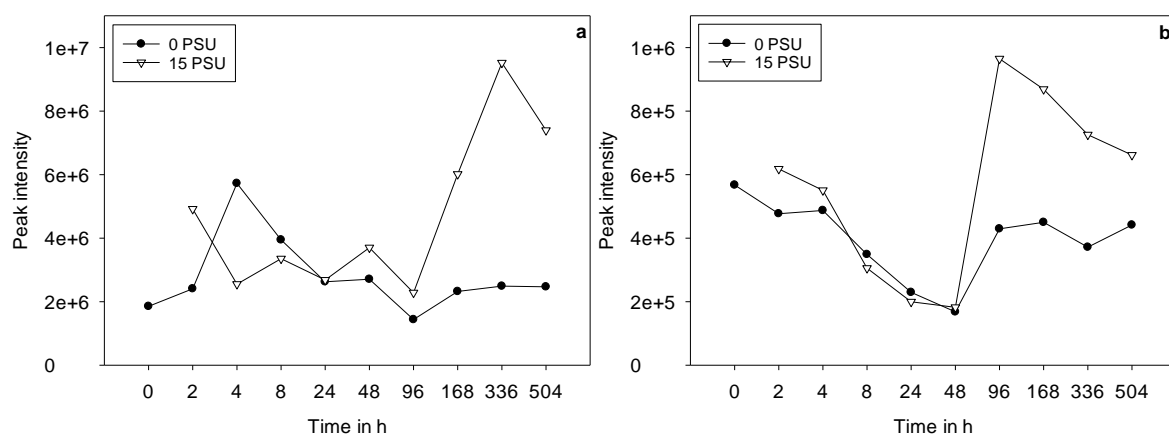
Figure 2. Temporal pattern of (a) *p*-coumaroylquinic acid isomers and (b) the unknown substance D (n.y.i.) in *C. maritimum* grown at 0 and 15 PSU. Values represent the peak area of a single measurement (n=1).

The differences between the values of *p*-coumaroylquinic acid isomers were small in the first 96 h but increased to fourfold higher values at 336 h for plants grown at 15 PSU compared to plants grown at 0 PSU. This is similar to the temporal pattern of the unknown substance D (n.y.i). There were just small differences in the first 48 h of salt stress, but at 96 h the peak area for substance D was twice as large in the stressed plants (15 PSU) compared to the

unstressed plants (0 PSU). These data are just peak areas, the concentration of the components need to be calculated for a better comparison. This was done exemplary with proline (Fig. 3). The proline concentration of plants grown at 15 PSU is relatively low at the beginning of the experiment but increased from 48 h onwards on to a maximum at 336 and 504 h, while the proline concentration of plants grown at 0 PSU had one outlier at the beginning of the experiment, but remained at a low concentration for the rest of the time.
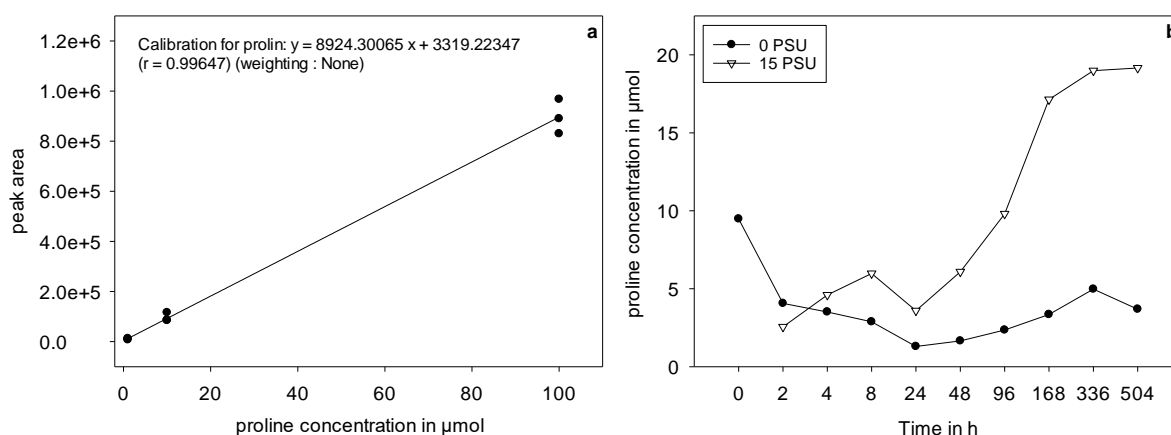

Figure 3. (a) Standard series for proline of three technical repetitions; (b) temporal pattern of the proline concentration for plants grown at 0 and 15 PSU (n=1).

Discussion

The creation of a list with known compounds in the literature helped a lot with the identification of unknown substances. But with this identification, only the mass of the compounds found, are compared to known masses from the literature. Further steps to ascertain the compound identification need to be done. The comparison of the fragmentation pattern of compounds and isotopic distribution are further steps to increase the certainty of the identification, but fragmentation patterns depend on the methodology (e.g. collision energy) and can therefore be different despite the same substance. It is possible to combine the LC-MS with a nuclear magnetic resonance (NMR) for the identification, but this requires an NMR device (Wu *et al.* 2007; Saldanha, Vilegas and Dokkedal 2013). The easiest solution for certainty is to buy standards from all substances identified, if available. These would be necessary for the quantification at least; however, it is assumed that there is a linear correlation between the peak area and the concentration for most substances, as the peak

area indicates the abundance of a substance. A library of all (poly)phenols is too expensive considering the number of 8000 identified structures (Del Rio *et al.* 2013). Also, the creation would take far too long. The best option would be the putative identification of substances, as described above, followed by a selective comparison of commercial standards, if the first results are promising. In this study, there is no certain identification necessary for some components, as they are not changing under the influence of salt. It has to be mentioned that this methodology is slightly biased, because it is set in advance, which substances may be present. However, this is based on literature and this should be correct. With this method, it was not possible to determine some compounds like the sugars and sugar alcohols, because they have the same mass and fragmentation pattern. Furthermore, it was not possible to distinguish between the isomeric forms of the quinic acid esters but that should be possible as Clifford *et al.* (2006) demonstrated fragmentation patterns, with different intensities and fragments for them. This needs to be investigated, especially for *p*-coumaroylquinic acid isomers, to see if one isomeric form is influenced by salinity more than the others. The reason for the enormous increase of *p*-coumaroylquinic acid isomers under salinity is currently unknown and needs further investigation.

Still some compounds which are known to be present in *C. maritimum* were not found in our study. This is due to the fact that method for extraction was limited to hydrophilic substances and the volatile oils which are present in many varieties are therefore excluded (Pateira *et al.* 1999). Furthermore, falcarindiol and falcarinol were found as formerly described (Cunsolo *et al.* 1993), but falcarindiol was first eluted at a retention time of 36 min during the purge step of the column, this leads to a high background and irreproducible results, and was therefore omitted.

In Boestfleisch and Papenbrock (2017) it was shown that salt stress increased the amount of the antioxidative (poly)phenols and flavonoids in *C. maritimum*, though uncertain which substances exactly. It was now demonstrated that the phenolic compounds mainly consist of quinic acid and quinic acid esters and the flavonoids consist of hesperidin and diosmin. While the flavonoids increased together under salinity stress, *p*-coumaroylquinic acid isomers from the phenols increased much more compared towards the other quinic acids. This needs confirmation through additional measurements and quantification and a reason needs to be found. The identification of the unknown substances, especially D needs further effort.

Combining the biomass data for *C. maritimum* from Boestfleisch and Papenbrock (2017) and the peak area of *p*-coumaroylquinic acid isomers and the unknown substance D (n.y.i.) a preliminary estimation for the yield of these substances can be made: From 96 h onwards the yield for *p*-coumaroylquinic acid isomers would be higher in salt-stressed plants, and in the unknown substance D (n.y.i.) the yield would be higher from 96 h towards 336 h in salt stressed plants. Therefore, it was shown that it is possible to increase the concentration and probably yield of single metabolites through saline cultivation and the identification of putative (poly)phenols is fast with the help of literature screening.

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Chapter 6

General discussion

Mankind started cultivation of crop plants, like barley (*Hordeum vulgare*), about 10.000 years ago (Badr *et al.* 2000). This is a very long time compared to the use of halophytes as crop plants. Although the process of cultivation of halophytes is difficult and costly (Yensen 2008), it is already practiced on a small scale (Ventura and Sagi 2013), but still a lot of work has to be done in agricultural practices and marketing (de Vos *et al.* 2013). Nevertheless, the number and potential uses for halophytes increase, solving many problems caused by salinity (Panta *et al.* 2014; Ventura *et al.* 2014). This study contributes significantly to this field.

Difficulties with the measurements of stress and growth

Direct stress measurements

To see if the salt is causing stress reactions in plants, it is a good idea to measure the stress directly. There are a few possibilities to do this; one is the measurement of the chlorophyll fluorescence. Debez *et al.* (2010) showed an influence of salt on the electron transport rate (ETR) of *Batis maritima*, but only at 800 mM NaCl and no influence on photochemical (qP) and non-photochemical quenching (NPQ). In another experiment, a reaction in fluorescence parameters first occurred after 4 months of salt exposure (Jimenez and Gonzalez-Rodriguez 1997). Maricle *et al.* (2007) evaluated this among other studies and postulated that there appears to be no strong relationship between fluorescence parameters and salt stress. Furthermore, the measurement is time consuming so that it would have been impossible to measure the chlorophyll fluorescence in-between the first points of time in our experiments. Furthermore, infrared thermography can be used to measure stress, as it was shown that areas of elevated leaf temperature reflect closed stomata, compared to areas non-elevated temperature which reflect open stomata (Jones 1999). This phenomenon was proven for the measurement of salt stress in barley (Sirault, James and Furbank 2009), however, there is a lack of studies concerning infrared thermography measurements of salt stress in halophytes. This could indicate that either the stomata are not closed, or that the NPQ which produces the heat is not significantly increased. This might indicate that the photochemistry is more resistant in halophytes as indicated by Maricle *et al.* (2007).

Indirect stress measurements

The measurement of growth of the plants is at the same time also a measurement of stress, as growth is inhibited by salinity stress in plants (Flowers, Munns and Colmer 2015). This could be done non-invasive by measuring the size, or invasive by measuring the weight, which is very quick and simple. Plant growth at optimal conditions should have a maximal gain in biomass. The results of halophyte growth in this study were concurring with the literature. Growth is elevated under low salinity, compared to higher salinity or non-saline conditions. However, the optimum varies for the different halophyte species. *Lepidium latifolium* had an optimal growth at 15 PSU, whereas *C. maritimum*, *H. portulacoides* and *T. maritima* had a maximal growth at 5 PSU. Higher salinity reduced the growth of the halophytes indicating stress for the plants. The lower tolerance of monocotyledonous halophytes (*T. maritima*) towards salinity stress, compared to dicotyledonous halophytes (*C. maritimum*, *L. latifolium* and *H. portulacoides*), as Flowers and Colmer (2008) reported for many species, was not observed, probably because they have measured dry weight and not fresh weight. However, both fresh weight and dry weight are not optimal growth parameters, as water and accumulated ions can make up much of these and the increase in organic matter is the best index for growth (Yeo and Flowers 1980). Furthermore, the use of a control with 0 PSU is questionable, as this is suboptimal for the halophytes (Flowers and Colmer 2008). It is unclear whether gain of fresh weight is a valuable indicator for stress.

Another possibility to determine the stress triggered by salinity is on the molecular level: The gene expression. The expression of enzymes from the enzymatic antioxidative system, like ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), peroxidise (POD) and superoxide dismutase (SOD) were suggested to be good stress markers for salt and have already been tested (Sharma *et al.* 2010; Parida and Jha 2010). However, this requires information about the genome of the halophyte which is a very difficult task if there is no sequence information available.

Some metabolites, like the osmolyte proline act as a biochemical stress marker. An increase in the proline concentration under salt stress was shown in *Spartina anglica* (Diggelen *et al.* 2006), and was also clearly observed in *C. maritimum* (chapter 4). However, there are some exceptions; as generally accumulated under osmotic stress, proline concentration decreases

if free proline is incorporated into proteins for seeds, at least in barley (Wenzel *et al.* 2015). In some species, several other osmolytes are used instead of proline. Additionally, an upregulation is not always observed, suggesting a constitutive synthesis of these osmolytes (Slama *et al.* 2015). *Triglochin maritima* showed signs of a constitutive synthesis of proline and as well as with *H. portulacoides,* however, both might have accumulated other osmolytes as well (chapter 4). It was shown that proline acts for some species as a salt stress marker, and is also applied as a marker for drought stress, but it does not accumulate in every halophyte species.

Metabolites, especially antioxidants as possible stress marker

The challenging measurement of ascorbic acid

The measurements of metabolites in this study were always linked to the measurement of antioxidants, since all measured metabolites as (poly)phenols including flavonoids, ascorbic acid (AA), glucosinolates (GSL) and even proline (Slama *et al.* 2015) are non-enzymatic antioxidative compounds.

The difference between dehydroascorbic acid (DHA) and ascorbic acid is one of the first indicators for antioxidative stress and also an important one. (Foyer, Trebst and Noctor 2006) This phenomenon was shown in the halophyte *Salicornia brachiata*, where the concentration of AA decreased after the induction of salt stress while the concentration of DHA increased (Parida and Jha 2010). In contrast to this, Hernández *et al.* (2000) showed a decrease in AA and DHA of a salt tolerant and a salt sensitive *Pisum sativum* cultivar and not a ratio change of AA towards DHA. A ratio change of AA towards DHA was not observed in our experiments, only concentration changes of AA were observed at salinity stress. Only in *T. maritima* the AA concentration first decreased at 2 h, then increased at 4 h with elevated salinity. This could indicate a consumption of AA trough oxidative stress, followed by an adaption shortly after. However, there was no increase in DHA and the reaction was very fast compared to Parida and Jha (2010), where the decrease in AA concentration was measured after one week. There is a possible reason for the absence of a ratio change: The establishment of a measurement of AA and DHA was difficult, due to the high number of samples, the test had to have a high throughput. The use of enzymatic assays was tested at first, in which the absorption of AA was measured directly under UV-light and ascorbate oxidase oxidizes AA to DHA for the blank. The

maximum AA amount was measured with the help of DTT, which reduces all DHA to TAA. Space for four different reactions on a microtiter plate (including blanks) was required and this would have been expensive, considering technical replications, as of the consumption of many pricey UV-microtiter plates. The use of another protocol and the measurement with the help of ferric chloride seemed more practical for high throughput, as only two wells for two reactions were required, and it was also cheaper as no UV-microtiter plates were needed. For a comparison of the two methods see Ueda, Wu and Frei (2013). However, problems occurred with the ferric chloride method, but were only recognized after the AA measurements of chapter 2 and 3. The use of trichloroacetic acid (TCA) and a low-grade $FeCl_3$ led to a wrong, lower determination of the AA concentration. Either, the pH of TCA was probably not low enough to protect the AA or the $FeCl_3$ was possibly already oxidized prior to the start of the experiment and impurities, like $CuSO_4$ could interfere with the measurement (Harel 1994; Ueda, Wu and Frei 2013). Therefore, the amount of DHA was overrepresented, as it was calculated as the total ascorbic acid (TAA) minus the AA concentration. It was a challenging task to find and solve the problem, but is was finally achieved by using metaphosphoric acid (MPA) and a new and higher grade of $FeCl_3$ as described in chapter 4. This together might explain the lack of a ratio change in *L. latifolium* (chapter 3) of DHA and AA as described by Parida and Jha (2010). In chapter 4 this problem was already solved but there was still no ratio between DHA and AA. But in these experiments, the salt concentrations were much lower compared to the ones of Parida and Jha (2010). Supposedly this is the reason that no ratio changes were observed. The various concentration changes of AA and correlations to other metabolites are currently unknown and need further investigation

A mathematical operation increased the precision of the ORAC

The ORAC gives an overview of the antioxidative capacity, which is valuable to know, if the plant is addressed as a nutraceutical for human consumption. However, it does not indicate which metabolites are responsible for the antioxidative effect. Furthermore, it was shown (chapter 1 and 4) that the ORAC cannot measure every possible antioxidant at the same time, either hydrophilic or hydrophobic and mainly chain-braking ones are determined (Ou *et al.* 2002). There were some problems with the ORAC during the experiments of chapter 2, which are mentioned in chapter 3 and 4. A critical step is the addition of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) to the mixture of analyte and fluorescine, because the

reaction starts at that time. Addition of warm (37°C) AAPH results in lower values at the started well and higher ones in the finished well. To prevent this, the AAPH was kept at 0°C minimizing the radical production. After the addition of AAPH, the plate was incubated in the microtiter plate reader at 37°C and red every minute. However, the microtiter plate heated up faster in the corners leading to a gradient from the outside of the plate towards the middle. But this was more controllable than the pipetting speed and direction of the user. Nevertheless, this influenced the values since the ones further outside were underrepresented, but this problem was solved by a mathematical compensation of the values which reduced the standard deviation by 50% leading to more precise results.

The stepwise analysis of (poly)phenols to flavonoids and to individual substances

The flavonoid and therefore the (poly)phenol concentrations was increased by salinity in this study (chapter 2-4). The responsible reasons (over reduced electronic transport chain) were addressed in chapter 1. In this study, the main class of metabolites contributing to the ORAC seemed to be the one of (poly)phenols. This was indicated by the high correlations between the values of ORAC and (poly)phenols. However, to prove this, all components need to be identified and analyzed individually for their ORAC.

The flavonoids make up around 20-22% of the (poly)phenols in *L. latifolium* and *H. portulacoides,* and 34-38% in *C. maritimum* and *T. maritima*. This is a basic calculation, only comparing the molarities of the substances which were used as the standards, gallic acid for (poly)phenols, and catechin for flavonoids. Due to the difference in percentages, the correlations of flavonoids towards ORAC in *C. maritimum* and *T. maritima* should be higher, but this was not the case. That is because the Pearson correlation is slightly biased since it just indicates if values are influenced in the same or opposite direction, not considering the amount. Furthermore, the radical scavenging capacity of different flavonoid compounds and phenolic compounds differs (Ou, Hampsch-Woodill and Prior 2001). Even if single components are analyzed for their ORAC, it may not be possible to simply add the values as there are synergistic and antagonistic effects (Jacobo-Velazquez and Cisneros-Zevallos 2009). It was possible to analyze the individual (poly)phenols and flavonids in *C. maritimum* as shown in chapter 5. The main part of the phenols was quinic acid and its esters with ferulic acid, *p*-coumaric acid and with caffeic acid. These substances are presumable responsible for the antioxidative effect, as the radical scavenging efficiency of chlorogenic acid (3-caffeoylquinic

acid) was previously shown (Meot-Duros and Magné 2009). The flavonoid components present in *C. maritimum* are diosmin and hesperidin. These were proven to have an antioxidative effect, but lower compared to the phenolic compounds (Rice-Evans, Miller and Paganga 1996). The antioxidative effects of these substances alone and in combination need further investigation, to see whether synergistic or antagonistic effects are present, or whether it is possible to "add up" the antioxidative effect.

The measurement of metabolites via LC-MS has many advantages compared to the determination of (poly)phenols and flavonoids. Single compounds are analyzed and not a group of substances. Therefore, a more precise picture about the change of the metabolites at stress conditions is obtained and the determination of a possible following application is easier. But there are drawbacks, at first, the availability, which was also not given till the end of this study. If the sample contains many unknown substances it is a difficult and time-consuming task, but libraries and literature research as described in chapter 5 might help. If a metabolite is identified, the structure gives hints, but no certainty about the antioxidant capacity. Other tests like the ORAC have to fulfil this part.

Evaluation of the metabolite data, advantage of halophyte crops

Biofortification through saline cultivation

It was shown (chapter 2-4) that it is possible to enhance the metabolite concentration through saline cultivation. All metabolites were enhanced at some point in time through salinity, even though not at the same point in time. This could be described as biofortification, albeit the term of biofortification was previously described as the procession of staple crops, so that they are capable of delivering the daily necessary micronutrients (Hirschi 2009). This was done by fertilizer application, conventional breeding or by genetical modification, but conventional breeding takes years and genetic modification is rejected by a large part of the society (Gaskell *et al.* 2010). A typical example for a biofortified crop is "golden rice" (Paine *et al.* 2005). However, the biofortification through saline cultivation does not have the deficits of the techniques mentioned above. It should be taken in to consideration to expand the term of biofortification for the increase of antioxidants through saline cultivation.

Halophytes as possible nutraceuticals

In comparison to crops like potatoes, corn and beans, and even vegetables, like spinach and broccoli, halophytes have a much higher ORAC (Tab.1). The amount of (poly)phenols is on average, similar to other crops. *Crithmum maritimum* can compete with blueberries, which are well known for their high (poly)phenol concentration (Prior *et al.* 1998). With elevated salinity, *C. maritimum* and *L. latifolium* reach a (poly)phenol concentration of about 4 mg GAE g^{-1} which is higher than the concentration in blueberries. Outstanding is *T. pannonicum* with an average (poly)phenol concentration of 3.8 mg GAE g^{-1} which increase to values of 4.5 mg GAE g^{-1} under salt stress. The average requirement of AA for men and women are 110 and 95 mg per day (German Nutrition Society (DGE) 2015). Hence around 50 g of *L. latifolium*, or 100 g of the other halophytes are sufficient to fulfil the AA requirement per day.

The GSL content of *L. latifolium* is 2.3-7.6 mg g^{-1} FM, around the same level, or even 20 times higher than the GSL concentration of broccoli, depending on the considered source (López-Berenguer *et al.* 2008; López-Berenguer *et al.* 2009; Khan, Ulrichs and Mewis 2010; Zaghdoud *et al.* 2012). If broccoli is grown at saline conditions (80 mM NaCl), the GSL increases, therefore reaching two times higher GSL concentration compared to *L. latifolium*. However, the salt causes severe reductions in the growth of broccoli, at conditions which are below an optimum for *L. latifolium* (López-Berenguer *et al.* 2008; López-Berenguer *et al.* 2009). Therefore, halophyte crops can be rated as nutraceuticals because they are more valuable than just food; their high (poly)phenolic, ascorbic and GLS content can prevent diseases (Del Rio *et al.* 2013; Rodriguez-Mateos *et al.* 2014). The analysis and determination of the single components provide important information about the potential use. For example, diosmin and hesperidin found in *C. maritimum* are used as medicine in combination, like Daflon 500 mg (Servier Laboratories, France), for the treatment of chronic venous deficiency (Rosa, Alvarez-Parrilla and Gonzalez-Aguilar 2010). Quinic acid, also found abundant in *C. maritimum* has a positive effect on DNA repair and/or immune enhancing properties (Pero, Lund and Leanderson 2008).

Table. 1 Staple crops, vegetables, healthy foods and halophytes compared for ORAC (µmol TE g^{-1}), (poly)phenols (mg GAE g^{-1}) and AA (mg g^{-1}). Values taken from Prior *et al.* (1998), Haytowitz and Bhagwat (2010) and US Department of Agriculture (Agricultural Research Service and Nutrient Data Laboratory 2015). Values of the halophytes present the mean of the experiments.

Antioxidant / crop	potato	corn	beans	spinach	broccoli	blueberries	apples	*L. latifolium*	*T. maritima*	*H. portulacoides*	*C. maritimum*	*T. pannonicum*
ORAC	10	5.9	2.1	15.1	13.5	47	30	52	30	25	82	130
Phenols	1.6	2.1	0.1	2.1	3.16	3.1	2.5	2.2	1.4	1.1	2.9	3.8
AA	0.2	0.07	0.1	0.08	0.81	0.1	0.04	1.9	0.9	0.8	0.8	0.9

Consumer needs should be met

If halophytes should be bought by people as food or nutraceutical, the consumer needs should be addressed. The consumer needs vary in the different regions of the world, but luckily healthy food is the number one attribute consumers appreciate in every region. Appearance alongside smell is the second attribute in the western world (Moser *et al.* 2011). Halophytes are certainly a healthy choice, but it is questionable, if odour or appearance satisfies consumers. Astonishingly the taste was not mentioned as decisive criteria, but the taste is still important as de Vos *et al.* (2013) stated and it might become more important in the future. The procession of the halophytes should be kept in mind. If a vegetable is new on the market and the consumer does not know how to prepare it, he might skip it for a better known one. Old traditional recipes might be very useful, if marked simultaneously like the pickled *C. maritimum*. Furthermore, sometimes other cultural groups already consume halophyte species on a regular basis, like *L. latifoium* in India (Kaur *et al*. 2013). There is certainly much more to be learned in case of processing of halophytes.

Extrapolation of halophyte cultivation

This was a small-scale experiment. Before planting a field with halophytes watered with brackish water on a large scale; some further steps have to be taken into consideration. There

might be other species or ecotypes with higher or different metabolite concentration, as was shown for GSL (Kliebenstein 2001), or a faster growth, resulting in a higher biomass production, or multiple harvests. Mature plants, ready to harvest, should be tested for an increase of metabolites though severe salt stress. The possibilities of an increase of secondary metabolites in halophytes, grown in brackish water, through drought or high radiation should be investigated as well. A screening for the best suited ecotype must be made. Furthermore, agricultural practices and marketing need to be examined as de Vos *et al.* (2013) indicated. Larger scale flavour studies should be carried out, as well as studies towards seasonal availability and storage properties of halophytes. The fields of application should be maximal for a species. For example, if a field of *C. maritimum* is not used completely for food production it can be bought to bloom and oil can be obtained from the seeds (Pateira *et al*. 1999; Jallali *et al*. 2014).

Possible criticism

Halophytes adapt to salt stress by accumulation of Na^+ and Cl^- in their vacuole (Flowers *et al.* 2015). This was also shown in our experiment (chapter 3). One could argue that halophytes are not suitable for daily consumption because of the high salt concentration. In our study *C. maritimum*, *H. portulacoides* and *T. maritima* accumulated 5, 11 and 11 mg Na g^{-1} FM respectively. It is suggested that the average salt intake per day should be less than 5-6 g per day (currently 12 g in Europe) (Strazzullo *et al.* 2009). But this is not much compared to other foods, as bread contains about 5 mg Na g^{-1} and meat products 11 mg Na g^{-1} (USDA 2015). An easy solution to reduce the salt concentration of *T. maritima* and *H. portulacoides* is a sturdy wash, as they have developed glandular cells and bladder hairs (Koyro *et al.* 2010), which are presumably rinsed off. Nevertheless, for consumption it is better to use halophytes that do not accumulate Na in high amounts.

Toxic substances, like oxalic acid and nitrate may be present in halophyte species (Guil, Rodríguez-García and Torija 1997). It should be monitored that these substances do not accumulate. No toxic substances were found in *C. maritimum* with the LC-MS analysis, however, not all components are identified yet. The other halophyte species should also be screened for toxic substances.

Introduced plants can become invasive species, like *L. latifolium* in Northern America (Francis and Warwick 2007). Therefore, fields must be monitored and the uncontrolled spread of plants must be prevented. A better solution would be the use of native plant species in the according area.

Conclusions

It was demonstrated that it is possible to manipulate (poly)phenols including flavonoids, ascorbic acid, and consequently the ORAC through moderate salt stress, within 24 h (chapter 2). A longer application of salt stress from 4 days onward let to an adaption of the plants, resulting in a decrease in growth and therefore a lower metabolite yield (chapter 3). However, the GSL concentration started to increase after a few days rising further with every week. But the yield of total GSL did not increase (chapter 3).

Mild salt stress did increase the concentration of the metabolites slightly, but that was overcompensated by the loss of biomass (chapter 4). Single components were influenced so strongly by salt stress, like proline, glucoiberin, *p*-coumaroylquinic acid isomers and a substance which is not yet identified, that the yield was still higher despite reduced growth (chapter 2-5). So, the method to increase metabolites depends on the preferred metabolite. Choose either severe salt stress for a short amount of time, in order to achieve high amounts of antioxidants, or mild salt stress for a prolonged time, to obtain an increased concentration of individual components.

In addition, halophytes grow better with low salinity than the control plants without salt, making halophyte agriculture with brackish water very attractive, even more if stress is applied.

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Danksagung

Hiermit möchte ich mich ganz herzlich bei allen bedanken, die mich bei dieser vorliegenden Arbeit unterstützt haben.

Dazu möchte ich mich als aller erstes bei **Prof. Dr. Jutta Papenbrock** bedanken, die es mir ermöglicht hat, an diesem interessanten und herausfordernden Thema zu arbeiten. Aber auch für die anregenden Diskussionen und guten Vorschläge möchte ich mich bei ihr, sowie bei **Prof. Dr. Bernhard Huchzermeyer** bedanken, auch für die Übernahme des Koreferats. Ein großes Dankeschön geht an **PD Dr. Carsten Zeilinger** für die Übernahme des Vorsitzes der Promotionskommission.

Ein ganz besonderes Dankeschön gebührt **Yvonne Leye** und **Lutz Krüger** für die tatkräftige Unterstützung in der Pflanzenanzucht, dem Vorbereiten der hydroponischen Kultur und der Versorgung während den Versuchen und den Exkursionen zur Saatgutgewinnung.

Bei **Pamela von Trzebiatowski**, **Julia Volker** und **Birgit Lippmann** bedanke ich mich für die Hilfe bei den vielen technischen Fragen im Labor.

Weiterhin möchte ich mich bei allen Mitarbeitenden der Arbeitsgruppe bedanken, besonders bei **Annekathrin Rumlow**, **Yvana Glasenapp**, **Ariel Turcios** und **Felix Hirschmann**, **Johann Hornbacher**, **Jana Theilmann** und den früheren Mitarbeitenden **Anne Kathrin Buhmann** und **Nico Wagenseil**, sowie allen weiteren Mitarbeitenden der Arbeitsgruppe und des Instituts für Botanik für die Unterstützung und eine tolle Arbeitsatmosphäre.

Schließlich möchte ich mich noch bei meinen Freunden und meiner Familie bedanken, für ihre Unterstützung und das entgegengebrachte Verständnis in der letzten Phase dieser Arbeit.

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Education and career history

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10/2010 - 11/2012	Studies of plant biology (M.Sc.), Gottfried Wilhelm Leibniz University Hannover Germany; Topic of the thesis: "Biofiltration and utilization potential of *Spartina* spp."
10/2006 - 9/2009	Studies of biology (B.Sc.), Gottfried Wilhelm Leibniz University Hannover, Germany; Topic of the thesis: "Investigations on the phytoremediation potential of *Sesuvium portulacastrum* L."
10/2005 - 09/2006	Studies of mechanical engineering, Technical University Braunschweig
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International experience

03/2012   Four-week stay abroad at the Chilika Lagoon, Orissa, India, work on a project for the study of halophytes in marine wetlands as biofilters for nutrients from aquaculture effluents and other possible uses of halophytes, which was sponsored by the International Bureau of the BMBF.

Conference participation and presentations

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