Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE E TECNOLOGIE AGRARIE, AMBIENTALI E ALIMENTARI

Ciclo 34

Settore Concorsuale: 07/B1 - AGRONOMIA E SISTEMI COLTURALI ERBACEI ED ORTOFLORICOLI

Settore Scientifico Disciplinare: AGR/02 - AGRONOMIA E COLTIVAZIONI ERBACEE

PHYSIOLOGICAL AND BIOCHEMICAL ADAPTATION TO SALINITY IN WILD HALOPHYTES SUITABLE FOR MEDITERRANEAN AGRICULTURE

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Esame finale anno 2022

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Abstract

Due to the accelerating processes of soil salinization and shortage of fresh water, the practice of saline agriculture is gaining momentum in many areas of the world, especially the arid and semiarid regions.

However, there are some concerns that using saline water for irrigation may be non-environmentally sustainable, with potential to cause irreversible soil degradation. In addition to this, there is a lack of information on the morphological, physiological, and biochemical changes that can occur in plants when irrigated with saline water.

In light of the above, the major aim of this work was to investigate the effects of a range of water salinity levels and irrigation regimes on the performances of some salt tolerant species promising as future crop plants for saline agriculture. The following objectives were addressed:

- To determine the effects of different water regimes (leaching irrigation vs. no leaching irrigation) with water at increasing salinity concentrations on the growth, ion accumulation and water relations of S*orghum bicolor* plants grown under saline soil conditions.
- To describe the germination response of *Salicornia europaea* seeds across a wide range of water salinity levels through six reliable indices for screening salinity tolerance at the seed germination stage.
- To explore the different physiological responses of six wild halophytes commonly found in the Mediterranean area (*Artemisia absinthium, Artemisia vulgaris, Atriplex halimus, Chenopodium album, Salsola komarovii, and Sanguisorba minor*), and rank their tolerance after exposure to growing levels of water salinity.
- To identify the main adaptation mechanisms that distinguish C3 from C4 halophytes when exposed to increasing salinity in the growth media, through a comparative study between the C3 species *Atriplex hortensis* and the C4 species *Atriplex halimus*.
- To identify the main adaptation mechanisms that distinguish annual from perennial halophytes when exposed to severe conditions of salinity and drought, through a comparative analysis between two annual *Salicornia* spp. and the perennial *Sarcocornia fruticosa*.

One of the pillars for a successful and long-time sustainable saline agriculture is the proper management of salt leaching though irrigation. Working out this concept, a pot experiment on *Sorghum bicolor* was implemented. The experimental design involved three levels of soil salinity (0, 3, and 6 dS m^{-1}) combined with three levels of water salinity (0, 2–4, and 4–8 dS m^{-1}, depending on time) and two water regimes: no salt leaching (No SL) and salt leaching (SL). The plant response to the treatments was assessed through a series of biometric and physiological measurements. High soil salinity associated with high water salinity impaired plant growth, water relations, and nutrient uptake and translocation to a greater extent in No SL than SL. Higher water availability (SL), although causing higher salt input when associated with high water salinity, determined higher water use efficiency and selective uptake of Ca over Na, and limited the Na translocation to the leaf, thereby preventing salt induced injuries to the photosynthetic organs.

Besides applying adequate agricultural and irrigation practices, saline agriculture relies on the selection of appropriate crop species. Screening salinity tolerance at germination, one of the growth stages most sensitive to salinity, represents a robust method for crop selection, as plants showing tolerance at this stage remain generally tolerant during the further growth stages. Keeping this in mind, a germination trial on *S. europaea* seeds exposed to 6 levels of salinity (0, 100, 200, 300, 400, and 600 mM NaCl) was set up with the aim to compare the response of eight common germination indices: germination percentage (GP), germination energy (GE), germination value (GV), coefficient of germination velocity (CVG), germination rate index (GRI), germination peak value (GPV), mean germination time (MGT), and time to 50% germination (T_{50}). The first six of the eight indices showed a consistent outcome and allowed us to identify two salinity thresholds to describe *S. europaea* germination under salinity, the first causing a slight germination decline (100 mM NaCl) and the

second causing a severe germination drop (600 mM NaCl), with no significant germination variation between the intermediate salinity levels.
Once the delicate phase of germination has been passed, there are several strategies that halophytes can deploy to continue growing under saline conditions. To explore this diversity, an experimental study was set up on six halophytes commonly found in the Mediterranean area, different in habitus, ecology, and life cycle: *Artemisia absinthium, Artemisia vulgaris, Atriplex halimus, Chenopodium album, Salsola komarovii, and Sanguisorba minor*. The plants were subjected to increasing NaCl concentration (control, 100, 200, 300 and 600 mM NaCl) for 161 days. Thereafter, fresh weight (FW), leaf stomatal conductance (GS), relative water content (RWC) and water potential (WP) were measured. Among the six plants, the best performing was *Atriplex halimus*, which showed the highest FW, GS, and RWC levels under low-intermediate levels of salinity, in contrast to *S. minor* and *A. absinthium*, which exhibited the most severe effects, i.e. a steep drop in GS and RWC.
The remarkable resilience to salinity stress demonstrated by *A. halimus* may also depend on this species' C4 photosynthetic pathway. To verify the reliability of this hypothesis, a comparative study between the C4 *Atriplex halimus* and the C3 *Atriplex hortensis* (this latter with three cultivars: green, red and scarlet) was carried out. The four genotypes were grown for 35 days with water salinity ranging from 0 to 360 mM NaCl. A series of parameters related to plant growth, water relations, ion accumulation, photosynthesis, stomatal conductance, and membrane peroxidation were measured at the middle and the end of the experiment.
Overall, both species showed to be extremely resistant to salinity. Stomatal conductance and transpiration rate were more severely affected by salinity in the C4 *A. halimus* than in the C3 *A. hortensis*. Nevertheless, a lower leaf water potential, indicating stronger osmotic adjustment, was recorded in *A. halimus* than *A. hortensis*, which was reflected in a higher relative water content in the former genotype. FTIR spectroscopy revealed in both species a reduced amount of pectin, lignin, and cellulose under salinity, indicating a weakened cell wall structure due to salinity.
Besides the photosynthetic pathway, another characteristic distinguishing the two *Atriplex* species is the length of the life cycle, being *A. halimus* a perennial shrub and *A. hortensis* an annual plant. This observation raised the question of whether the length of the life cycle may affect the adaptive response and the recovery capacity from severe environmental stresses.
To address this question, a further comparative study between two annual species *Salicornia europaea* and *S. veneta* and the perennial species *Sarcocornia fruticosa* was set up. The plants were exposed to high salinity (SS, irrigation with 700 mM NaCl water solution) and drought conditions (WS, complete withholding of water) for 30 days. Thereafter, they were allowed to recover (Recovery, non-limiting freshwater supply) for the following 15 days. A series of morphological and biochemical parameters related to ion accumulation, photosynthetic pigment content, osmolyte synthesis, and antioxidant activity were assessed before and after the recovery.
The results indicated that the two annual *S. europaea* and *S. veneta* and the perennial *S. fruticosa* were highly tolerant to SS but sensitive to WS, although the latter species to a lesser extent. Indeed, the drought impact on *S. fruticosa* biomass was milder and its content of photosynthetic pigments remained unchanged. This could be attributed to the fact that *S. fruticosa* seeds were collected from an area characterized by a warmer and drier climate than that from where the seeds of the two *Salicornia* species were gathered and, hence, that *S. fruticosa* has evolved a more robust system to cope with drought conditions. Nevertheless, we cannot exclude that the slower metabolism of perennial plants could offer an adaptive advantage for survival under unfavourable conditions since it allows to reduce the consumption of water and other resources while enhancing the synthesis of protective compounds. This may have contributed to the better performance of the perennial *S. fruticosa* under water deficit with respect to the annual *S. europaea* and *S. veneta.*
The high salinity tolerance shown by all three species seemed to depend mostly on a controlled transport of ions to the aerial parts and on the biosynthesis of organic osmolytes (glycine betaine, proline, and soluble sugars) for intracellular osmotic adjustment. These mechanisms, in fact, were sufficient to preserve the stressed plants from oxidative stress.

All three species were able to recover from drought stress, although the fresh weight of the water-stressed plants of *S. europaea* and *S. veneta* remained significantly lower than in control plants.
Altogether, the results of this project reveal that, at the whole plant level, the halophyte ability to cope with salinity is mainly related to the capacity to accumulate and safely compartmentalize into vacuoles the soluble ions naturally present in the growth media, and to use them for osmotic adjustment. This strategy allows these plants to avoid the serious growth impairments and subsequent yield penalties experienced by glycophytes due to their need to redirect a major pool of photosynthates for de novo synthesis of organic osmolytes.
The C4 photosynthetic pattern appeared to favour halophytes by allowing them to continue photosynthesize even at very low leaf water potential and reduced stomatal gas exchange.
The perennial life cycle might have conferred more resilience to salinity and, above all, to drought stress, by slowing down the stress-induced senescence processes activated by annual plants, which prioritize seed development in order to guarantee the multiplication of the species, to the detriment of plant growth. Further studies will be required to confirm this hypothesis. Potassium accumulation and glycine betaine production resulted to play a major role in conferring drought tolerance.
In summary, this work has demonstrated the possibility in principle of sustainable crop production under saline conditions, using a variety of species as potential new crop types for saline agriculture ranging from slightly salt-tolerant (*S. bicolor*, *S. minor*, *Artemisia* spp.) and moderately to highly salt-tolerant species (*C. album, S. komarovii*) to very high salt-tolerant halophytes (*Atriplex* spp, *Salicornia* spp., *S. fruticosa*).

Chapter 1: General Introduction

1.1. Background

Salinization can be defined as the accumulation of water-soluble salts to a level that impairs agricultural production, environmental health, and economic welfare [1]. It is considered a land-use issue when the salt or sodium concentration degrades the soil structure and/or becomes detrimental to plant growth. It turns into a water issue when potential uses of water are limited by its salt content.

Soil and water salinization have always been a threat to the Earth's ecosystems and agricultural productivity.

Indeed, historical records show that many civilizations failed due to increased salinity in agricultural fields; one of the best-known cases is that of the ancient Mesopotamian civilization (located in modern Iraq), whose decline was led by three major salinization events: the first and most severe was from 2400 BC to 1700 BC, the second was between 1300 and 900 BC and the third occurred after 1200 AD. In all three cases, the main cause was attributed to the improper use of agricultural and irrigation practices [2].

Nowadays, salinization is occurring at an unprecedented rate and geographic scale, posing a serious risk to food security and ecosystems integrity.

Salinization can be driven by either natural (primary) or anthropic (secondary) processes. Primary salinization involves accumulation of salts through natural processes such as physical or chemical weathering and transport from parent material, geological deposits, or groundwater. Secondary salinization is caused by human interventions such as irrigation mismanagement, use of low-quality and salt-rich irrigation water, poor drainage conditions, inappropriate use of fertilisers, discharge of saline wastewater into rivers from industries and mining activities, and the periodic application of road de-icing agents in snow prone regions of developed country [3]. However, many of the factors leading to soil salinization are being exacerbated by climate change.

Salt-affected soils can be found across different climatic zones and altitudes, from territories below sea level, e.g., the district of the Dead Sea, to mountains rising over 5000 m as the Tibetan Plateau or the Rocky Mountains [4].

In arid and semi-arid areas, high evapotranspiration contributes steadily to the formation of saline soils, and the lack of rainfall impedes the washing of salts out of the soil. As a result, salts dissolved in soil solution build up at the soil surface after water evaporation. This, in turn, withdraws water from the underlying soil layers by capillary rise, feeding the salinization cycle. This form of salinization is common in the Mediterranean regions where the evaporation can reach 8-10 mm day^{-1} and the limited rainfall can reduce the extent of the watercourses, inducing a more and more severe transition to arid and saline environments [5].

Along the costal and subcostal areas, the current sea level rise is increasing the occurrence of flooding and seawater seepage into areas lying below the sea level [6]. Besides, it can boost lateral seawater intrusion into river outlets and coastal aquifers hydraulically connected to the sea, which in the long-term can contribute to soil salinization through irrigation and capillary rise. The groundwater over-exploitation for civil, industrial, and agricultural purposes may further increase this trend, contributing to groundwater salinization [7].

Saline and sodic soils can be encountered also in internal and continental lands, mostly derived from salt rich parent materials, coming from marine, fluvial, or lacustrine sediments, deposited far from the current seashores, on plains but especially on hilly environments [8].

Up to date, different estimates have been produced but the localization and extension of the areas affected by salinization are still controversial. Indeed, as in the last decades the climate warming has sped up the described salinization processes, the comparison of data taken from areas surveyed at different times becomes unreliable.

According to Dudal and Punell [9], salt affected soils occupy nearly 7% of the worlds land area. Massoud [10] estimated an extension of 932 Mha, of which about 16 in North America, 2 in Central America, 129 in South America, 80 in Africa, 85 in South Asia, 211 in North and Central Asia, 20 in Southeast Asia, 358 in Australia and 51 in Europe. Later on, Balba [11] quoted only 600 Mha, of which 30 Mha in Africa, 340 Mha in Asia, 140 Mha in Australia, 1 Mha Europe, 26 Mha in North America, and 60 Mha in South America.

More recently, Pessarakli and Szabolcs [12] estimated that saline and sodic soils cover an area of 954.8 Mha, Shahid et al. [13] approximated that salinity is likely to affect 10% of the world arable land, while Negacz et al. [14] produced a world map of soil affected by salinity as shown in Figure 1.

According to the FAO global map of salt-affected soils [15], 424 million hectares (Mha) of topsoil (0-30 cm) and 833 Mha of subsoil (30-100 cm) are salt-affected around the world, of which 316 Mha are in developing countries. Finally, based on the World Atlas of Desertification [16], the Earth's areas affected by primary salinization have an extension of around 1 billion ha. Secondary salinization, instead, affects approximately 77 Mha, of which 58 % are irrigated lands. It is estimated, indeed, that fully 20 % of all the world irrigated areas are affected by salinity, with the greatest incidence in the intensive farming regions of India, Pakistan, China, Iraq, and Iran. Among the regions more highly prone to salinization, there are the Mediterranean Basin, Australia, Central Asia, the Middle East, Northern and Eastern Africa, and South Asia.

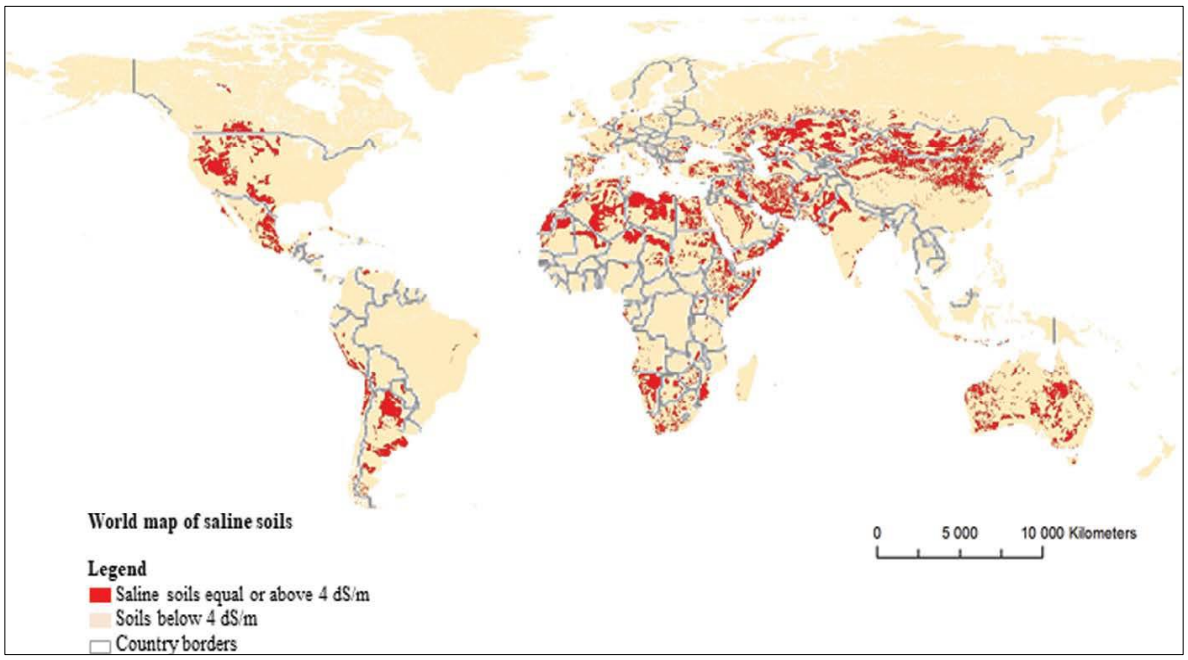


Figure 1. Word map representing countries with salinity problem [14].

In Europe, as seen, the estimate of the extension of saline and sodic areas is highly variable: 6.7 Mha according to the FAO-UNESCO soil map of the world [17], 3.8 Mha according to Stanners [18] or 30.7 Mha according to Rengasamy [1].

In 2008, the EU Joint Research Centre (JRC) updated the Soil Geographical Database of Europe (SGDBE), producing a map which represents the limitations to agricultural posed by salinity and sodicity in Europe (figure 2A).

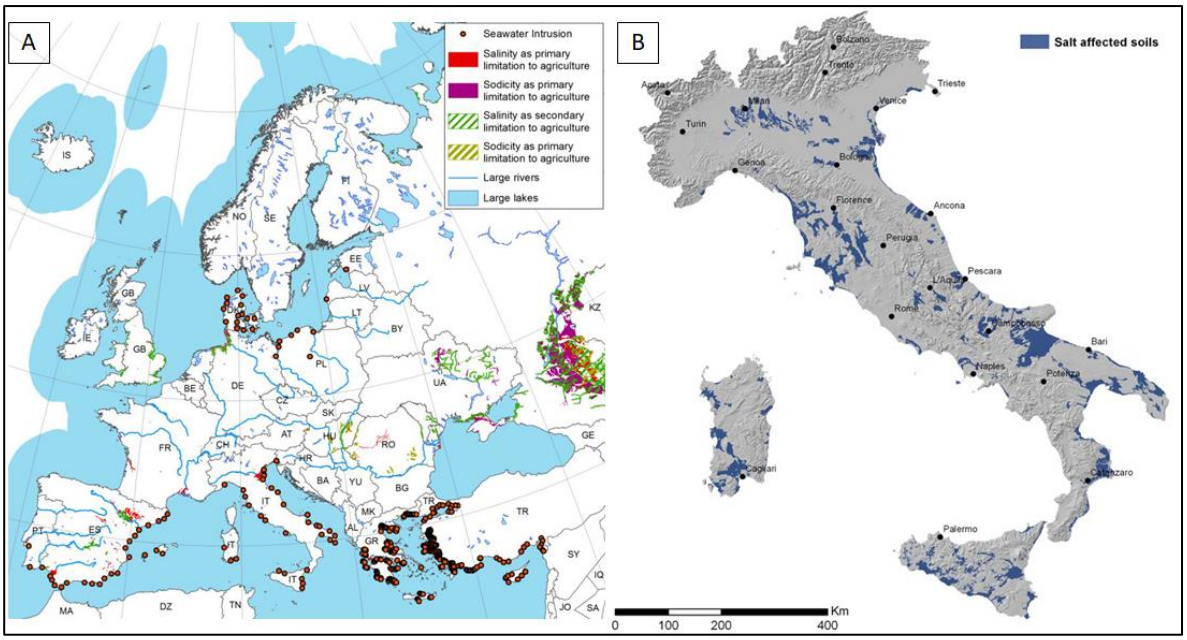


Figure 2: (A) Saline and sodic soils as primary and secondary limitations to agricultural use, and areas of seawater intrusion in the European Union [19]. (B) In blue the Italian territories affected by soil salinity [20].

A more recent study published by Hassani et al. [21] estimated that the area of salt spoiled soils in Europe is 24 Mha, representing approximately 2.05 % of the total salt affected area at worldwide level.

In the Mediterranean basin, 25% of irrigated cropland is affected by moderate to high salinization [22] caused by scarcity of precipitation, use of low-quality water for irrigation and undisciplined groundwater exploitation, which has caused over-pumping and consequent sea-water infiltration into the freshwater aquifers.

In Italy, one of the most salt-affected countries of the Mediterranean basin [23], salinization affects about 3.2 Mha of soil, distributed with different incidence in almost all the regions (figure 2B).

Beside the primary need of generating updated and homogeneous information on the global and regional extent of salinization, it is also essential to address salinization economic impact.

However, also in this case, there is a lack of a robust and harmonic method to assess the costs associated to salt-induced land degradation and restoration; indeed, the estimates produced so far are highly heterogenous and scarcely comparable as based on different criteria, scale, and economic context [24].

The last assessment formulated by the United Nations University (UNU) estimates that the inflation-adjusted cost of salt-induced land degradation was 441 US$ ha^{-1} in 2013, which raises the global economic losses at 27.3 billion US$ per year. The estimation was computed by reviewing more than 20 studies performed over the last 20 years in Australia, India, Pakistan, Spain, central Asia, and the United States.

Besides the cost associated to yield losses and field restoration, there are other cost components that must be considered: employment losses, increase in human and animal health problems, infrastructure deterioration (including roads, railways, and buildings), losses in property values of farms, and higher emission of carbon.

Besides salinization, another global concern is represented by the availability of fresh water as it is already a scarce resource in many countries. Up to date, the amount of brackish water worldwide is about equal to the amount of available fresh water (both 1 %), whereas the remaining 97.5 % is seawater [25].

The water scarcity can be categorized into physical and economic water scarcity (figure 3) [26].The former case refers to all the conditions in which water availability cannot fulfil the human

water demand, whereas the latter case refers to the case in which, even though water availability is not limiting, factors such as human, institutional, and financial capital limit access to water.

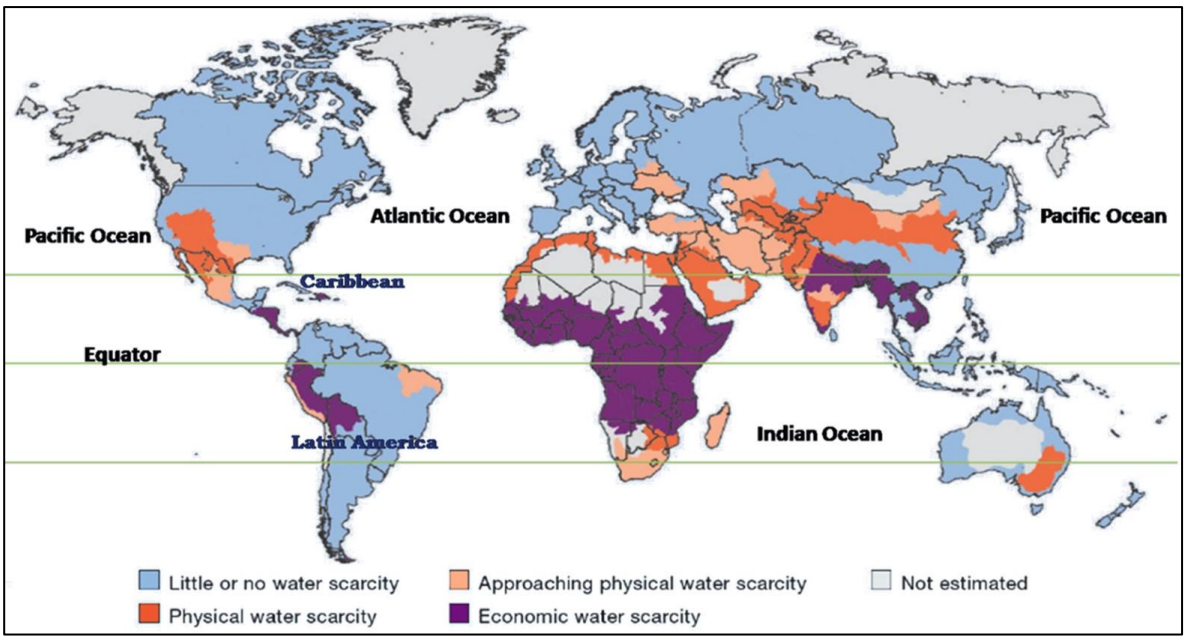


Figure 3: Areas of physical and economical water scarcity at the basin level in 2007. Modified from [27].

Mekkonen and Hoekstra [28] have developed a global map (figure 4) indicating the number of months in which water demand exceeds the supply. Fresh water shortage is foreseen to increase in the nearby future, mainly due to the rapid growth of the world population [29].

The world population, indeed, is projected to reach 9.6 billion in 2050 [30] implying that global food production will need to increase up to 70% by this time. Among other options, this could be achieved through the expansion of agricultural lands, both rainfed and irrigated.

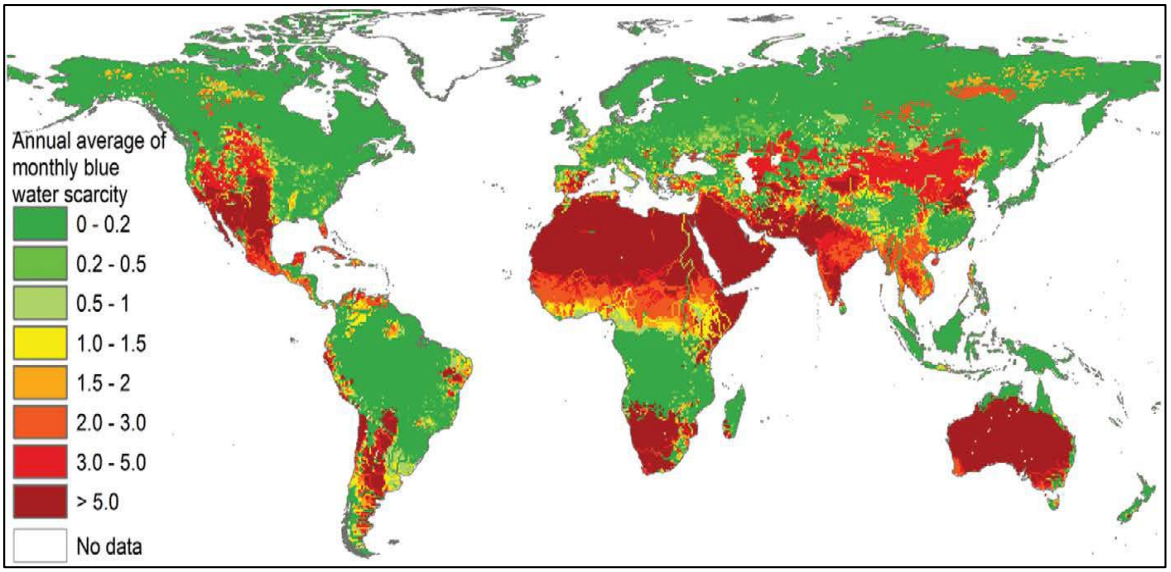


Figure 4 The number of months per year in which blue water scarcity exceeds 1.0 for period: 1996–2005 [28].

However, as the irrigated lands yield twice as much as rainfed lands [31], the irrigated croplands are projected to expand the most, increasing by 20 Mha (or 6.6 %) within 2050, mostly across central and southern Africa [32].

This will further increase the water consumption in agriculture, which is already responsible for 70% of the freshwater withdrawals [29], and the situation may get worse considering that the irrigation demand will increase with the higher global temperature [33], while water is likely to become even more saline, due to concentration following evaporation.

Considering these emerging issues, the key question arising is whether the available fresh water and soil resources can meet the future agricultural, industrial, and domestic demand?

The intense competition for good-quality land and freshwater from the urban and industrial sectors, will inevitably push agriculture more and more to the use of marginal lands and poor-quality waters for irrigation. This emphasizes the importance to explore the use of brackish water for cultivating salt affected areas by the means of saline agriculture.

1.2. Saline agriculture

From the foregoing discussion, there are no doubts that the recovery and reuse of salt-affected soils and waters are of paramount importance to support food production and ease the pressure on arable land and freshwater. According to the world Food and Agricultural Organization [34], even modest improvements in the agricultural productivity of saline and sodic lands will contribute to alleviating poverty and hunger in most disadvantaged regions of the world.

Mitigation and adaptation are terms commonly used to distinguish the climate change management approaches. These two terms, however, can also be adopted to differentiate the potential strategic approaches for the rehabilitation of salinized soil and water. Mitigation ordinarily refers to any kind of intervention made to block/contain salinization occurrence. Adaptation, in contrast, refers to the measures aimed at adjusting the agricultural practices so that salinity-affected areas can continue to be cultivated and their productivity is enhanced [13].

Mitigation measures can be either shorter term or annual practices and can be grouped in three main kinds: physical (levelling, salt scraping, tillage, subsoiling and sanding), chemical (use of soil amendments such as elemental S, acids, gypsum, etc., based on gypsum requirements to rectify soil sodicity problems and to improve soil health), and hydrological (irrigation systems for salt leaching and drainage).

These techniques are often costly, and their usefulness is highly dependent on the salinization's cause, extent, and depth of the water table. When the groundwater table is shallow, many mitigation procedures become unpracticable because the soil is too wet, and the water does not drain. If the sea-level rises, the water table gets ever closer to the soil surface in coastal areas, and mitigation becomes less and less efficient.

If the salinity source is continuous and the salinization condition is already chronic, then landowners have no choice but to integrate the mitigation techniques with adaptation measures, through the saline agriculture approach.

The concept of saline agriculture is not new as the use of sea or brackish water for crop cultivation in coastal, arid, and deserts zones has been proposed already by several decades, but it was practiced to a limited extent [35,36].

Initially, indeed, the agricultural research community did not respond positively to the prospect of developing saline agriculture. In the 1950s, institutions such as the USDA Salinity Laboratory in Riverside, California, considered irrigation water of 2.25 dS m^{-1} already too saline for irrigation under conventional conditions [37]. According to the recommendations formulated in the 1970s, a huge leaching fraction would have been necessary to maintain crop productivity under saline conditions, combined with surface or subsurface drainage systems to convey saline leachate away from the fields for off-site disposal [37,38]. Of course, these considerations were made on the assumption that almost all the available crop species were glycophyte, characterized by very low levels of inherent salt tolerance.

Early on, attitudes towards the use of saline water and soil began to change. First, it was understood that while sodic water can cause clay particles dispersion and loss of soil structure, this effect disappears when the total electrolyte conductivity exceeds about 1.32 dS m^{-1} [39]. Secondly, scientists recognized that improvement of marginal areas productivity could be achieved by using alternative salt tolerant crop species, e.g. halophytes [40], both through direct cultivation or through programs of genetic improvement.

Consequently, the crop leaching recommendations proposed in the earlier guidelines were resized as it was realized that they did not account for the high degree of self-regulation of the plant-soil-water system, overestimating the amount of water actually needed [41].

Another constraint creating scepticism in the agronomist community was the expected uneconomical costs associated with the agricultural practices necessary to allow saline agriculture. However, evaluations of the high pumping costs support the use of seawater for irrigation, even in large amounts under certain conditions [42]. Pumping costs depend on the volume of water pumped and the lift of the well. Generally, the depth of agricultural wells goes from 20 m to 100 m, whereas the lift of typical coastal seawater wells is only 3 - 10 m. Furthermore, in some locations, tides can be exploited to irrigate crops without the need for pumping. A demonstrative experiment conducted by Gleen et al. [43] remarked the economic and ecological advantages of saline agriculture, proving that the carbon cost of cultivating, harvesting, baling, and delivering halophytes for biofuel production is just one-third of the amount of carbon fixed, and this proportion is similar to that of conventional biofuel crops.

Furthermore, farms located in coastal or sandy soils generally have unimpeded drainage back to the sea, preventing groundwater salt contamination. In other cases, the groundwater is already saline per se and so is not damaged by saline drainage water.

The saline agriculture success is based on two major components, namely the selection of the right crop (salt-tolerant plants and/or halophytes) and the application of tailored agricultural practices to preserve and optimize the resources use efficiency.

When these two pillars are managed correctly, saline soil and water can become profitable resources rather than a burden, and can offer viable strategies to deal with the growing food, fodder and biofuel demands.

The economic analysis carried out by Qadir et al. [24] further reinforces the idea that, whereas reversing salt-induced land degradation would require several years without leading to real ameliorations, salinity adaptation strategies could be much more cost-effective in countries facing salt-induced land degradation.

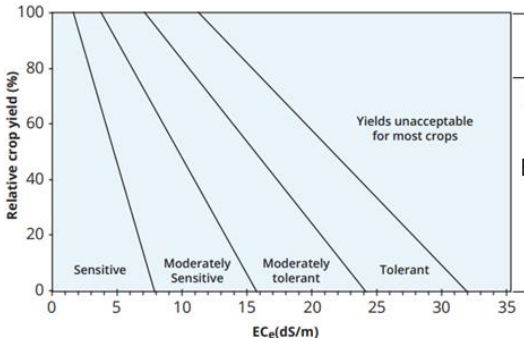
Besides crop production purposes, the sustainable use of saline agriculture provides additional recognized ecosystem services as regreening degraded areas, offering habitat for wildlife, reinforcing the endemic biodiversity pool, and storing large amounts of carbon, thereby increasing the ecosystem resilience to climate change.

In the next two paragraphs, the two pillars for establishing a sustainable saline agricultural system, namely the criteria for crop selection and agricultural practices, are described.

1.3. Saline agriculture: the crop selection

A broad variation in salinity tolerance exists among crop plants and even within cultivars. In general, most conventional freshwater crops requires irrigation water with a total salinity of less than 2.5 dS m^{-1}. Broccoli, peas, and cucumber belong to this category. Crop species like grass, sugar beet, and wheat, instead, can tolerate that level of salinity. On the other side, crops like pepper and lettuce are much more sensitive to saline or brackish water; showing a yield decrease already at 1.25 dS m^{-1} salinity [14].

According to their yield response to growing levels of salinity, plants can be grouped in four main classes (figure 5A and 5B): sensitive, moderately sensitive, moderately tolerant, and tolerant to salinity.



Tolerant	Moderately tolernat	Moderately sensitive	Sensitive
Barley (grain)	Barley (forage)	Alfalfa	Strawberry
Cotton	Sugar beet	Peanut	Bean
Bermudagrass	Wheat	Corn	Potato
	Oat	Rice	Tomato
	Soybean	Sweet clover	Pineapple
	Sorghum	Sweet potato	Onion

Figure 5: (A) Classification of crop tolerance to salinity [44]; **(B)** List of the major crops falling within each of the four classes of salt tolerance

Most agricultural crops are able to tolerate very low levels of salinity and are known as glycophytes, while those adapted to live in saline environment are named halophytes.

Over a century ago, halophytes were simply defined as essences able to thrive in saline habitats [45]. More recently, Flowers et al. elaborated a more scientifically robust description, defining them as plants that can complete their life cycle at 300 mM NaCl [46], or later, 200 mM [47]. Other authors fixed lower thresholds of 70 mM [48] or 85 mM [35].

Due to the aleatory definition, the number of species belonging to this category is not yet perfectly clear. Glenn et al. [35] indicated a number as high as 6000 species. During the 1980s, James Aronson compiled a comprehensive database of 1554 salt tolerant species, named HALOPH, now converted into the interactive eHALOPH repository [49], whereas Saslis et al. [50] identified a slightly higher number of 1653. Generally, however, it is considered that halophytes account for 1–2% of the world flora, which still represents more than 600 taxa, widely distributed among different plant genera and families [51].

Flowers and Yeo [40] and Mullan and Bannett-Lennard [52] proposed three possible strategies for the development of salt tolerant crops: (i) screening salt-tolerant accessions within domesticated crops; (ii) incorporation of genetic information from salt-tolerant relatives and halophytes into crop species through classical and molecular breeding; (iii) domestication of naturally salt-tolerant plants.

1.3.1. Screening salt-tolerant accessions from domesticated crops

Although not being halophytes, some plants species are able to grow under saline condition.

Among field domesticated crops, it is worth mentioning rye, oilseed rape, guar, wheat, kenaf, barley, pearl millet, triticale, and cotton [53]. Among vegetables, purslane and artichoke, and among fruit trees, guava, guayule, different genera of palms, pomegranate, olive, grape, and mango can also be considered as moderately salt-tolerant [54].

The salt tolerance degree of these plants may vary along the phenological growth stages and according to the soil properties, types of rhizobacteria colonizing the medium, and management

practices including use of salt-resistant rootstocks, and application of microbial biostimulants and other chemical agents [55].

Indeed, independently from the crop species, there are varieties better adapted to salinity, as they, thanks to the early establishment, can avoid critical periods of the year like summertime, when the higher evaporation rate increases salt built up at the shallow soil layers.

The advantage of rootstocks in enhancing crop growth under biotic and abiotic stresses in fruit crop production is well-known since the antiquity, probably starting about the beginning of the first millennium. Plinius the Elder documented its use in ancient Greece in his Natural History. Nowadays, the use of salt tolerant rootstocks on Mediterranean tree such as olive, pomegranate or fig is still negligible while its adoption for temperate-subtropical fruit trees represent an excellent tool for their cultivation in degraded soils [56]. More recently, there is an increasing use of grafting also in horticultural crops, especially in the families Cucurbitaceae and Solanaceae [57]. The use of symbiotic biological agents, as well, has been proposed as a means to sustain plant growth in saline habitats [58].

Worth to be mentioned for his extraordinary salt tolerance is the seed-crop *Chenopodium quinoa*, whose cultivation in saline agriculture is growing exponentially. Considered by some authors as a facultative halophyte [59], this species was domesticated in the Andean region of South America as early as 7000 years ago and has some varieties able to cope with levels of salinity as high as those present in seawater (approximately 50 dS m^{-1}).

The seeds of quinoa have exceptional nutritional qualities. They are rich in minerals and vitamins and their content of protein ranges from 12 to 17% depending on variety, environment, and crop management practices. This amount is higher than that of conventional cereal crops like rice (6 - 7%), wheat (10.5 - 14%) and barley (8 - 14%). More importantly, quinoa seeds lack gluten but are rich in essential amino acids like lysine and methionine which are the two amino acids absent in cereals and pulses, respectively [60].

1.3.2. Incorporate genetic information from salt-tolerant wild relatives and halophytes into crop species through classical and molecular breeding

As already mentioned, there are numerous salt-tolerant varieties and landraces in the world germplasm. Plant breeders have exploited this genetic variation at intraspecific, interspecific, and intergeneric levels to produce salt tolerant lines. However, crop improvement for saline environments through conventional breeding is a slow and challenging pursuit, as the plant physiological response to salinity is multifaceted and its genetic basis is still partly unknown [61]. Indeed, the plant response to salinity is mediated by various complex metabolic processes from the production of osmolytes and non-enzymatic and enzymatic antioxidative compounds, to the activation of ion transporters, ion channels and others signalling pathways and transcriptional factors. Identifying the genes involved in these processes and distinguishing their specific responses requires high effort and may involve the risk of transferring undesirable traits [62]. To ease this identification, multiple salinity stress indices have been developed, which can be very specific or evaluate the overall genotypes' ability to maintain growth and biomass production under salinity stress [63].

Notwithstanding the difficulties, some encouraging results have been obtained during the last decades and various salt tolerant crop cultivars/lines have been produced. For example, the Central Soil Salinity Research Institute (CSSRI) in India has developed several high-productive and salt-tolerant varieties of rice, wheat, Indian mustard and Chickpea.

Nowadays, however, molecular breeding, namely marker-assisted selection and other genetic engineering approaches, is preferred to conventional breeding, as it is faster and only deals with specific genes transfer [64]. Conventional breeding, indeed, requires 15–20 years to develop a new crop variety and may present limitations due to the low magnitude of variation in the genetic pool of some domesticated crops, and due to the reproductive barriers that may hamper the gene transfer from a wild relative of a crop to the domesticated cultivar.

However, despite the technological progress in genetic engineering, successful results in salt tolerance breeding are hardly achieved at field level. Indeed, most of the transgenic salt-tolerant plants are tested under controlled condition, in laboratory at seedlings stage or in greenhouse at vegetative and reproductive stage [65]. At field level, however, salinity stress is often mixed with other stresses and environmental factors undergoing fluctuating conditions that may affect the plant response and its tolerance degree.

1.3.3. Halophyte based agriculture

Halophytes are described as plants that naturally inhabit saline environments and profit from having abundant quantities of salt in the external media. Halophytes can be found in a variety of locations from coastal sand dunes, wetlands, and mudflats to inland deserts, salt flats and steppes [66]. They can belong to different plant families, with the Amaranthaceae being the most representative [67].

Halophytic species possess a range of highly efficient morphological, physiological and anatomical adaptations to bear and even benefit from saline environments [68].

The major hallmark of these plants is their capacity of exploiting the inorganic ions naturally present in the saline growth media, mostly Na^+ and Cl^-, to osmotically adjust their tissues. This mechanism is more energy-effective than synthesizing organic osmolytes ex novo, as these ions can be taken up passively along the electrochemical gradient without consuming extra energy (ATP) to drive the process.

Halophytes can tolerate these ions even at concentrations normally cytotoxic for glycophytes thanks to the ability to compartmentalize them in root and leaf cell vacuoles, or to sequester them in specific organs such as salt glands or bladders [68].

Thus, the growth of halophytic plants would offer a sustainable solution to recover saline soil and exploit unutilised water resources, thereby allowing agriculture to move into barren areas, salt marshes, and coastal saline and sodic soils. *Suaeda fruticosa,* one of the most representative halophyte, gives us an example of the climate change mitigation potential of this plant category, as it can survive and complete its life cycle under soil salinity of 65 dS m^{-1}, pH of 10.5, and under little or no irrigation, offering a large range of potential applications [69].

Hugo and Elisabeth Boyko, respectively a geophysicist and a plant-ecologist, were among the first scientists pioneering the research on halophyte cultivation under full-strength seawater irrigation. In the 1958, they proved the feasibility of cultivating two halophytes for fibre production on dunes irrigated with different seawater dilutions during a multi-year plot trial [70]. Subsequently, they demonstrated the ability of certain local barley strains to complete their life cycle with irrigation water at ca 34 dS m^{-1} [71]. Nearly simultaneously, successful investigations were carried out in saline wetlands to explore the feasibility of cultivating halophytes for ground cover and grazing [72,73]. Qadir and Oster conducted over 30 years of experimentation around the world proving that high salinity water may be used as part of sustainable agricultural systems in salt affected areas [74].

Plenty of studies on halophyte field cultivation (table 4) were then executed demonstrating that halophyte cultivation can yield as much as conventional crops, even with seawater irrigation. However, scepticism about their use as alternative crops or forage species still remains.

Anyway, market development for saline agricultural products has just started and increased demand in the future is likely to occur as the price of traditional crop products is increasing and the availability of good quality land and water is always less [75].

Table 1 Field biomass production of some forage halophytes cultivated under saline conditions [76,77].

Species	Biomass production	References	Cultivation conditions	Uses
Salicornia bigelovii, Distichlis palmeri, Batis maritima, Atriplex spp.	14-18 t DM ha^{-1}	[78]	Coastal desert environment, Sonora, Mexico	Salad greens, vegetable, oil, forage
Spartina alterniflora	40 t FW/ha	[79]	Low intertidal zone of estuaries	Ecosystem restoration
Salicornia bigelovii	13-25 t FW ha^{-1} 1-2.5 t $ha^{-1}$of seed	[80]	Full seawater irrigation	Salad greens, vegetable, oil
Atriplex spp,	13–21 t FW ha^{-1}	[79]	Full seawater irrigation	Salad greens, vegetable, forage
Salicornia europaea	20 t FW ha^{-1} 2 t ha^{-1} of seeds	[81]	Mexico, Egypt, United Arab Emirates	Salad greens, vegetable, oil
Distichlis spicata	5-10 t FW ha^{-1}	[82]	Saline water irrigation (13 dS m^{-1})	Forage
Spartina patens	14 t FW ha^{-1}	[82]	Saline water irrigation (47 dS m^{-1})	Forage
Atriplex triangularis	21 t FW ha^{-1}	[83]	Full seawater irrigation	Fresh vegetable
Inula crithomoides	4 t DM ha^{-1}	[83]	Saline water irrigation (40 dS m^{-1})	Forage
Atriplex halimus	14 t DM ha^{-1}	[82]	Saline water irrigation (20 dS m^{-1})	Forage
Leymus triticoides	10-14 t DM ha^{-1}	[84]	Saline water irrigation (13 dS m^{-1})	Forage
Festuca arundinacea	4.5 t DM ha^{-1}	[84]	Saline water irrigation (12 dS m^{-1})	Forage
Sporobolus airoides	6.7 t DM ha^{-1}	[84]	Saline water irrigation (12 dS m^{-1})	Forage
Sporobolus virginicus	45 t DM ha^{-1}	[85]	Saline water irrigation (30 dS m^{-1})	Forage
Distichlis spicata	45 t DM ha^{-1}	[86]	Saline water irrigation (30 dS m^{-1})	Forage
Allenrolfea occidentalis	17 t DM ha^{-1}	[87]	Saline water irrigation (14 dS m^{-1})	Forage
Bassia hyssopifolia	4-17 t DM ha^{-1}	[87]	Saline water irrigation (14 dS m^{-1})	Forage
Spartina gracilis	8.5 t DM ha^{-1}	[87]	Saline water irrigation (14 dS m^{-1})	Forage

Halophytes can be classified according to different criteria, summarized in figure 6.

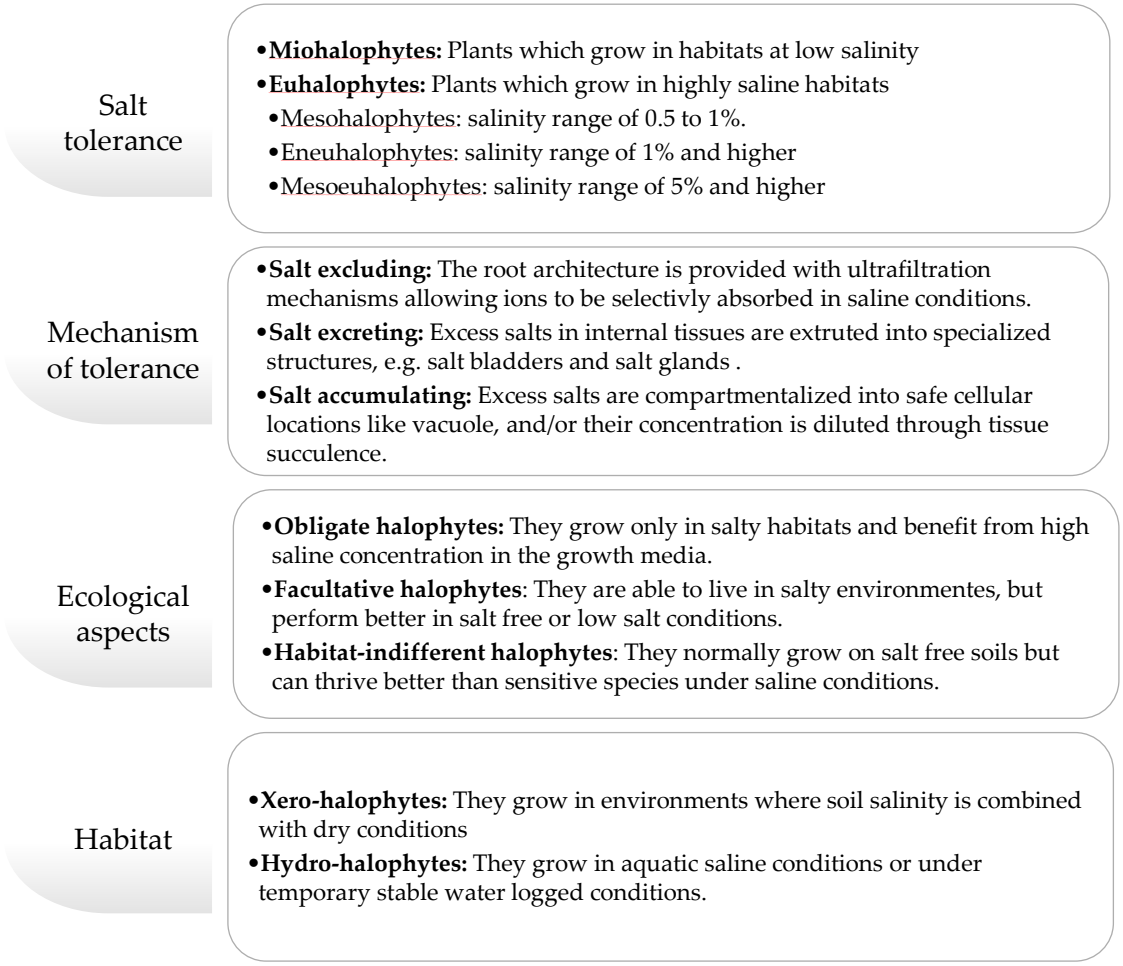


Figure 6: Classification of halophytes based on four major criteria: salt tolerance, mechanism of salt tolerance, ecology and habitat [88].

In the following sub-paragraphs are described the main application fields of these salt tolerant species.

a. Halophytes for fodder production

Biomass production, high palatability, digestibility, absence of anti-nutritional compounds and good nutritional value (high protein and low fibre, ash and oxalate contents) are the main parameters for the selection of a good quality fodder. In the past, halophytic grasses, shrubs and trees, containing high digestible protein levels, were planted for grazing or harvesting for fodder [89]. However, in the last decades, the use of halophytes species as fodder crops, even in combination with other energy sources, became always more limited since their nutritional value and feeding quality are reduced by salt accumulation [90].

Indeed, the elevated osmotic pressure caused by the increased load of salts within the ruminal environment is assumed to be critical for protozoa survival. Moreover, nearly all the salt ingested requires to be expelled through the kidneys, which increases the digestion metabolic costs and the need for freshwater [91].

Subsequently, the researchers' approach changed as it was recognized the importance of feeding livestock with balanced fodder mixtures, such as halophytes combined with herbaceous species and annual grasses, which together can reach a nutritional level equivalent to conventional fodders [92].

Recent trials and economical studies in various countries suggest that, under proper irrigation management and cultivation practices, some halophytes can be profitably used in mixed feeding regimes or for the extraction and production of leaf protein concentrates that are being increasingly used in animal feeds, e.g. in aquaculture or for horses, ostrich and poultry [91].

The usability of these plants in feed composition also depends on the animal species. The most salt tolerant farm animal is the camel, followed by sheep and goat, beef-cattle, whereas the least tolerant are pigs and poultry [86].

Among the most promising halophyte genera for fodder production are mentioned the grasses *Puccinellia* spp., *Spartina* spp., *Sporobolus* spp., and *Distichlis* spp. while, among the shrubs, the genera *Atriplex* spp., *Salsola* spp., *Salicornia* spp., and *Suaeda* spp. [93].

b. Halophyte for carbon sequestration

Carbon sequestration is defined as the removal of carbon from the atmosphere through relatively stable storage in terrestrial systems. Halophyte-based ecosystems may give a pivotal contribution in mitigating the human carbon footprint and maximizing the so-called blue carbon storage [94]. Indeed, they provide more effective carbon sinks (approximately 3.9 kg C m^{-2} $year^{-1}$), both in the short- and long-term storage of carbon, than typical terrestrial ecosystems, thus contributing to lowering greenhouse gas levels [93]. The carbon stored in the first meter of topsoil in marsh ecosystems equals approximately 259 Mg C ha^{-1} [94]. Considering a reference permanent carbon sequestration of 2.1 Mg C ha^{-1} and applying the carbon emission reduction (CER) price of the European emission trading system (ETS), € 12.38 Mg^{-1}, the value of these agroecosystems in terms of carbon content was estimated to be around € 26 ha^{-1} $year^{-1}$ [95].

In this regard, special attention was posed to halophyte based silvipastoral systems. Apart from reclamation of saline lands, indeed, these systems aid in improving carbon sequestration, reducing greenhouse gas emissions, increasing soil rhizospheric activity, and reinforcing the overall ecosystem resilience to changing climates [96]. Among the reported successful silvipastoral combinations established in saline and sodic soils can be mentioned *Desmostachya bipinnata* associated with *Prosopis juliflora* or *Acacia nilotica* or *Dalbergia sissoo*, or *Prosopis juliflora* with *Leptochloa fusca* [97].

Eucalyptus tereticornis, *Syzygium cumini*, *Pongamia pinnata* and *Populus deltoides* are other salt tolerant trees which have received special attention as carbon sinks thanks to their high growth rate, attractive wood quality and bio drainage properties [98]. Among shrubs, perennial, large, shrubby genera such as *Atriplex* and *Halocnemum* are preferred [99].

c. Halophytes as energy crops

Halophytes have been thoroughly investigated as sources of bioethanol, biodiesel, and fuelwood [100], as they can live in harsh environments under full-strength seawater irrigation without experiencing significant biomass or seed yield reduction, thereby relieving the competition on good quality water and soil for non-food production. To that aim, salt excluding halophytes are preferred as salt is non-combustible and would reduce the fuel power.

Several studies reported that halophytes like *Tamarix chinensis, Halopyrum mucronatum, Katropha curcas, Desmostachya bipinnata, Phragmites australis, Phragmites karka, Miscanthus* spp., *Panicum turgidum, Typha domingensis* and *Spartina alterniflora* have potential for ethanol production [100–103]. A particularly striking example is given by the halophytic grass *Panicum virgatum* that achieved an ethanol yield equivalent to that of corn, which is among the major conventional food crops cultivated for ethanol production [104].

Salicornia spp., *Suaeda* spp., *Atriplex* spp., *Distichlis* spp., and *Batis* spp. are another set of promising halophytes rich in lignocellulose content [101,105,106], while sugar beet, nipa palm, and kallar grass were identified as good sources of gaseous fuel [107].

The National Aeronautics and Space Administration (NASA) of the Unites States of America has set up a GreenLab research facility for optimizing the biomass production of alternative energy crops,

among which the halophytes *Salicornia virginicam*, *S. bigelovii, S. euphoraea*, *sea grass*, and two mangroves, i.e. *Rhizophora mangle* and *Avicennia berminans* [108].

Another interesting initiative was launched by a consortium made up of some airline companies and the Masdar Institute of Science and Technology, which is currently working for the development of a sustainable aviation biofuel starting from halophytic herbs. They built an integrated seawater energy and agricultural system (ISEAS) where biofuel feedstock is cultivated with aquaculture and mangrove silviculture.

d. Halophytes for Phytoextraction

Phytoextraction, namely the removal of pollutants and salts by plants, is a rapidly developing tool to reclaim contaminated soil, sludge, and mining sites.

The physiological and molecular mechanisms used by halophytes to survive high sodium and chloride concentrations may confer tolerance also to other toxic elements, heavy metals, and anthropogenic sources of pollutants as polycyclic aromatic hydrocarbons (PAHs), asphalt, or radio nuclides. Indeed, the ability to limit the entry of ions into the transpiration stream, to compartmentalize ions, to synthesize organic solutes, and to activate effective antioxidative systems, are processes shared by heavy metal tolerant species [109].

An increasing number of investigations have successfully demonstrated the phytoremediation capacity of deep rooted, accumulator or excretory halophytes characterized by a large biomass such us *Atriplex* spp., *Spartina* spp., *Sesuvium* spp., *Salicornia* spp., *Limoniastrum* spp., *Phragmites* spp., *Mesembryanthemum* spp., and *Tamarix* spp. [110–112].

As the goal of phytoextraction is to remove a contaminant from the environment, halophytes used for phytoremediation must be harvested and disposed properly. The appropriateness of today's disposal procedures, however, is still controversial. Incineration, direct disposal, ashing, and liquid extraction are the main methods tested so far, whose the first is the most widely accepted because of the feasible and economic procedure [113], even though pyro-gasification is considered to have lower environmental impact [114].

e. Halophytes as source of food and nutraceutical products

Some halophytes are being studied or are already cultivated as alternative raw, pickled, or cooked vegetables. Their seeds are also drawing growing attention, as they generally do not accumulate salts and can be immediately used without any pre-treatment [115]. Indeed, although halophyte seeds are rather small, which could be seen as a disadvantage, they are produced in relatively high amount per hectare [106].

Fatty acid profile of some halophyte seeds holds great promise for production of high polyunsaturated vegetable oils like commercial vegetable oils from canola and sunflower [116]. In this regard, *Sarcocornia fruticosa*, *Aster tripolium*, *Suaeda maritima,* and *S. vera* emerge as some of the most suitable halophytes for food and/or feed industry [76], thanks to the abundant content of fatty acids as linolenic and linoleic acid, being part of the omega-3 and omega-6 categories, respectively, which are essential for human health [117]. Seeds of *Salvadora oleoides* and *S. persica*, instead, are a good source of industrially important lauric and myristic acids, usually used for soap and candle making [106].

Halophytes are also promising protein-rich food sources. *Halimione portulacoides*, *Spartina maritima,* and *Sarcocornia fruticosa* show very high protein contents (up to 4 mg g^{-1} FW) in their edible parts [118]. If we consider producing the same amount of proteins, halophytes occupy less than 10% of the land normally required for the corresponding animal production and, adding to this the advantage that halophyte species can be grown in marginal lands, the potential of these marine plants increases greatly [119].

Furthermore, halophytes have an elevated concentration of antioxidant compounds in their tissues [120]. Among these, phenolic compounds and flavonoids are secondary metabolites covering

different biological activities in the plants, including pigmentation, resistance to pests, predators, and oxidative stress. Polyphenolic extracts of species such as *Mesembryanthemum crystallinum* and *M. nodiflorum*, *Puccinellia maritima, Spartina maritime,* and *Spartina patens* have revealed a significant antioxidant, anti-acetylcholinesterase, antibacterial and antifungal activities, supporting the nutritional and medicinal potential of these halophytic grasses [121].

However, regardless of the high nutritional value, the success of halophytes as agroecological solutions depends greatly on consumer acceptance of new tastes and textures in their diet. At present, although not documented by actualized statistics, the number of enterprises selling halophytes like *Saliconia* spp., *Sarcocornia* spp. *Salsola* spp., *Aster* spp., and *Atriplex* spp. in European markets is growing, indicating an increased consumption and acceptance of alternative halophyte crops from the general public [115].

f. Halophytes as source of medicines

A review of the ethnobotanical literature indicates that halophytes have been used in traditional medicine for treating a number of pathological conditions as digestive system disorders, inflammation, viral and microbial infections, and ageing processes particularly in the rural areas, where folk medicine remains the primary form to treat minor ailments [121]. Indeed, as halophytes normally have to deal with extremely harsh conditions, they have developed high physiological plasticity and adaptative mechanisms based, among others, on the production of bioactive molecules such as polyunsaturated fatty acids, carotenoids, vitamins, sterols, polysaccharides, glycosides, and phenolic compounds.

In recent years, pharmacologists and researchers of medicinal plants have recognized the therapeutic potentials of these plants [122] taking into account also that, compared to their glycophytic counterparts, the concentration of useful biomolecules is much higher in halophytes or is specific only of certain species [123].

Medicinal halophyte applications cover a broad range of cases, such as asthma (*Evolvulus alsinoides*), as a diuretic (*Portulaca quadrifida, Salsola baryosma*), for the eyes (*Zygophyllum simplex*), liver and stomach disorders (*Tamarix articulata* and *Cress cretica*), gonorrhea (*Portulaca oleracea*), for heart diseases (*Capparis decidua, Kochia indica, Pandanus odoratissimus*), piles and constipation (*Salicornia* spp., *Capparis decidua, Salvadora persica*), as pain killer (*Solanum surattense, Salvadora persica*), as antibiotic (various mangroves), for pneumonia (*Corchorus depressus*), as sedative (*Withania somnifera*), for skin diseases (*Salsola imbricata*), snakebites (*Rumex vesicarius*), ulcers (*Ceriops tagal*), diabetes (*Salicornia* spp.), for cancer (*Salicornia* spp., *Catharanthu* spp.) [121,124,125].

1.4. Saline agriculture: the agronomic practices

Before going more in depth with the description of agronomic practices for saline agriculture, some notes are given here on the main indicators of soil salinity. Salinity can be expressed as percentage, total dissolved solutes (TDS, mg l^{-1} or ppm), total soluble salts (TSS, meq l^{-1}), or as electrical conductivity of the medium at a standard temperature of 25 °C (EC, mS cm^{-1} or dS m^{-1}).

According to two common classifications [126,127], soil and water having a salinity respectively below 4 dS m^{-1} and 0.7 dS $m^{-1}$are considered as non-saline (table 2). Above 2 dS m^{-1} m, irrigation water starts to be considered saline, given that the salinity of seawater is around 50 dS m^{-1} NaCl, while an extreme level of soil salinity starts from 14-16 dS m^{-1} NaCl.

Table 2. Classification of soil and water salinity. Soil salinity is expressed as electrical conductivity of the soil saturated extract paste (EC_e), while water salinity is expressed as electrical conductivity of water (EC_w)

Soil salinity class	**EC (dS m^{-1})**	**Effects**
Non-saline	0-2	Salinity effects negligible
Slightly saline	2-4	Yield of sensitive crops may be restricted
Moderately saline	4-8	Yield of many crops are restricted
Strongly saline	8-16	Only tolerant crops yield satisfactorily
Very strongly saline	>16	Only a few very tolerant crops yield satisfactory (halophytes)
Water salinity class	**EC (dS m^{-1})**	
Non – saline	<0.7	Drinking and irrigation water
Slightly saline	0.7-2	Irrigation water
Moderately saline	2-10	Not suitable for irrigation
Strongly saline	10-25	Not suitable for irrigation
Very strongly saline	25-45	Not suitable for irrigation
Brine	>45	Seawater = 55 dS m^{-1}

Two different criteria are currently recognized in the scientific literature as indices of sodicity: the Sodium Adsorption Ratio (SAR), and the Exchangeable Sodium Percentage (ESP), defined as in Eq. 1 and Eq. 2:

$$SAR = \frac{[\mathrm{Na}]}{[\sqrt{(\mathrm{Ca} + \mathrm{Mg})}]^{-2}} \quad (1)$$

Where Na^+, Ca^{2+}, Mg^{2+} are the measured exchangeable Na^+, Ca^{2+}, and Mg^{2+}, respectively.

$$ESP(\%) = \frac{\mathrm{Na}^+ \text{ (cmol/kg)}}{\mathrm{CEC} \text{ (cmol/kg)}} * 100 \quad (2)$$

Where Na^+ is the measured exchangeable Na and CEC is the Cation Exchange Capacity.

As noted by Weil and Brady [128], a soil is classified as saline when the electrical conductivity of the saturated paste extract is greater than 4 dS m^{-1}, the ESP is less than 15%, the SAR is less than 13 and the pH <8.5 (table 3), since the exchange complex is dominated by Mg^{2+} and Ca^{2+} and not by Na^+. Sodic soils, on the other hand, have EC values lower than 4 dS m^{-1}, but high ESP (> 15%), SAR (> 13) and pH values (generally exceeding 8.5). Extreme pH values are due to the presence of sodium carbonate, responsible for the high concentrations of bicarbonate and carbonate ions in the soil solution.

Table 3: Classification of salt affected soil based on EC, ESP, SAR, and pH [129].

Soil type	Soil property			
	EC (dS m^{-1})	**SAR**	**ESP (%)**	**pH**
Non-saline, non-sodic	<4	<13	<15	<8.5
Saline	>4	<13	<15	<8.5
Sodic	<4	>13	>15	>8.5
Saline - Sodic	>4	>13	>15	>8.5

1.4.1. Irrigation

A proper irrigation management is crucial for saline agriculture. Compared to non-saline farming conditions, frequent irrigations are recommended to sustain crop productivity under saline agriculture. Just after an irrigation, indeed, the osmotic pressure exerted by salts dissolved in soil solution is minimal and the water availability is maximum. This is the most favourable condition for plant growth. The more the soil dries out due to evapotranspiration losses, the more the osmotic pressure exerted by salts increases. This process makes the soil solution increasingly difficult to be absorbed by the plants, with a consequent reduction in crop yield [126]. Thus, frequent irrigations are fundamental to keep an adequate soil moisture and minimize the adverse effects of salinity on plants.

However, one undesirable effect that needs to be prevented when irrigation is done with saline water is the long-term accumulation of salt in the root zone. For this reason, supplementary water must be applied, in addition to the water required to replenish evapotranspiration losses, to remove salts accumulated during previous irrigations [130].

Nevertheless, giving extra water within each irrigation is not necessary. Excess irrigation, indeed, may also have drawbacks by leaching nutrients and other agrochemicals applied to the soils [131], which in turn can damage the water bodies receiving them. Consequently, leaching reduces water and nutrient use efficiency because increases the amount of water applied but diminishes the availability of fertilizers in the root zone [132].

Extra water should be applied only if the level of salinity in the active rootzone passes the maximum salt concentration permissible in the soil solution which, in turn, depends on the salt tolerance of the specific crop [132]. Much of the leaching demand can be met between one crop cycle and another or during pre-irrigation and early growth-stage irrigation, when soil permeability is ordinarily higher. In sub-humid climates, for example, rainfall alone often provides the required leaching [133].

Prevention of excessive salt accumulation can be easily addressed in coarse- and medium-textured soils, especially if they are characterised by a good structure and reside above a sand or gravel aquifer, which facilitates the water drainage and removal. The leaching management is generally more difficult in fine-textured, stratified, and scarcely permeable soils.

When leaching is practiced, an adequate drainage network is needed to take away the saline effluents, therefore preventing or reducing the upward stream of salts. Improved drainage system can also be used to control or lower the level of saline water tables, thereby halting the capillary rise of saline water [134]. For a smarter management of saline effluents, the drainage network should be connected to retention ponds where the effluent quantity and quality can be monitored over the crop cycle [135].

The extent to which the leaching fraction can be reduced depends on the salinity of the irrigation water, the salt tolerance of the cultivated crops, the irrigation method, and the soil infiltration rate. However, the aquifers collecting the drainage water may not always benefit from reduced leaching. Indeed, with no other sources of water recharge than the drainage flows, the groundwater salt concentration may even increase.

As much as saline drainage water may still have value for transpiration use by crops with higher salt tolerance, this water should be intercepted before it is returned to water bodies, to be used again

for irrigation. This would also contribute to safeguard the water resources associated with irrigated saline farms.

One integrated strategy to enhance the reuse of such saline drainage waters for irrigation is the "dual rotation cyclic" management strategy proposed by Rhoades [136]. In this system, sensitive crops (such as lettuce, alfalfa, etc.) and salt-tolerant crops (such as cotton, sugar beet, wheat, etc.) are cultivated in rotation and irrigated respectively with low salinity water (the former group) and with saline drainage water (the latter group). In the latter case, preplant and initial irrigations are made with low-salinity irrigation water and the switch to saline water is usually done after seedling establishment. The secondary drainage resulting from such re-use can again be isolated and reused for irrigating crops of increasingly greater salt tolerance (including halophytes and tolerant trees). The ultimate unusable drainage water should be, then, disposed to some appropriate outlet or treatment facility.

A common situation conducive to the reuse of saline drainage water for irrigation, for example, takes place in India and Pakistan where fresh water is available during the early growing season, but its supply is either too costly or limited to fulfil the whole season requirements. In these scenarios, moderate to high salt tolerant crops could be irrigated with saline drainage or groundwater, especially at later growth stages, with economic benefits despite some yield reduction.

Another case where saline drainage reuse is recommended is when drainage water disposal is impractical due to physical, environmental, social, economic, or political factors. Many farming enterprises in the San Joaquín Valley of California are practicing the reuse of drainage water in order to reduce the drainage amount, and meet the discharge restrictions imposed for protecting the quality and ecology of receiving water systems [137].

Another point of concern in the long-term feasibility of using saline water for irrigation is the possibility of detrimental effects on soil permeability. The incidence of this problem rises as SAR increases and EC decreases. Therefore, adverse effects are most likely to occur when irrigation with sodic waters are followed by periods of rainfall and irrigation with low-salinity water. The consequence is the formation of impermeable and crusted soils.

In 2018, the Salt Farm Foundation [138] produced a scheme providing a simplified version of the possible scenarios when we combine the presence or absence of soil salinity, two types of soil (sand or clay) and the irrigation with fresh or brackish water (table 4). Of course, when the soil is not salinized, and the irrigation water is fresh, we are talking about conventional agriculture.

Table 4: Possible environmental scenarios under which saline agriculture can be suitable or is not recommended

		Irrigation water	
Soil type	**Soil salinity**	**Fresh**	**Salt/brackish**
Sand	**Yes**	Good possibilities	Good possibilities
	No	Conventional agriculture	Good possibilities
Clay	**Yes**	Tricky	Not recommended
	No	Conventional agriculture	Not recommended

Concerning the irrigation system, the main methods of water application are basin flooding, furrow irrigation, sprinkling, subirrigation, and drip irrigation. Flood irrigation may be good for salinity control when land surface is adequately levelled, although problems of aeration, crusting and rising water table may occur, especially in clay and loamy soils, where deep drainage is impeded [138]. Aeration and crusting problems can be minimized by using furrow irrigation. However, since water converges from the two furrows towards the centre of the bed, any salts dissolved in the water tend to accumulate in the centre of the bed. Thus, a periodic freshwater irrigation by sprinkler or flooding should be used as salinity-control measure. Irrigation by sprinkling may ensure a better

control of the amount and distribution of water; however, sometimes the volume applied may be not sufficient to leach the salts beyond the rootzone.

Frequent light sprinkler irrigations associated to specific tillage techniques may also help to overcome germination and emergence problems in case of crusting. However, sprinkler irrigation, by wetting the green tissues of the plant, may cause extensive damages on susceptible crop organs or during susceptible growth stages. Micro-irrigation and subsurface drip irrigation techniques may help overcoming this problem. If properly designed, these techniques are recommended in saline agriculture because they provide small amount of water regularly, and therefore allow the wetted soil volume and salt concentration to be controlled, thereby minimizing matric stress and drainage below the rooting zone [138].

1.4.2. Nutrient Management

The fertilization management in saline agriculture should consider on one side the effect of the fertilizers on the electrical conductivity of the soil and, on the other side, the effect of salts on the nutrient status and availability for plants. The fertilizer application can further increase the osmotic stress associated with salinity. This effect may be more or less marked according to the fertilizer type. The application of potassium chloride, for example, showed adverse results in terms of crop yield by favouring the accumulation of salts in the soil, while potassium sulphate resulted to have a less negative impact [139].

Crop response to fertilizers under saline or sodic conditions is complex, since it is influenced by the interaction of many factors. Indeed, salt accumulation can affect the nutrient form and availability through several mechanisms: i) by altering the status in which nutrients are normally found in soil; ii) by inducing nutrient depletion due to the irrigation aimed for leaching; iii) by inducing denitrification, precipitation and other processes that reduce nutrient bioavailability; iv) through the competition effects played by non-nutrient ions on nutrient uptake; and v) by decreasing the fertilizer use efficiency.

Also, in the management of fertilization it is recommended to employ fertigation techniques, as they enhance a more precise and site-specific distribution of the fertilizers, thereby containing the consequences associated to their dispersion. Furthermore, as salinity interferes with the uptake and translocation of the main cations, the foliar application can offer an alternative viable solution [140].

The overall nutrient condition of soil can be improved by the periodical application of farmyard manure and green manuring through leguminous crop incorporation. These not only provide organic matter and other nutrients, but also increase soil porosity, aeration and moisture absorption, thus enhancing soil microorganisms [141].

1.4.3. Soil management

Soil management in saline agriculture includes a broad umbrella of practices aimed at avoiding the salt build up in the rootzone, reducing evaporation from the soil surface, and regulating the water flow from and to the water-table.

Tillage operations are usually carried out during seedbed preparation to enhance soil permeability and break up surface crusts. Nevertheless, when executed on sodic soils, heavy machinery traffic should be avoided to prevent further soil compaction. Besides superficial soil practices, crops can markedly benefit from the application of deep ploughing (up to 100 cm) every three or four years. Deep ploughing can improve the physical conditions of stratified soils having impermeable layers lying between permeable layers. Deep ploughing up to 60 cm loosens the aggregates, increases soil-water storage capacity, and helps to control salt accumulation when using saline water for irrigation [142]. Special equipment can invert even soil profiles at 2.5 m depth, breaking the layers that impede deep percolation in order to improve drainage ability. However, in sodic soils this technique should be applied only after reclaiming the sodicity; otherwise, it may further worsen the soil structure.

The addition of chemical amendments to saline soils can be performed to neutralize the medium reaction, to release calcium by solubilization of $CaCO_3$, and promote the replacement of exchangeable Na^+ by Ca^{2+} and Mg^{2+}. They can also decrease the SAR of irrigation water if added in the irrigation system. Gypsum and calcium chloride are among the chemical amendments most used to replace the excess exchangeable sodium with calcium in sodic soils to improve soil infiltration. Other minor compounds like lime and sulphur-containing amendments, if distributed together with large amount of organic manure, can contribute to improve soil aggregate stability and permeability, and prevent crust formation. Addition of such amendments is generally followed by a leaching irrigation to remove Na and other reaction products from the rooting zone.

Incorporating organic matter and green manures into the soil is a complementary measure that helps improving soil permeability and boosts the release of carbon dioxide and certain organic acids during its decomposition. In such way organic matter and green manures, as the chemical amendments, will help in lowering soil pH and solubilize $CaCO_3$ [143].

Different studies have revealed that reducing soil evaporation by allowing the persistence of a crop residue layer at soil surface would notably decrease the salt build up in the shallow soil layers, regulate soil water and salt movement [144]. In this regard, straw mulching is a promising option for farmers to reduce evaporation losses and decrease the risk of increasing soil surface salinization. Thus, whenever feasible, mulching to reduce the upward flux of soluble salts should be encouraged.

1.5. Saline agriculture: some examples and initiatives

In the last few decades, there have been several initiatives exploring the feasibility of growing salt-tolerant species and wild halophytes under saline conditions.

The U.S. Salinity Laboratory, launched in 1954, was one of the earliest initiatives that gave the go-ahead to research feasible solutions for augmenting agricultural productivity under saline conditions. In 1977 the Seawater Foundation was established, a non-profit organization composed by a group of scientific, political, and commercial partners from all over the word. The Seawater Foundation is pursuing from several decades research and basic implementation of seawater agriculture and aquaculture technologies, including the plantation of mangrove forests for carbon sequestration. In 2003, they installed the first commercial-scale integrated seawater farm in Eritrea. They also introduced at Bahia Kino (Mexico) a new integrated aquaculture, agriculture and forestry system utilizing seawater effluent from a shrimp farm to irrigate salt-tolerant crops and produce additional products for feeding people and livestock.

More recently, the International Centre for Biosaline Agriculture (ICBA) was founded, initiated in the United Arabic Emirates in 2000 [145].

Among various initiatives, in 2010, ICBA scientists ran a five-year project in West Asia and North Africa, one of the most water-scarce areas of the world, where agriculture consumes over 75% of freshwater resources. During the project, nearly 8000 accessions of more than 20 forage species were screened and evaluated to identify genotypes with better stress tolerance and productivity under marginal conditions. Crops like safflower and quinoa were also introduced into several regions.

Furthermore, between 2017 and 2018, hundreds of smallholder farmers in Egypt, Morocco, and Kyrgyzstan were trained and furnished with equipment by ICBA for cultivating quinoa on abandoned land, thereby improving the livelihood of many local populations. Today, these farmers have become an important link in the national quinoa value chain, selling their products across the country.

Likewise, ICBA trained hundreds of farmers in Kazakhstan on the profitable cultivation and use of local salt-tolerant ecotypes of crop species, by releasing and patenting one variety of pearl millet and two varieties of sorghum.

Many innovative ways to boost halophyte application for saline agriculture, bioremediation, ecological restoration, and rehabilitation of degraded wetlands were also proposed through the COST action “Putting Halophytes to Work, From Genes to Ecosystems”.

In 2016 the Salt Farm Foundation was instituted, which is currently running multiple tests, together with the participating farmers, at two experimental stations of Texel (The Netherland) and Bonaire (Venezuela), on different crop varieties for salt tolerance. At the Texel Salt Farm research centre, they are currently experimenting with several conventional crops and their salt-tolerant relatives. In their experimental fields, they are investigating various aspects of salinization and saltwater irrigation related to the growth and quality of these crops. They discovered that specific varieties of some conventional crops, such as potatoes, carrots, and cabbage can thrive on salinized soils and under irrigation with a blend of fresh and saltwater. At the Bonaire research centre, instead, participating farmers have successfully grown tomato plants and rocket salad with brackish water.

In 2018 the Salt Farm Foundation founded the Knowledge Centre on Saline Agriculture for sharing with farmers, NGO’s, students, and scientists worldwide knowledge, solutions, and training modules to promote saline agriculture.

In 2017 Seawater Solutions was also initiated, an association whose mission is to restore degraded coastlines and reform healthy and productive shore ecosystems through the introduction of salt-tolerant species. The association is currently carrying out several projects in Ghana, Malawi, Namibia, Spain, and Vietnam.

In 2018 the first international forum on biosaline agriculture was held in Laayoune, Western Sahara, Africa in accordance with the Arab Water Security Strategy, and within the framework of the FAO Regional Water Scarcity Initiative, as well as the ICBA/Phosboucraa cooperation on “Sustainable Management of Brackish Water Agriculture Use in Desert Areas”.

During the forum, several themes were addressed, e.g. sustainable soil management under saline waters irrigation, mapping and monitoring salinity at regional and field scales, the individuation of the best practices for brackish water uses in agriculture, and the development of alternative crops with improved salt tolerance.

In June 2021 a project was launched called SALAD (Saline AgricuLture for ADaptation) that aims at improving the resilience of food production by upscaling the cultivation of New Zealand spinach, potatoes, quinoa, and tomatoes under saline agriculture, through the combined action of a consortium of eight countries, four from Europe and four from Africa.

1.6. Saline agriculture: monitoring

Under saline agriculture, monitoring of salt and water status within the crop root zone must be performed regularly to assess the adequacy of the irrigation and drainage systems, and evaluate the whole system water and nutrient use efficiency.

For mapping soil salinity, two electrical methods are conventionally used: the aqueous electrical conductivity or the soil-paste, and the bulk soil electrical conductivity. However, direct monitoring can be challenging due to the salinity spatial variability, implying that numerous samples are needed to characterize an area. This task may be further complicated by the salinity's dynamic nature, as salt status is affected by changing weather patterns, agronomic practices, and fluctuations in the water table levels. If we consider that repeated sampling is necessary during the year, it becomes obvious that conventional soil sampling procedures are not time and cost effective.

For these reasons, new integrated monitoring systems that couple proximal tools for measuring soil salinity with mobile transport vehicles, remotely sensed imagery, GIS modelling and other computer mapping techniques are being developed.

Remote detection of soil salinity can be done through indirect or direct methods. In the first case, the salinity of the rooting zone is inferred looking at the status of the cultivated crops, usually described through canopy spectral reflectance or thermographic data. The reflectance of certain visible or infrared spectra, indeed, generally differs from healthy to stressed leaves [146]. Thus, if a correlation between soil salinity and crop spectral response can be determined, regression or models can be established to quantify or tag soil salinity levels.

Direct methods, instead, detect salinity in bare soils by measuring the reflectance in the visible part of the spectrum of salt covered areas and crusts [147].

The use of these procedures should be further embedded with solute-transport models in order to forecast saline flows within the soil profile and generate irrigation schedules that take into account the environmental dynamics influencing the water and salt balance. For a more efficient management of these integrated solutions, a network of meteorological stations, piezometers, and soil salinity sensors should be installed within the area at different soil depths [141].

Precision agriculture (PA) is a farming practice increasingly being adopted in saline agriculture that, by using the above described integrated tools of proximal and remote sensing, optimizes input (water, fertilizers, amendments, pesticides) application by taking into account spatial and temporal variation across the field (soil texture, salt concentration and composition, moisture, nutrient content, and plant health status), with the aim of maximizing input use efficiency while, at the same time, reducing the saline agriculture environmental footprint.

1.7. Aims of the research

Soil salinity will continue to threaten crop production and food security in the future. Cultivation of salt-tolerant crops is the most effective way to overcome this environmental issue.

Salt tolerant and halophyte species have developed special structural, physiological, and biochemical adaptations to tolerate high concentration of salts in their growth media. Shifting toward saline agriculture, i.e. the cultivation of salt tolerant and halophyte plants in saline environments, would allow the recovery of marginal saline soils and waters for food, fodder and biomass production, thereby reducing the pressure on fertile land and fresh water. Additionally, understanding the mechanisms underlying the salt tolerance of these plants can potentially lead to applications in breeding programs of salt-sensitive plants.

The research carried out during my PhD touched two different themes related to saline agriculture, e.g., the water management and the crop choice, with the main aims to:

- Elucidate the effects of variable leaching levels on soil at increasing salinity and with irrigation water at rising saline concentrations, through a greenhouse pot experiment on *Sorghum bicolor*, a grain and biomass crop characterized by a good level of salt stress tolerance (Chapter 2).
- Individuate the more appropriate indices to evaluate salinity tolerance at seed germination level, through a growth chamber study on *Salicornia europaea*, one of the most representative halophyte species commonly used as model plant for studying salt tolerance mechanisms (Chapter 3).
- Discern the main differences in the saline stress responses implemented by six wild halophytic species commonly found in the Mediterranean area (*Artemisia absinthium, Artemisia vulgaris, Atriplex halimus, Chenopodium album, Salsola komarovii, and Sanguisorba minor*), exposed to growing levels of water salinity (Chapter 4).
- Identify the distinctive physiological mechanisms adopted by C3 and C4 halophytes when exposed to rising levels of salinity through a greenhouse pot experiment, using as model plants the C3 *Atriplex hortensis* and the C4 *Atriplex halimus* (Chapter 5).
- Investigate the difference in tolerance and capacity of recovery from severe salt and drought stress between annual and perennial halophytes through a greenhouse pot experiment, using as model crops respectively two annual species of *Salicornia* spp. and the perennial *Sarcocornia fruticosa* (Chapter 6).

The above listed research points are summarized in a flowchart in figure 7.

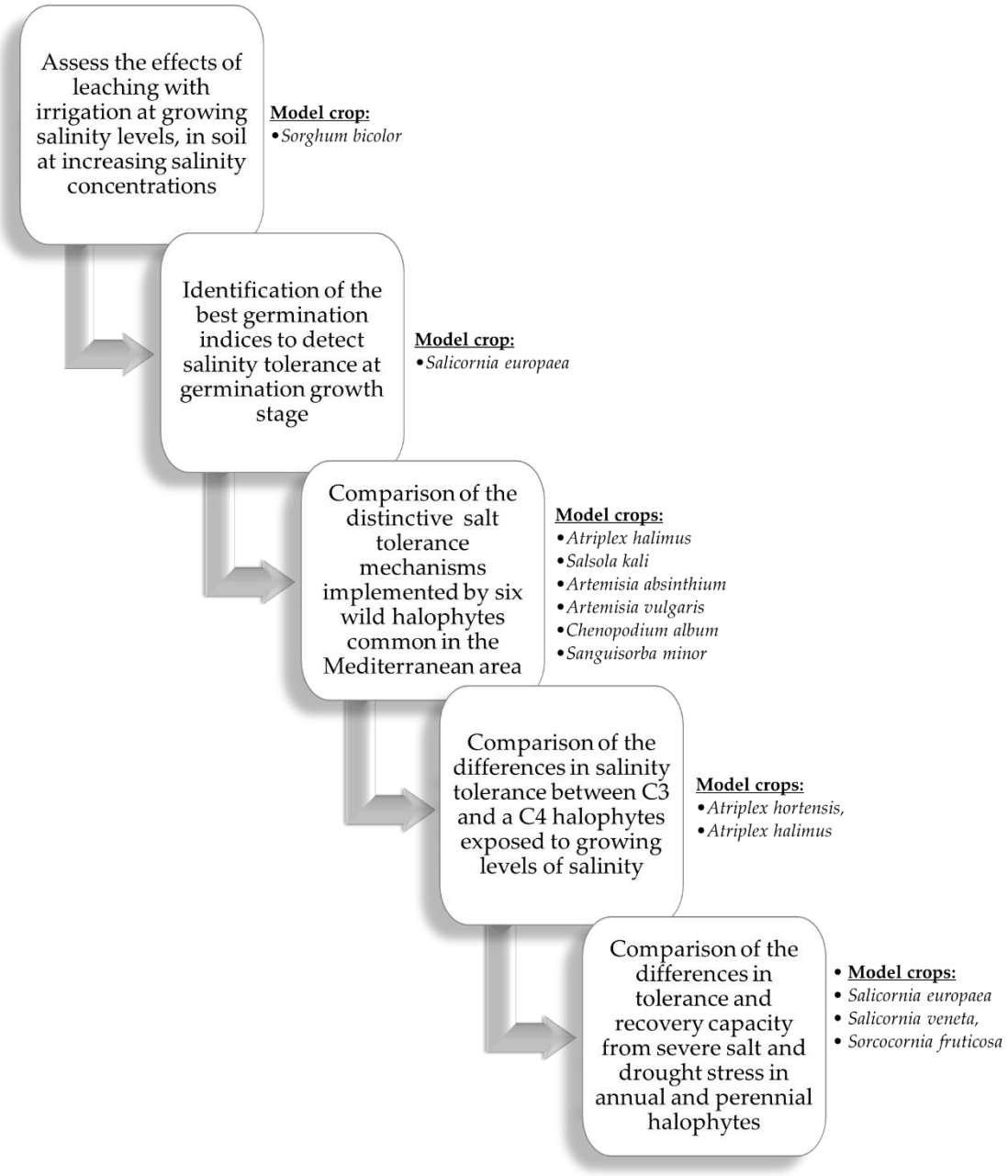


Figure 7: Flowchart of the main research topics addressed during my PhD research activity

Chapter 2

Article published in *Plants* 2020, 9, 561

Salt Tolerance and Na Allocation in *Sorghum bicolor* under Variable Soil and Water Salinity

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Received: 3 April 2020; Accepted: 24 April 2020; Published: 28 April 2020

Abstract: Salinity is a major constraint for plant growth in world areas exposed to salinization. *Sorghum bicolor* (L.) Moench is a species that has received attention for biomass production in saline areas thanks to drought and salinity tolerance. To improve the knowledge in the mechanisms of salt tolerance and sodium allocation to plant organs, a pot experiment was set up. The experimental design combined three levels of soil salinity (0, 3, and 6 dS m^{-1}) with three levels of water salinity (0, 2–4, and 4–8 dS m^{-1}) and two water regimes: no salt leaching (No SL) and salt leaching (SL). This latter regime was carried out with the same three water salinity levels and resulted in average +81% water supply. High soil salinity associated with high water salinity (HSS-HWS) affected plant growth and final dry weight (DW) to a greater extent in No SL (−87% DW) than SL (−42% DW). Additionally, HSS-HWS determined a stronger decrease in leaf water potential and relative water content under No SL than SL. HSS-HWS with No SL resulted in a higher Na bioaccumulation from soil to plant and in translocation from roots to stem and, finally, leaves, which are the most sensitive organ. Higher water availability (SL), although determining higher salt input when associated with HWS, limited Na bioaccumulation, prevented Na translocation to leaves, and enhanced selective absorption of Ca vs. Na. At plant level, higher Na accumulation was associated with lower Ca and Mg accumulation, especially in No SL. This indicates altered ion homeostasis and cation unbalance.

Keywords: *Sorghum bicolor*; salinity; salt leaching; sodium translocation; element balance

2.1. Introduction

Salinity is a major cause of soil degradation in agricultural land worldwide, and arid and semi-arid climate zones are the most affected [1]. In Europe, saline soils account for about 3.8 million ha, mostly located in the Mediterranean area [2]. Coastal areas are experiencing groundwater salinization due to seawater intrusion into the shallow water table [3,4]. This condition is progressively leading to secondary salinization [5], with consequent loss of soil fertility and crop productivity [6,7].

In particular, salinity affects soil biodiversity, microbial activities, and biochemical cycles, interfering with soil respiration, organic residue decomposition, nitrification, and denitrification [8]. Additionally, salinity alters the soil physicochemical properties, leading to organic matter reduction and sodification, with consequent clay particle dispersion and loss in aggregate stability. This makes soil less structured and undermines soil hydraulic conductivity and water storage/drainage capacity, increasing surface runoff and wind erosion vulnerability [9].

Under salt stress conditions, stronger, i.e., more negative, osmotic potential in the soil solution affects seed germination, seedling establishment, and crop growth [10]. According to the biphasic

model proposed by Munns [11], plant response to salinity is articulated in two phases: the first phase is an immediate growth reduction due to osmotic effect, which is similar to what happens under water stress; the second phase is a slower effect due to progressive salt ion accumulation. The first osmotic effect is due to the decrease in the leaf osmotic potential, which is necessary to counterbalance the decrease in the soil osmotic potential and allow plants to take up water and nutrients. The subsequent ion-specific effect, instead, is the consequence of toxic build-up of saline ions in plant organs, causing nutritional disorders, membrane disorganization, and oxidative stress, followed by reduction in cell division and expansion. Generally, salt-tolerant plants differ from the sensitive ones, especially in their ability to control salt accumulation and endure its deleterious effect [12,13].

Under climatic change, soil salinization is expected to increase in the Mediterranean region, because extreme heat and drought events are becoming more and more frequent [14,15].

Many strategies are envisaged to preserve soil productivity, based on irrigation management and choice of salt-tolerant species [16–18]. In particular, salt leaching through excess irrigation is a practice often used to leach soluble salts out of the root zone. However, when only brackish water is available, the efficiency of this method and its effect on plant growth are debated.

A good candidate for investigations on salinity is *Sorghum bicolor* (L.) Moench (Poaceae), which is commonly referred to as sorghum. Thanks to C_4 metabolism, sorghum can sustain photosynthetic activity and dry matter production in stressful conditions such as high temperature, drought and salinity [19,20]. Owing to this, sorghum is the fifth most cultivated cereal crop in arid and semiarid world regions [21] and is regarded as a tolerant crop plant for marginal conditions, including saline soils.

Sorghum is believed to tolerate soil and water salinity up to 6.8 and 4.5 dS m^{-1} of electrical conductivity, respectively [22]. Above these thresholds, a 16% yield reduction is expected per each soil salinity unit increase [23]. In a saline environment, sorghum showed a certain ability to exclude Na [24] and limit Na transport from the roots to the leaves by unloading Na from the xylem into the roots [25–28]. Additionally, sorghum can compartmentalize Na into the cell vacuoles as an osmolyte to adjust osmosis at the cellular level and thereby compensate the potential drop in the growing medium [29]. Selective uptake and translocation of K and Ca versus Na were identified as further mechanism for salt tolerance in this plant [30]. However, above a certain threshold, Na can lead to a toxic accumulation in sorghum leaf and affect K, Ca, and Mg uptake and translocation [31,32], hampering photosynthetic activity and plant development [33,34]. The accumulation of osmoprotectants such as proline [35] and sugars [36], the increase in pigment levels (chlorophylls and carotenoids) [37], and the enhanced antioxidant enzymatic activity [38] are additional sorghum strategies to maintain cellular osmotic pressure, defend plant metabolism against reactive oxygen species (ROS), and protect the assembly of photosystems under salinity.

The goal of this study is to investigate sorghum response to the combined effects of soil and water salinity, and salt leaching through irrigation, on (i) plant growth and biometry (plant height, basal stem diameter, leaf number, final dry weight); (ii) leaf water relations (relative water content, water use efficiency, leaf water potential and its components); and (iii) ion (Na, K, Ca, and Mg) assimilation and allocation to plant organs. We expected leaching to reduce salt stress in sorghum, although plant acclimation processes to salinity and salt leaching are still not sufficiently known.

2.2. Materials and Methods

2.2.1. Acronyms

The acronyms used in this study are defined as follows: electrical conductivity of the saturation soil extract at 25 °C (EC_e), electrical conductivity of water at 25 °C (EC_w), leaching fraction (LF), salt leaching (SL), relative water content (RWC), root-to-shoot ratio (R:S), water use efficiency (WUE), dry weight (DW), fresh weight (FW), osmotic adjustment (OA), water potential (WP), osmotic potential (OP), turgor potential (TP), bioaccumulation factor (BAF), translocation index (TI), selective absorption (SA).

2.2.2. Experimental Set Up

The experiment was carried out in a greenhouse at the Department of Agricultural and Food Sciences (DISTAL), University of Bologna, Italy. *Sorghum bicolor* cv. Bulldozer (fiber sorghum) was grown for 103 days from 31 May to 11 September 2017. During this time, maximum and minimum air temperature and relative humidity remained consistently at 31.3 ± 3.1 °C, 25.5 ± 2.1 °C, and 52.9% ± 4.1%, respectively.

The three factors, namely soil salinity (three levels), water salinity (three levels), and water regime (two levels), were cross-combined, resulting in 18 treatments (Table 1). Three completely randomized replicates were set up, totaling 54 pots. The 7 L pots were filled with sandy soil (80% sand, 13% silt, and 8% clay), previously sieved and mixed with table salt (NaCl) at 97% purity [39], to obtain the following treatments: control with no added salt (Ctrl), low soil salinity (LSS), and high soil salinity (HSS). LSS and HSS corresponded to electrical conductivity of the saturation soil extract at the standard temperature of 25 °C (EC_e) [12] of 3 and 6 dS m^{-1}, respectively. Soil EC_e in Ctrl was 0.27 dS m^{-1}.

Table 1. Scheme of the 18 treatments obtained by combining three levels of soil salinity (EC_e), three levels of water salinity (EC_w), and two water regimes (SL). Shaded rows indicate the four corner treatments whose data are discussed in this paper.

Treatment No.	Soil Salinity EC_e (dS m^{-1})	Water Salinity EC_w (dS m^{-1})	Salt Leaching (SL)
1	0	0	No
2	0	2–4	No
3	0	4–8	No
4	3	0	No
5	3	2–4	No
6	3	4–8	No
7	6	0	No
8	6	2–4	No
9	6	4–8	No
10	0	0	Yes
11	0	2–4	Yes
12	0	4–8	Yes
13	3	0	Yes
14	3	2–4	Yes
15	3	4–8	Yes
16	6	0	Yes
17	6	2–4	Yes
18	6	4–8	Yes

In the first two-thirds of the experiment (until August 8, i.e., 68 days after seeding), low water salinity (LWS) and high water salinity (HWS) were set at water electrical conductivity at the standard temperature of 25 °C (EC_w) of 2 and 4 dS m^{-1}, respectively; then, EC_w was increased to 4 and 8 dS m^{-1}, respectively, until the end of the experiment. EC was measured with the benchtop CDM210 Conductivity Meter (Meter Lab). The amount of salt added to soil/water in order to reach the aforementioned salinity levels was calculated according to the following equation (1):

$$\text{TSS}\ (g_{NaCl}\ kg^{-1}\ \text{soil/water}) = \text{ECe}\ (\text{dS}\ m^{-1}) \times 0.640 \quad (1)$$

NaCl concentration in tap water was 0.028 g L^{-1} (according to water supply company).

The pots were watered manually 2–3 times a week, determining the amount of water on a gravimetric basis. Two water regimes were imposed: one was maintaining pots close to field capacity

while avoiding percolation and salt leaching (No SL), and the other was overirrigation to determine water drainage and, thereby, salt leaching (SL).

2.2.3. Plant Growth

Plant height, basal stem diameter. and leaf number were measured weekly. At harvest, shoots were divided into stems and leaves, and roots were recovered from the sandy soil. Root, stem, and leaf samples were oven-dried at 60 °C and weighed to determine the dry weight (DW) of the three plant organs and total plant biomass. The root-to-shoot ratio (R:S) was assessed on a DW basis.

2.2.4. Leaf Water Status

Leaf water potential (WP) (MPa) was assessed in the uppermost fully expanded leaf before harvest, through the WP4-C dewpoint potentiometer (METER Group, Pullman, WA, USA). The measurement was repeated after freezing and subsequently thawing the leaf to determine the osmotic potential (OP). Turgor potential (TP) was assessed as the difference between WP and OP.

The relative water content (RWC) (%) was determined on the same leaf. A small leaf disc of 2 cm diameter was cut from the leaf. It was weighed to determine fresh weight (FW) and was put in a 15 mL vial with distilled water in the dark. After 24 h, the turgid weight (TW) was measured, and then the sample was oven-dried at 105 °C for 24 h to assess the DW. The RWC (%) was calculated according to the following equation [40]:

$$RWC = \frac{FW - DW}{TW - DW} \times 10 \quad (2)$$

Leaf osmotic adjustment (OA) (MPa) was calculated according to the following formula [41]:

$$\mathrm{OA} = (\mathrm{RWC_C} \times \mathrm{OP_C}) - (\mathrm{RWC_{ST}} \times \mathrm{OP_{ST}}) \quad (3)$$

where RWC_C and RWC_{ST} indicate the RWC in the control and saline treatment, respectively, and OP_C and OP_{ST} indicate the OP in the control and saline treatment, respectively.

Water use efficiency (WUE) (kg DW L^{-1} H_2O) was determined at harvest according to Equation (4) [42].

$$\mathrm{WUE} = \frac{\text{plant DW}}{\mathrm{V_{H_20}}} \quad (4)$$

2.2.5. Mineral Elements

Dry samples of plant organs were ground, and the concentration of the main cationic elements (Na, K, Ca, and Mg) was quantified by inductively coupled plasma spectrometry (ICP-OES) (Spectro Arcos, Ametek, Kleve, Germany).

2.2.6. Bioaccumulation Factor

The bioaccumulation factor (BAF) is defined as the ratio between the concentration of a given element in the plant (mg kg^{-1} DW) and its concentration in the soil (mg kg^{-1} soil DW). It was calculated for Na according to Equation (5):

$$\mathrm{BAF} = \frac{\mathrm{C_{Na}\ plant\ tissue}}{\mathrm{C_{Na}\ soil}} \quad (5)$$

where C_{Na} is Na concentration (mg kg^{-1} DW). Greater BAF values indicate lower ion retention in soil colloids or higher root ability to extract ions [43].

2.2.7. Selective Absorption

The selective absorption (SA) of K and Ca quantifies the root ability to adsorb K and Ca over Na and is calculated according to Equation (6):

$$SA_{(Ca^1)} = \frac{Na/Ca^1 \text{ soil}}{Na/Ca^1 \text{ plant}} \times 100 \quad (6)$$

where the superscript 1 refers to the concentration of Ca, K or Mg (g kg^{-1} DW). Higher SA values indicate stronger exclusion of Na^+ and selective absorption of Ca, K or Mg by the roots [44].

2.2.8. Translocation Index

The translocation index (TI) is defined as the ratio between the content (element concentration × DW) of a given element in a plant organ and the content in the whole plant. The TI was calculated to quantify element partitioning to roots (TI_R), stem (TI_S), and leaves (TI_L) according to the following equations [45]:

$$TI_R = \frac{C_{Na^1} \text{ roots}}{C_{Na^1} \text{ roots} + C_{Na^1} \text{ stem} + C_{Na^1} \text{ leaves}} \times 100 \quad (7)$$

$$TI_S = \frac{C_{Na^1} \text{ stem}}{C_{Na^1} \text{ roots} + C_{Na^1} \text{ stem} + C_{Na^1} \text{leaves}} \times 100 \quad (8)$$

$$TI_L = \frac{C_{Na^1} \text{ leaves}}{C_{Na^1} \text{ roots} + C_{Na^1} \text{ stem} + C_{Na^1} \text{ leaves}} \times 100 \quad (9)$$

where C is the ion content (mg) in the specific plant organ and the superscript 1 refers to Na, Ca, K or Mg.

2.2.9. Vector Analysis of Dry Weight and Element Concentration and Content

The dynamics of the aforementioned elements in the plant's tissues triggered by the saline treatments were represented through a vector analysis diagram [46]. This system shows the simultaneous changes in total plant biomass (DW) and element concentration and content in the plants exposed to salinity. DW and element concentration and content were expressed as relative data with respect to the Ctrl No SL, which was set at 100%. The three-dimensional vector analysis diagram has the element content on the horizontal axis and the element concentration on the vertical axis, while DW intervals are plotted as diagonal axes. The observation of the three parameters' shifts in a single graph facilitates the assessment of each element's status, i.e., element dilution, deficiency, sufficiency, luxury uptake, toxicity, and multielement interactions [46].

2.2.10. Statistical Analysis and Data Presentation

To better highlight the key effects of the experiment, we report data from the four corner treatments, i.e., those encompassing the full range of the three factors' levels (Table 1): Ctrl No SL (Control + No Salt Leaching), HSS-HWS No SL (High Soil Salinity + High Water Salinity + No Salt Leaching), Ctrl SL (Control + Salt Leaching), and HSS-HWS SL (High Soil Salinity + High Water Salinity + Salt Leaching).

Data of plant growth, leaf water status, and mineral elements were analyzed in a one-way analysis of variance (ANOVA) using the CoStat 6.4 package (CoHort Software, Berkeley, CA, USA). Prior to statistical analyses, all data were tested for homogeneity of variance through the Bartlett test. Wherever necessary, data were log-transformed to ensure homogeneity of variance. The LSD test at $p \leq 0.05$ was used to indicate significant differences among treatments.

Data of final plant morphology, growth, and leaf water traits in the 18 original treatments and the *P*-levels in the three-way ANOVA are reported in Table S1 of the Supplementary Materials.

A principal component analysis (PCA) was performed with JMP 15 (SAS Institute Inc., Cary, NC, USA) on biomass (DW), morphological (PH, SD, LF, R:S) and leaf water traits (WUE, RWC, WP,

OP, TP), and element accumulation indices (BAF, SA, TI) to reduce the number of variables into a smaller number of principal components accounting for most of variance in the original dataset.

2.3. Results

2.3.1. Water and Na Input to the System

The total amounts of water and Na supplied during the experiment are reported in Table 2. The two treatments Ctrl SL and HSS-HWS SL received +63% and +100% more water, respectively, than the corresponding No SL treatments. The water outputs, i.e., the amount percolated, were negligible under No SL, while they amounted to 15.9–21.5 L under SL. The leaching fraction (LF), i.e., the amount of water lost in percent of the amount supplied, was 28.8% and 43.8% in Ctrl SL and HSS-HWS SL, respectively.

Table 2. Total amount of water and Na supplied to the system.

Treatment	Water Input (L)	Water Output (L)	LF (%)	Na Input Soil (g)	Na Input Water (g)	Na Output with Leaching (g)	Na Output–Input (g)
Ctrl No SL	33.8	0.8	2.4	3.6	0.4	0.02	4.0
HSS-HWS No SL	24.6	0.5	2.1	15.2	10.0	1.59	23.6
Ctrl SL	55.1	15.9	28.8	3.6	0.6	0.33	3.8
HSS-HWS SL	49.1	21.5	43.8	15.2	27.9	16.09	27.1

LF, leaching fraction; Ctrl, control; HSS-HWS, high soil salinity and high water salinity; SL, salt leaching.

The total Na input with soil and water (Table 2) was negligible in the two Ctrl groups (~4 g pot^{-1}), whereas it reached 25.2 and 43.2 g pot^{-1} in HSS-HWS No SL and SL, respectively. In HSS-HWS No SL, the soil Na input was higher that the water Na input; the opposite occurred in HSS-HWS SL. The loss of Na through leaching was negligible in the Ctrl under both No SL and SL; it was small in HSS-HWS No SL, and it was relevant in HSS-HWS SL. Based on Na input (soil and water) and output (leaching), the amount of Na remaining in the system at the end of the experiment was an average 3.9 g in the two Ctrl groups (No SL and SL) and an average 25.3 g in the two HSS-HWS treatments (No SL and SL).

2.3.2. Morphological Traits

Soil and water salinity determined stunted growth resulting in a reduced plant height, number of leaves, and stem diameter (Figure 1). However, under HSS-HWS, SL had a mitigating effect on plant height and leaf number, compared to No SL. On the contrary, in the Ctrl, the extra amount of water supplied with SL did not significantly determine higher measures of morphological traits; this indicates that the amount of water supplied with No SL was nonlimiting for plant growth.

At the end of the experiment, plant height was reduced by 47% and 76% under HSS-HWS SL and HSS-HWS No SL compared to the averaged Ctrl SL and No SL, respectively. Leaf number decreased by 30% and 23% under HSS-HWS SL and HSS-HWS No SL, respectively. Stem diameter decreased by 35% and 20% under HSS-HWS SL and HSS-HWS No SL, respectively.

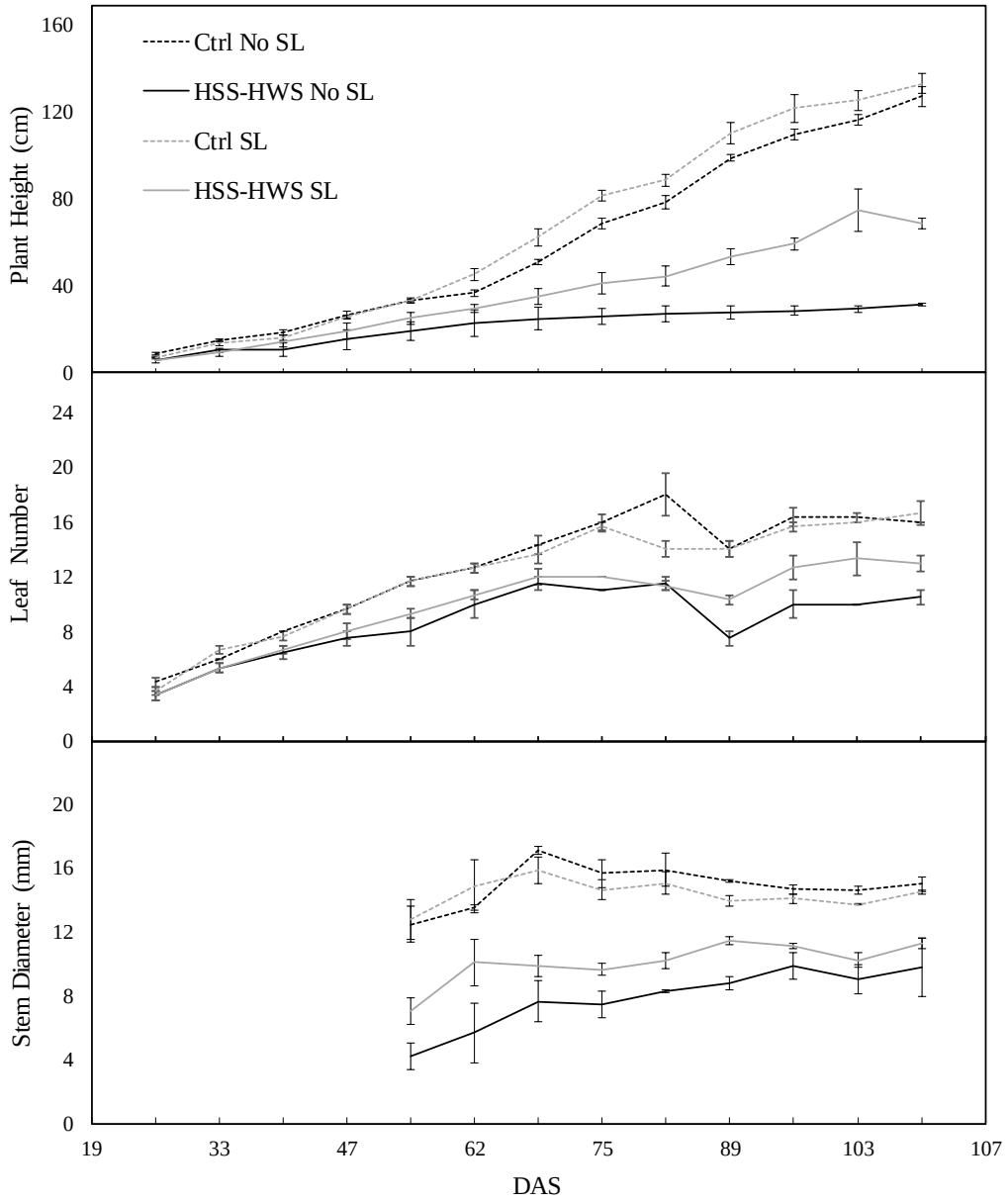

Figure 1. Time trend of sorghum morphological traits in four treatments at variable soil and water salinity and salt leaching. Ctrl, control; HSS-HWS, high soil salinity and high water salinity; SL, salt leaching; DAS, days after seeding. Vertical bars indicate ± standard error (n = 3).

2.3.3. Final Plant Growth

Soil and water salinity had a negative effect on plant biomass, root-to-shoot ratio, and WUE. HSS-HWS had a stronger impact on final dry weight under No SL than SL (Figure 2A), in accordance with morphological traits (Figure 1). Under control condition, final dry weight was comparable between SL and No SL, indicating that the amount of water distributed in this latter treatment was nonlimiting for plant growth. The root-to-shoot ratio was significantly lower only in HSS-HWS No SL, while that of HSS-HWS SL was comparable with the two Ctrl groups (Figure 2B). WUE was greatest in the Ctrl No SL, followed by the Ctrl SL (Figure 2C). This was due to similar plant biomass in these two treatments (Figure 2A), in contrast to a higher amount of water used by Ctrl SL (Table 1). WUE dropped more heavily under HSS-HWS with No SL than SL (Figure 2C). This was due to a stronger reduction in plant biomass (Figure 2A) than in the amount of water used (Table 1) under No SL.

2.3.4. Leaf Water Status

Soil and water salinity affected leaf water status. RWC was lower in HSS-HWS, and the decrease was stronger under No SL than SL (Figure 2D). WP and OP also decreased due to HSS-HWS (Figure 2E). However, under No SL both WP and OP were more negative than under SL. A certain decrease in the two potentials was also registered in the Ctrl No SL. The turgor potential (TP) (not shown) was not significantly affected by soil salinity, water salinity, or SL, likely because of osmotic adjustment (OA). However, OA did not vary significantly in the two salinity treatments (Figure 2F).

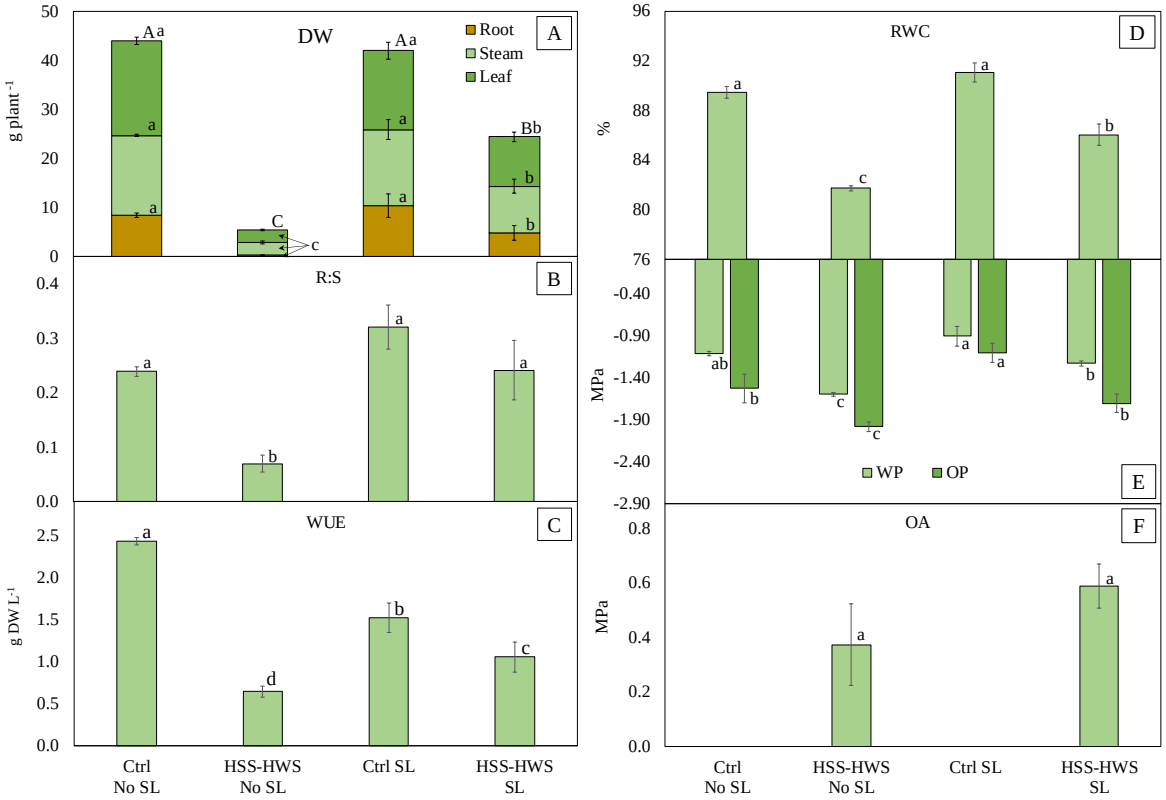

Figure 2. (**A**) Dry weight (DW); (**B**) root-to-shoot ratio (R:S); (**C**) water use efficiency (WUE); (**D**) relative water content (RWC); (**E**) water and osmotic potential (WP and OP, respectively); and (**F**) osmotic adjustment (OA) in the four treatments. Ctrl, control; HSS-HWS, high soil salinity and high water salinity; SL, salt leaching. Vertical bars indicate ± standard error (n = 3). Different letters indicate significant differences at $p \leq 0.05$. In Figure 1A (DW), lowercase and uppercase letters indicate statistical differences ($p \leq 0.05$) among treatments in single organs and their totals, respectively.

2.3.5. Cation Accumulation and Translocation

High soil and water salinity (HSS-HWS) determined sizeable increases in Na concentration in roots, stem, and leaves with respect to nonsaline Ctrl, both under No SL and SL (Table 3). HSS-HWS No SL also showed a higher leaf Na concentration than HSS-HWS SL. Potassium concentration counter-balanced Na concentration, but only at root level (Table 3): in fact, higher $K_{(R)}$ concentrations were found in the two Ctrl groups (average 7.60 mg kg^{-1}) vs. the two HSS-HWS (average 3.99 mg kg^{-1}). Lastly, Ca and Mg concentrations were influenced by salinity only at shoot, i.e., stem and leaf, level (Table 3): the concentration of these two elements decreased in both organs under salinity; however, HSS-HWS No SL suffered a stronger decrease than HSS-HWS SL.

Sodium bioaccumulation peaked in HSS-HWS No SL (BAF 3.16), compared to the other three treatments, which were statistically similar (average BAF 1.67) (Table 3). This indicates that Na plant concentration significantly increased with respect to Na soil concentration only under No SL.

Table 3. Na, K, Ca, and Mg concentrations (mg kg^{-1} DW) in roots (R), stem (S), and leaves (L); Na bioaccumulation factor (BAF); and K, Ca, and Mg selective absorption (SA). Different letters indicate significant differences at $p \leq 0.05$.

Treatment	$Na_{(R)}$	$Na_{(S)}$	$Na_{(L)}$	$K_{(R)}$	$K_{(S)}$	$K_{(L)}$	$Ca_{(R)}$	$Ca_{(S)}$	$Ca_{(L)}$	$Mg_{(R)}$	$Mg_{(S)}$	$Mg_{(L)}$	$BAF_{(Na)}$	$SA_{(K)}$	$SA_{(Ca)}$	$SA_{(Mg)}$
Ctrl No SL	3.19 b	0.44 b	0.20 c	7.39 a	4.76	7.62	8.62	8.31 a	6.25 a	1.68	5.20 a	3.40 a	1.72 b	0.79	0.07 a	0.31 a
HSS-HWS No SL	8.66 a	8.47 a	6.59 a	3.32 c	8.11	7.83	9.74	3.89 c	4.19 b	1.69	2.08 b	1.70 c	3.16 a	0.51	0.02 b	0.08 b
Ctrl SL	2.50 b	0.25 b	0.18 c	7.82 a	6.97	8.08	7.39	6.48 b	5.89 a	1.56	3.39 b	3.08 ab	1.58 b	1.04	0.06 a	0.26 a
HSS-HWS SL	8.58 a	5.99 a	0.71 b	4.66 b	5.73	7.58	8.62	4.66 bc	3.91 b	1.61	3.06 b	2.44 bc	1.73 b	0.73	0.05 a	0.23 b
P	0.001 **	0.001 **	0.001 **	0.001 **	0.338 ns	0.833 ns	0.651 ns	0.022 **	0.018 *	0.962 ns	0.080 **	0.0043 **	0.018 *	0.054 (+)	0.003 **	0.002 **

Ctrl, control; HSS-HWS, high soil salinity and high-water salinity; SL, salt leaching. ns, (+), *, and ** mean not significant and significant at $P \leq 0.10$, $P \leq 0.05$, and $P \leq 0.01$, respectively.

Selective absorption of K was mildly ($p \leq 0.10$) reduced by salinity (Table 3), indicating loss of plant ability to select this macronutrient under Na-enriched environment. The same pattern was shown for $SA_{(Mg)}$ and, only in HSS-HWS No SL, for $SA_{(Ca)}$.

Translocation indices address the relationships in element contents (Figure 3), while the above-described BAF and SA relate to relationships in element concentrations. More than 50% of the amount of Na taken up by the plant remained in the roots under no salinity, whereas the saline environment (HSS-HWS) determined an upsurge of this element to stem and leaves ($TI_{Na,}$ Figure 3A). This was especially true in HSS-HWS No SL, where leaves, which are the most delicate of the three plant organs, received almost 40% of the total amount of Na. The strongest differences in TI_K among treatments concerned roots (Figure 3B). HSS HWS No SL had a similar effect on TI_K in the three organs as it did on TI_{Na} (Figure 3A). The other three treatments allocated more K to leaves. Small differences among treatments were observed for TI_{Ca} and TI_{Mg} (Figure 3C and Figure 3D, respectively): for both elements, the stem was more important than roots for the allocation of these elements.

The vector analysis combines changes in biomass (Figure 2A) and element concentration (Table 3) and content (Figure 3) into a comprehensive picture of plant response to Na input (Figure 4). Overall, the strongest variations were associated with Na, the element that we directly supplied in soil and water (Figure 4A). However, changes were also observed for K, Ca, and Mg (Figure 4B).

High salinity without leaching (HSS-HWS No SL) determined Na toxicity, i.e., strong increase in Na concentration and concurrent drop in biomass, resulting in approximately the same Na content in the whole plant as found in the nonsaline reference treatment (Ctrl No SL) (Table 4). K and Ca were not influenced in terms of concentration, whereas their content decreased proportionally with biomass reduction. In the Ctrl SL, the extra amount of water resulted in water excess, i.e., no biomass increase and no changes in element concentration and, therefore, content. Lastly, high salinity with salt leaching (HSS-HWS SL), involving extra amounts of both water and salt with respect to HSS-HWS No SL (Table 1), determined Na and water excess, i.e., biomass reduction and a more than proportional increase in Na concentration, resulting in higher Na content. In contrast to Na, the other elements had the same response as in the HSS-HWS No SL treatment.

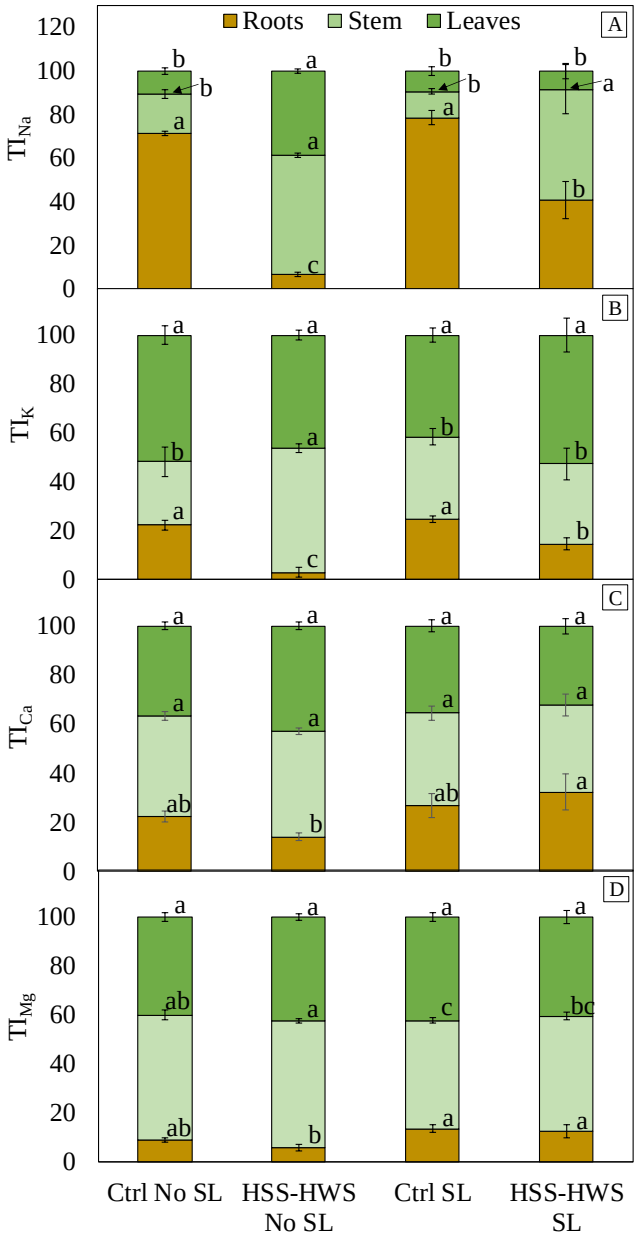

Figure 3. Translocation Index (TI) of **(A)** sodium, **(B)** potassium, **(C)** calcium, and (**D**) magnesium to the roots, stem, and leaves. Ctrl, control; HSS-HWS, high soil salinity and high water salinity; SL, salt leaching. Vertical bars indicate ± standard error (n = 3). Different letters indicate significant differences among treatments at $p \leq 0.05$.

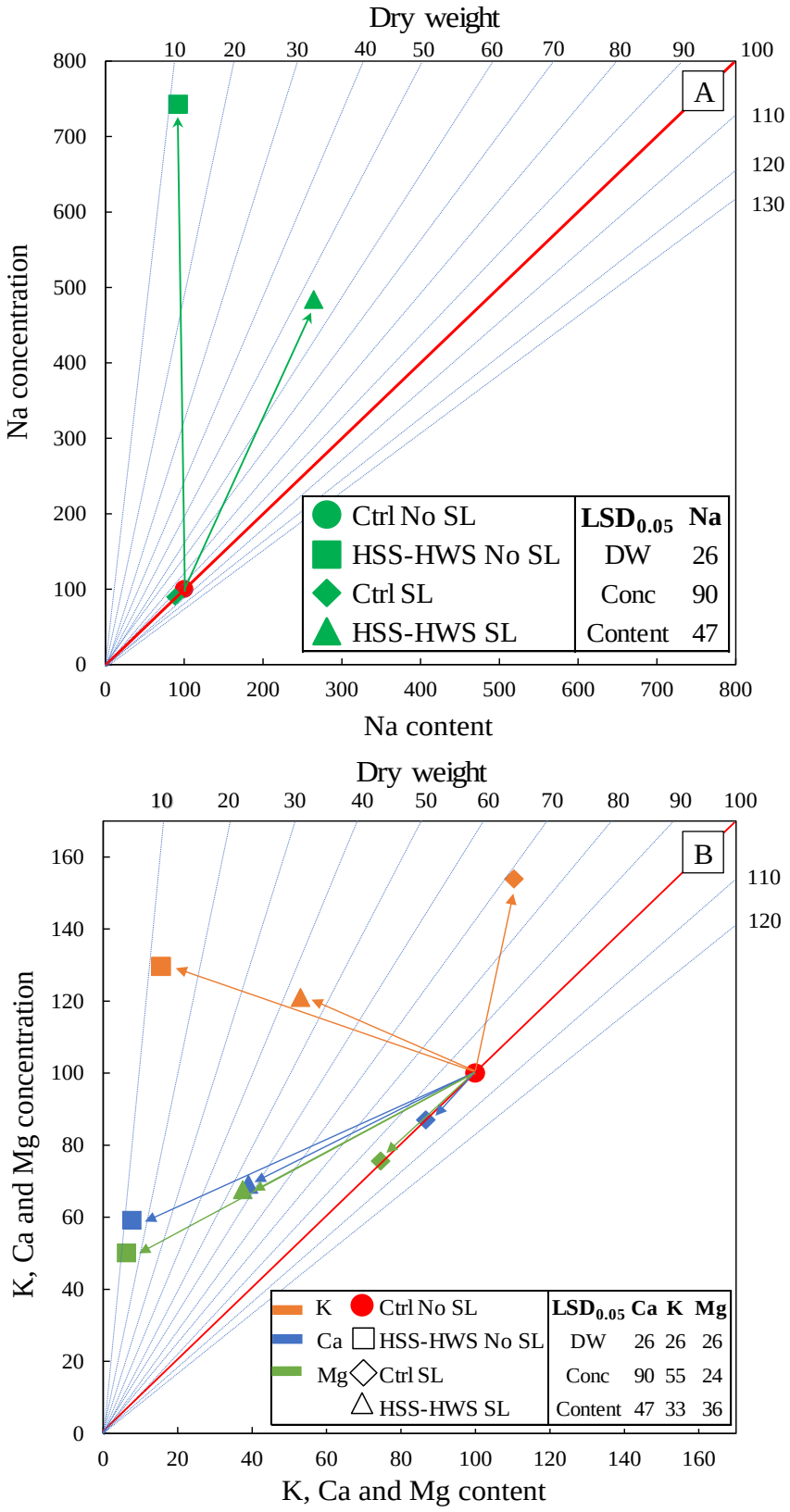

Figure 4. (**A**) Vector analysis showing directional changes in biomass and Na content and concentration in sorghum plants and, (**B**) vector analysis showing directional changes in relative biomass and K, Ca, and Mg content and concentration in sorghum plants. Dry weight and element content and concentration are expressed as relative data with respect to the Ctrl No SL treatment, which is set at 100% and is indicated by a red filled circle.

Table 4. Interpretation of the directional changes in relative dry weight (DW) and element concentration and content with respect to the reference treatment (Ctrl No SL), shown in Figure 4. Upwards and downwards arrows indicate significant changes, and (~) indicates insignificant changes. The three increasing arrow slopes indicate an increasing amplitude of the variation (from >1 LSD to >3 LSD).

Treat.	DW	Elem.	Conc.	Cont.	Interpretation
HSS-HWS No SL	↘	Na	↗	~	**Na toxicity** Excess Na associated with normal soil moisture caused a strong decrease in biomass and K, Ca, and Mg content. However, the concentration of all these elements except Mg remained constant, meaning that the reduction in their content was proportional to biomass reduction. The drop in Mg concentration indicates a reduction in Mg uptake proportionally greater than biomass reduction. Na content remained unvaried but, due to the drastic biomass reduction, its concentration increased dramatically.
		K	~	↘	
		Ca	~	↘	
		Mg	↘	↘	
CTRL SL	~	Na	~	~	**Water excess** Water availability exceeding the soil water holding capacity did not determine extra biomass gain, nor did it influence Na, K, Ca, and Mg concentration and content.
		K	~	~	
		Ca	~	~	
		Mg	~	~	
HSS-HWS SL	↘	Na	↗	↗	**Na and water excess** Irrigation with saline water exceeding the soil water holding capacity slightly reduced K, Ca, and Mg concentration and content and plant biomass. The concentration of all these elements except Mg remained constant, meaning that the reduction in their content was proportional to biomass reduction. The drop in Mg concentration indicates a reduction in Mg uptake proportionally greater than biomass reduction. Na concentration and content, on the contrary, increased considerably.
		K	~	↘	
		Ca	~	↘	
		Mg	↘	↘	

2.3.6. Principal Component Analysis of Plant Traits

The PCA of biomass, leaf physiological traits, and element concentrations in sorghum organs extracted two main principal components (eigenvalues and loadings in Tables S2 and S3, respectively), constituting 72.4 % of the total variation.

The biplot of PC1 and PC2 (Figure 5) showed that they contributed to 56.2% and 16.2 % of the total variation, respectively. It showed a net separation between the Ctrl groups (blue triangles and green squares), placed in the positive side of PC1 axis, and the HSS-HWS treatments (red dots and purple rhombuses), placed in the negative side of PC1 axis.

The PC1 had high negative loadings for $Na_{(R)}$, $Na_{(S)}$, $Na_{(L)}$, and TP and positive loadings for R:S ratio, meaning that these parameters separated HSS-HWS No SL from SL and separated these two treatments from the controls. Hence, it is sensed that PC1 represents the effects of salinity on plant growth.

PC2 separated SL from No SL treatments, although the separation was imperfect. The parameters that accounted for PC2 are $Ca_{(R)}$ and $Mg_{(R)}$, which had high positive loadings. This is consistent with the ANOVA of Ca and Mg translocation indices (TI) to the roots, which were higher in HSS-HWS SL than in HSS-HWS No SL (Figure 3C,D). The analysis also identified $K_{(L)}$ and $K_{(S)}$ as important components of PC2, with high negative loadings on PC2. Potassium concentration (Table 2) and translocation (Figure 3) to the leaves were not statistically different between HSS-HWS SL and No SL. Notably, K concentration and translocation to the roots decreased significantly under HSS-HWS SL and, to a greater extent, under No SL. Potassium translocation to the stem increased significantly only in HSS-HWS No SL, although K concentration resulted as comparable with the other three treatments. This circumstance and the weak loadings of $K_{(S)}$ and $K_{(L)}$ on the PC1 (salinity)

suggest that salinity mainly alters K homeostasis at the root level, whereas K status in the stem and leaves depends more on SL.

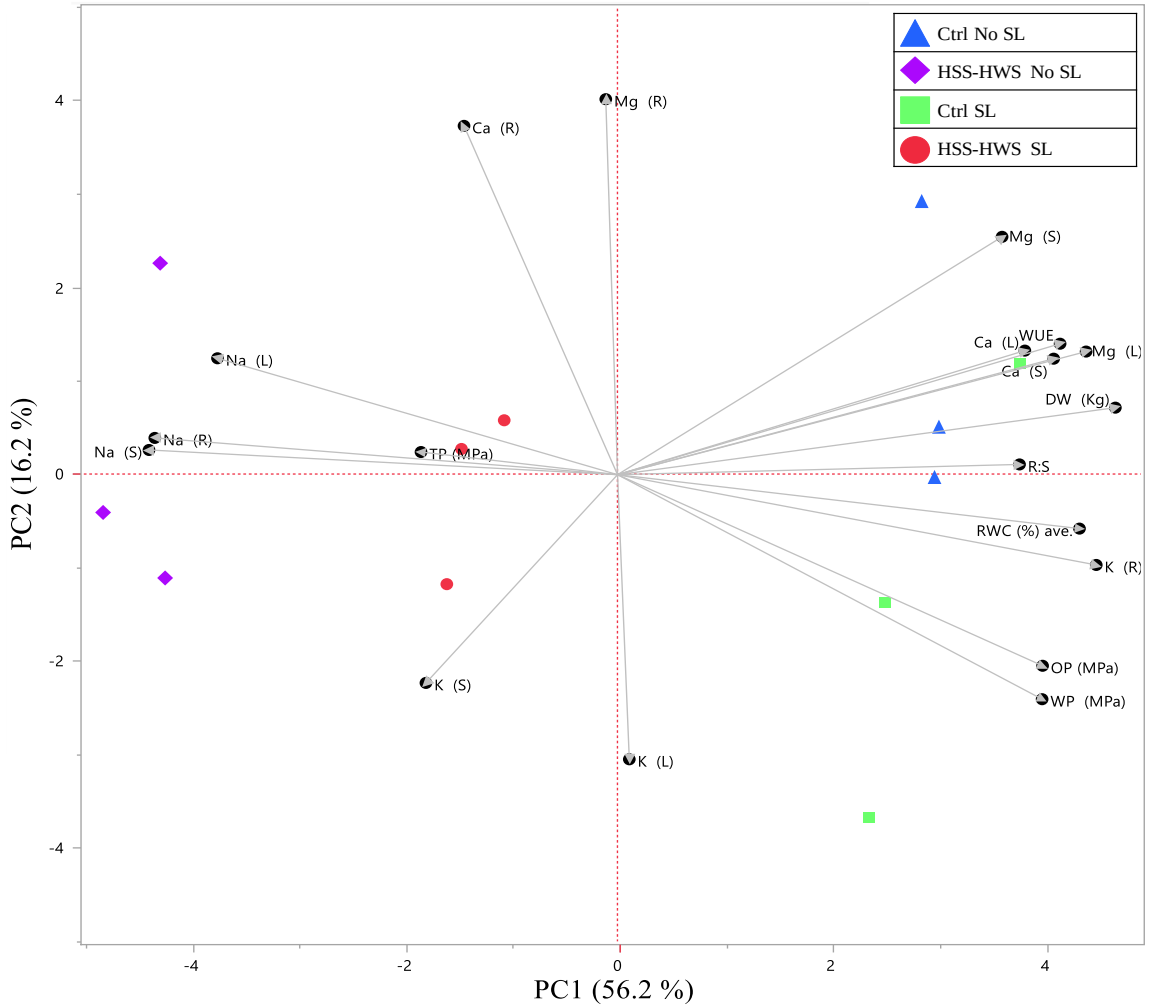

Figure 5. Biplot of the principal component analysis of biomass, morphological and leaf water status traits, and element translocation indices in the four treatments. The amount of variation associated with each PC is indicated in brackets. Ctrl, control; HSS-HWS, high soil salinity and high water salinity; SL, salt leaching; Na(R), Na(S), and Na(L), Na concentration in roots, stem, and leaves, respectively; K, Ca, and Mg followed by (R), (S), and (L) indicates K, Ca, and Mg concentrations in the respective organs; DW, plant dry weight; R:S, root to shoot ratio; RWC, relative water content; WP, leaf water potential; OP, osmotic potential; TP, turgor potential.

2.4. Discussion

Under high soil and water salinity (HSS-HWS), sorghum incurred a significant reduction in plant height and leaf number, as observed in a previous study [47]. These effects were stronger in No SL: in fact, SL introduced 71% more Na into the system (Table 2) but promoted Na leaching from the soil profile. This resulted in only a 15% higher amount of residual Na in the soil–plant system at the end of the experiment.

In saline soils, leaching decreases the osmotic potential of soil solution, consequently increasing soil moisture and permanent wilting point [22]. High soil moisture prevents plant wilting, so a higher irrigation volume is a strategy to increase water availability with saline water. On the contrary, under no salt leaching, water uptake and plant water status are rapidly impaired by salinity. Salt stress delays cell division and elongation [48], affecting foliar differentiation, expansion, and internode length, while concurrently accelerating leaf senescence [49,50]. This explains why in our study stem elongation and leaf development were less severely affected by salinity under SL than No SL, despite the higher amount of salt supplied with SL (Table 2).

In contrast to our results, Joardar et al. [34] did not observe any change in sorghum height, leaf number, and stem diameter with EC_w up to 7.18 dS m^{-1}, while Jafari et al. [51] observed a significant reduction in plant height but not leaf number at 80 mM EC_w (≈ 8 dS m^{-1}). The inconsistency between our study and these sources may at least partially be due to differential genotype tolerance within the *Sorghum bicolor* species [52,53] Additionally, in our study the combined effect of soil and water salinity could lead to a stronger impact than that of water salinity alone in the cited sources.

The stunted plant growth determined by salinity resulted in lower DW and R:S (Figure 2), as observed by several sources [31,53,54,55]. Biomass reduction may be due to increased respiration in response to salt stress [56] or due to toxic ion accumulation [57], while the higher release of ethylene under stress may have inhibited root and shoot growth and decreased their ratio [58]. The R:S decrease under salinity indicates plant reaction to reduce root exposure to the hostile environment. This is in contrast to drought stress that drives plants to expand their root system to explore a larger soil volume in search of water [59]. However, this hypothesis is not supported by the findings of Jafari et al. [51], De Lacerda et al. [60], and Al-Amoudi and Rashed [61], who reported a sorghum R:S increase at salinities up to 90, 100, and 240 mM NaCl, respectively, corresponding to 9, 10, and 24 dS m^{-1} of EC_w. Aishah et al. [62], instead, found a sorghum R:S increase up to 10 dS m^{-1} of EC_w, followed by a drop at higher values. Lastly, Mahmood et al. [63] did not observe any sorghum R:S change up to 24 dS m^{-1}. According to Shannon et al [64], a R:S increase under salinity is the premise for a better use of soil moisture and nutrients. Comparing our study with these sources where higher EC_w levels were tested, it is sensed that the plant only allocates more resources to the root system above a certain salinity threshold, as a mechanism to escape salt stress. Conversely, at salinity levels similar to our case, the plant reacts by reducing its root biomass to minimize salt exposure and control Na uptake [51]. Moreover, the R:S decrease may indicate a stronger carbon allocation to the photosynthetic organs in order to increase carbon assimilation, as mechanism of acclimation to salt stress [65].

Guimarães et al. [66] found a 50% WUE decline with an EC_w of 6.9 dS m^{-1} in sorghum, in accordance with the sharp WUE decrease observed in our experiment (Figure 3D). Richardson and McCree [67] and Yan et al. [68] argued that sorghum reduces stomatal conductance and transpiration under salinity, potentially leading to WUE increases. However, reduced stomatal conductance limits photosynthesis and final biomass. Reduced biomass was the main cause for WUE loss in our study.

The decrease of leaf RWC, WP, and OP under salinity (Figure 2D,E) reflects the findings of Netondo et al.[31], who obtained similar results in sorghum at EC_w up to 25 dS m^{-1}. However, the strongest drop in that study was observed between 0 and 100 mM NaCl.

In our study, the strongest reduction in RWC was observed in No SL, although less salt was supplied compared to SL (Table 2). A decrease in RWC is normally associated with turgor loss, because of limited water availability [69]. In our study, the plant was able to adjust osmotically (Figure 2F) and maintain leaf water balance and turgor potential. However, plant growth was seriously impaired by salt stress. In salt-exposed plants, the cations supplied with saline water (Ca, K, Na) play a key role in OA [70], as their uptake and use in plant tissues are less energy consuming than the production of organic solutes to be used as osmoregulating compounds for OA [63,64]. The OP reduction observed in salt-treated plants (Figure 2E) is the likely consequence of intracellular accumulation of osmoregulating compounds and cations, which is a key mechanism, together with intercellular compartmentalization, to perform OA [71]. Lower OP values generally indicate higher OA and water retention in plant tissues [72]. In our experiment, the higher water volume supplied with HSS-HWS SL did not determine a higher OA (i.e., stronger OP reduction) compared to HSS-HWS No SL. However, both OP and RWC benefitted from higher water supply (SL), as they decreased less than under No SL. Therefore, it is evinced that with higher soil moisture and SL, the plants were less hampered by salt stress and necessitated a smaller OA to cope with the stress.

Sodium was the cation that accumulated most steeply in sorghum plants under salinity (Figure 4A). Na accumulation in roots is a tolerance strategy: the consequent reduction in root OP can sustain water uptake. Conversely, the leaf accumulation of potentially toxic Na could slow down, or even stop, photosynthesis [73]. Our results suggest that under salinity sorghum roots were saturated with Na, forcing Na translocation to the stem. When salinity (HSS-HWS) was associated with No SL, Na reached stem saturation and a significant amount of Na was translocated to the leaves (Figure 4A). De Lacerda et al. [74] observed a similar increase in

Na allocation to the shoot in sorghum genotypes irrigated with water at 100 mM NaCl (10 dS m^{-1}). This was in contrast to Niu et al. [54], who did not observe changes in Na uptake in sorghum genotypes irrigated with water at 8 dS m^{-1} EC_w.

The maintenance of low cytosolic Na concentration and Ca/Na and K/Na homeostasis is another mechanism of salt tolerance [75]. Ca plays a key role in the response to abiotic stress, acting as second messenger in the pathway of stress signal transduction [76,77]; it also acts in exocytosis [77] to exclude toxic ions. K is involved in turgor control: inhibition of K uptake leads to stunted growth [73]. The K and Na ions have similar radius and hydration energy [78] and can be taken up jointly under sodic conditions. K/Na selective absorption depends on cell wall and plasma membrane (PM) integrity. Salinity promotes Ca accumulation at the root level in order to increase Na exclusion and preserve K accumulation [79]. However, highly concentrated Na can displace Ca in the cell wall fibrils and PM binding sites, causing membrane depolarization and cell wall instability [80]. The resulting K/Na imbalance prompts uncontrolled Na influx and cytosolic K leakage from the cell [81]. Indeed, higher doses of Ca, K, and Mg under salinity help the plant to contrast nutrient imbalance [61].

Higher water availability in HSS-HWS SL maintained $SA_{(Ca)}$ at the same level as nonsaline Ctrl (Table 3), while increasing Ca allocation at the root level (Figure 3C). Under HSS-HWS, the higher Ca accumulation in roots vs. shoots may indicate the plant's attempt to maintain selective transport across membranes. Furthermore, restricted root growth can limit Ca uptake and transport from root to shoot, in spite of the transpiration stream [82]. Inhibition of Ca flux in the phloem was also observed under salinity [31], causing leaf deficiency and reduced photosynthetic rate.

As it concerns K, restrained allocation to shoots was observed in sorghum under salinity [32,57], which was in contrast to no change found in sorghum K uptake and allocation to the upper organs in another study [65] and also in contrast to increased leaf K concentration with salinity [53]. In our study, K concentration and translocation decreased only in the roots under salinity (Table 3, Figure 3B), reaching the lowest value in No SL. It may be assumed that K was more translocated to the shoot in order to maintain high K concentration in the leaves, where this ion plays a key role in maintaining leaf turgor. However, the PC2 showed that leaf K concentration was related to a combined effect of salinity, water availability, and SL, rather than salinity alone (Figure 5).

Additionally, the ability to selectively adsorb K and Ca over Na is not sufficient to assure cation homeostasis. In fact, Na can also be transported through the apoplastic transpiration stream, bypassing all filter barriers imposed by cell membranes [83].

The reduction in plant Mg content was proportional to biomass reduction (Figure 4B). Mg root allocation was significantly lower in HSS-HWS No SL. However, Mg translocation to the stem was not related to salinity, as it was lower in SL treatments with both saline and nonsaline water (Figure 3D). Despite unaltered Mg translocation to the leaves (Figure 3D), Mg leaf concentration declined dramatically with HSS-HWS (Table 3), supporting the findings by Netondo et al. [31] who reported a constraint in Mg leaf incorporation under salinity.

2.5. Conclusions

The present study demonstrates that salt leaching, although performed with saline water, alleviates salt stress in *S. bicolor* by reducing the detrimental effects exerted by salinity on plant growth, leaf water status, and cation homeostasis across plant organs.

Sodium input to the soil and irrigation water resulted in higher Na concentration in plant organs. This was especially true in the case of no salt leaching. Under this circumstance, the plant had to deploy a special effort to maintain cation (K, Ca, and Mg) homeostasis and counter Na upsurge from the root apparatus to the leaves. The stem appeared to act as a buffer organ, trying to maintain cation balance and prevent Na from reaching the delicate photosynthetic organs.

In the frame of soil and water salinity, a higher irrigation volume determined a higher salt input to the system but nevertheless is able to mitigate the noxious effects associated with Na accumulation in plant organs. In contrast to this, conservative irrigation, i.e., limiting salt input to the system by avoiding the extra

supply of water needed for salt leaching, was proved to be a losing strategy that worsened Na effects by hampering plant water uptake and cation selective absorption.

Although the practice of salt leaching when using saline water leads to a more tolerable rhizosphere environment, further research is needed to evaluate the long-term sustainability of this method, assess Na fate in the soil–plant system, and investigate Na impact on soils and aquifers.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Final plant morphology, growth and leaf water traits in the 18 treatments obtained by combining three levels of soil salinity (EC_e), three levels of water salinity (EC_w) and two water regimes (SL), and statistical significance of the three simple factors and their interactions, Table S2: Eigen analysis of the correlation matrix, Table S3: Factorial loadings for principal component.

Author Contributions: Conceptualization, R.C. and L.B.; methodology, L.B. and R.S.; formal analysis, R.C. and L.B.; investigation, R.C., L.B., and R.S.; resources, M.S. and L.V.A.; data curation, R.C., L.B., and G.V.; writing—original draft preparation, R.C. and L.B.; writing—review and editing, R.C., L.B., and C.L.; supervision, L.B., M.S., and L.V.A. All authors have read and agreed to the published version of the manuscript.

Funding: The work has been financed by the European LIFE 2014–2020 Programme, Project LIFE AGROWETLANDS II—Smart Water and Soil Salinity Management in Agro-Wetlands (LIFE15 ENV/IT/000423). The publication cost is covered by this project.

Acknowledgement: Marco Bittelli, Department of Agricultural and Food Sciences (DISTAL) of the University of Bologna, is gratefully acknowledged for instrumental support.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3

Article Published in *Agriculture* 2020, 10, 29

Assessing *Salicornia europaea* Tolerance to Salinity at Seed Germination Stage

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Received: 10 December 2019; Accepted: 19 January 2020; Published: date

Abstract: *Salicornia europaea*, a halophytic species, was investigated to assess its ability to withstand salinity during seed germination, and to identify suitable indices to interpret salt tolerance at this delicate stage. Seed germination indices (germination percentage (GP), germination energy (GE), germination value (GV), coefficient of germination velocity (CVG), germination rate index (GRI), germination peak value (GPV), mean germination time (MGT), and time to 50% germination (T_{50})) were calculated under increasing salinity (0, 100, 200, 300, 400, and 600 mM NaCl). Principal component analysis (PCA) was used to describe the relationships involving the variables that account for data variance. Two salinity thresholds were identified (100 and 600 mM NaCl) determining significant decreases in all the indices, except for T_{50} and MGT. In fact, PCA based on generated correlation circle showed significant negative correlations (r close to −1) between salt stress and GP, GE, GRI, PV, GV, and CVG, whereas no correlation was observed with T_{50} and MGT (r close to zero). Based on this, GP, GE, GRI, PV, GV, and CVG can be considered useful traits to assess salt tolerance during germination in *S. europaea*, while T_{50} and MGT, that were not affected by the range of salinity levels investigated, should not be used for this purpose.

Keywords: *Salicornia europaea*; salinity; seed germination; PCA; correlation circle

3.1. Introduction

Salinity in soil or water is one of the stresses most severely limiting crop production [1]. More than 20% of cultivated land worldwide is affected by salt accumulation, and this figure is feared to increase up to 50% by 2050 [2]. Salinity impairs seed germination, delays plant development, and reduces crop yield [3]. As a result, the decline in food availability, and the quest for more sustainable sources of food and forage are stirring the interest in halophyte plants. Halophytes are naturally evolved salt-tolerant plants that represent almost 2% of terrestrial species [4]. Halophytes are currently being studied for wider commercial applications, including as a source of food and forage, but also aromatic, cosmetic, and nutraceutical compounds for human uses [5]. *Salicornia* is a halophyte genus belonging to the Amaranthaceae family. It is known as pickleweed, glasswort, sea beans, sea asparagus, or crow's foot greens [6]. Besides *Salicornia europaea,* several other species of *Salicornia* are well known, such as *S. bigelovii*, *S. brachiata*, *S. virginica*, *S. maritima*, *S. ramosissima*, *S. herbacea*, and *S. persica.* These plants are commonly found at the edges of wetlands, marshes, seashores, and mudflats. They have been reported to be able to tolerate up to 500 mM salinity, as in the case of *Salicornia europea* [7], and are considered good candidates for reclamation of barren lands, salt flats, and seashores [6].

Salicornia spp. has been historically used for both edible and non-edible purposes. The aerial parts of the plant are consumed in salads or processed into pickles, beverages, or vinegar [8,9]. On the other hand, the use of this plant as a source of soda (sodium carbonate) for glass and soap making has been a common practice for several centuries [10]. Recently, additional potential uses have been proposed. Some *Salicornia* species (e.g.,

S. bigelovii) are grown at the commercial scale to produce biofuel, livestock feeding, and for salt and oil extraction [11]. A recent study reports the suitability of some *Salicornia* species as bio-indicators of zinc and copper, also emphasizing their potential for soil phytoremediation from these metals [12]. The possibility of using *S. persica* as biofilter for the treatment of the effluent released by a recirculating maricultural system has been studied in Israel [13].

Moreover, the medical and nutraceutical properties of this genus are drawing attention, contributing to the growing interest in it [14]. The efficacy of *S. herbacea* against oxidative stress, inflammation, diabetes, asthma, hepatitis, cancer, gastroenteritis has already been reported [15]. Additionally, the powder of *S. herbacea* has been transformed into spherical granules showing the potential to be used as dietary NaCl [16]. *S. herbacea* has also proved that seed oil is stable to oxidation and eligible to be used in food processing [17]. Crude, as well as purified, polysaccharides from *S. herbacea* have demonstrated cell antiproliferation in human colon cancer [18]. Furthermore, various options to control hyperglycemia have been studied using *S. herbacea* powder on diabetic-induced rats [19].

However, despite the plentiful benefits of *Salicornia*, the consumption of these plants may also determine adverse effects. For instance, the Amaranthaceae family is known for a high oxalate content, which might be harmful to consumers [20]. A study reports *S. brachiata* as being able to accumulate heavy metals, such as cadmium, nickel, and arsenic salts [21], therefore posing a potentially serious risk to consumer health [22].

Despite the potential multiple applications, the use of halophytes as cultivated plants is still restricted due to several impediments, among which is the difficult and uneven germination. In fact, some halophytes are salt-tolerant when adults, but have a differential ecotypic response to salinity during seed germination [23,24]. Typically, germination is higher in fresh water and declines as salinity increases, albeit for some species, low salt concentrations may stimulate germination [25,26]. Many halophytes have developed mechanisms of avoidance based on seed dormancy in order to germinate when salinity is the lowest in their natural environment [27]. Often, indeed, germination occurs after a rainy period when soil salinity is diluted, and the risk of salt stress is reduced [28].

Therefore, the domestication efforts addressing *Salicornia* should include understanding its germination behavior.

Ungar [28] observed that *S. europaea* has low germination when treated with NaCl solutions between 1% (170 mM) and 5% (860 mM), and a germination level similar to control (distilled water) when treated with solutions not exceeding 1% NaCl. In nature, *S. europaea* germinates during the winter and spring season, when the salt concentration is the lowest [28]. Additionally, Orlovsky et al. [29] studied the germination response of *S. europaea* dimorphic seeds under growing salinity and demonstrated that large seeds keep a 90% germination up to 2% NaCl concentration (342 mM), with a drastic drop to 20% at 3% NaCl (513 mM) and no germination at 5% and 7% NaCl concentration. Small seeds, instead, showed germination below 10% at 2% NaCl concentration. This explains why, in the early phase of halophyte cultivation, freshwater irrigation was recommended to ensure good germination and seedling establishment (Gallagher, 1985).

On the other hand, a growing piece of literature, as reviewed by Jisha et al. [30], demonstrates the potential of NaCl seed priming in conferring glycophyte species a higher salt tolerance, which is essentially due to the acquisition of a higher osmotic adjustment capacity. Nonetheless, negative effects with seed osmo-priming were also detected in halophytes, as reviewed by Gul et al. [27]. Therefore, in view of promoting the cultivation of *S. europaea* in Mediterranean areas affected by soil and water salinity, further investigations on germination under saline conditions are needed. In this light, this work was conducted to study the influence of salinity on *S. europaea* seed germination through different indices, with the aim of evaluating their performance and reliability, in order to detect the most suitable ones to assess salt tolerance at this delicate stage.

3.2. Materials and Methods

3.2.1. Plant Material and Germination Conditions

The experiment was set up at the Department of Agricultural and Food Sciences (DISTAL), University of Bologna, Italy. Commercial *Salicornia europaea* seeds were purchased from B & T World Seeds (Aigues-Vives, Gard, France). Healthy seeds were surface-sterilized with a 3% sodium hypochlorite solution for 2 min, rinsed in deionized water for 5 min, and dried at room temperature. The cold stratification method [10] was used to overcome seed dormancy. Seeds were then placed on damp filter paper in 9-cm Petri dishes that were wrapped in transparent plastics and stored in a dark refrigerator at 6 °C for 30 days. During this time, the seeds were dampened with distilled water.

Thereafter, Petri dishes were moved into an incubator at 24 °C, 70–80% relative humidity, and 16/8 h light/dark period for 18 days. At this stage, the filter paper was dampened with distilled water (0 mM NaCl—control), and five treatments at increasing salinity (100, 200, 300, 400, and 600 mM NaCl). Two replicates of 50 seeds per Petri dish were set up for each treatment. The number of seeds germinated under the given conditions was counted every other day until no more germination was observed (up to 18 days). Seeds were considered germinated when the protruding radicle was ≥2 mm long.

3.2.2. Germination Indices

The following indices were calculated: Germination percentage (GP), germination energy (GE), germination value (GV), coefficient of germination velocity (CVG), germination rate index (GRI), peak value (PV), mean germination time (MGT), and time to 50% germination (T_{50}). For each index, the formula and the source are given in Table 1.

Table 1. Description of the various germination indices used in this study.

Index	Unit	Formula	Ref.
Germination Percentage (GP)	%	$GP = \frac{No.of\ germinated\ seeds}{No.of\ seeds}\ x100$	[31]
Germination Energy (GE)	------	$GE = \frac{N_1}{D_1} + \frac{N_2 - N_1}{D_2} + \cdots + \frac{Nj - Ni}{D_j}$	[32]
		where *N* is the number of germinated seeds on the counting date and D the number of days	
Peak Value (PV)	------	$PV = \frac{M_{ag}}{No.of\ seeds} \times 100$	[33]
		where M_{ag} is the maximum of seeds accumulative germination	
Germination Value (GV)	------	$GV = PV \times MDG^1$	[33]
Coefficient of Germination Velocity (CVG)	------	$CVG = (N_1 + N_2 + \cdots N_n)/100 \times [(N_1 \times D_1) + (N_2 \times D_2) + \cdots + (N_n \times D_n)]$	[34]
		where *N* is the number of germinated seeds every day and D is the number of days from seeding corresponding to *N*.	
Germination Rate Index (GRI)	% day^{-1}	$GRI = \frac{G_1}{D_1} + \frac{G_2}{D_2} + \cdots + \frac{G_n}{D_n}$	[31]
		where G_1 is the germination percentage on day 1 (D_1), and so on	
Time to 50% germination (T_{50})	days	$T_{50} = t_i + \frac{(N/2 - n_i) \times (t_j - t_i)}{(n_j - n_i)}$	[35]
		where *N* is the final number of germinated seeds, and n_i and n_j are the total number of seeds that had germinated (by adjacent counts) at times t_i and t_j, when $n_i < N/2 < n_j$.	
Mean Germination Time (MGT)	days	$MGT = \frac{\sum(N \times D)}{\sum N}$	[36]
		where *N* is the number of seeds germinated on day D	

[1] Mean Daily Germination (MDG): No. germinated seeds/No. of days.

3.2.3. Statistical Analysis

Data of the eight germination indices were subjected to a one-way ANOVA for the six salinity levels (from zero to 600 mM NaCl), using the CoStat, 6.4 statistical package (CoHort Software, Berkeley, CA, USA). Tukey's HSD test at $p \leq 0.05$ was used as a mean separation test for significant indices. Additionally, the Statistica 7 program (StatSoft, Tulsa, OK, USA) was used to perform the principal component analysis (PCA). Significant covariance between the studied parameters was defined by the correlation circle using the Pearson correlation coefficient (r) at $p \leq 0.05$ to indicate the similarity.

3.3. Results

3.3.1. Salt Effects on Seed Germination Indices

Figure 1 reports the variations determined by salt stress on germination indices. A significant decrease was detected in all indices at increasing NaCl concentration, except for MGT and T_{50} that did not exhibit any significant change. This decreasing trend could be described by combining three linear functions, whose slopes vary at the salinity levels causing significant reductions in the surveyed indices. The first significant decline was generally observed between 0 and 100 mM NaCl, but the strongest reduction occurred between 400 and 600 mM NaCl.

Optimal GP took place in distilled water (72%), and then seed germination declined to an average 45%, remaining constant between 100 and 400 mM NaCl (Figure 1A). A further decrease was evidenced at 600 mM NaCl, although germination was not totally inhibited, as the residual 28% GP demonstrates (Figure 1A).

GE showed a similar trend (Figure 1B): After the first initial drop between zero and 100 mM NaCl, seeds subjected to a salinity range between 100 and 400 mM NaCl showed a similar GE reduction (35% on average), while the strongest drop in GE was observed at 600 mM NaCl (−61%). PV, instead, decreased quite consistently across the range of salinity (Figure 1C), losing more than 50% of the initial value at 600 mM NaCl (Figure 1C).

A much stronger variation was shown in GV (i.e., the product of PV by mean daily germination) (Figure 1D). The fall in both PV (Figure 1C) and MDG (not shown) determined a multiplicative effect on GV, resulting in an almost 85% drop between 0 and 600 mM NaCl (Figure 1D).

CVG exhibited the same trend of GP, GE, and PV (Figure 1E). The highest CVG value was recorded under control conditions (77). A substantial decline was registered at the salinity level between 100 and 400 mM NaCl (−60%), and a further drop was evidenced at 600 mM NaCl (Figure 1E). GRI, which reflects the daily germination percentage, staged a similar trend, and also the final drop at 600 mM NaCl was the same as CVG (−60%) (Figure 1F).

Contrarily, T50 was unaffected by salinity, as three to four days were needed for all the tested seeds to reach 50% germination, regardless of the salt level (Figure 1G). Likewise, MGT did not exhibit any considerable variation in response to salt concentration (Figure 1H), and the mean time seeds require to initiate and complete germination was six days either under control condition or at the highest salt level (600 mM NaCl).

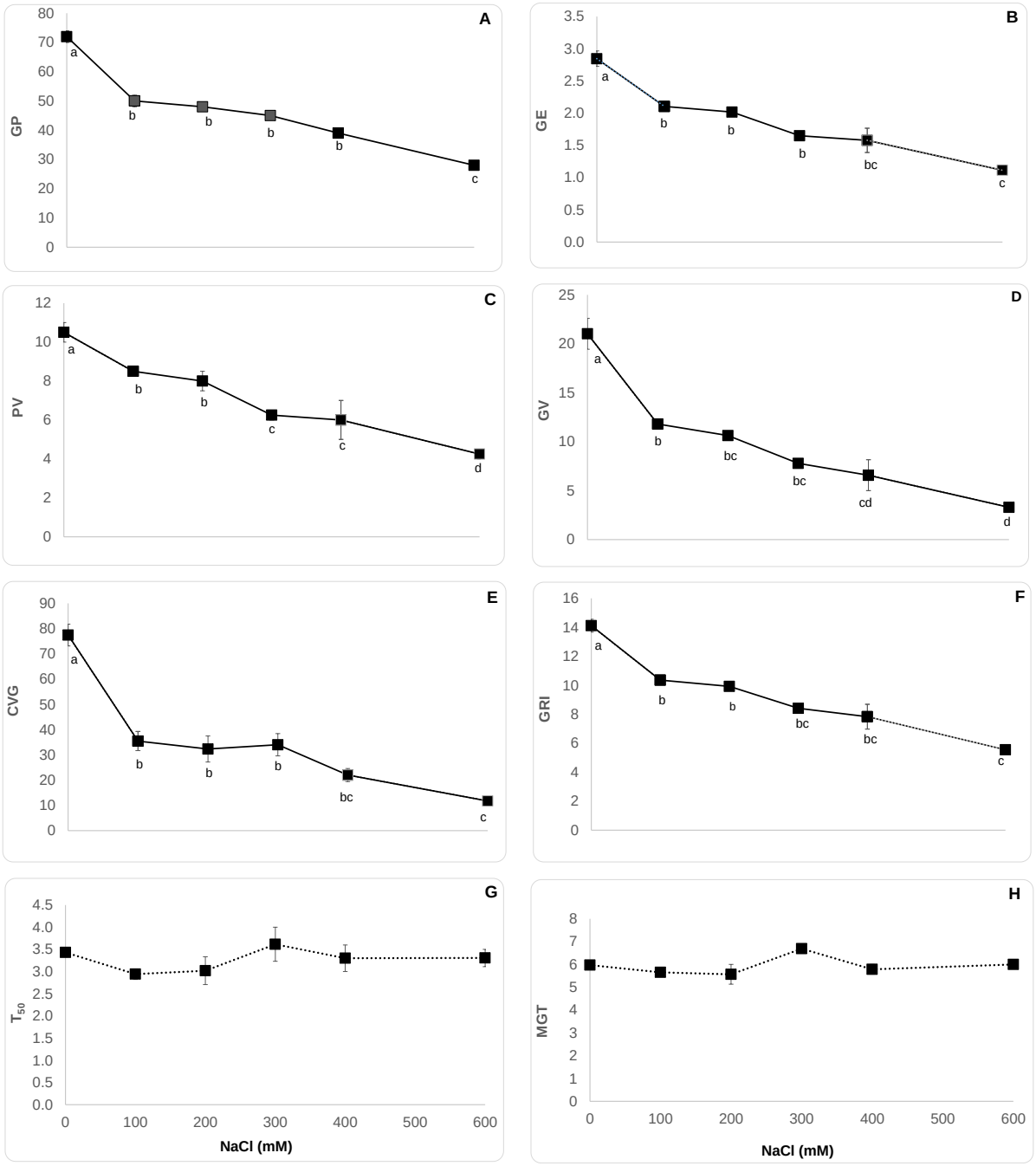

Figure 1. Effects of different salt concentrations on germination indices of *Salicornia europaea* seeds. GP, germination percentage (**A**), GE, germination energy (**B**), PV, peak value (**C**), GV, germination value (**D**), GRI, germination rate index (**E**), CVG, coefficient of velocity of germination (**F**), T50, time to reach 50% of germination (**G**), MGT, mean germination time (**H**). Data presented are means ± SE.

3.3.2. Principal Component Analysis of Germination Indices

Principal component analysis was carried out to establish the relationship among the variables that account for the observed data variance. Eigenvalues higher than 1 were used to determine the number of principal components (Table 2). The first two principal components (PC1 and PC2) jointly explained 98% of the observed variance and were, therefore, represented in a two-dimensional space (Figure 2). PC1, plotted on the horizontal axis, explained the largest share of variance (73.8%), while PC2, plotted on the vertical axis, represented an additional 24.4% of the total variance (Table 2). Variable squared cosines were used to define variable contributions to the respective PC1 and PC2 (Table 2).

Table 2. Eigen analysis of the correlation matrix.

Principal Component Analysis	PC1	PC2
Eigenvalue	5.905139	1.954265
Total variance (%)	73.81423	24.42831
Cumulative Eigenvalue	5.905139	7.859403
Cumulative variance (%)	73.8142	98.2425
Variable Squared Cosines		
Variable	**PC1**	**PC2**
GP	0.98	1.00
GE	1.00	1.00
PV	0.97	0.99
GV	0.99	0.99
CVG	0.93	0.99
GRI	1.00	1.00
T_{50}	0.00	0.95
MGT	0.03	0.94

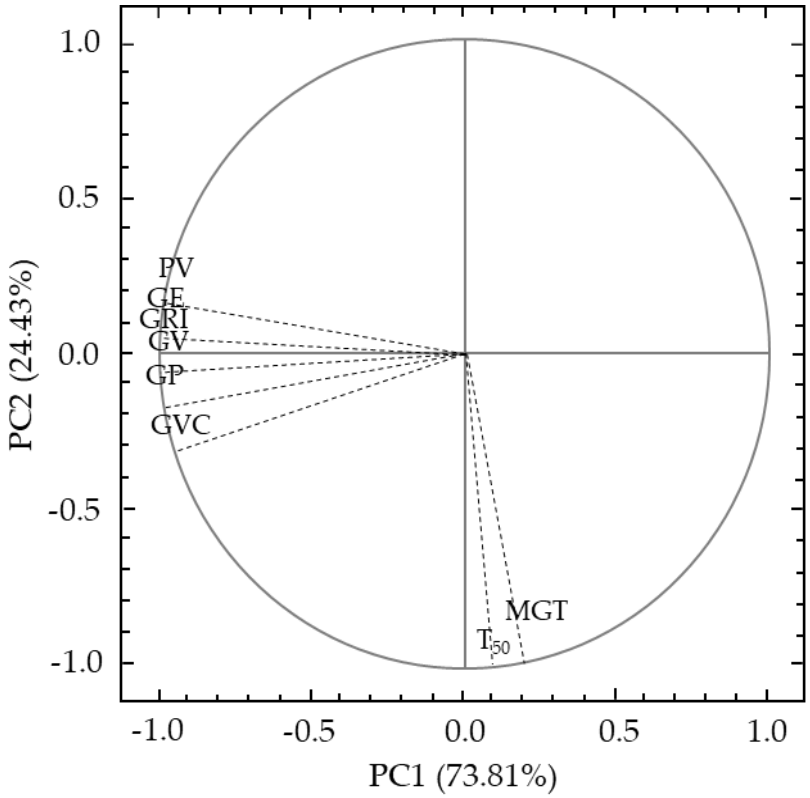

Figure 2. Site score plot of the studied variables on the first two principal components (PC1 and PC2) of *Salicornia europaea* seeds exposed to salt stress. GP, germination percentage; GE, germination energy; PV, peak value; GV, germination value; GRI, germination rate index; CVG, coefficient of velocity of germination; T_{50}, time to reach 50% of germination; MGT, mean germination time (MGT).

Figure 2 represents the site score plot of the eight indices on the two first PC of *Salicornia europaea* seeds. GP, GE, PV, GV, CVG, and GRI appeared to be negatively correlated with salt stress, being positioned on the negative side of the horizontal axis representing PC1. The highest correlations were especially observed between salt stress and GP, GE, PV, GV, and GRI (r between −0.99 and −1.00). CVG was also shown to be well correlated with salt stress (r = −0.96). On the other hand, T_{50} and MGT that are located on the positive side of

the horizontal axis, very close to zero, express a very low correlation with salinity stress (r = 0.18 and r = 0.05, respectively).

Figure 3 illustrates each variable's specific contribution to PC1 (total contribution = 1). The contribution of GP, GE, PV, GV, CVG, and GRI was relatively high and uniform, whereas T_{50} and MGT contribution to PC1 was negligible.

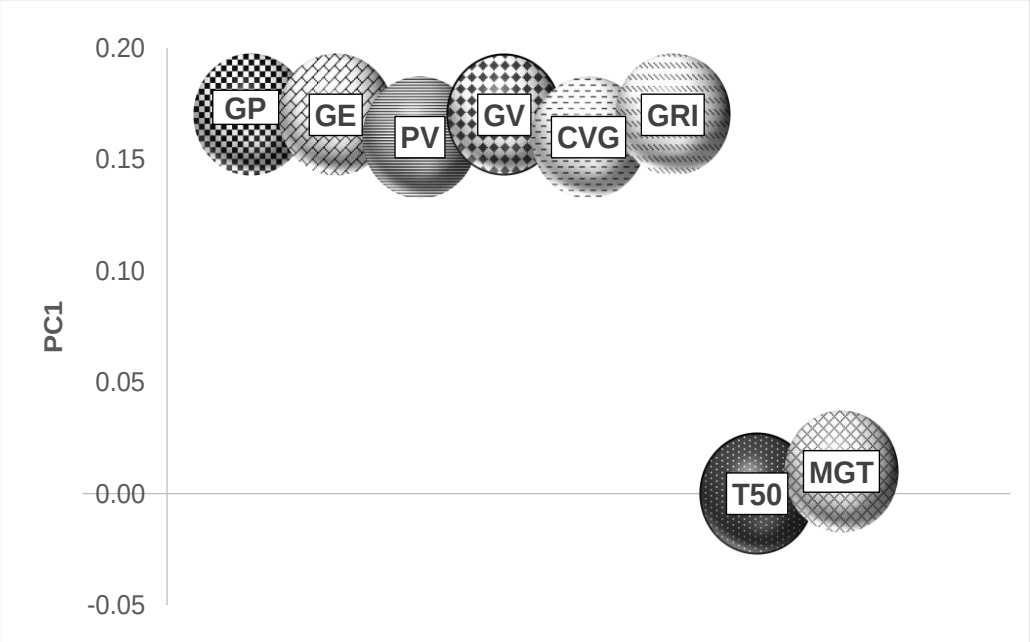

Figure 3. Contribution of the studied variables to the first principal component (PC1) of *Salicornia europaea* seeds exposed to salt stress. GP, germination percentage; GE, germination energy; PV, peak value; GV, germination value; GRI, germination rate index; CVG, coefficient of velocity of germination; T_{50}, time to reach 50% germination; MGT, mean germination time.

3.4. Discussion

Germination characteristics are among the most suitable criteria for assessing salt tolerance in plants [37]. Salinity is a serious constraint hindering seed germination [38], and the fact that germination indices are adversely affected by salinity is generally acknowledged [39]. In the present work, various indices were focused on assessing *Salicornia europaea* germination performance, each having a slightly different focus. Two salinity thresholds were identified: The first one was at 100 mM NaCl (low salinity threshold), where most of the indices showed the first decline, with the exception of T_{50} and MGT (Figure 1). The second critical drop was observed at 600 mM NaCl (high salinity threshold), again with the exception of T_{50} and MGT that remained substantially unaffected up to this level (Figure 1). Hence, most indices exhibited a consistency of the effect in a relatively wide range of salt doses (between 100 and 400 mM NaCl).

It could be argued that low-medium salt stress (up to 400 mM NaCl) might break *S. europaea* seed dormancy and promote germination. Sanoubar et al. [40] identified two thresholds of salinity response in white cabbage, respectively, at 100 mmol L^{-1} NaCl (moderate salinity threshold) and 200 mmol L^{-1} NaCl (high salinity threshold). Maggio et al. [41] proposed that the relationship between yield and salinity in tomato could be represented by a bilinear response function, suggesting the existence of a second physiological threshold. Such a threshold may be used to identify functional shifts between different adaptation mechanisms.

However, at a high salt concentration (600 mM NaCl), GP was severely reduced but not completely inhibited, and seeds were still able to germinate (28% vs. 72% of the control) (Table 1, Figures 1–2). This suggests a high tolerance of *S. europaea* towards salinity stress, proving its ability to germinate even under high salt concentration. Such tolerance was identified also in other halophytes, such as *Haloxylon ammodendron* (200 mM) [25], *Salsola affinis* (400 mM), [42], and *Bromus inermis* (200 mM) [43]. Compared to this, for *Artemisia*

annua, a species that is not acknowledged to be a halophyte, Bijeh Keshavarzi [44] reported a GP of 77.5% under no salinity, vs. 8.75% at 100 mM NaCl, and zero at only 150 mM NaCl. Seed germination of halophytes under salinity was also reported by Li et al. [45], who found that GP of *Apocynum venetum* increased with NaCl concentrations up to 150 mM, supporting the assumption that low salt stress could promote seed germination in this species. In *S. europaea* germination of small seeds was also reported to be slightly improved at 0.5 and 1% NaCl vs. no salt added [29].

Compared to this, other studies on halophytes such as *Atriplex isatidea*, *Phragmites australis*, *Sesbania cannabina*, and *Limonium bicolor* reported germination levels of 5% or lower at 300 mM NaCl [46]. Similar results were also observed in species as *Reaumuria trigyna* [47], and *Salsola vermiculata* [48]. High salinity might lead to ionic imbalance, with excess Na^+ and Cl^- ions determining irreversible damage of function and structure of cell membranes, in turn, leading to cell death [49].

Furthermore, PCA supported our quest to identify the traits contributing to explain the variable behavior in response to salinity. The first two principal components, PC1 and PC2, accounting for almost all the observed variance (Table 2), were used to plot two-dimensional scatter plots (Figure 2) [50,51]. As already mentioned, the first principal component PC1 alone expressed almost three quarters of the variability in our data set (Table 2). Consequently, PC1 could be considered the main salinity-related component. Some of the selected germination indices (GP, GE, PV, GV, CVG, and GRI) were located at the extreme left side of the horizontal axis in the loading plot, meaning that they were negatively correlated with salt stress (r close to −1) (Figure 2). This distribution of GP, GE, PV, GV, CVG, and GRI can be ascribed to the salinity factor, and the negative correlation indicates adverse effect exerted by salinity in each of these traits. Therefore, it is perceived that these indices represent a robust parameter for evaluating *Salicornia europaea* seed salinity tolerance. In contrast, T_{50} and MGT were loaded orthogonally (r close to 0) (Figure 2), indicating unsuitability to reveal salt stress. Therefore, they cannot be considered useful indices for salt tolerance screening in seed lots of this species.

3.5. Conclusions

The achievement of the highest germination in distilled water (control) suggests that *S. europaea* does not have a real physiological need for salt during germination. Conversely, salinity progressively affects its germination, without ever completely suppressing it. Eight seed germination indices were selected to study *S. europaea* germination under non-saline control and five levels of increasing salinity. Two salinity thresholds (100 and 600 Mm NaCl) where identified, at which all the surveyed indices were significantly reduced, with exception of T_{50} and MGT.

A principal component analysis (PCA) was carried out to identify and group the variables accounting for the largest share of data variance. PCA results showed a significant negative correlation (r close −1) between salt stress and all measured indices, with the exception of T_{50} and MGT (r close 0). Accordingly, GP, GE, PV, GV, CVG, and GRI may be considered appropriate parameters for the evaluation of salinity tolerance in *Salicornia europaea* seed lots, while T_{50} and MGT should not be addressed in salt stress assessment, as they did not reveal sufficient sensitivity to this factor.

Author Contributions: The following statements should be used "Conceptualization, R.S..; methodology, R.S..; formal analysis, R.S..; investigation, R.C.; resources, L.B. and E.N.; data curation, R.C..; writing—original draft preparation, R.C. and R.S.; writing—review and editing, R.C., L.B., E.N. .; visualization, R.C., L.B.,.; supervision, L.B.. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: All seed germination tests were carried out at the Seed Research and Testing Laboratory (LaRAS) of DISTAL, University of Bologna. Giovanni Urso of LaRAS is gratefully acknowledged for the support provided during the experiment.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 4

Article published *in Plants* 2021, 10, 309.

Physiological Adaptation to Water Salinity in Six Wild Halophytes Suitable for Mediterranean Agriculture

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Received: 30 November 2020; Accepted: 2 February 2021; Published: 5 February 2021

Abstract: Owing to the high interspecific biodiversity, halophytes have been regarded as a tool for understanding salt tolerance mechanisms in plants in view of adapting to climate change. The present study addressed the physiological response to salinity of six halophyte species common in the Mediterranean area: *Artemisia absinthium, Artemisia vulgaris, Atriplex halimus, Chenopodium album, Salsola komarovii*, and *Sanguisorba minor*. A 161-day pot experiment was conducted, watering the plants with solutions at increasing NaCl concentration (control, 100, 200, 300 and 600 mM). Fresh weight (FW), leaf stomatal conductance (GS), relative water content (RWC) and water potential (WP) were measured. A principal component analysis (PCA) was used to describe the relationships involving the variables that accounted for data variance. *A. halimus* was shown the species most resilient to salinity, being able to maintain FW up to 300 mM, and RWC and WP up to 600 mM, followed by *C. album*. Compared to them, *A. vulgaris* and *S. komarovii* showed intermediate performances, staging the highest FW and GS values, respectively, under salinity. Lastly, *S. minor* and *A. absinthium* exhibited the most severe effects with a steep drop in GS and RWC. Lower WP values appeared to be associated with best halophyte performances under the highest salinity levels, i.e. 300 and 600 mM NaCl.

Keywords: Halophytes; Salt stress; Salinity levels; Relative water content; Water potential.

4.1. Introduction

The world population is expected to reach 9.7 billion people by 2050 and about 10.9 billion by 2100 [1]. To feed this growing population, food production shall increase by up to 70% by 2050 [2], although the progressive loss of fertile land due to soil degradation and urbanization will make this objective ever harder to achieve. This is further hampered by the rise in global warming, which is already increasing the occurrence and severity of drought events in formerly very productive land all over the world, increasing the pressure on high-quality water for irrigation [3], and highlighting the urgency of more resilient agricultural systems, especially in agricultural land suffering soil degradation and loss. On a global scale, salinity is one of the most severe factors of soil degradation, which affects over 100 countries and a land surface larger than 1 million hectares [4].

Natural soil salinization, known as primary salinization, occurs in arid and semi-arid climatic areas because of seawater intrusion, wind salt deposition, or parent material dissolution. Conversely, secondary salinization is induced by anthropogenic actions and is caused by the application of

agrochemicals and by the use of low-quality irrigation water such as saline groundwater and wastewater [5]. Secondary salinization is expanding worldwide with a rate of 2 Mha $year^{-1}$, posing a serious threat to agricultural productivity [6].

Unfortunately, even a minimum quantity of sodium chloride in irrigation water can cause severe yield losses in most agricultural crops. For example, the yield of bean, pepper, maize and potatoes was reported to decrease by 19%, 14%, 12% and 12%, respectively, at a salinity level of 2 dS m^{-1} [7]. There is thus the urgent need to identify alternative salt-resistant crops for farming and for the restoration of salt-affected areas in order to achieve the 2050 objective [8]. The state of the world's plants and fungi 2020 report [9] of Kew Royal Botanic Gardens, drawn up by 210 researchers in 97 institutions across 42 countries, lists about 7000 edible plant species of which only 417 are included in the Food and Agriculture Organization's (FAO) lists of major crops. Overlooked and underutilized plants may transform food production systems into more robust and ecologically sustainable ecosystems, through the cultivation of pre-adapted plants and the diversification of the spectrum of species used, while protecting biodiversity and essential ecosystem services.

Plants able to grow and thrive in saline environments are known as halophytes, and account for less than 2% of vascular plants. These species are able to grow in extreme environments characterized by high temperature, drought and salinity, such as coastal sand dunes, salt marshes and pans, and steppes where no crop would survive [10]. Halophytes are a source of fresh vegetables, medicinal and nutraceutical compounds, and provide multiple ecosystem services including soil protection from erosion and salinization, ornamental landscaping, wildlife support [11], and can mitigate the adverse effects of soil and water salinization on agricultural lands.

In this group of plants, *Artemisia absinthium* L., *Artemisia vulgaris* L., *Atriplex halimus* L., *Chenopodium album* L., *Salsola komarovii* Iljin, and *Sanguisorba minor* Scop. are six wild species with varying degrees of salt tolerance and of high potential agricultural value in the Mediterranean area.

Artemisia is a genus belonging to the Asteraceae family that is common in the temperate regions of the world. It includes around 500 species including perennial herbs and shrubs which were historically used in pharmaceutical and cosmetic preparations [12], thanks to their high content of active compounds such as essential oils, tannins, organic acids, carotenoids, ascorbic acid, and glycosides [13] with antioxidant, antiviral, and bactericidal activity [14,15]. *A. absinthium* has already been successfully used for salt- and heavy metal- phytoremediation [16–19]. *Artemisia* spp. are considered glycohalophytes, i.e., halfway between true halophytes and glycophytes, as they can control salt uptake by reducing root permeability to inorganic ions [20].

The two annual species *C. album* and *S. komarovii*, and the perennial *A. halimus* belong to the Amaranthaceae family (previously Chenopodiaceae) and occur in many world regions. *C. album* is an underexploited weed that, besides providing minerals, fibers, vitamins and essential fatty acids, was historically used for its medical properties as a blood purifier, diuretic, sedative, hepatoprotective, antiscorbutic laxative and as an anthelmintic drug [21,22]. Previous studies demonstrated the ability of *C. album* seeds to germinate in conditions of up to 400 mM soil salinity [23], to be able to select K^+ over Na^+ [24], and to produce osmo-compatible solutes and antioxidant enzymes that can prevent membrane lipids peroxidation under salt stress [25].

Salsola komarovii, also known as "agretti" in the Mediterranean region, was the main source of soda ash in past times [26]. Nowadays, it is sold as sea vegetable and salad crop at relatively high prices [27]. *Salsola komarovii* and *S. soda* are also used for soil salt and heavy-metal phytoremediaton [28–30], and in intercropping systems to increase the performance of glicophyte species [31–33]. Studies on the nutraceutical properties of *Salsola* spp. have also demonstrated their hypoglycemic effect [34,35] and their potential to contrast hypertension, constipation, inflammation, and Alzheimer's disease [36,37].

Atriplex halimus is a shrub that is highly resistant to drought [38,39], salinity [40], and heavy-metal stress [41,42]. *Atriplex* species, indeed, are capable of excreting salts into special bladders located on the leaf [43]. A previous study documented increasing biomass under increasing salinity

levels [44] in *Atriplex* spp., indicating that these plants can be key species in agro-ecosystems for saline soils cultivation and restoration, as well as for pasture and fodder production [45,46].

Sanguisorba minor is a perennial wild herb belonging to the Rosaceae family and mainly distributed in temperate areas of Europe. Known as salad burnet, it is a drought- and cold-tolerant species [47,48] that can also tolerate mild saline soils [49]. *S. minor* can be consumed as a fresh or boiled vegetable [50], or used for its medicinal and functional properties due to its high content of antioxidant bioactive compounds which provide digestive, antioxidant, astringent, carminative and diuretic properties [51,52].

Despite their multiple uses and a promising market, these species have a limited agricultural relevance at a local scale and are poorly known to farmers, as well as to agronomists and breeders.

The aim of this study is to increase the knowledge on how these salt-tolerant born species cope with salinity at the ecophysiological level and which saline conditions they prefer, can tolerate or are vulnerable to. Of these taxa, *Atriplex* and *Salsola* were already proposed as model organisms on which to focus the research in salt-adaptation in halophytes [53]. However, given the multitude of strategies evolved by halophytes, the comparison of species responses to salinity is more informative than studies concerning species-specific response of a single species and provide multiple reference systems that can serve as models to advance the research in salt-tolerance [53]. By comparing the ecophysiology of six halophytes in response to increasing salinity, this study establishes fundamental knowledge on which to build agronomic research in salt-tolerant crops and crop traits, and is the first step towards the resilient and sustainable agro-ecosystems that we all claim for the future.

4.2. Materials and Methods

4.2.1. Experimental set up

The experiment was carried out at the Department of Agricultural and Food Sciences (DISTAL), University of Bologna, Italy, and lasted 161 days, from 15 June to 23 November 2017. Commercial seeds of *Artemisia absinthium* L., *Artemisia vulgaris* L., *Atriplex halimus* L., *Chenopodium album* L., *Salsola komarovii* Iljin, and *Sanguisorba minor* Scop. were purchased from B & T World Seeds (Aigues-Vives, France) and Chiltern seeds (Wallingford, England) online shop. Seeds were surface-sterilized by immersion in a 3% sodium hypochlorite solution for 2 min, rinsed in deionized water for 5 min, and dried at room temperature. Seeds were then put in 9-cm Petri dishes on damp filter paper and placed into an incubator at 24 °C, 70–80% relative humidity, and 16/8 h light/dark period for 18 days. The filter paper was either dampened with distilled water (0 mM NaCl—control), or with four water solutions at increasing salinity (100, 200, 300, and 600 mM NaCl). The amount of salt added to water (TSS) to reach the salinity levels (EC_w) was calculated according to equation 1:

$$TSS\ (g_{NaCl}\ L^{-1}\ water) = EC_w\ (dS\ m^{-1}) \times 0.640 \quad (1)$$

The germinated seeds (protruding radicles longer than 3 mm) were picked from the Petri dishes and moved into 2 L pots filled with peat moss growing media (26% organic carbon, pH_{H2O} = 7, and EC at 25 °C = 0.6 dS m^{-1}). Germination was reduced under 300 mM NaCl and severely inhibited under 600 mM NaCl. Owing to this, seeds germinated in the range 0 – 300 mM NaCl were used for pots at various salinity levels according to this scheme: 0 mM NaCl (Petri dishes) → 0 and 100 mM NaCl (Pots); 100 mM NaCl (Petri dishes) → 200 mM NaCl (pot); 200 mM NaCl (Petri dishes) → 300 mM NaCl (pot); 300 mM NaCl (Petri dishes) → 600 mM NaCl (pot). Pots were transferred to a greenhouse.

The 6 species (HS) × 5 salinity levels (WS), totalling 30 combinations, were set up with three completely randomized replicates. This number of replicates is quite commonly adopted in pot experiments on this topic [24,54–57].

Ammonium nitrate (N, 26%) was added at 0.1 g pot^{-1} prior to seedling transplant; a second dose was supplied at mid-experiment by placing the granular fertilizer directly on the substrate surface prior to watering.

The pots were manually watered 2–3 times a week up to the end of the experiment. Around 9 L of saline solutions were distributed per pot, corresponding to a NaCl amount of 57.6 g, 115.2 g, 172.8 g, and 345.6 g per pot in the four respective salinity levels. The maximum and minimum air temperature and relative humidity in the greenhouse were 28.47 ± 3.4 °C, 21.6 ± 4.6 °C, 71.0 ± 12%, and 49.8 ± 8.2%, respectively. Photosynthetically active radiation (PAR) of 200 μmol m−2 s−1 was provided by high pressure sodium lamps with a 16-h light and 8-h dark cycle.

4.2.2. Physiological parameters measurement

Leaf stomatal conductance (GS, mmol m^{-2} s^{-1}) was measured at vegetative peak 28 days before harvest using a leaf porometer (AP4Porometer, Delta-T Devices Ltd., Cambridge, England) equipped with a leaf chamber. Measurements were performed on the upper fully expanded leaf, on the middle portion of the blade between the midrib and the leaf margin.

Leaf water potential (WP, MPa) was assessed 21 days before harvest on a disk cut picked from the upper fully expanded leaf, using the WP4-C dewpoint potentiometer (METER Group, Pullman, WA, USA). Leaf cuts were stored in sealed plastic cups and measurements were started within a short time of sampling [58]. Concurrently, leaf relative water content (RWC, %) was determined on the same leaf: a small disc of 2 cm diameter was cut from the leaf and weighed to determine fresh weight (FW). Then, it was put in a 15 mL vial with distilled water in the dark and after 24 h the turgid weight (TW) was measured. The sample was finally oven-dried at 105 °C for 24 h to assess the dry weight (DW). The RWC (%) was calculated according to Equation (2) [59]:

$$RWC = \frac{\mathrm{FW - DW}}{\mathrm{TW - DW}} \times 100 \quad (2)$$

Since that *S. komarovii* has succulent threadlike leaves, a piece of branch from the upper part of the plant, with a surface area of about 2 cm^2, was used for WP and RWC measurements.

At harvest, shoots were separated from roots and weighed to determine the plant fresh weight (FW, g $plant^{-1}$). In *A. halimus* and *S. komarovii*, one of the three replicates treated with 600 mM and 200 mM WS, respectively, died before harvest. Therefore, we removed the incomplete FW data of these treatments from the dataset.

4.2.3. Statistical analysis

The data of the four physiological traits (FW, GS, RWC and, WP) were submitted to ANOVA for the six species under control and the four saline treatments. We first analysed the overall effect of salinity in the 6 species vs. control, considering all the salinity levels together, i.e., the 12 salinity data collected for each trait for each species. This analysis, a one-way ANOVA, was meant to highlight the variation in salinity response and in tolerance extent among the six halophytes. Given the species-specific differences detected by this analysis, the measured traits, i.e. FW, GS, RWC and, WP, were normalized vs. their respective controls prior to the subsequent analyses (Eq. 3):

$$Y_r = \frac{X_i - X_c}{X_c} \times 100 \quad (3)$$

where Y_r is the percentage difference of the parameter with respect to the control at the i salinity level, X_i is the value of the parameter at i salinity level and X_c is the mean value of the parameter in the control treatment.

Differences due to the specific salinity levels were analyzed by a two-way ANOVA using the species and the saline treatments as factors and testing their interaction. Since the interaction between the two factors resulted always significant at $p \leq 0.05$ (except for RWC, $p \leq 0.1$), meaning that the response to the saline treatment varied across the six species, the data were analyzed also with a one-way ANOVA within each species, to evaluate specific response to saline water treatment. The

Tukey's HSD post-hoc test at $p \leq 0.05$ was used to indicate significant differences among species and/or treatments.

Two Principal Component Analyses were carried out to summarize the performances of the six halophytes under control (Control PCA) and salt stress conditions (Control-normalized PCA). The Principal Components (PCs) were obtained from centered and scaled quantitative variables, through diagonalization of the correlation matrix and extraction of the associated eigenvectors and eigenvalues. In the Control PCA, the variables FW, GS, RWC, and WP were set as active quantitative variables, while the six halophyte species (HS) were used as supplementary categorical variables, i.e. variables that were not used in the computation of PCs. In the Control-normalized PCA, instead, the control-normalized FW, GS, RWC and, WP data were used as quantitative variables while the four saline water treatments (WS) and the six halophyte species (HS) were set as supplementary categorical variables.

A Hierarchical Clustering on the first two PCs (HCPC) was then realized both on control (Control HC) and saline treatments (Control-normalized HC) PCAs. The effect size (η^2) was calculated for quantitative variables, i.e. plant physiological traits, to assess the proportion of the total variance associated with the extracted clusters that was explained by each trait. η^2 was calculated as follows (Eq. 4):

$$\eta^2 = \frac{SS\ effect}{SS\ total} \quad (4)$$

where *SS effec*t is the sum of squares for the quantitative variable effect, and *SS total* is the total sum of squares.

Clusters were then characterized using both quantitative and categorical variables through a test value (v-test). For quantitative variables, the cluster mean (x_q) was compared with the overall mean (x), to ascertain if there was a significant difference within the cluster. The following quantity (Eq. 5) was calculated:

$$u = \frac{x_q - x}{\sqrt{\frac{s^2}{n_q}\left(\frac{N-n_q}{N-1}\right)}} \quad (5)$$

where n_q is the number of individuals in cluster q, N the total number of individuals, s the global standard deviation. The value of u was then compared to the corresponding quantile of the normal distribution; therefore, an absolute value greater than 1.96 implies a significant difference at $p < 0.05$, which in turn indicates the given variable as a characterizing one to discriminate the cluster. The sign indicates the direction of the deviation from the global mean.

For categorical variables, we aimed at identifying the category levels being over- or under-represented within the clusters. A χ^2 test was performed between each categorical variable and the cluster variable. For the significant cases, the frequency N_{qj} (number of individuals of the group q in the category level j) was represented in a hypergeometric distribution with the parameters N, n_j, $\frac{n_q}{N}$ (where n_j is the number of individuals that have taken the category j), and a p-value was calculated. The p-value was then transformed into the corresponding quantile value of the Gaussian distribution. Positive and negative signs mean over- and under- representation, respectively, of the referred category within the specific cluster.

All the statistical analyses were performed with the R 6.3.6 statistical software, using Car [60] and Emmeans [61] packages for the analysis of variance and post-hoc test, and the FactoMineR package for principal component analysis and hierarchical clustering on principal components [62]. Charts were created with the ggplot2 [63] R packages.

4.3. Results

4.3.1. Control vs saline water treatments

The performance of the six halophyte species under saline conditions (100 – 600 mM NaCl) vs. control are displayed in Figure 1. Under control condition, *A. vulgaris*, *A. absinthium* and *S. minor* showed, in decreasing order, high FW, while the other three species had a similar, low FW. Under salinity, *A. vulgaris*, *A. absinthium* and *A. halimus* were top ranked with respect to FW, which means that the first two species plummeted with respect to the control (-68% and -49%), while the third species soared (+133%). They were followed by *C. album*, whose FW did not substantially vary from the control, while *S. komarovii* and *S. minor* were bottom ranked and showed the strongest drops vs. control conditions (-75% and -81%, respectively). The lack of the 600 mM and 200 mM WS data in *A. halimus* and *S. komarovii*, respectively, might have increased and decreased the FW mean value under salinity, in the two respective cases.

A. vulgaris, S. komarovii and *S. minor* showed the highest stomatal conductance values under control conditions, followed by the three remaining species having similar lower GS values. Under salinity, *S. komarovii* had the highest GS, followed by the group of *A. vulgaris*, *S. minor, C. album* and *A. halimus*. The lowest GS was shown by *A. absinthium*, resulting in the strongest drop from the control (-51%).

Relative water content outlined narrow, yet significant, differences under control conditions: *S. minor* had the highest RWC, while *A. halimus* had the lowest RWC. Under salinity, *S. minor* remained top ranked, followed by *S. komarovii*, *A. vulgaris* and *A. halimus*. Lastly, *C. album* and *A. absinthium* were the two bottom ranked species. In the case of *A. halimus* no RWC difference was observed, in practice, between control and salinity, while in the other species the decrease due to salinity ranged between 8% (*S. komarovii*) and 16% (*A. vulgaris*).

Water potential did not significantly vary under control conditions. Under saline treatments, *A. halimus* and *S. minor* dropped to significantly lower WP levels than *S. komarovii*, while the other three species were intermediate. The relative decreases vs. control conditions ranged from a minimum of 32% (*A. vulgaris*) to a maximum of 134% (*C. album*).

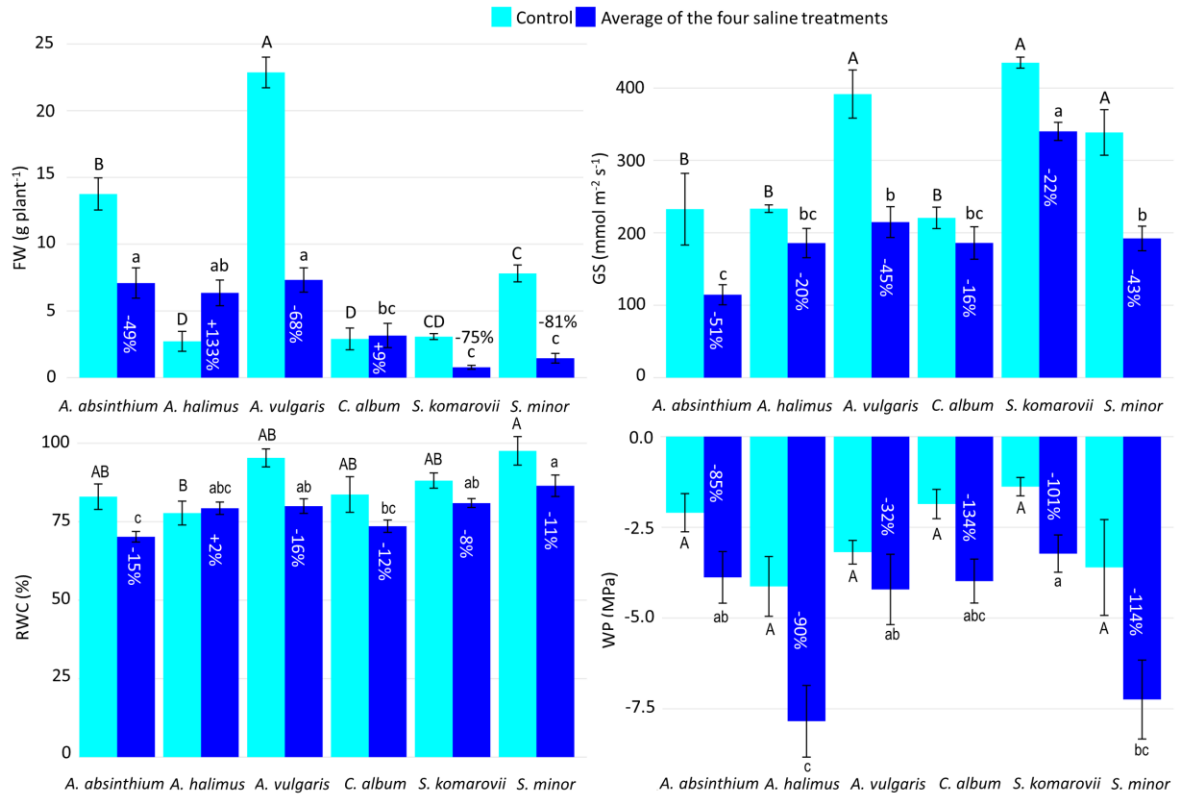

Figure 1. Fresh weight (FW), stomatal conductance (GS), leaf relative water content (RWC), and leaf water potential (WP) of the six halophyte species under control (cyan histograms), and the four saline treatments combined (blue histograms). The percent difference between control and the combined saline treatment is reported for each species as % in the saline treatment column. Uppercase and lowercase letters indicate statistical differences ($p \leq 0.05$) among the six species in controls and average saline treatment respectively. Vertical bars indicate ± one standard error.

4.3.2. Normalized water saline treatments

To better compare the performances of the halophyte species, the values of each physiological trait were normalized with respect to the mean control value. The two-way ANOVA (Table 1) of normalized data showed a significant HS × WS interaction for FW, GS, WP and, to a less extent ($p \leq 0.1$), RWC, indicating a different effect of saline treatments within species.

Table 1. F values and statistical significance in the two-way analysis of variance (Halophyte Species x Water Salinity levels) carried out on the control-normalized values of the four measured physiological traits: fresh weight (n-FW), stomatal conductance (n-GS), leaf relative water content (n-RWC), and leaf water potential (n-WP).

Source	dF	n-FW	n-GS	n-RWC	n-WP
HS	5	21.74**	15.67 **	7.36 **	2.18 (+)
WS	4	7.56 **	61.88 **	9.99 **	21.07 **
HS x WS	20	3.91**	3.18 **	1.67 (+)	1.91 *

WS =Water salinity; HS=Halophyte species. Significance codes: (+), * and ** mean significant at $p \leq 0.1$, $p \leq 0.05$ and $p \leq 0.01$, respectively.

Accordingly, a one-way ANOVA (Table 2) was performed to assess the effects of salinity within each species for the above-mentioned physiological traits. Salinity determined significant variations

in all traits and species, with the exception of n-FW, n-RWC and n-WP in *A. halimus*, n-RWC in *C. album*, and n-WP in *A. vulgaris*. The effects across increasing salinity levels are displayed in Figure 2.

Table 2. F values and statistical significance in the one-way analysis of variance carried out on the control-normalized percent values of the four measured physiological traits: fresh weight (n-FW), stomatal conductance (n-GS), leaf relative water content (n-RWC), and leaf water potential (n-WP).

Halophyte species	dF	n-FW	n-GS	n-RWC	n-WP
A. absinthium	4	24.05**	6.14 **	3.60*	6.28**
A. vulgaris	4	90.46**	15.60 **	2.93 (+)	1.41 ns
A. halimus	4	2.02 ns	38.19 **	0.17 ns	1.26 ns
C. album	4	3.76 (+)	27.62 **	3.10 ns	3.48 (+)
S. komarovii	4	46.41 **	14.60 **	4.64 *	19.40 **
S. minor	4	29.28 **	22.15 **	8.11 **	24.73 **

Significance codes: ns, (+), * and ** mean non-significant and significant at $p \leq 0.1$, $p \leq 0.05$ and $p \leq 0.01$, respectively.

Saline treatments determined significant n-FW decreases in all species except *A. halimus* and *C. album.* Higher FW values in salt treatments than controls may indicate that lack of salt, rather than salinity, is a limiting factor in *A. halimus* and *C. album*. However, for *A. halimus* we cannot assess the effect of the 600 mM NaCl treatment, owing to missing data. Only in *A. vulgaris* the highest salinity level, 600 mM NaCl, had a significant stronger negative effect than the lower saline levels, whereas in *A. absinthium, S. komarovii* and *S. minor* there was no significant effect beyond 100 mM NaCl (*S. komarovii* and *S. minor*) or 200 mM NaCl (*A. absinthium*) (Figure 2). For *S. komarovii,* hence, the lack of the 200 mM NaCl treatment data appears less critical than the lack of 600 mM for *A. halimus*, because higher salinity levels (300 and 600 mM NaCl) did not exhibit a different FW response compared to the 100 mM NaCl treatment.

Salinity determined significant n-GS decreases in all species, and the effect was quite proportional to the salinity levels (Figure 2). Salinity, as well, induced significant n-RWC decreases in *A. absinthium, S. komarovii* and *S. minor*. The effect was consistent only in the highest salinity treatment (600 mM NaCl) in *S. Komarovii* and *S. minor* (Figure 2). Salinity also determined a significant n-WP decreases in these three species; the effect was significant from 300 mM NaCl (*S. komarovii* and *S. minor*) or with 600 mM NaCl (*A. absinthium*) (Figure 2). *A. vulgaris, A. halimus*, and *C. album* did not show RWC and WP changes across salinity levels.

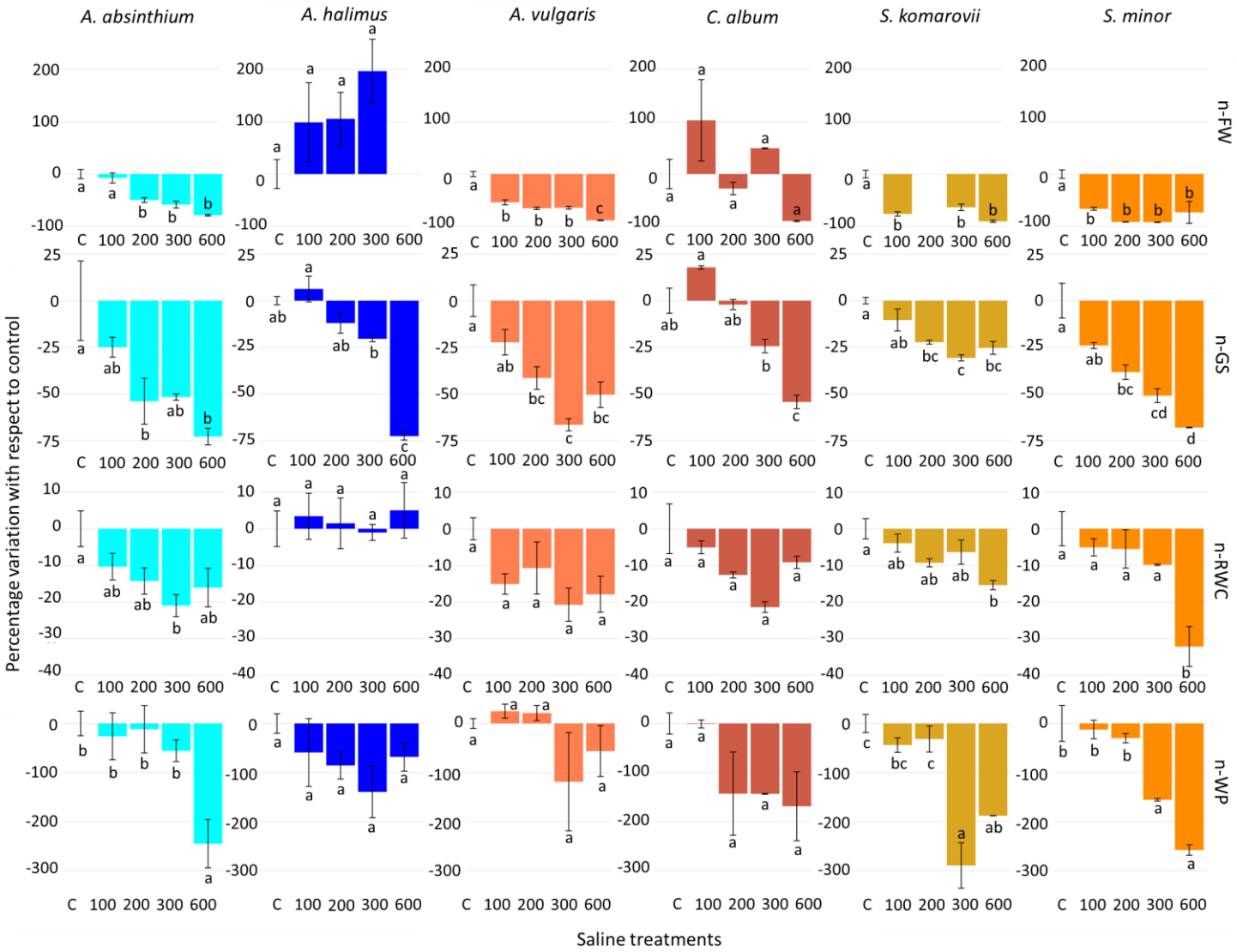

Figure 2. Control-normalized percent differences in fresh weight (n-FW), stomatal conductance (n-GS), leaf relative water content (n-RWC), and leaf water potential (n-WP) within species due to the different saline treatments experimented in the study. Different letters indicate significant differences at $p \leq 0.05$. Vertical bars indicate ± one standard error. In *A. halimus* and *S. komarovii*, one n-FW level is missing due to dead plants at harvest.

4.3.3. Results of the multivariate analysis

Two PCAs were performed to summarize with a multivariate approach the performance of the six halophyte species under control (Control PCA) and salt stress treatments (Control-normalized PCA).

The first two PCs (eigenvalues are reported in Table S1 of the Supplementary Materials) were used for PCA interpretation, explaining 74.4% and 72.2% of the total variance in the respective Control PCA and Control-normalized PCA (Figure 3). The correlation coefficients were calculated between the PCs and each quantitative (the four physiological traits) and qualitative, i.e. categorical (the six halophytes species and the four saline treatment) variables (PCAs correlation circles in Figure S1 of the Supplementary Materials). The associated *p*-values were computed to rank the variables according to their relevance (Table S2 of the Supplementary Materials).

Under both control (Control PCA) and salt stress treatments (Control-normalize PCA), FW, GS and RWC were positively correlated with PC1, suggesting a high degree of multicollinearity among these parameters (red barycenters in Figure 3). WP, instead, resulted to be positively correlated with PC2 in both PCAs, and negatively correlated with PC1 under saline treatments (Table S2). WP, indeed, was located on the negative side of PC1 (Figure 3), at the opposite side of FW, GS, and RWC which are placed at positive values. This is in agreement with the fact that a reduction in WP (more negative values) should contrast FW, GS and RWC reductions caused by the NaCl related osmotic stress and ion toxicity.

The position of the barycenters of the six species in the two PCs changed drastically from control to saline treatments (Figure 3). In the Control PCA, the PC1 had negative loadings for *A. halimus* and *C. album*, and positive loadings for *A. vulgaris* and *S. minor* (green barycenters in Figure 3A). Conversely, in the Control-normalized PCA, the PC1 had negative loadings for *A. vulgaris* and *S. minor*, and positive loadings for *A. halimus* and *C. album* (green barycenters in Figure 3B). This suggests that the last two species were the best performing under salt stress condition (no substantial FW, GS and RWC drop) although they resulted to be the worst performing under control condition (lowest FW, GS, and RWC values). The barycenters of *S. komarovii* and *A. absinthium* in the PCA biplot did not vary substantially along PC1 due to saline treatments. *S. komarovii* was still located on the positive side of the PC1, as it showed high-intermediate ranking both under control (highest GS) and salinity condition (highest GS and WP). *A. absinthium*, as well, remained located on the negative side of the PC1, showing an intermediate performance under control and a low-intermediate performance under salinity (lowest GS and RWC). *A. absinthium* was likely located on the negative side of the PC1 because, despite the restrained FW drop, this species was the last ranked for RWC and GS under salinity (Figure 1).

Under salt treatments (Control-normalized PCA), hence, the HS barycenters (green square in Figure 3B) followed a linear trend, with the best performing species (*A. halimus* and *C. album*) located in the upper-right quadrant, in the same direction as RWC, FW, and GS (red dot in Figure 3B), opposite to the worst performing species placed in the bottom-left quadrant. The barycenters of the water saline treatments (WS) (blue triangles in Figure 3B), instead, followed an inverse gradient, crossing the HS linear trend, with the two lowest WS treatments (100 and 200 mM NaCl) in the right-bottom quadrant, and the two highest WS treatments (300 and 600 mM NaCl) in the upper-left quadrant, at the opposite side of the FW, RWC and GS quantitative variables. The best performing species (*A. halimus*, *C. album* and *S. komarovii*) and the two highest saline treatments (300 and 600 mM NaCl), hence, resulted to be located on the positive side of the PC2. As already mentioned, the PC2 was strongly correlated with the WP (Table S2), thereby suggesting that *A, halimus*, *C album* and *S. komarovii* respond to high salinity by lowering WP, i.e. by osmotic adjustment.

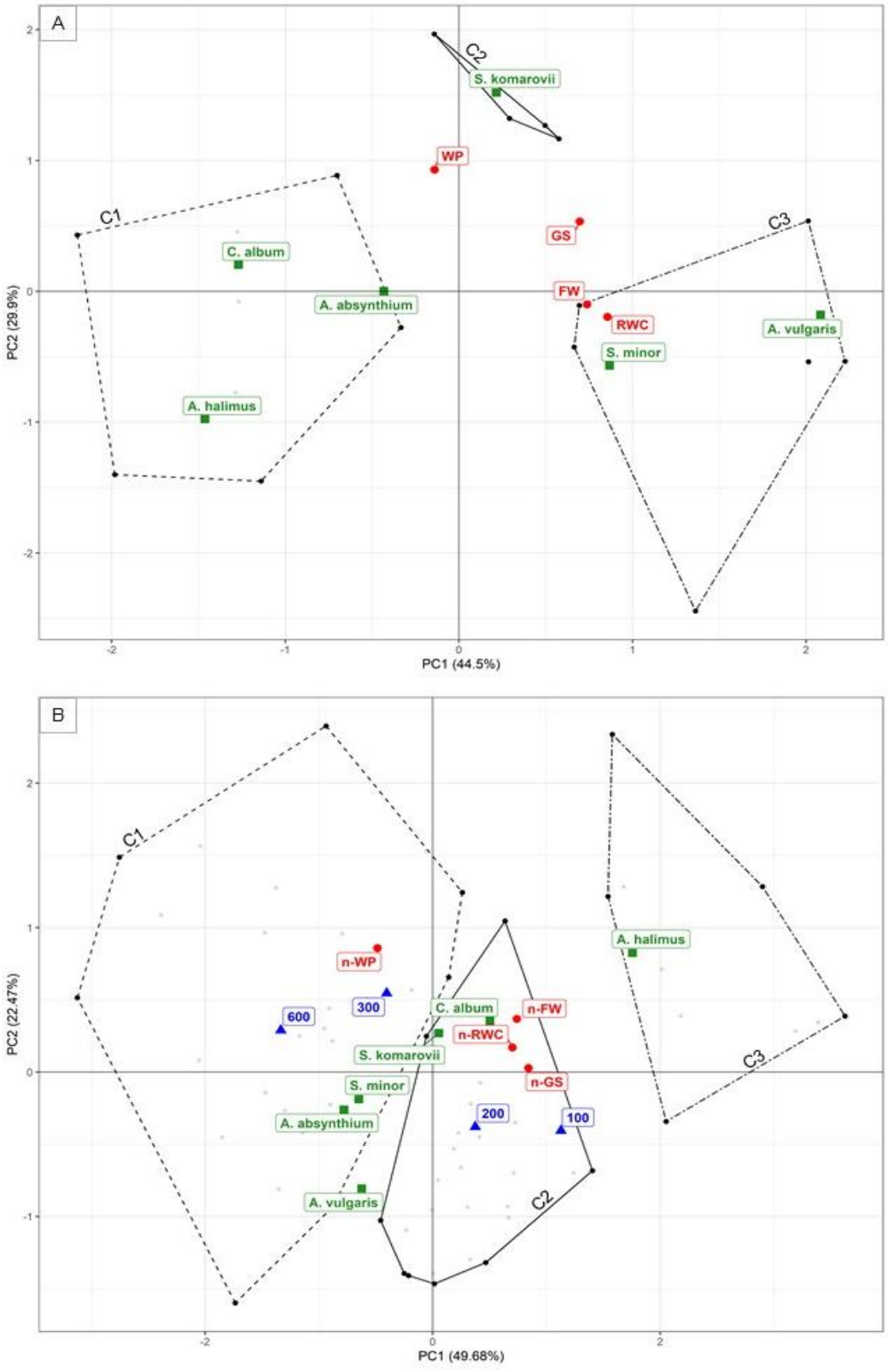

Figure 3. (A) Control and **(B)** Control-normalized PCA biplot of variables. Green points show the barycenters of the Halophytes Species (HS), blue point show the barycenters of the saline treatments (WS), red points show the quantitative variables, i.e. fresh weight (FW), stomatal conductance (GS), leaf relative water content (RWC), and leaf water potential (WP). The lowercase n indicates control-normalized data. The polygons show the three extracted clusters in the first two PCs space. Grey dots represent the individuals of the two categorical variables, i.e. WS and HS. C1, C2 and C3 = Cluster 1, 2, and 3.

4.3.4. Cluster Analysis

The hierarchical clustering (HC) extracted three clusters in the first two PCs space, both for control (Control HC) and salt stress treatments (Control-normalized HC) (Fig. 4A and 4B). Effect size (η^2) and χ^2 test values, expressing the link between the cluster variable and the respective quantitative and categorical variables, are reported in Table S2 and S3, respectively, of the Supplementary Materials.

In the Control HC, the first cluster (C1) was characterized by average RWC and GS values lower than the overall mean (80.25 vs 87.55% and 220 vs 308.67 mmol m^{-2} s^{-1}, respectively) (Table S5). None of the categorical variables significantly characterized this cluster, although 100% of *A. halimus* and *C. album* individuals were linked to it at p = 0.068 (+), (Table S6). The second cluster (C2), instead, was characterized by GS mean values higher than the overall mean (425 vs 308.67 mmol m^{-2} s^{-1}), in exchange for WP mean values lower than the overall mean (-1.39 MPa vs -2.71 MPa) (Table S7). This cluster was mainly represented by *S. komarovii* (100% of the individuals) (Table S8). The third cluster (C3), finally, was characterized by FW and RWC mean values higher than the overall mean (16.5 vs 8.86 g $plant^{-1}$ and 96.54 vs 87.55%, respectively) (Table S9). *A. vulgaris* was primarily present in this cluster (100% of the individuals) (Table S10).

Under salt treatments (Control-normalized HC), the WS categorical variables (i.e., water saline treatments) characterized the clusters better than HS categorical variables (i.e. halophytes species). The C1 mainly contained WP, FW, RWC and GS mean values lower than the overall mean (-170.05% vs -90.54%, -62.40% vs -27.01%, -16.22% vs -10.28%, and -50.86% vs -34.10%, respectively), (Table S4). This cluster, indeed, was described by the 300 mM and 600 mM NaCl WS levels (82.35% and 85.71% of the respective individuals), whose frequency in C1 was much higher than their frequency in the complete dataset (50% vs 26.15% and 42.86% vs 21.54%, respectively), (Table S6). C1, hence, may be indicated as the cluster describing the most severe effects associated with the highest salinity levels.

Cluster C2 was characterized by FW and WP mean values lower than the overall mean (-53.77% vs -27.02% and 17.27% vs 90.54%, respectively) (Table S7), and mainly grouped the two WS levels 100 mM and 200 mM NaCl (76% and 71% of the respective individuals), whose frequency in C2 was much higher than in the whole dataset (48.15% and 44.44% vs 26.15% global mean for the two respective categories) (Table S8). C2, hence, may be indicated as the cluster describing the intermediate effects connected to moderate salinity levels.

Cluster C3, finally, was characterized by FW, GS, and RWC mean values higher than the overall mean (144.25% vs -27.01%, -3.54 vs -34.10%, and 0.39% vs. -10.28%) (Table S9). This was the only cluster that was meaningfully described by a HS category, i.e. the *A. halimus* species (with 72.73% of the individuals), whose frequency in C3 was 80% against 16.92% in the whole dataset (Table S10). The other species did not reveal any clear pattern according to the three clusters. C3, hence, may be summarized as the cluster representing the most effective physiological reactions involved in salt tolerance.

4.4. Discussion

Halophytes are a small group of plants naturally adapted to survive salinity in areas where almost all other terrestrial species cannot grow. This work compares some physiological traits of six common halophytes in the Mediterranean area whose cultivation may promote the recovery and reuse of salt-degraded lands. Additionally, they may provide an alternative to, or can diversify, conventional crops [64], and reduce agricultural pressure on good quality land and water resources.

The six species showed different strategies to cope with salinity associated with their specific functional and life history traits. *Artemisia* spp. and *A. halimus* are for example perennial shrubs growing up to 1–2 m height, *C. album* is an annual herbaceous plant 1 to 1.5 m tall, *S. minor* is a perennial rhizomatous forb with erect stems 2 to 70 cm tall, while *S. komarovii* is an annual species growing up to 30 cm, with long needle-like succulent leaves. The study showed also species-specific salinity thresholds beyond which stress became evident, in different ways, making this dataset an important base of knowledge for the research in salt-tolerance in plants and for the selection of species for future crops.

A. vulgaris and *A. halimus* showed, respectively, the highest and lowest FW (∽14 and 3 g $plant^{-1}$, respectively) under control. *A. vulgaris* and *A. halimus* have different life history traits; indeed, *A. vulgaris* is a fast growing species, while *A. halimus* has been classified as a medium growth rate species [65]. This may

explain why the former produced a higher FW over the same period. However, while *A. vulgaris* FW dropped by 68% under water salinity, *A. halimus* FW increased up to 300 mM NaCl (+133%), although not significantly, in accordance with previous studies that showed no reduction in *A. halimus* biomass up to 600 mM NaCl [66,67]. However, other studies showed a significant decrease in *A. halimus* biomass with salinity above 200 mM NaCl [38,46]. Like *A. halimus*, *C. album* growth was not affected by salinity. This is in contrast with the results of Rasouli et al. [68], who found that FW dropped already at 100 mM NaCl in *C. album*; conversely, it is in agreement with the findings of Ivanova et al. [69], who did not record biomass variations up to 200 mM NaCl.

By contrast with the other four species, GS did not decrease in *A. halimus* and *C. album* up to 200 mM NaCl, but on the contrary increased at 100 mM NaCl (Figure 2), although the GS values of these two species were among the lowest under control treatment. In contrast to our results, Pérez-Romer et al. [40] did not observe any GS change in *A. halimus* up to 513 mM NaCl, whereas Rasouli et al. [68] observed a significant GS drop in *C. album* already at 100 mM NaCl.

Halophytes exposed to increasing salinity may activate a partial stomatal closure as a mechanism to limit both transpiration and transport of salts to the leaves [70]. A decline in GS, however, is usually associated with a reduced diffusion of CO_2 leading to a lower carboxylation efficiency. Rasouli et al. [68], however, observed that the CO_2 assimilation rate in salt-grown halophytes is largely unrelated to stomatal conductance since they were able to sustain FW notwithstanding the limited GS. This was attributed to a faster, thus more efficient, stomatal opening and closure regulation and to a faster RuBisCo carboxylation rate under salinity.

This mechanism may explain the steadiness in FW shown by *A. halimus* and *C. album* at increasing salinity, despite the GS drop above 200 mM NaCl.

The two *Artemisia* species reacted differently to salinity, as *A. absinthium* showed a stronger decline in GS (−51%) than that shown by *A. vulgaris* (−45%) (Figure 1), resulting to be the last ranked species for this parameter. Under salinity, indeed, GS value dropped below 200 mmol m^{-2} s^{-1} in *A. absinthium* (Figure 1), similarly to what observed by Aftab et al. [71] in *Artemisia annua* under 200 mM NaCl.

Another trait characterizing *A. halimus* and *C. album*, and shared also by *A. vulgaris,* was RWC steadiness under salinity (Figure 1). Similarly, Paulino et al. [72] and De Araújo et al. [73] observed, no RWC decrease up to 600 mM NaCl in *Atriplex nummularia* despite decreased transpiration rates. Ivanova et al. [69], as well, remarked the ability of *C. album* to maintain unaltered water status up to 350 mM NaCl water salinity, while Lu et al. [74] observed a reduction in its water content already at 300 mM NaCl. Likewise, *S. minor* showed a steadiness in RWC up to 300 mM NaCl, but then experienced a drastic RWC drop with the highest NaCl level. This is in contrast with the findings of Shariat et al. [75] who observed a *S. minor* RWC reduction at already 100 mM NaCl. *A. absinthium,* instead, reached a significant RWC drop already at 300 mM NaCl, contrary to the findings of Sharifivash et al. [76], who observed a first significant reduction in RWC at 150 mM NaCl in *A. absinthium.*

The ability to keep unaltered leaf RWC under salt stress suggests that *A. halimus*, *C. album* and *A. vulgaris* evolved adequate mechanisms to ensure sufficient water uptake in saline soils. According to Ben Amor et al. [77], salt tolerance of dicotyledonous halophytes is strongly related to their ability to accumulate high levels of osmolytes in their tissues and, thus, to adjust osmotically by lowering the osmotic potential in the cytoplasm. A decrease in WP, however, could also originate from tissue dehydration, although this does not seem to be the case for A. *halimus*, *C. album*, and *A. vulgaris* that did not change in RWC with saline treatments. The osmotic adjustment (OA) allows root water uptake and leaf turgor to be maintained under conditions of low soil water potential [78]. When turgor is maintained, all the turgor-dependent processes, i.e., stomatal conductance, assimilation rate and cellular wall expansion, can be maintained, albeit to a reduced rate due to the OA-associated metabolic costs [79]. This mechanism is fully supported by the results of the PCAs (Figure 3), that placed WP loadings in an opposite distinct position from the other physiological traits, supporting the hypothesis that the capability to sustain RWC, FW and GS depends on the magnitude of WP lowering.

Hence, although *A. halimus, C. album*, and *A. vulgaris* did not show a significant WP decrease with salinity, their similar and low WP values may have favoured root water uptake and in turn RWC maintenance (Figures 1 and 2*).*

S. komarovii showed the highest WP under control and this may be due to its succulent habit. Succulent plants, indeed, have larger vacuole where they store water and this likely prevents the development of low water potentials in their photosynthetic tissues [80]. Succulence usually tends to increase under saline conditions [66] in such a way that the increasing vacuole volume may serve to compartmentalize and dilute salts and help handle temporary imbalances due to NaCl introduction into the plant [64]. Despite this, however, *S. komarovii* showed a WP decrease under 300 mM NaCl and was not able to preserve its RWC over this salinity threshold.

Overall, the discrepancy sometimes observed in the response to salinity across the six species may be attributed to intraspecific variation in populations originating from different habitats, as well as to different growth conditions in the cited experiments. The latter circumstance supports the need for harmonization in the protocol of salinity experiments, as a premise for more consistent results.

4.5. Conclusions

Our results indicate that *A. halimus* and *C. album* are the best adapted species to salinity, followed by the group of *S. komarovii* and *A. vulgaris*, whereas *S. minor* and *A. absinthium* emerged as the least capable to adapt to increasing salinity levels.

A. halimus had low-intermediate FW, RWC and WP values under control treatment, and these traits remained almost stable under saline treatments. As a matter of fact, *A. halimus*, a C4 species, constituted a clearly distinctive cluster under salinity with respect to the other species, all at C3 photosynthetic pathway, and displayed the most consistent tolerance to salinity. *C. album* was also resilient (no FW, RWC and WP reduction), but less tolerant to salinity than *A. halimus*, as *C. album* data were split between the first and third cluster. These two species, however, appeared to be stimulated by weakly saline environment, as their FW and GS slightly increased. *S. komarovii* was characterized by the highest GS values both under control and the highest salinity level, although with a significant drop in FW, RWC and WP. *A. vulgaris* showed the highest FW under control, but its FW was halved under salinity; however, its RWC and WP were not affected by salinity. *S minor* showed the highest RWC under control and was able to preserve it up to 300 mM NaCl; however, it showed a severe FW, GS, and WP drop. Finally, *A. absinthium* had one of the highest FW both under control and salinity, but suffered the highest GS and RWC drop already at 100 mM NaCl.

The physiological parameters addressed in this study are essential to assess salt-tolerance and appreciate differences in salt-adaptation among species. However, our study is far from being exhaustive as it concerns the processes associated with salinity response in halophytes. Although incomplete, this study opens several research windows and perspectives concerning (a) the actual production and/or accumulation of organic and inorganic osmolytes, in order to evaluate their contribution to the plant's osmoregulation, (2) the effective changes in stomata shape and density with salinity, and the overall salinity effect on the photosynthetic capacity; and finally, (3) the cellular wall elasticity changes, which play an important role in plant ability to regulate its water relations.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Figure S1:** Correlation circle of active quantitative variables in (A) Control PCA and (B) Control-normalized PCA on the four physiological traits: fresh weight (FW), stomatal conductance (GS), leaf relative water content (RWC), and leaf water potential (WP); the lowercase n indicates control-normalised data. The amount of variation explained by each principal component (Dim) is indicated in brackets, **Table S1**. Eigen analysis of the correlation matrix in Control and Control-Normalized PCA, **Table S2**. Correlation coefficients between quantitative and categorical variables, and the first two PCs in Control PCA and Control-normalized PCA. FW = fresh weight, GS = stomatal conductance, RWC = leaf relative water content, and (WP) leaf water potential; the lowercase n indicates control-normalised data. The Control PCs were computed using 18 input data, while the Control-normalized PCs were computed using 65 input data, **Table S3**. η^2 values calculated for each quantitative variable, indicating the amount of explained between-clusters variance; only significant results are showed, **Table S4**. P values of the χ^2 tests performed between the categorical variables and the extracted clusters, **Table S5**. v-test results for Cluster I quantitative variables; only significant results are showed. A positive or negative test statistic indicates a Cluster mean significantly higher or lower, respectively, than the overall mean. Cluster and Overall mean and standard deviation are also reported. Variables are ordered by value of v-test. **Table S6.** v-test results for Cluster I categorical variables.; only significant results are showed. Within-cluster (Mod/Cla) and across-cluster (Cla/Mod) distributions, and overall cluster mean of categorical variables are also reported. Variables are ordered by value of v-test, **Table S7**. v-test results for Cluster

II quantitative variables; only significant results are showed. A positive or negative test statistic indicates a Cluster mean significantly higher or lower, respectively, than the overall mean. Cluster and Overall mean and standard deviation are also reported. Variables are ordered by value of v-test, **Table S8.** v-test results for Cluster II categorical variables; only significant results are showed. Within-cluster (Mod/Cla) and across-cluster (Cla/Mod) distributions, and overall cluster mean of categorical variables are also reported. Variables are ordered by value of v-test, **Table S9.** v-test results for Cluster III quantitative variables; only significant results are showed. A positive or negative test statistic indicates a Cluster mean significantly higher or lower, respectively, than the overall mean. Cluster and Overall mean and standard deviation are also reported. Variables are ordered by value of v-test, **Table S10.** v-test results for Cluster III categorical variables; only significant results are showed. Within-cluster (Mod/Cla), and across-cluster (Cla/Mod) distributions, and overall cluster mean of categorical variables are also reported. Variables are ordered by value of v-test.

Author Contributions: Conceptualization, Roberta Calone, Rabab Sanoubar and Lorenzo Barbanti; Data curation, Roberta Calone, Simone Bregaglio and Lorenzo Barbanti; Formal analysis, Simone Bregaglio; Investigation, Roberta Calone and Rabab Sanoubar; Methodology, Roberta Calone and Rabab Sanoubar; Resources, Enrico Noli; Software, Simone Bregaglio; Supervision, Lorenzo Barbanti; Validation, Lorenzo Barbanti; Visualization, Roberta Calone, Simone Bregaglio and Carla Lambertini; Writing – original draft, Roberta Calone; Writing – review & editing, Roberta Calone, Enrico Noli, Carla Lambertini and Lorenzo Barbanti.

Funding: The work has been partially funded by the European LIFE 2014–2020 Programme, Project LIFE AGROWETLANDS II—Smart Water and Soil Salinity Management in Agro-Wetlands (LIFE15 ENV/IT/000423).

Institutional Review Board Statement: Not applicable as the study did not involve humans or animals.

Informed Consent Statement: Not applicable as the study did not involve humans.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest

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Chapter 5

Article published *in Agronomy* 2021, 11, 1967

The C4 *Atriplex halimus* vs. the C3 *Atriplex hortensis*: Similarities and Differences in the Salinity Stress Response

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Received: 2 August 2021; Accepted: 22 September 2021; Published: 29 September 2021

Abstract: Soil properties and the ability to sustain agricultural production are seriously impaired by salinity. The cultivation of halophytes is seen as a solution to cope with the problem. In this framework, a greenhouse pot experiment was set up to assess salinity response in the perennial C4 species *Atriplex halimus*, and in the following three cultivars of the annual C3 *Atriplex hortensis*: green, red, and scarlet. The four genotypes were grown for 35 days with water salinity (WS) ranging from 0 to 360 mM NaCl. Plant height and fresh weight (FW) increased at 360 vs. 0 WS. The stomatal conductance (GS) and transpiration rate (E) were more severely affected by salinity in the C4 *A. halimus* than in the C3 species *A. hortensis*. This was reflected in a lower leaf water potential indicating stronger osmotic adjustment, and a higher relative water content associated with more turgid leaves, in *A. halimus* than *A. hortensis*. In a PCA including all the studied traits, the GS and E negatively correlated to the FW, which, in turn, positively correlated with Na concentration and intrinsic water use efficiency (iWUE), indicating that reduced gas exchange associated with Na accumulation contributed to sustain iWUE under salinity. Finally, FTIR spectroscopy showed a reduced amount of pectin, lignin, and cellulose under salinity, indicating a weakened cell wall structure. Overall, both species were remarkably adapted to salinity: From an agronomic perspective, the opposite strategies of longer vs. faster soil coverage, involved by the perennial *A. halimus* vs. the annual *A. hortensis* cv. scarlet, are viable natural remedies for revegetating marginal saline soils and increasing soil organic carbon.

Keywords: halophytes; gas exchanges; chlorophyll fluorescence; FTIR spectroscopy; element content; C:N ratio; electrolyte leakage

5.1. Introduction

The global population is growing at a rate of 1.1% per year and it is with 95% certainty that by 2050 it will reach between 9.4 and 10.1 billion people [1]. Recent projections, which use 2014 as a baseline, estimate that crop production should increase by 25–70% to meet food demand in 2050 [2]. Agricultural topsoil and soil organic carbon (SOC) are key ingredients for intensive food production [3–5]. SOC, indeed, plays a crucial role in the maintenance of soil health and productivity, due to its significant contribution to the physical, chemical, and biological properties of soil [6]. However, besides enhancing crop yield, SOC can act either as a source or a sink of atmospheric CO_2, and thereby, can influence the global process of climate change [7].

Soil holds about 80% (2500 GT) of the terrestrial carbon stock. Of this, nearly 1550 GT are in the form of SOC, and the remaining in the form of soil inorganic carbon (SIC), that mainly consists of elemental carbon and carbonate rocks such as calcite, dolomite, and gypsum. The soil carbon reserve is around three times that

currently found in the atmosphere (800 GT) and four times that fixed in living plants and animals (560 GT). Only oceans have a larger carbon pool (about 38,400 GT), mostly in the inorganic form [8].

Over the last 10,000 years, however, the conversion of semi-natural or natural ecosystems into human-managed agro-ecosystems has caused a 50–75% depletion of their carbon stock, with around 135 GT C released into the atmosphere [9,10].

Nevertheless, a recent study estimated that the soil carbon capacity for C sequestration can be expanded up to 1.45–3.44 GT C per year (around 5.3–12.6 GT CO_2) [11]. Due to this large carbon storage capacity, increasing atmospheric CO_2 sequestration into long-life soil carbon is considered one of the most cost-effective solutions to combat climatic changes, contrast land degradation, and ensure food security [12].

Soil organic carbon content is a function of C input and of its turnover. The main sources of SOC include crop residues, dead roots, and livestock manure; the decomposition of this organic matter (SOM) is mainly driven by soil microorganisms such as bacteria and fungi. The processes of carbon gain and storage, however, are affected by numerous factors including soil texture and pH, irrigation and management practices, and several environmental factors such as high temperature, drought, salinity, etc. [13]. Consequently, the implementation of soil carbon sequestration strategies requires tailored options accounting for site-specific trade-offs and management opportunities.

As secondary soil salinisation is expanding worldwide with a rate of 2 Mha per year [14], there is a growing interest in exploring the potential of saline lands for carbon sequestration and storage. Indeed, the revegetation of saline areas can sequester substantial amounts of carbon [15], besides providing important ecosystemic benefits as wildlife habitats, biodiversity pools, and land regreening for grazing [16].

In saline soils, generally, the SOC content is lower than in non-saline soil, mainly due to sparser plant cover and lower microbial activity [17], which implies a reduced input of organic matter and slower decomposition rates [18].

On the other hand, SIC in the form of carbonate salts is higher in sodic and saline-sodic soils, while soil C loss due to microbial respiration and leaching may be much lower than in low salinity environments [19].

Hence, revegetating these areas with salt-tolerant crops can increase organic matter deposition and enhance SIC dissolution through their root respiration [20,21], and can aid in the maintenance of soil structure and SOC accumulation [22,23]. Additionally, growing halophytes as food and fodder crops could, at the same time, indirectly contribute to the atmospheric carbon mitigation by reducing deforestation to create new cropland [24].

The choice of halophyte species for saline area cultivation will depend on their salt tolerance level, agronomic value, ease of cultivation, water and nutrient requirement, and biomass potential.

In a previous study [25], the perennial C4 shrub *Atriplex halimus* resulted as the most salt tolerant species among six wild halophytes common in the Mediterranean area.

The wide range of salt stress responses described in *Atriplex* spp., indeed, makes this genus an attractive taxon for saline land reclamation and SOC accumulation in highly disturbed areas, as also proposed in other studies [26–30].

In this study, we compare the performance of the C4 *Atriplex halimus* L. with that of a closely related species, the C3 annual *Atriplex hortensis* L. Plenty of studies have investigated the two species taken separately, but little has been done to compare their mechanisms of salt tolerance and understand which conditions are more suitable for one species or for the other.

Atriplex halimus is a xerohalophytic shrub common in the Mediterranean basin. This species has been investigated for its ability to ameliorate soil properties and enhance carbon sequestration [30–34], restore highly calcareous sodic soil [35], improve degraded rangeland [36–38], remove heavy metals and salts from contaminated soil [34,39–44], and sustain the growth of consociated salt sensitive crops [45,46]. *Atriplex halimus* leaves were also traditionally used as a food dressing for their salty flavour [35]. However, their consumption mainly occurred at times when other sources of food were unavailable [32].

Atriplex hortensis, also known as orach or mountain spinach, is a species adapted to brackish marshes in temperate environments. It is a sodium-accumulating halophyte [47,48] studied for saline soil phytoremediation [42,49] and, above all, as a leafy vegetable for the human diet thanks to its high nutritional value and medicinal properties [50–53].

The goal of the present study is to compare these two used halophytes, in order to evaluate their biomass production, carbon content, and Na accumulation capacity, and determine how physiological traits are shaped by salt tolerance in two species that are closely related but very different in their life cycle and photosynthetic pathway. We selected a red, a scarlet and a green cultivar of *A. hortensis* and a common *A. halimus* species for our experiment.

5.2. Materials and Methods

5.2.1. Acronyms

The acronyms used in this study are defined as follows: fresh weight (FW), dry weight (DW), plant height (PH), electrolyte leakage (EL), specific leaf area (SLA), carbon isotope ratio ($\delta^{13}C$), net photosynthesis (A), leaf transpiration (E), stomatal conductance (GS), spad value (SPAD), effective quantum yield efficiency of PSII (ΦPSII), level of photochemical quenching of PSII (qP), PSII maximum efficiency (Fv'/Fm'), electron transport rate (ETR), leaf relative water content (RWC), leaf water potential (LWP), and intrinsic water use efficiency (iWUE).

5.2.2. Plant Material and Growth Conditions

The experiment was carried out at the Department of Agricultural and Food Sciences (DISTAL), University of Bologna, Italy. Commercial seeds of *Atriplex halimus*, *A. hortensis* cv. red, *A. hortensis* cv. Scarlet, and *A. hortensis* cv. green were purchased online from The Original Garden (Valladolid, Spain) and Seedaholic (Galway, Ireland) online shops.

Seeds were surface-sterilised by immersion in a 3% sodium hypochlorite solution for 2 min, rinsed in deionised water for 5 min, and dried at room temperature. Prior to sowing, *A. halimus* seeds were soaked for 12 h in deionised water and, thereafter, scarified by manual bract removal in order to interrupt dormancy. Due to their slower germination, seeds of *A. halimus* were sown 30 days before *A. hortensis* spp. seeds.

Seeds were sown manually in plastic seedling trays and placed into a growth chamber with a 16/8-hour light/dark cycle, day/night temperatures of 27/22 °C and 70–80% relative humidity. The trays were dampened with distilled water once per day.

After germination, plantlets of similar size (4 leaves stage) were transplanted into plastic pots of 3-litre volume (1 plant/pot) filled with a mix of river sand, peat moss (26% organic carbon, pH_{H2O} = 5.8, and salt content = 1.6 g L^{-1}), and perlite (7:2:1 *v*/*v*).

Pots were transferred to a greenhouse, placed over benches, and irrigated automatically three times per week with 200 mL of fresh water up to the beginning of the salt treatment, to ensure adequate substrate moisture. Ammonium nitrate (N, 26%) was added at 0.1 g pot^{-1} prior to seedling transplant; a second dose was supplied mid-experiment by placing the granular fertiliser directly on substrate surface prior to watering.

In the greenhouse, temperatures ranged between 22.2 ± 1.1 and 27.7 ± 16 °C, and RH between 42 ± 4% and 82 ± 3.7%. Photosynthetically active radiation (PAR) of 200 mol m^{-2} s^{-1} was provided by high pressure sodium lamps with a 16-hour light and 8-hour dark cycle.

5.2.3. Treatments and Experimental Design

In all plants, excluding those grown at 0 mM NaCl, salt treatments were started 20 days after transplanting, on the 20 July, when plants had six to eight leaves. Salt stress was induced by incremental increases of 90 mM in the irrigation water every 3 days, until the final concentrations of 90, 180, and 360 mM NaCl were reached on the 27 July.

The amount of salt added to water (TSS, g_{NaCl} L^{-1} H_2O) to reach the four salinity levels (EC_w, dS m^{-1}) was calculated according to Equation (1) as follows:

$$TSS = EC_w * 0.640 \quad (1)$$

A total of 200 ml of water solution was automatically distributed to each pot three time per week until the end of the experiment.

The 4 halophyte genotypes (HG) × 4 water salinity levels (WS), totalling 16 combinations, were set up with 8 randomised replicates, totalling 128 pots. Four plants per treatment were used for non-destructive measurements and final harvest, while other four plants were used for destructive measurements (SLA, EL, WP, RWC), as below described.

The four genotypes were abbreviated AH (*A. halimus*), AR (*A. hortensis* red), AS (*A. hortensis* scarlet), and AG (*A. hortensis* green). The four water salinity treatments were named Ctrl, WS90, WS180, and 360 WS.

5.2.4. Growth and Yield Assessment

On the 31 August, 35 days after salt stress initiation (DAS), four plants per each treatment were randomly selected and harvested. Shoots were separated from roots and weighed to determine the plant fresh weight (FW, g plant^{-1}) and the plant height (PH, cm). Shoot samples were oven-dried at 60 °C and weighed to determine the dry weight (DW, g plant^{-1}). Specific leaf area (SLA, cm^2 g^{-1}) was measured one day before harvest on four plants per treatment by dividing the leaf area of three randomly selected leaves by their dry weight.

5.2.5. Plant Water Relations

Water potential (Ψw) was measured on a leaf disk from the uppermost fully expanded leaf, using the WP4-C dewpoint potentiometer (METER Group, Pullman, WA, USA). Leaf cuts were stored in sealed plastic cups and measurements were started within a short time of sampling [54]. Concurrently, leaf relative water content (RWC, %) was determined on the same leaf: a small disc of 2 cm diameter was cut from the leaf and weighed to determine fresh weight (FW). Then, it was put in a 15-millilitre vial with distilled water in the dark and, after 24 h, the turgid weight (TW) was measured. The sample was finally oven-dried at 105 °C for 24 h to assess the dry weight (DW). The RWC (%) was calculated according to Equation (2) as follows [55]:

$$\mathrm{RWC} = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100 \quad (2)$$

All the plant water relation measurements were performed at 7 and 27 days after salt stress initiation (DAS).

5.2.6. Electrolyte Leakage

To determine cell membrane permeability, a leaf sample of 2 cm^2 was cut from the upper fully expanded leaf, rinsed 3 times with demineralised water and immersed into distilled water in 10-millilitre flasks. Electrical conductivity (EC) was measured after 2 h of floating at room temperature using a conductivity meter.

Total conductivity was obtained by repeating the procedure after keeping the flasks in an oven (90 °C) for 2 h. Results were expressed as a percentage of total conductivity. The measurement was executed one day before harvest on four plants per treatment.

5.2.7. Leaf Gas Exchange and Chlorophyll - Fluorescence

Leaf transpiration (E, in mmol m^{-2} s^{-1}), stomatal conductance (GS, in mmol m^{-2} s^{-1}), net photosynthesis (A, in μmol m^{-2} s^{-1}), maximum (Fm′) and minimum (Fo′) fluorescence with light-adapted leaf, and steady state fluorescence (Fs) were measured using a portable photosynthesis system (Li-Cor 6400, LI-COR Biosciences, Lincoln, NE, USA), after setting a CO_2 concentration similar to the external environment (400 ppm), and a LED light source (90% red and 10% blue) similar to the natural irradiance occurring inside the greenhouse (200 μmol m^{-2} s^{-1}). The chamber block temperature was 28 ± 2 °C and the leaf temperature inside the sensor head was 28 ± 3 °C. The chamber oxygen concentration was equal to the external environment.

The effective quantum yield efficiency of PSII (ΦPSII), which represents the capacity for photon energy absorbed by photosystem II (PSII) to be utilised in photochemistry under light-adapted conditions, was calculated as (Fm′—Fs)/Fm′. The ΦPSII was broken down in its two components, as follows: the level of photochemical quenching of PSII (qP) and the PSII maximum efficiency (Fv′/Fm′). The qP, which is related to the actual fraction of photochemically active PSII reaction centres, was calculated as (Fm′—Fs)/(Fm′—Fo′). The

Fv′/Fm′ was calculated as (Fm′—Fo′)/Fm′ and describes the maximum operating efficiency in the light adapted state, with any decrease in this parameter reflecting an increase in non-photochemical quenching.

The intrinsic water use efficiency (iWUE, μmol CO_2 $mmol^{-1}$ H_2O) was determined as the molar ratio between photosynthetic assimilation of CO_2 (A) and water loss by transpiration (E). All gas exchange and fluorescence parameters were measured at 7 and 27 days after salt stress initiation (DAS) on the youngest fully expanded leaf of four plants per treatment.

5.2.8. Mineral Elements

Dry samples of plant shoots were ground, and the concentration of the main elements (Na, K, Ca, Mg and P) was quantified using Inductively Coupled Plasma Spectrometry (ICP-OES) (Spectro Arcos, Ametek, Kleve, Germany). Three analyses per treatment were performed.

5.2.9. Total Carbon and Nitrogen Content and Carbon Isotope Ratio

The C and N concentration of the dry and ground shoot samples was determined using the Flash 2000 elemental analyser (Thermo Fisher Scientific, Waltham, MA, USA).

The stable carbon isotope ratio was measured on the same sample using Continuous Flow-Isotope Ratio Mass Spectrometry (CF-IRMS), by introducing the combustion gases (CO_2) from the elemental analyser into the Isotope Ratio Mass Spectrometer (IRMS, Delta V Advantage, Thermo Fisher Scientific).

The C isotope ratio was expressed as δ ^{13}C values (‰) according to following formula:

$$\delta\,^{13}\mathrm{C}\ ‰ = \left[\frac{\text{R sample}}{\text{R standard}} - 1\right] \times 1000 \quad (3)$$

where R sample and R standard are the $^{13}C/^{12}C$ ratios of sample and of the international standard (Vienna Pee Dee Belemnite standard, VPDB), respectively. Three analyses per treatment were performed.

5.2.10. Spectroscopic Characterisation

FT-IR spectra of samples were recorded by using a Bruker Tensor FT-IR instrument (Bruker Optics, Ettlingen, Germany) provided with an accessory for analysis in total reflectance attenuated (ATR), single reflection and a 45° angle of incidence (Specac Quest ATR, Specac Ltd., Orpington, Bromley, UK).

The spectra were acquired from 4000 to 400 cm^{-1}, with a spectral resolution of 4 cm^{-1} and 64 scans. Offset normalisation was performed to adjust the baseline and move the spectra intensities so that the minimum absorbance value was 0. A background against air before each measurement was performed. Spectra were processed using the Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH, USA).

5.2.11. Statistical Analysis

The data of the measured traits were submitted to two-way ANOVA for the two factors HG and WS, and the HG × WS interaction. The Tukey's honest significant difference (HSD) post hoc test at $p < 0.05$ was used to indicate significant differences among ANOVA sources. The ANOVA results are reported in Tables S1–S3 of the supplementary materials.

To better highlight the key effects of the experiment, only the data from the two corner salinity treatments (Ctrl and 360 WS) are reported in the Results; the intermediate salinity levels (90 and 180 WS) exhibited an intermediate behaviour.

We investigated the relationships among traits measured at 27 DAS by computing the pairwise Spearman's rank correlation coefficients (ϱ), and then testing their significance with $\alpha = 0.05$ [56].

A principal component analysis (PCA) was carried out on the physiological data collected at harvest to summarise with a multivariate approach the performances of the four genotypes under Ctrl and 360 WS treatment.

The principal components (PCs) were obtained from centred and scaled quantitative variables, through diagonalisation of the correlation matrix and extraction of the associated eigenvectors and eigenvalues. All the

measured traits were set as active quantitative variables, while the four halophyte genotypes (AH, AG, AR, AS) and the two treatments (Ctrl and 360 WS) were used as supplementary categorical variables, i.e., variables that were not used in the computation of PCs.

All the statistical analyses were performed with the R 6.3.6 statistical software, using Car [57] and Emmeans [58] packages for the analysis of variance and post hoc test, and the FactoMineR package for principal component analysis [59]. Charts were created with the ggplot2 [60] and corrplot [61] R packages.

5.3. Results

5.3.1. Plant Growth

Under the Ctrl conditions, *Atriplex hortensis* cv. green and scarlet exhibited the highest fresh weight (FW), while *A. hortensis* red showed the lowest FW. All the four genotypes, however, recorded a significant biomass increase under 360 WS, with the greatest increment in *A. hortensis* red and scarlet (+38 and +33%, respectively) (Figure 1A). Despite the increased FW, the plant DW did not show a significant HG × WS interaction (Figure 1B), although the resulting effects of the two single factors were significant (Table S4 of the Supplementary Materials).

Under the Ctrl condition, the three *A. hortensis* cultivars had a similar and statistically higher plant height (PH) than *A. halimus* (Figure 1C). The impact of salinity on PH was almost negligible in all the halophytes except *A. hortensis* cv. red, which showed a significant PH decrease (−31%). Likewise, the three *A. hortensis* cultivars showed a statistically higher specific leaf area (SLA) than *A. halimus* under the control condition (Figure 1E). Under salinity, however, *A halimus* did not undergo a significant SLA decrease, while *A. hortensis* green and scarlet showed higher, significant reductions (−35 and −20%, respectively).

Salinity induced a significant increase in leaf electrolyte leakage (EL) in all of the four halophytes (Figure 1D), with the highest increment in the two green-leaved genotypes, *A. hortensis* green (+131%) and *A. halimus* (+105%).

The three C3 *A. hortensis* genotypes showed a similar (−33‰ on average) and statistically lower (more negative) $\delta^{13}C$ compared to the C4 *A. halimus* genotype (Figure 1F). The 360 WS induced a $\delta^{13}C$ shift towards less negative values in the three *A. hortensis* genotypes with no effects, instead, in *A. halimus*.

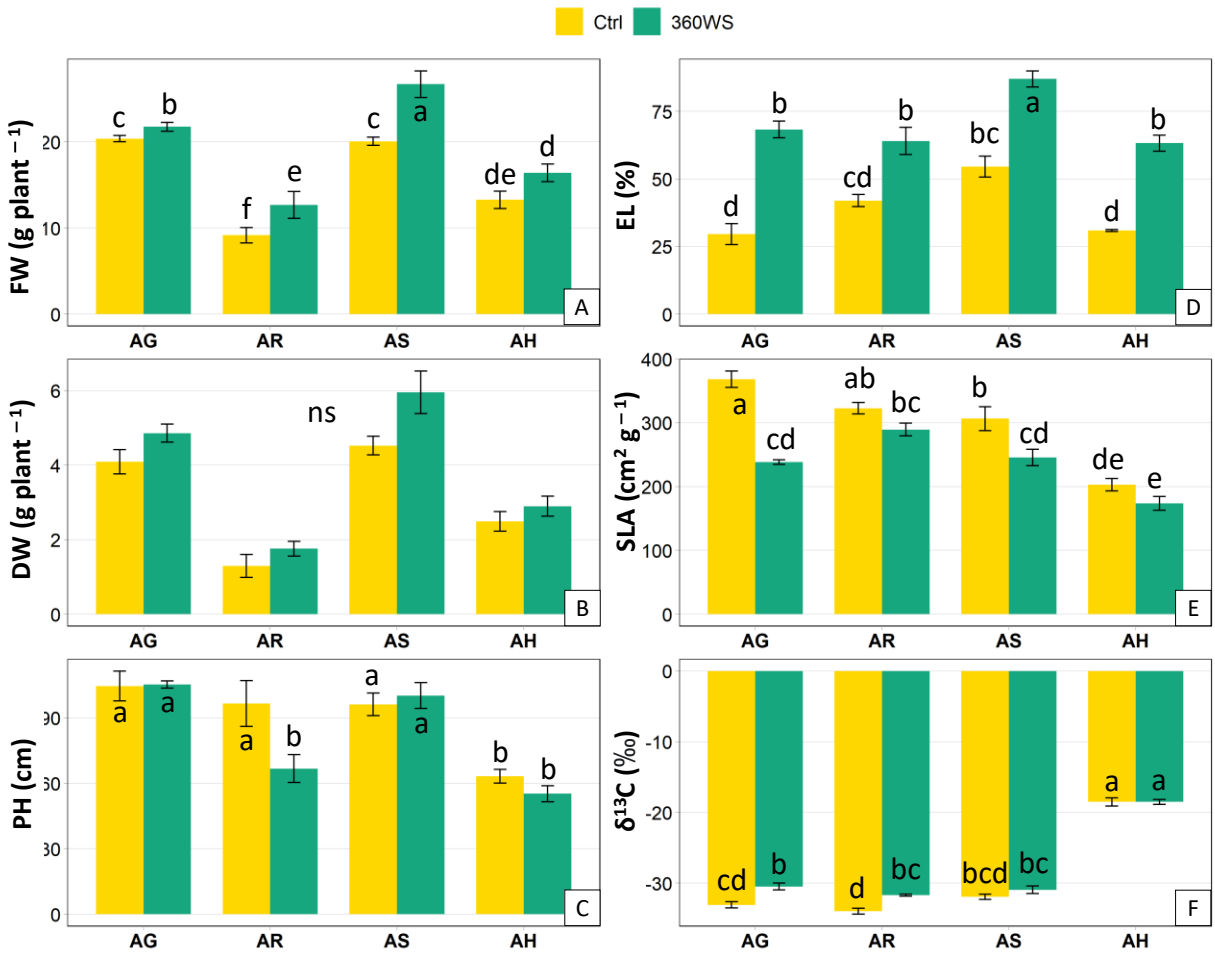


Figure 1. (**A**) Fresh weight, (**B**) dry weight, (**C**) plant height, (**D**) electrolyte leakage, (**E**) specific leaf area, and (**F**) ^{13}C isotope ratio under control (Ctrl) and 360 mM water salinity (360 WS) in the four genotypes *A. hortensis* cv. green (AG), *A. hortensis* cv. red (AR), *A. hortensis* cv. scarlet (AS), and *A. halimus* (AH). Vertical bars indicate standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$.

5.3.2. Photosynthesis and Leaf Gas Exchange

Seven days after the salt stress initiation (7 DAS), the two genotypes showing the highest photosynthetic activity (A) under Ctrl, i.e., *A. hortensis* scarlet and green, were those recording the highest A drop under salinity (-32 and -29%, respectively; Figure 2A), while the other two genotypes had milder, non-significant A drops (Figure 2A).

The stomatal conductance (GS), on the contrary, decreased significantly in all of the four genotypes, with the sharpest drop in *A. hortensis* green, which was the genotype showing the highest GS under the Ctrl condition (Figure 2B). The plant transpiration (E) followed a similar pattern as the GS (Figure 2C,G). Salinity did not affect the chlorophyll content (SPAD value), whereas remarkable differences occurred among genotypes under Ctrl, with the highest SPAD values in *A. hortensis* scarlet (39.5), followed by *A. halimus* (28.9) (Figure 2D,H).

Twenty days later, at 27 DAS, the four genotypes did not show significant differences in A values between Ctrl and HWS (Figure 2E) with, however, a considerable drop in A values in *A. hortensis* scarlet compared to 7 DAS, especially under 360 WS. Under Ctrl, the stomatal conductance (GS) decreased in all three *A. hortens*is genotypes, while it remained almost unchanged in *A. halimus* (Figure 2F). As already observed at 7 DAS, the salinity significantly decreased the GS, with the greatest decrease in the two green-leaved genotypes, *A. halimus* (−60%) and *A. hortensis green* (−55%). Similarly, at 27 DAS, E dropped under Ctrl in all the three *A. hortensis* genotypes and increased in *A. halimus* (Figure 2G). The effects of salinity on E were similar to that

observed in the GS (Figure 2F). The SPAD value was not affected by salinity at 27 DAS (Figure 2H), as it was not affected at 7 DAS.

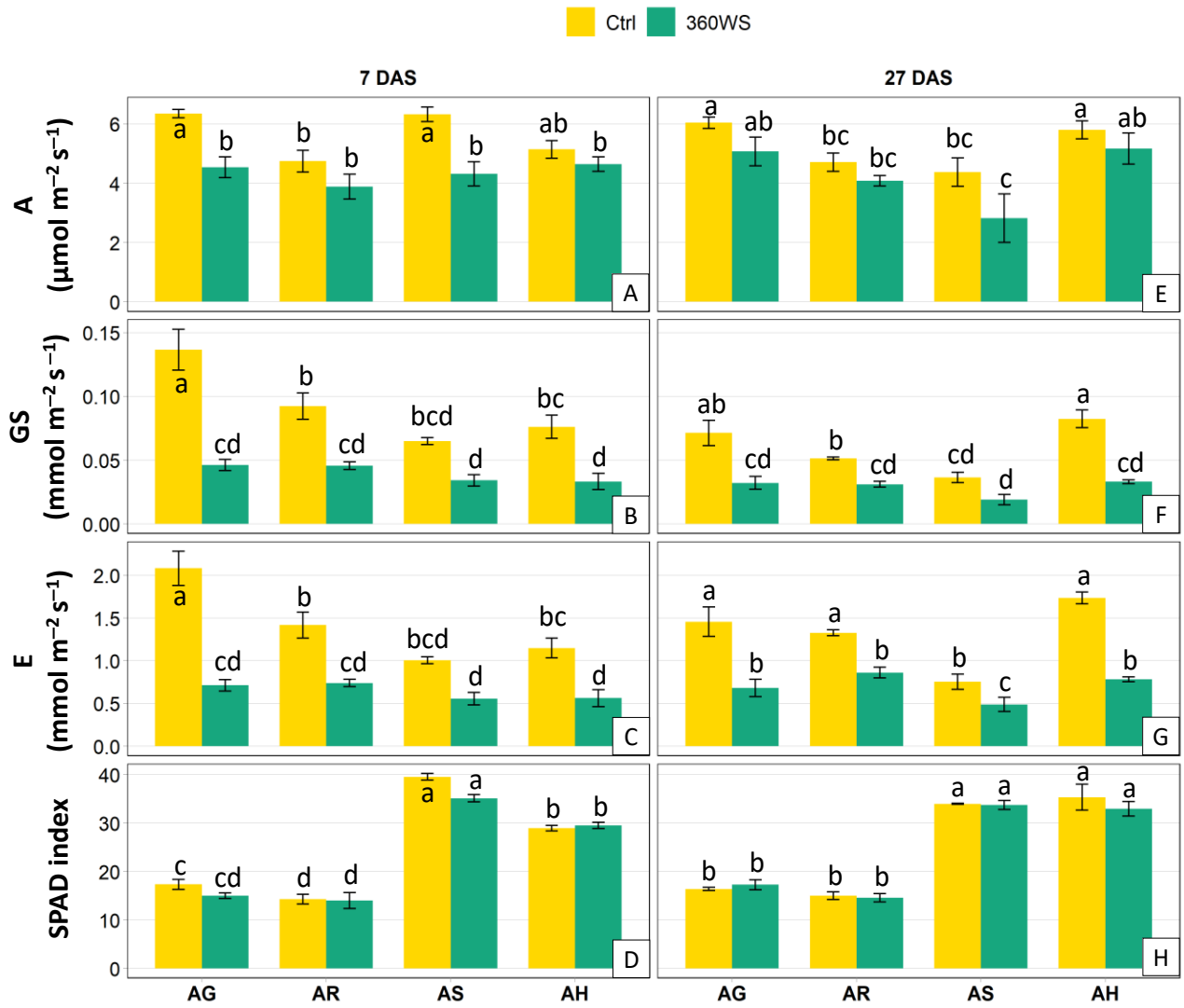


Figure 2. Photosynthetic activity (**A**), stomatal gas exchange (GS), transpiration rate (**E**) and, chlorophyll content (SPAD value) at 7 days after salt stress initiation (DAS) (**A**, **B**, **C**, **D**, respectively) and at 27 DAS (**E**, **F**, **G**, **H**, respectively) under control (Ctrl) and 360 mM water salinity (360 WS) in the four genotypes *A. hortensis* cv. green (AG), *A. hortensis* cv. red (AR), *A. hortensis* cv. scarlet (AS), and *A. halimus* (AH). Vertical bars indicate standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$.

5.3.3. Chlorophyll Fluorescence

At 7 DAS, the operating efficiency of PSII photochemistry (ΦPSII) and its qP component, i.e., the level of photochemical quenching of PSII, did not yet show a significant HG × WS interaction, although the single effect of HG was. The same was observed for the electron transport rate (ETR) (Table S5 of the Supplementary Materials). The only fluorescence parameter recording a significant interaction between the two experimental factors was Fv'/Fm', which is the second component of the ΦPSII expressing the maximum efficiency of PSII photochemistry in the light (Fv'/Fm') (Figure 3B). Under the Ctrl condition, Fv'/Fm' reached the highest value in *A. hortensis* green and scarlet. These latter, however, were the only two genotypes showing an Fv'/Fm' decrease with 360 WS.

The single WS factor, however, did not determine any significant changes in any of the four described fluorescence parameters.

At 27 DAS, instead, all the four fluorescence parameters presented a significant HG × WS interaction (Table S6 of the Supplementary Materials). In contrast to the other three genotypes, *Atriplex hortensis* scarlet was the sole genotype increasing the ΦPSII, Fv'/Fm', and ETR under 360 WS (Figure 3E–G), while *A. halimus* decreased in Fv'/Fm'. In the other genotypes, 360 WS did not induce significant fluorescence changes compared to the Ctrl.

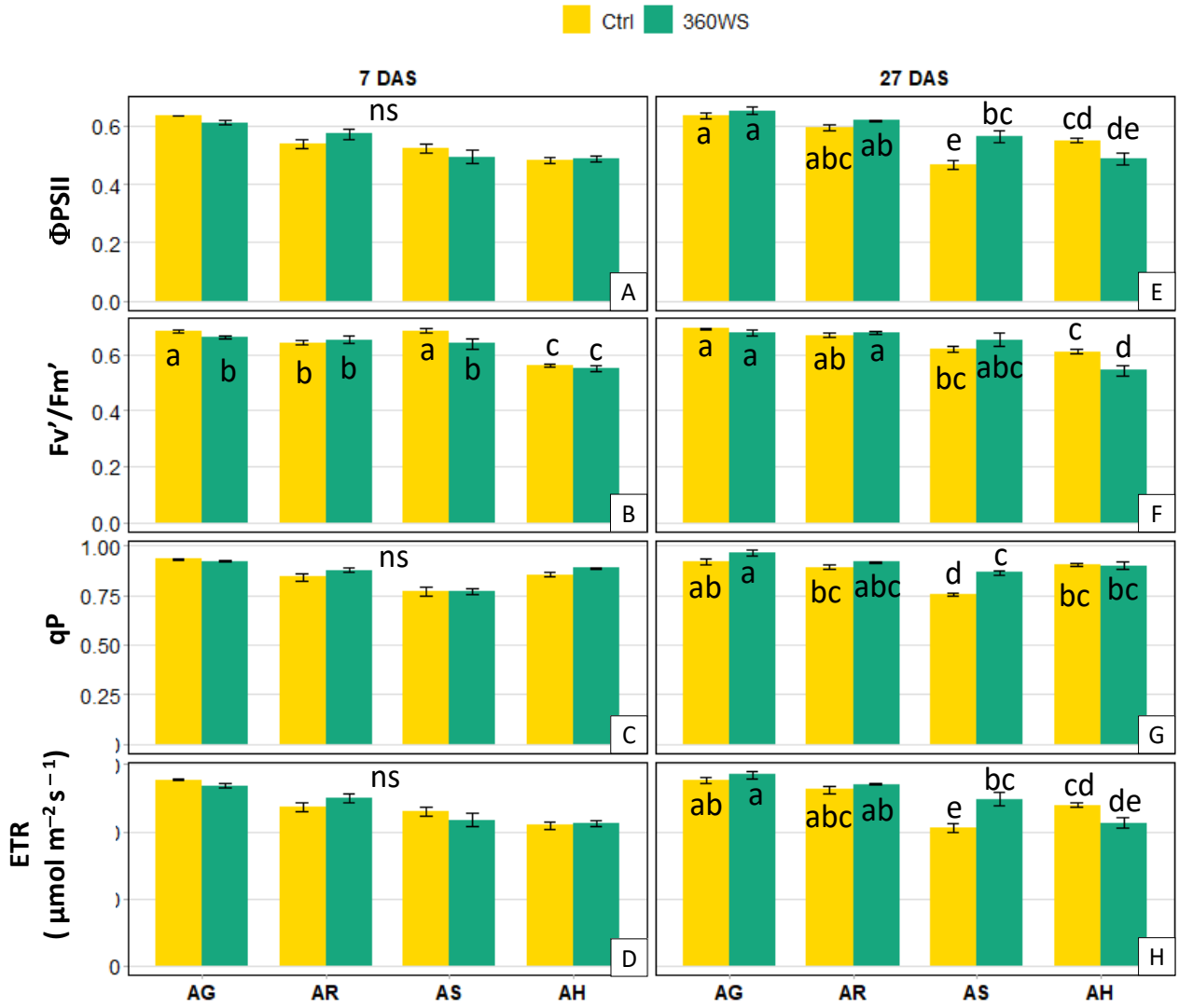


Figure 3. Operating efficiency of PSII photochemistry (ΦPSII), maximum efficiency of PSII photochemistry in the light (Fv'/Fm'), level of photochemical quenching of PSII (qP), and electron transport rare (ETR) at 7 days after salt stress initiation (**A**, **B**, **C**, **D**, respectively) and at 27 days after salt stress initiation (**E**, **F**, **G**, **H**, respectively) under control (Ctrl) and 360 mM water salinity (360 WS) in the four genotypes *A. hortensis* cv. green (AG), *A. hortensis* cv. red (AR), *A. hortensis* cv. scarlet (AS), and *A. halimus* (AH). Vertical bars indicate standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$.

5.3.4. Water Relations

At 7 DAS, the plant relative water content (RWC) and intrinsic water use efficiency (iWUE) did not show any significant HG × WS interaction, although the resulting effects of the two single factors were significant (Table S5 of the Supplementary Materials). The leaf water potential (WP) outlined narrow, yet significant, differences under Ctrl with the lowest value in *A. halimus* (Figure 4B). The 360 WS determined a WP decrease in all of the four genotypes, with the greatest reduction in *A. hortensis* scarlet and *A. halimus* (−154 and −107%, respectively).

Twenty days later, at 27 DAS, the four genotypes had similar RWCs under Ctrl, but the 360 WS determined a RWC decrease in the two red-leaved halophytes (*A. hortensis* scarlet and *A. halimus*) (Figure 4D).

The WP exhibited similar values among the four genotypes under Ctrl, but the 360 WS determined a severe WP decrease in *A. halimus* and *A. hortensis* green (−192 and −254%, respectively) and negligible drops in the two red-leaved species.

Under Ctrl, the iWUE result was similar among the four genotypes but the 360 WS determined a iWUE increase in the two green-leaved species, although only in *A. hortensis* green this change was significant (Figure 4F).

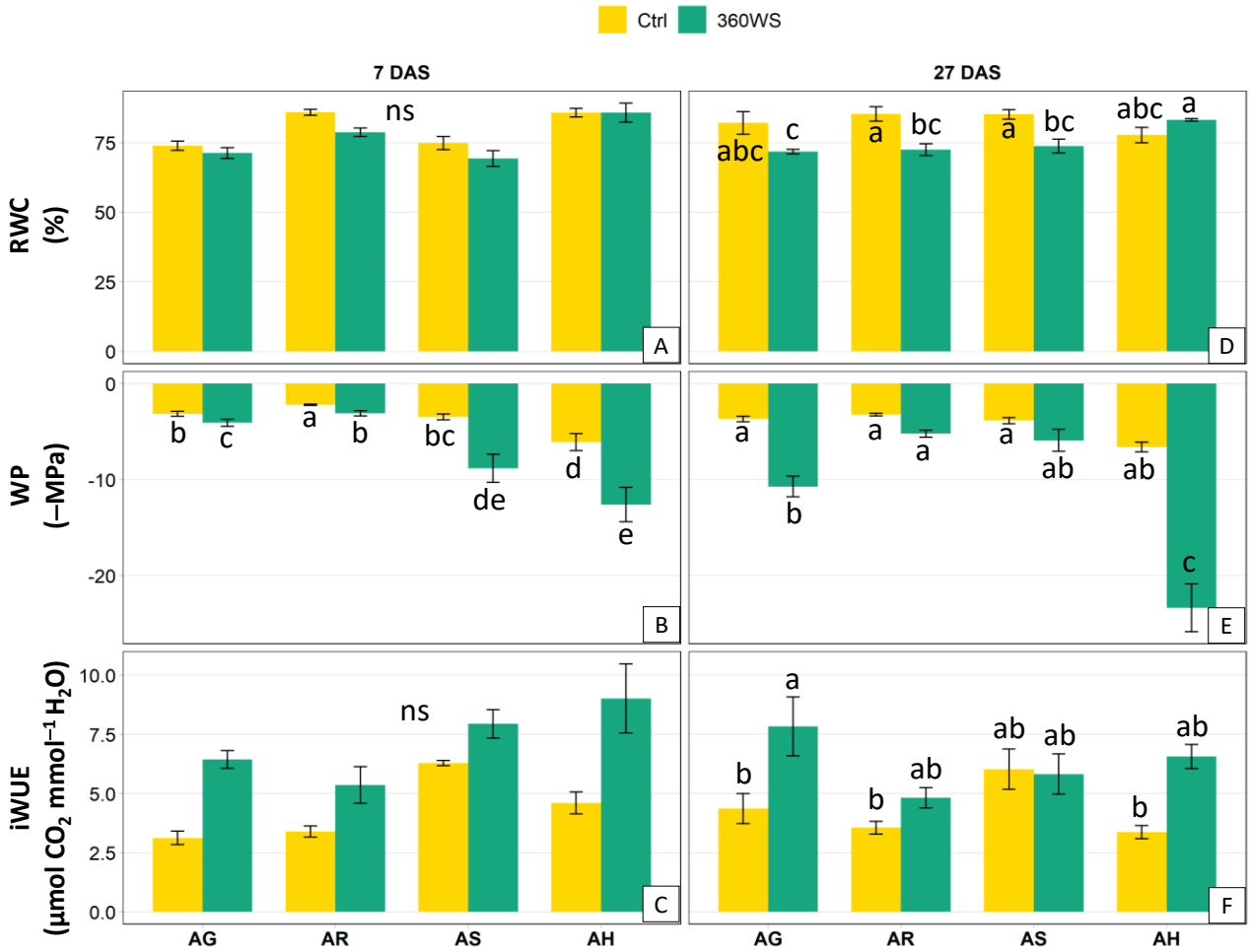


Figure 4. Leaf relative water content, leaf water potential, and intrinsic water use efficiency at 7 days after salt stress initiation (**A**, **B**, **C**, respectively) and at 27 days after salt stress initiation (D, **E**, **F**, respectively) under control (Ctrl) and 360 mM water salinity (360 WS) in the four genotypes A. *hortensis* cv. green (AG), *A. hortensis* cv. red (AR), *A. hortensis* cv. scarlet (AS), and *A. halimus* (AH). Vertical bars indicate standard error (n = 4). Different letters indicate significant differences at $p < 0.05$.

5.3.5. Element Vector Analysis

The vector analysis combines changes in biomass and element concentration and content into a comprehensive picture of plant response to Na input (Figure 5A).

High salinity (360 WS) did not determine a significant dry weight change in any of the four genotypes. Hence, the strong increase in the Na content and concentration may indicate an enhanced and selective uptake of this nutrient in all of the four *Atriplex* accessions, with no detrimental effect on overall plant growth.

Minor changes were also observed in the other elements. The potassium uptake appeared to be not limited by salinity (Figure 5B). However, *A. hortensis* cv. red showed a significant drop in the K concentration, indicating a possible dilution effect related to a plant DW increase, although the latter was not significant. *A.*

hortensis cv. scarlet was the only genotype showing a strong K content increase with 360 WS, confirming that K availability was not limited by Na competition and a preferential K accumulation occurred in the plant.

Ca availability, instead, appeared to be more affected by salinity as all of the four genotypes showed a Ca concentration decrease (Figure 5B). This decrease may be interpreted as a symptom of Na antagonism, in those genotypes also showing a Ca content drop (*A. halimus* and *A. hortensis* cv. green), or dilution, as in the case of *A. hortensis* cv. scarlet and red, which showed the highest, although not significant, DW increase under 360 WS.

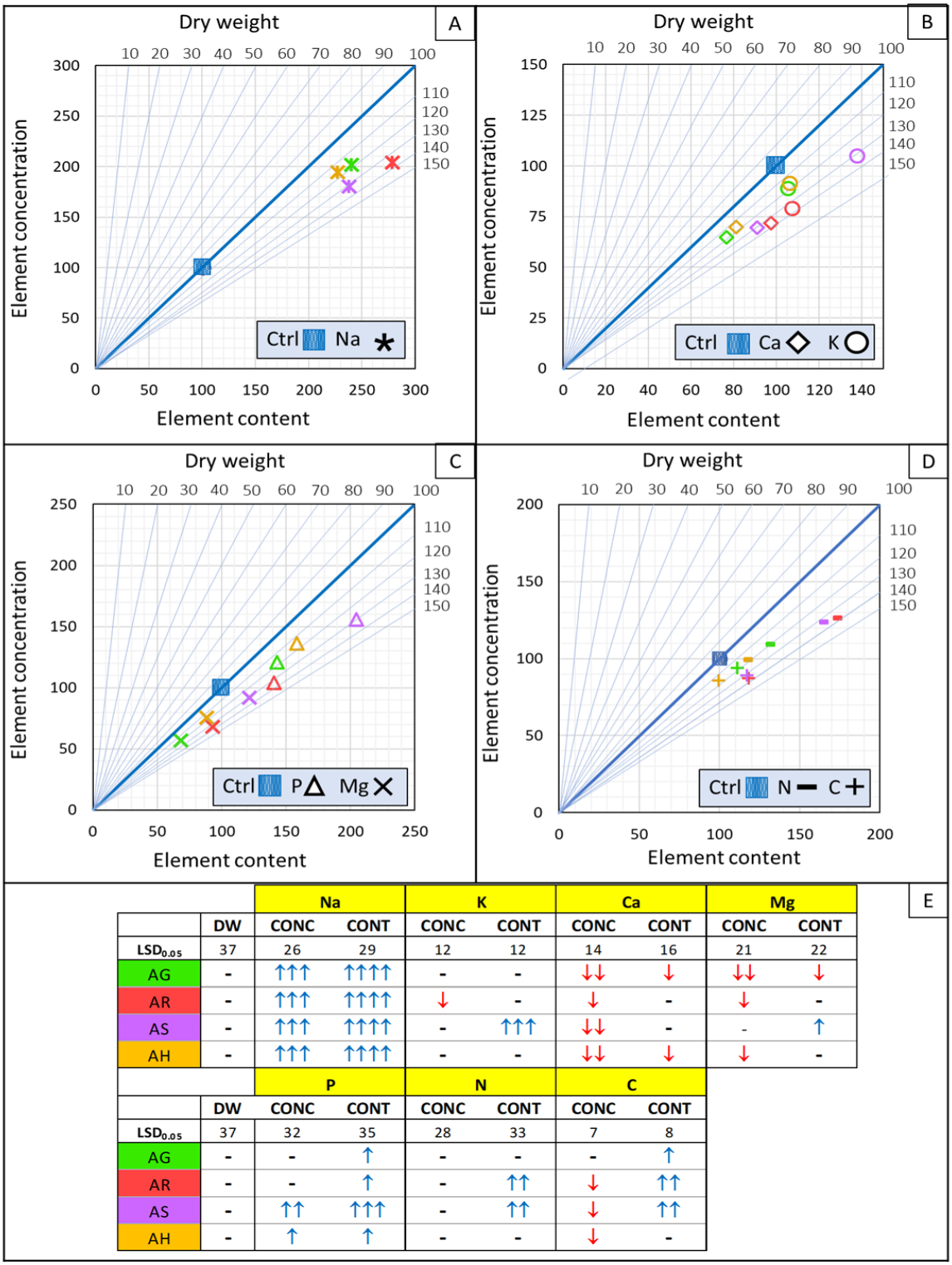
Three out of the four genotypes, as well, outlined a Mg concentration decrease that may be ascribed to Na antagonism in *A. hortensis* cv. green (a significant Mg content decrease, too) or to a dilution effect in the case of *A. halimus* and *A. hortensis* cv. red (no Mg content decrease) (Figure 5C). In the case of *A. hortensis* cv. scarlet, again, the rise in Mg content combined with the steadiness in Mg concentration may be ascribed to a dilution effect.

Contrarily to the three cations, the P uptake appeared to be boosted by 360 WS (Figure 5C). In all of the four genotypes, indeed, the P content increased, with the highest accumulation in *A. hortensis* cv. scarlet. The lack of P concentration increases in *A. hortensis* cv. red and green may indicate that the increase in the P content was proportional to the dry biomass increase, thereby resulting in no significant change in element concentration.

Nitrogen content increased with salinity, although the increase was significant only in *A. hortensis* cv. red and scarlet (Figure 5D). As before, the unchanged value in N concentration in all of the four genotypes may indicate that the N content increase was proportional to the dry biomass increase, thereby resulting in no significant change in element concentration.

Finally, the carbon content increased in all of the genotypes except *A. halimus* (Figure 5D). The observed drop in element concentration in *A. halimus* and in *A. hortensis* cv. red and scarlet may indicate that the increase in the biomass was proportionally higher that the increase in the element content, resulting in a C concentration drop.

Thus, among the four genotypes, *A. hortensis* cv. scarlet stands out for its higher content of K, P, and Mg as a likely result of selective uptake as osmoregulation compounds. *A. hortensis* cv. red, instead, is characterised by the higher N and Na increase. Compared to the former two genotypes, *A. halimus* and *A. hortensis* cv. green did not show a significant change in neither the K nor N content and concentration (Figure 5E).



E

		Na		K		Ca		Mg	
	DW	CONC	CONT	CONC	CONT	CONC	CONT	CONC	CONT
$LSD_{0.05}$	37	26	29	12	12	14	16	21	22
AG	-	↑↑↑	↑↑↑↑	-	-	↓↓	↓	↓↓	↓
AR	-	↑↑↑	↑↑↑↑	↓	-	↓	-	↓	-
AS	-	↑↑↑	↑↑↑↑	-	↑↑↑	↓↓	-	-	↑
AH	-	↑↑↑	↑↑↑↑	-	-	↓↓	↓	↓	-

		P		N		C	
	DW	CONC	CONT	CONC	CONT	CONC	CONT
$LSD_{0.05}$	37	32	35	28	33	7	8
AG	-	-	↑	-	-	-	↑
AR	-	-	↑	-	↑↑	↓	↑↑
AS	-	↑↑	↑↑↑	-	↑↑	↓	↑↑
AH	-	↑	↑	-	-	↓	-

Figure 5. Vector analysis showing directional changes in *Atriplex* spp. dry weight and (**A**) Na content and concentration, (**B**) K and Ca content and concentration, (**C**) Mg and P content and concentration, (**D**) N and C content and concentration. Dry weight and element content and concentration are expressed as relative data with respect to the Ctrl treatment, which is set at 100% and is indicated by a blue square; the colours of the element symbols are related to the four genotypes, as shown in the ensuing interpretation of the directional changes in relative dry weight (DW) and element concentration and content with respect to the reference treatment (Ctrl) (**E**). Upwards and downwards arrows indicate significant changes, and (**-**) indicates insignificant changes. The number of arrows indicate an increasing amplitude of the variation (from >1 to >4 LSD).

5.3.6. Physiological Traits Relationships and Results of the Multivariate Analysis

The plant fresh weight (FW) and electrolyte leakage (EL) results were negatively correlated with the transpiration rate E (ϱ = −0.52 and ϱ = −0.85, respectively) and stomatal conductance (GS) (ϱ = −0.37 and ϱ = 0.86, respectively), suggesting that, although the gas exchange reduction had a detrimental effect on the plant membrane stability, it represents a key strategy adopted by the plant to maintain its growth (Figure 6). Indeed, the positive correlation between the FW and EL (ϱ = 0.52) indicates that increased membrane permeability did not hamper plant growth.

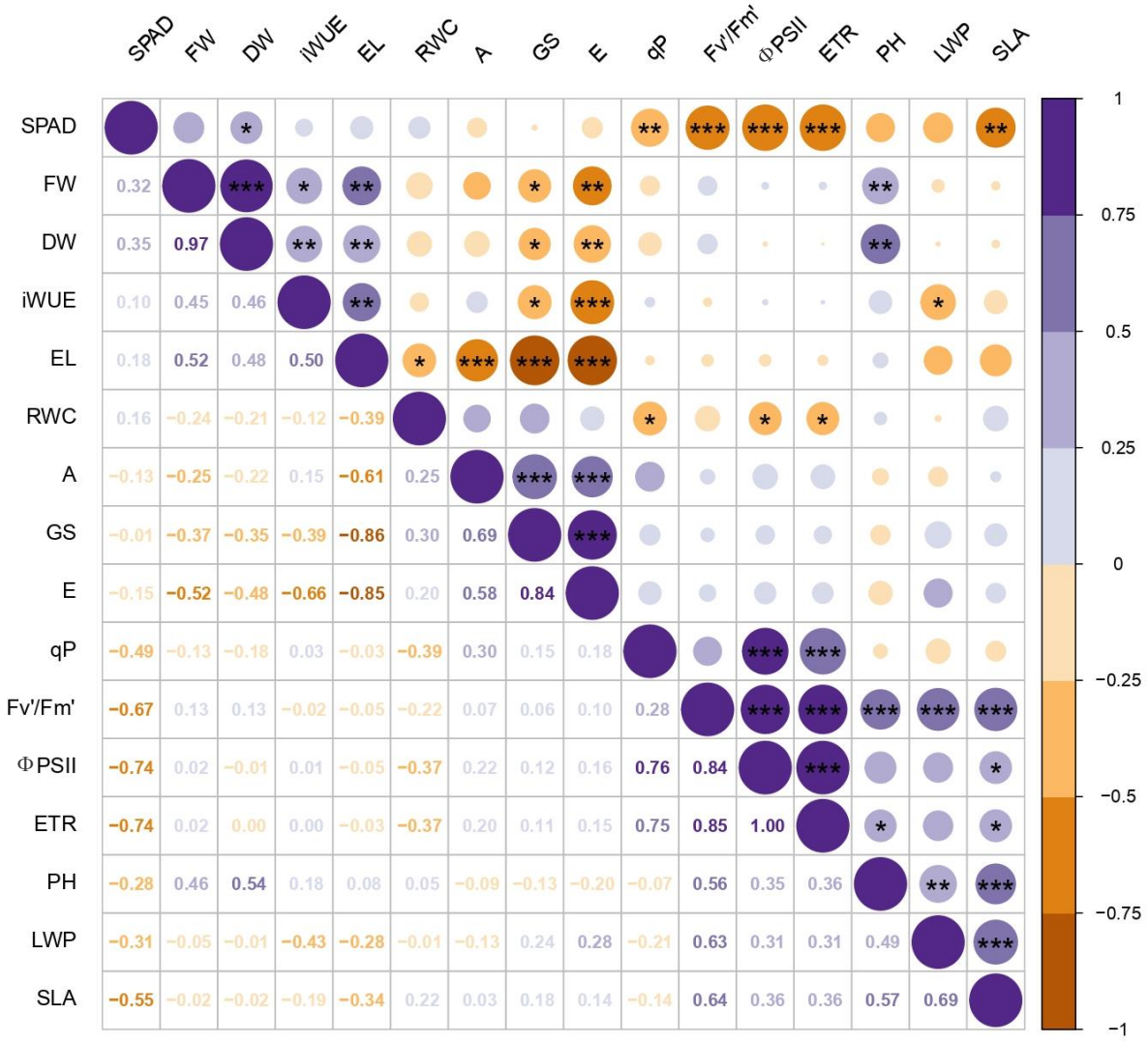


Figure 6. Pairwise Spearman's rank correlation coefficients calculated for the surveyed traits. Above the diagonal: graphical representation, with the colours and size of the circles referring to the direction and intensity of the correlations, while the symbols *, **, *** express significant correlations at $p < 0.05$, 0.01, and 0.001 Below the diagonal: numerical coefficients.

Consistently, the plant intrinsic water use efficiency (iWUE) outlined a negative association with the plant GS (ϱ = −0.39), E (ϱ = −0.66), and leaf water potential (LWP) (ϱ = −0.43), meaning that a decrease in these parameters allowed plant growth to be sustained under conditions of reduced water availability.

These observations are further corroborated by the negative, although bland, correlation between the leaf RWC and qP (ϱ = −0.39), ΦPSII (ϱ = −0.37), and ETR (ϱ = −0.37), suggesting that a lower activity of the plant photosynthetic machinery served to maintain the plant RWC.

The LWP, in turn, result was positively associated to the plant height (PH) and the specific leaf area (SLA) (ϱ = 0.49 and ϱ = 0.69, respectively), indicating that a decrease in the plant size and leaf area per unit of dry

matter contributed to the lowering of the osmic potential through the concentration of the cells' fluids. The SLA result was negatively correlated to the SPAD index, suggesting that an increase in leaf thickness (i.e., a decrease in the SLA) may have favoured chlorophyll concentration.

The SLA and PH, in turn, results were positively correlated to the Fv'/Fm', indicating a reduction in the photosystem II photochemical efficiency in parallel with the whole plant size.

We further explored the associations among traits with Principal Component Analysis (PCA), to assess the strength and direction of correlations between the original traits and the extracted Principal Components (PCs).

The first three PCs, explaining 72% of the total variance (eigenvalues reported in table S7 of the Supplementary Materials), were used for PCA interpretation. The correlation coefficients were calculated between the PCs and each quantitative (the 24 physiological traits) and qualitative, i.e., categorical, variables (the four *Atriplex* genotypes and the two water treatments). The associated *p*-values were computed to rank the variables according to their relevance (Table S8 of the Supplementary Materials).

The correlation circle and the PCA biplot of variables are reported in Figure 7.

PC1 synthesised the direct inter-relations involving plant gas exchanges, content of the main favourable cations, and plant growth. We found the strongest positive correlations with PC1 for K concentration (0.89), Ca concentration (0.85), Mg concentration (0.71), E (0.71), GS (0.67), $\delta^{13}C$ (0.63), and in contrast, the strongest negative correlation for EL (−0.68), FW (−0.67), and PH (−0.60).

PC2 synthesised the direct inter-relations involving Na content, plant water potential, and the PSII photochemical activity. The PC2 showed the strongest correlation with the SLA (0.81), LWP (0.79), Fv'/Fm' (0.73), C concentration (0.70), ΦPSII (0.58), ETR (0.57), E (0.68), and GS (0.64), and the strongest negative correlations with Na (−0.71) and the $\delta^{13}C$ (−0.66).

PC3 summarised the inter-relations involving plant growth, Na and N concentration, and chlorophyll fluorescence. The PC3 showed the highest positive correlation with the qP (0.81), N (0.64), ΦPSII (0.61), and ETR (0.61), and the highest negative correlation with the C (−54), SPAD (−0.54), and DW (−0.49).

The position of the barycentres (Figure 7B) of the three *A. hortensis* cultivars showed a modest gap from *A. halimus* (AH). AH barycentre, indeed, was in the lower-right quadrant in the same direction of δC, which is the parameter that mainly differentiated it from the other C3 species. AH, then, together with *A. hortensis* cv. scarlet (AS), were the genotypes showing the highest content of chlorophyll (SPAD value). Furthermore, AH had the same positive direction as the RWC, being the species showing the highest RWC under salinity.

A. hortensis cv. scarlet barycentre is in the lower-left quadrant in the same direction as the FW, DW, EL, and P, and together with the barycentre of the 360 WS treatment, as it was the species showing the highest value of these parameters under salinity.

The *A. hortensis* cv. green (AG) barycentre was located in the upper-right quadrant, being that showing the highest value in the fluorescence parameters despite salinity.

The proximity of the *A. hortensis* cv. red (AR) barycentre to the AG barycentre suggests their greater mutual similarity than the other two species.

The barycentre of the Ctrl treatments was located in the same direction as the gas exchange parameters, in the opposite quadrant of the fresh weight and water content parameters.

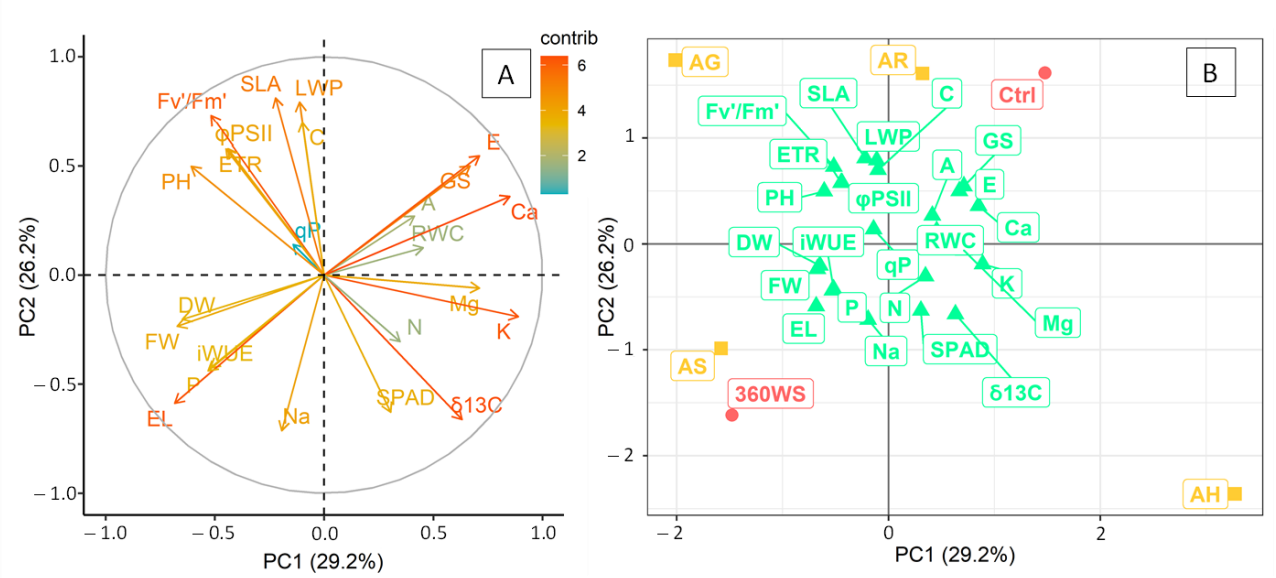


Figure 7. (**A**) PCA correlation circles; the increasing arrows' lengths and shades of colour from light blue to red indicate the increasing contribution of variables to the definition of the first two principal components; (**B**) PCA biplot of variables. Yellow squares show the barycentres of the four *Atriplex* genotypes (AG, AR, AS, and AH), the red points show the barycentres of the two salinity treatments (Ctrl and 360 WS), and the green triangles show the quantitative variables, i.e., the 24 measured traits.

5.3.7. FT-IR Spectroscopy

In order to obtain a detailed indication of the structural changes in the samples due to saline stress, the interval between 1800 and 400 cm^{-1} was shown (Figure 8). In general, all the spectra displayed similar functional groups, although the relative intensity decreased in the presence of NaCl.

A weak band at 1733 cm^{-1} is attributable to the uronic esters and acetyl groups of hemicelluloses, or the ester bonds of the carboxyl groups of the ferulic and *p*-coumaric acids of lignin [62]. In all the spectra, the band at 1630 cm^{-1} may be due to the Amide I (C=O) of proteins, unesterified uronic acids (-COO-) [63], and aromatic skeletal vibrations. Additional bands at 1550 (N-H) and 1240 (C-N) cm^{-1} may be assigned to Amide II and Amide III bands, respectively. The broad shoulder at 1430–40 cm^{-1} was attributed to the CH_2 bending of cellulose and lignin. The band at 1379–69 cm^{-1} was assigned to O-H bending [63]. The absorbance at 1320 cm^{-1} is typical of the aromatic skeletal vibrations [64] of the syringyl ring plus the guaiacyl ring, and the C=O stretching vibration in lignin. The strong band at 1030 cm^{-1} was assigned to the C-O-C pyranose skeletal vibration of cellulose [65]. The appearance of the band at 900 cm^{-1} is typical of β-glucosidic bonds between the glucose units in cellulose [66]. The bands appearing from 1000 to 500 cm^{-1} may be also due to the vibrations of the Si-O group.

As the spectral profile of all the samples is dominated by cellulose bands, a significant decrease in the bands of this compound was observed under salt stress. In AG, no structural modification was found.

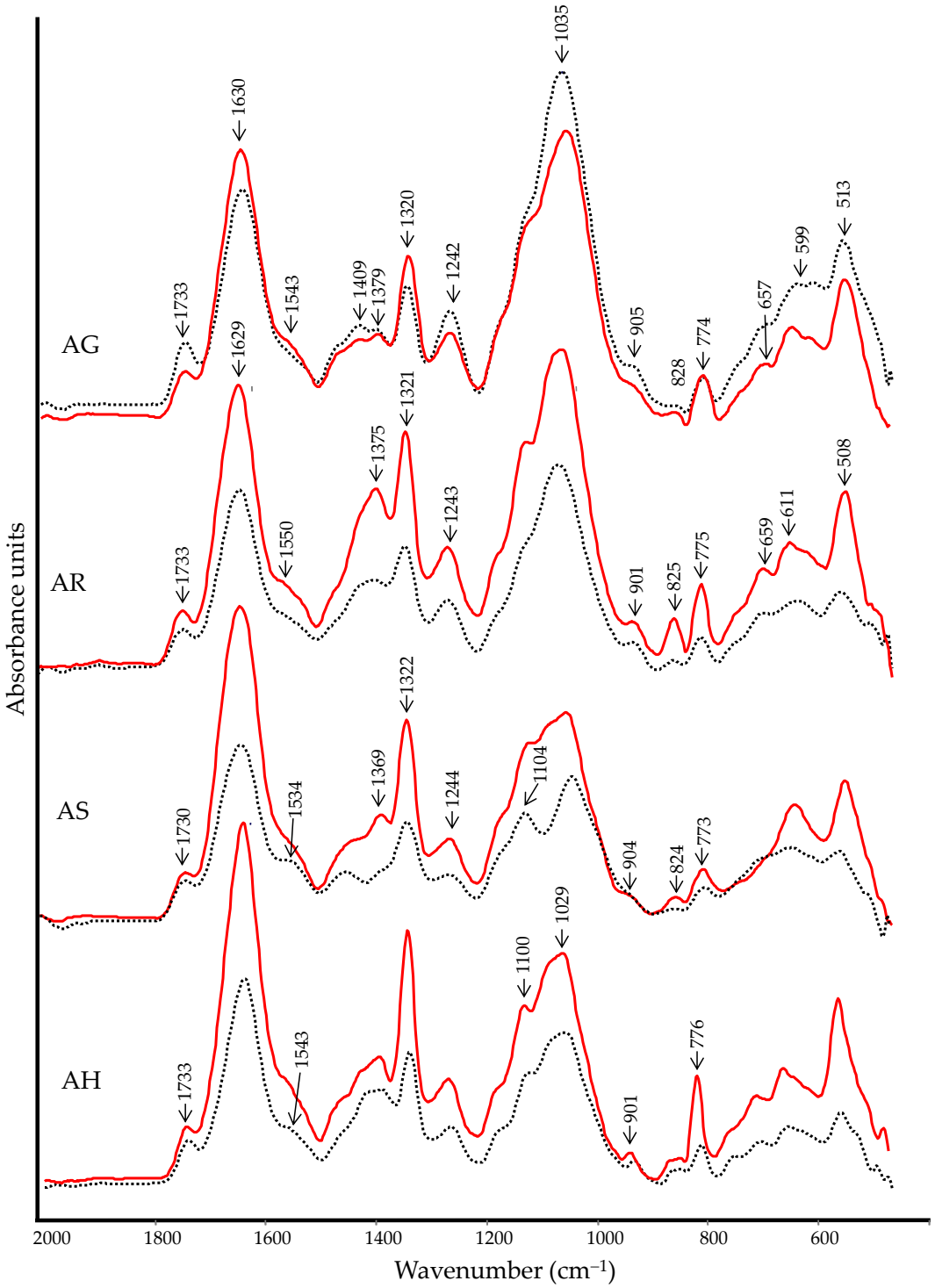


Figure 8. FTIR-ATR spectra of *A. hortensis* cv. green (**AG**), cv. red (**AR**), cv. scarlet (**AS**), and *A. halimus* (**AH**) samples under control (red line) and 360 mM water salinity (black dotted line) in the 1800 and 400 cm^{-1} wavelengths region.

5.4. Discussion

Salt-affected soils occupy around 932 million ha of the earth's surface [67]. Through the cultivation of salt-tolerant plants, these lands may be at least partially converted into important terrestrial ecosystems, with an associated carbon sink contributing to increase the soil organic carbon stock, while providing additional space to grow food and fodder for the future population.

This work compares some physiological traits of four *Atriplex* genotypes common in the Mediterranean basin, whose cultivation may be part of a strategy to promote the restoration of saline soil organic carbon and fertility. The four genotypes showed similar behaviours under salinity, although with differences in the intensity of salinity effects.

Under Ctrl, the FW was significantly lower in *A. halimus* than in *A. hortensis* cv. green and scarlet (Figure 1). This was expected, since *A. halimus* is a perennial plant, whereas *A. hortensis* is an annual one, having a faster growth rate [42]. This difference was encountered also in plant height, positively correlated with plant FW (Figure 6), as *A. halimus* showed the lowest height. *Atriplex hortensis* cv. red resulted as the smallest among the three C3 genotypes, probably due to an intrinsic difference in its plant size.

The growth of all of the four genotypes was enhanced under 360 WS, although not significantly in *A. halimus*. In agreement with this, a significant increase in carbon content was detected in the three annual species but not in *A. halimus* under salinity (Figure 5). These results are in contrast to the findings of previous studies that found a biomass decrease in *A. hortensis* already at 78 [47,68] and 250 mM [48,69], and in A. *halimus* already at 160 [70] and 300 mM [71–73], while Yepes et al. [74] did not detect a biomass decrease up to 300 mM in *A. halimus.*

The plant height did not vary significantly with salinity except for *A. hortensis* cv red. This is not in accordance with Kachout et al. [47], who observed a similar decrease both in green and red *A. hortensis* already at 78 mM.

Under control conditions, the three C3 *A. hortensis* showed values of net photosynthesis (A), stomatal conductance (GS), and transpiration rate (E) similar or even higher with respect to the C4 *A. halimus* (Figure 2). At 27 DAS, these parameters, especially GS and E, showed a decrease, more pronounced in the C3 than in the C4 *Atriplex* species (Figure 2). According to Nippert et al. [75], the higher resource use efficiency of C4 plants would allow them to remain active over a longer time and to persist through periods of resource limitations up to the late vegetative season when the C3 species may have already senesced.

Under salinity, a decrease in the GS and E was observed already after 7 days from the stress initiation (Figure 2), suggesting that an effective control of the stomata opening was the primary mechanism adopted by the plants to adapt to the stress.

The higher GS and E drop compared to the control observed in *A. halimus* at 27 DAS confirm that the C4 species have a lower stomatal conductance requirement per unit of fixed CO_2 [76] thanks to the ability to maintain photosynthesis also with a low internal CO_2 concentration [77]. The greater stomata closure, in turn, may also explain the higher RWC values found in *A. halimus* than in the three *A. hortensis* genotypes at 27 DAS (Figure 4).

The maintenance of the levels of net photosynthesis (A) and intrinsic water use efficiency (iWUE) in all of the genotypes up to the end of the experiment indicates that the positive effect of the increased stomatal resistance to water vapour deficit due to stomata closure was higher than the detrimental effect of the increased mesophyll resistance to CO_2 uptake.

This hypothesis is furtherly confirmed by the pathway in the carbon isotope ratio ($\delta^{13}C$) (Figure 1F), which decreased with 360 WS in two of the three C3 *A. hortensis* genotypes, while it remained unchanged in the C4 *A. halimus.*

The carbon isotope ratio, indeed, is related to the stomatal conductance, as a higher ^{13}C discrimination occurs when the stomata are open [78]. A lower ^{13}C discrimination (i.e., less negative $\delta^{13}C$ values), therefore, indicates the closure of the stomata and is associated with an increased iWUE [79].

The $\delta^{13}C$ to iWUE relationship is easily demonstrated in the C3 species [80], because of the larger discrimination toward the heavier $^{13}CO_2$ molecule with respect to the lighter $^{12}CO_2$, resulting from Rubisco fractionation [81]. The relationship is found also in the C4 species, but to a lesser degree because of the smaller fractionation during Rubisco carboxylation [80]. C3 plants, indeed, have $\delta^{13}C$ values between −32.6 and −19.2‰, while C4 plants between −10.4 and −16.6‰ [82].

The $\delta^{13}C$ increase with 360 WS observed in the C3 genotypes confirms the salinity-induced increase in stomata resistance that, in turn, augmented the plant iWUE.

Not surprisingly, the C4 *A. halimus* ^{13}C discrimination appeared to be less sensitive to salinity, although previous studies on the C4 *Atriplex confertifolia* [83] and *A. lentiformis* [84] showed a $\delta^{13}C$ decrease under salinity.

Despite the significant GS and E reductions already at 7 DAS, the mild, albeit significant, effects of salinity on the PSII photochemistry were detected only at 27 DAS (Figure 3).

High external NaCl concentrations could harm thylakoid membranes by denaturing the lipid bilayer and lipid–protein associations, thus impairing the electron transport chain [85]. Furthermore, salinity-induced stomata closure may lead to a reduction in the proportion of opened reaction centres, thereby reducing the photochemical quenching coefficient (qP) [86]. However, the absence of these symptoms, proved by the almost unchanged ΦPSII, Fv'/Fm', qP, and ETR values, indicates that there were no serious damages to PSII machinery under 360 WS. On the contrary, the slight increase in Fv'/Fm' and ETR in *A. hortensis* cv. scarlet may confirm the association of red plant pigment with an improved salinity tolerance [87,88].

Unlike previous studies that showed reduced pigment content in *A. halimus* [89] and *A. hortensis* [47] under salt stress, we did not observe salinity effects on the chlorophyll content in any of the four *Atriplex* genotypes. The negative correlation between the SPAD and the SLA (Figure 6) may in part explain this phenomenon, suggesting that a possible salt-induced reduction in chlorophyll may have been counterbalanced by the reduction in the leaf area, resulting in the unchanged concentration of the pigment.

Under salinity, the restricted water availability and the necessity to save water led to a reduced leaf area per unit of dry weight and an increased leaf thickness. In fact, the thicker cell walls and the increased concentration of osmolytes have been shown to enhance the cellular hydration maintenance, helping to overcome the salinity-induced osmotic imbalance [90]. In our experiment, this was confirmed by the positive correlation found between the SLA and the leaf water potential (LWP) (Figure 6), which suggests that a decrease in the SLA (i.e., in leaf area per unit of dry weight) contributed to the LWP lowering and, hence, to the cell osmotic adjustment.

The electrolyte leakage (EL) is a useful indicator of ROS production in plants subjected to salinity and enables us to evaluate the membrane injury caused by lipid peroxidation [91]. Thus, the results obtained in this study suggest that *Atriplex* membrane integrity was affected by 360 WS, and that *A. halimus* and *A. hortensis* cv. green were the genotypes most prone to oxidative stress. The negative correlation between the EL, GS, and E (Figure 6) is an indication that a reduced intracellular CO_2 concentration under salinity favoured the formation of ROS threatening the membrane stability.

Similarly, Nedjimi et al. [71] and Paulino et al. [92] observed an EL increase in *A. halimus* and *A. nummularia* under 100 and 300 mM NaCl, respectively.

Nevertheless, although Na concentration was positively correlated to EL (Figure 6), the absence of toxic NaCl effects on the plant photosystems indicates that the plant was able to keep Na compartmentalisation. Hence, in this case, we may assume that the high EL values may not be considered a sign of damaged plasma membranes, but rather of an NaCl-induced efflux of K [93,94]. Indeed, according to Demidchik et al. [94], under environmental stress conditions, the cytosolic K efflux plays a role in the metabolic switch by inhibiting consumptive anabolic reactions and stimulating energy-releasing catabolic processes that are necessary for stress adaptation and cell membrane reparation.

However, the absence of salinity effects on the K content in all of the four genotypes proves the *Atriplex*'s ability to keep a selective K uptake also in conditions of high external Na concentration [95,96].

All of the four genotypes already showed a leaf water potential (LWP) decrease after 7 days from the salt stress initiation, with *A. hortensis* cv. green and *A. halimus* reaching the lowest LWP levels at 27 DAS (Figure 4). As shown in the PCA correlation circle, the Na concentration was inversely correlated to the LWP, suggesting that an increase in the former caused a decrease in the latter. A higher uptake of Na under saline conditions is a common condition in many *Atriplex* species whose cells are able to use Na for the maintenance of cellular osmotic potential under high salinity [97,98]

Furthermore, the LWP appeared inversely correlated to the N concentration (Figure 7). Indeed, several nitrogen-containing compounds are accumulated in plants exposed to salinity [93,95]. These compounds contribute to the osmotic adjustment and scavenging of free radicals, favouring the maintenance of cellular macromolecules and pH. In our study, a significant increase in N content was detected in the two red leaved

A. hortensis genotypes, and this may also be related to their higher content in betacyanins, a class of pigments constituted by nitrogen-containing phenols that also play a key antioxidant role.

The increase in the phosphate concentration under salinity (Figure 5) and its positive correlation with Na (Figure 6) reveal, as well, the involvement of this anion in *Atriplex* leaf osmotic adjustment and indicate the ability of this species to augment selective phosphate uptake under high external NaCl concentrations [98]. In fact, salinisation has been found to increase the plant P requirement, likely due its major role in the energy transfer system, in the carbohydrate partitioning and transport, and in the constitution of most enzymes [99].

Finally, FTIR spectroscopy was used for a collective screening of the changes in carbohydrates, proteins, and cell wall components during salt stress. Overall, the spectra profile of all the samples between 1800 and 400 cm^{-1} was dominated by cellulose bands (Figure 8). Salt stress, indeed, can indirectly affect cell wall properties.

The absorption bands at 1743, 1430–40, 1320, and 1033 cm^{-1} most closely reflect the presence of pectin, lignin, and cellulose. These bands decreased in all the *Atriplex* genotypes under saline stress, in particular in AS and AH.

Based on these results, we can infer that cell wall biosynthesis was sensitive to salinity. Similarly, Wang et al. [100] observed a reduced cell wall lignification in *Atriplex prostrata* under salinity due to a possible substitution of lignin with extensin during salt stress adaptation. The same was observed in the Chenopodiaceae halophyte *Suaeda maritima* [101].

The decrease in the Ca content (Figure 5), probably due to Na competition, may be among the causes of the change in the cell wall structure. Calcium ions, indeed, are involved in the stabilisation of the cell wall structure and control of the wall enzymes' activities.

A general remark, however, is that salinity may increase lignification at root level, while it decreases it at stem level [102].

Proteins, as well, are involved in the maintenance of the cell wall structure and play a major role in the cell's physiological functions and homeostasis.

The interpretation of protein status as means to assess the degree of salt tolerance of plants should, however, be considered with caution, as the relationship between protein status and salinity is not always entirely conclusive. As reported by Ashraf and Harris [103], in some studies, a higher soluble protein content was observed in salt-tolerant species than in salt-sensitive species, while other studies found a decrease or no change in the protein content under saline conditions, regardless of the degree of tolerance of the surveyed species.

In our study, the amide reduction (Figure 8) was not supported by the N content (Figure 5), which was higher under salt stress conditions. However, we cannot exclude the possibility that the greater contribution of the band at 1630 cm^{-1} may be due to the uronic acid and aromatic ring vibrations, confirming that the *Atriplex* cell wall composition changed in response to salinity.

By considering that the carbohydrates region between 1100 and 900 cm^{-1} decreased from AR to AH, we can also deduce that carbohydrates contributed only modestly to osmoregulation. Indeed, as stated by Bennert et al. [104], inorganic ions (Na, K, Cl) and oxalate, an organic acid, are the main osmotically active solutes in *Atriplex* spp., while soluble carbohydrates, amino acids, and other organic acids are scarcely involved in the ionic balance maintenance.

5.5. Conclusions

Both *Atriplex halimus* and *A. hortensis* thrived under salinity, as the improved plant growth demonstrates. This is apparently due to mechanisms of physiological adaptation (reduced stomatal conductance (GS) and transpiration (E)) enabling the two species to preserve moisture and improve water use efficiency.

In this respect, *A. halimus* was shown to be a slightly better performer than *A. hortensis*, thanks to the C4 vs. C3 photosynthetic pathway allowing the former species to maintain net photosynthesis despite the stronger GS and E drop under salinity. This is further supported by a stronger drop in the leaf water potential under salinity, allowing *A. halimus* to maintain a higher relative water content.

The results obtained in this study indicate both *Atriplex* species to be potentially suited for cultivation in marginal/abandoned saline areas, thereby contributing to soil carbon sequestration, which is the premise for

the agricultural reclamation of these areas. In this strategy, the perennial *A. halimus* ensures a slower but longer lasting soil canopy coverage thanks to better plant homeostasis in the face of salinity, whereas the scarlet leaved genotype of *A. hortensis* is expected to provide faster soil coverage and higher biomass production.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1; Table S1: Effects of halophyte genotype (HG) and the four (Ctrl, 90, 180, 360 mM NaCl) water salinity levels (WS) on the physiological traits at seven days from the salt stress initiation (7 DAS). Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Different letters indicate significant differences at $p \leq 0.05$ ($n = 4$); Table S2: Effects of halophyte genotype (HG) and the four (Ctrl, 90, 180, 360 mM NaCl) water salinity levels (WS) on the physiological traits at twenty-seven days from the salt stress initiation (27 DAS). Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Different letters indicate significant differences at $p \leq 0.05$ ($n = 4$); Table S3: Effects of halophyte genotype (HG) and the four (Ctrl, 90, 180, 360 mM NaCl) water salinity levels (WS) on physiological traits, biomass, and element content at twenty-seven days from the salt stress initiation (27 DAS). Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Different letters indicate significant differences at $p \leq 0.05$ ($n = 4$); Table S4: Effects of halophyte genotype (HG) and the two corner (Ctrl and 360 mM NaCl) water salinity levels (WS) on physiological traits, biomass, and element content at twenty-seven days from the salt stress initiation (27 DAS). Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Different letters indicate significant differences at $p \leq 0.05$ ($n = 4$);Table S5: Effects of halophyte genotype (HG) and the two corner (Ctrl and 360 mM NaCl) water salinity levels (WS) on the physiological traits at seven days from the salt stress initiation (7 DAS). Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Different letters indicate significant differences at $p \leq 0.05$ ($n = 4$); Table S6: Effects of halophyte genotype (HG) and the two corner (Ctrl and 360 mM NaCl) water salinity levels (WS) on the physiological traits at twenty-seven days from the salt stress initiation (27 DAS). Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Different letters indicate significant differences at $p \leq 0.05$ ($n = 4$); Table S7. Eigen analysis of the PCA correlation matrix; Table S8. Correlation coefficients between quantitative and categorical variables, and the first three PCs. The PCs were computed using 32 input data.

Author Contributions: Conceptualisation, R.C., C.L., and L.B.; methodology, R.C. and C.L.; software, R.C.; validation, L.B.; formal analysis, R.C.; investigation, R.C., L.M., A.C., P.G., and A.S.; resources, L.B., L.M., and A.S.; data curation, R.C.; writing—original draft preparation, R.C.; writing—review and editing, L.B. and A.C.; visualisation, R.C.; supervision, L.B.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable

Data Availability Statement: Data is contained within the article or supplementary material.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 6

Article published *in Plants* 2022, 11, 1058

Recovery from salinity and drought stress in the perennial *Sarcocornia fruticosa* vs the annual *Salicornia europaea* and *S. veneta*

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Abstract:

Current agricultural problems, such as the decline of freshwater and fertile land, foster saline agriculture development. *Salicornia* and *Sarcocornia* species, with a long history of human consumption, are ideal models for developing halophyte crops. A greenhouse experiment was set up to compare the response of the perennial *Sarcocornia fruticosa* and the two annual *Salicornia europaea* and *S. veneta* to 30 days of salt stress (watering with 700 mM NaCl) and water deficit (complete withholding of irrigation) separate treatments, followed by 15 days of recovery. The three species showed high tolerance to salt stress, based on the accumulation of ions (Na^+, Cl^-, Ca^{2+}) in the shoots and the synthesis of organic osmolytes. These defence mechanisms were partly constitutive, as active ion transport to the shoots and high levels of glycine betaine were also observed in non-stressed plants. The three halophytes were sensitive to water stress, albeit *S. fruticosa* to a lesser extent. In fact, *S. fruticosa* showed a lower reduction in shoot fresh weight than *S. europaea* or *S. veneta*, no degradation of photosynthetic pigments, a significant increase in glycine betaine contents, and full recovery after the water stress treatment. The observed differences could be due to a better adaptation of *S. fruticosa* to a drier natural habitat, as compared to the two *Salicornia* species. However, a more gradual stress-induced senescence in the perennial *S. fruticosa* may contribute to greater drought tolerance in this species.

Keywords: halophytes; salt stress; drought stress; stress recovery; life cycle length; habitat; osmolytes; ions transport; oxidative markers.

6.1. Introduction

In response to the current increase in world population, agriculture is called to address two major but opposite needs: increasing food production while decreasing its negative environmental impacts. Boosting food security through sustainable agricultural practices represents a priority objective for the 2030 Agenda for Sustainable Development [1], a goal that, to date, is even more urgent, considering that, in 2020, the number of undernourished people worldwide has increased by 83–132 million due to the COVID-19 pandemic [2]. However, the growing competition for land and water caused by the dramatic expansion of cities [3], in

conjunction with the increasingly recurrent phenomena of soil erosion, water scarcity, and loss of agrobiodiversity, are posing serious obstacles to achieving this objective.

The Mediterranean basin is amongst the areas most threatened by salinisation in the world due to climate change [4]. According to the Intergovernmental Panel on Climate Change, in the Mediterranean region, temperatures will rise by 2–4 °C, and rainfall will decrease between 4% and 30% by 2050 [5], whereas sea level is expected to increase by approximately 35 cm by 2100 [6]. The projected climate changes will also exacerbate the salt accumulation processes driven by seawater intrusion in the coastal shallow aquifers, which in turn will constrain soil fertility and crop productivity.

In 2009, the World Bank introduced the concept of climate-smart agriculture (CSA), referring to an integrated approach to address the complex nexus of climate change, food security, and sustainable development [7]. Today, the FAO Strategic Framework 2022–2031 considers the transition to CSA imperative to improve agricultural resilience and productivity and lower its climate footprint and costs [8]. The CSA approach is implemented through three priority lines of action: firstly, boosting sustainable agricultural production to support increased incomes and food security; secondly, increasing agroecosystems' adaptive capacity; and thirdly, reducing greenhouse gas emissions while increasing carbon sequestration [9].

The CSA applications are context-specific, depending on the local socio-political, financial, and environmental context, and encourage the integration of new technologies and practices such as precision farming tools, decision support systems for land and water management, conservative and organic crop practices, integrated pest and disease management, and the introduction of drought-, salt-, and flood-tolerant crops [10]. In this last regard, the Mediterranean region represents a precious hotspot of biodiversity, with a remarkable richness in cultivated and native wild plants that have adapted to various unfavourable conditions such as prolonged drought, salinity, and flooding.

Halophytes are extremophile plants that can tolerate harsh conditions and salinity levels toxic to most plants. Within the CSA framework, the study of halophytes' stress tolerance mechanisms is an outlooking strategy for improving crop resilience to environmental stress. Besides providing valuable scientific models, these plants can be cultivated for the direct production of food, fodder, biomass and medicinal compounds, as well as for soil phytoremediation, carbon sequestration, and landscaping purposes, including the recovery of marginal saline soils and water [11,12]. About 1100 halophyte species occur in the Mediterranean Basin, when considered in its broadest meaning, i.e., from the Aral Sea to the Atlantic Ocean [13]. Taxonomical, biological, and ecological diversity is high here, and there are traditional and new potential uses of these plants.

The subfamily *Salicornioideae* includes around 100 species of succulent halophytes, the *Sarcocornia*/*Salicornia* lineage being one of the most important in terms of species diversity [14]. This lineage consists of hygro-halophytes diversified during the Middle Miocene [15] and was confirmed by transcribed spacer (ITS) and atpB–rbcL spacer sequences as monophyletic, being clearly separated from other taxa [15]. Molecular phylogenetic studies based on external transcribed spacer (ETS) sequence revealed that this lineage comprises three primary clades: *Salicornia*, American-Eurasian *Sarcocornia*, and South African-Australian *Sarcocornia* [16]. The genus *Sarcocornia* A.J. Scott was separated from *Salicornia* L. and A*rthrocnemum* Moq. on the basis of morphological characters [17]. The *Salicornia* and *Sarcocornia* genera are morphologically similar and can be distinguished only by inflorescence characters and their life form, the former including only annuals and the latter only perennials. *Salicornia* is clearly a monophyletic genus, as revealed by ETS sequence data [16], whereas *Sarcocornia* remains unresolved as possibly paraphyletic [14]. Annual *Salicornia* species evolved from the perennial *Sarcocornia* during Miocene, and their high self-fertility allowed their rapid expansion, colonising coastal and inland remote habitats [14,16].

Three species of the *Sarcocornia*/*Salicornia* lineage were selected for this study. *Salicornia europaea* L. belongs to a diploid clade including genotypes that show a wide geographical distribution. *S. veneta* Pign. et Lausi is a member of the well-supported monophyletic group of *Salicornia dolichostachya* Moss with very little genetic variation among its taxa [16,18]. The species is endemic to NE Italy in the area of the Lagoon of Venice and West Slovenia and is classified as vulnerable according to the UICN criteria [19]. The third species under study, *Sarcocornia fruticosa* (L.) A.J. Scott with Mediterranean distribution, belongs to the Eurasian clade of *Sarcocornia* [14]. The three species are morphologically similar, with succulent and articulate stems, reduced

leaves, and inflorescences of minute reduced flowers. Their young, fleshy tips are edible and commercialised with the name of "samphire", "sea asparagus", "pickleweed", or "poor man's asparagus" [20]. Thanks to the crunchy texture and salty taste, their succulent shoots are highly appreciated in gourmet cuisine [21–23]. Moreover, they are a good source of fibre, antioxidants, and anti-inflammatory metabolites, such as vitamin C and polyphenolic compounds, making them an ideal nutraceutical supplement [23,24]. These species are also appreciated as oil-seed crops. Indeed, oil extracted from their seeds is rich in polyunsaturated fatty acids, particularly oleic and linoleic acid, having valuable health properties [25]. Furthermore, these species can produce high amounts of biomass rich in lignocellulosic materials suitable for bioethanol production [26]. The high biomass production, combined with the high phytoextraction capacity, also makes these species very attractive for the phytoremediation of saline and heavy metal-contaminated soils [27]. Finally, several studies have demonstrated their suitability for the regreening of marginal areas to increase carbon sequestration and relieve soil erosion [28,29].

Without salt glands or salt bladders, the strategy of glassworts to tolerate the ionic and osmotic components of salt stress relies largely on the massive accumulation and vacuolar compartmentalisation of Na^+ and Cl^- [30–33], which allow them to maintain the osmotic potential necessary to drive water uptake into cells while preventing ion-related cytotoxic effects. Moreover, they have evolved the ability to increase succulence in shoots diluting the accumulated ions [34], synthesise compatible solutes for osmotic adjustment, especially glycine betaine [34–37], produce ROS-scavenging enzymes and compounds [38,39], maintain high K-Na selectivity [33], and effectively regulate ammonium detoxification processes under stress conditions [40]. Furthermore, glassworts have the ability to transit from green to reddish colouration through the accumulation of red-violet pigments and betacyanins, which allow them to cope with excessive light energy in the photosystems when the plants experience osmotic stress and photosynthesis declines by dissipating excess excitation energy into heat [41].

In their natural habitats, halophytes are subjected to wide seasonal oscillations in precipitations and temperature, and therefore in soil moisture and salinity, which result in periods of high and low stress intensity that alternate during the year [42]. Significantly stressful conditions at the field level, however, are often only transient and rarely cause plant death as more favourable conditions usually return, although they often result in reduced crop yield [43]. However, basic studies on stress tolerance in halophytes have generally focused on their responses to different applied stress treatments, and very little is known on the equally important mechanisms of stress recovery, which are essential for ensuring sustainable crop production under intermittent stress events.

The focus of the present study was to analyse differences between the three aforementioned Salicornioideae species in their responses to stress and stress recovery treatments, which could be due to differences in the plants' life cycle or native environments. For this, we determined growth parameters in plants of the investigated species after applying controlled salt and water deficit treatments in a greenhouse, followed by irrigation with non-saline water. To obtain insights into their stress tolerance mechanisms, growth responses were correlated with changes in the levels of specific biochemical stress markers, such as photosynthetic pigments, different mono and divalent ions and organic osmolytes, oxidative stress markers, and antioxidant compounds.

6.2. Materials and Methods

6.2.1.Plant material and experimental conditions

Seeds of *Salicornia europaea* and *Salicornia veneta* were collected from Pialassa della Baiona, a coastal lagoon located within the Po Delta Regional Park in Italy. Seeds of *Sarcocornia fruticosa* were collected from 'La Albufera' Natural Park, located near the city of Valencia, Eastern Spain. Mean annual values of climatic parameters from 2006 to 2021 in the two sampling areas are reported in Table 1. The experiments were carried out in the laboratories and greenhouses of the Institute for the Conservation and Improvement of Valencian Agrodiversity (COMAV), Polytechnic University of Valencia, Spain.

Table 1. Historical weather data (from 2006 to 2021) of the areas of 'La Albufera' Natural Park (Spain) and Piallassa della Baiona (Italy), provided, respectively, by the Spanish Agroclimatic Information System for Irrigation (SIAR) and the Italian Arpae-Simc meteorological network [44,45]. T: temperature; RH: relative humidity; Eto: evapotranspiration. Eto data of Piallassa della Baiona were calculated applying the Thornthwaite method [46].

Year	La Albufera Natural Park				Piallassa della Baiona			
	Mean T (°C)	Mean RH (%)	Rainfall (mm)	Eto (mm)	Mean T (°C)	Mean RH (%)	Rainfall (mm)	Eto (mm)
2006	17.53	69.13	464.4	1189.38	14.40	77.64	337.65	814.71
2007	16.81	68.13	894.4	1164.5	14.20	73.18	490.00	809.25
2008	16.88	68.35	674.4	1194.1	14.20	73.63	491.13	804.14
2009	17.34	68.6	446.2	1215.26	14.19	72.79	555.86	816.07
2010	16.78	68.31	565	1206.22	13.23	74.09	450.00	776.35
2011	17.57	70.32	472	1166.73	14.76	71.36	346.60	846.35
2012	17.31	67.58	503.61	1208.25	14.71	69.98	563.60	864.97
2013	17.55	63.26	263.8	1245.42	14.49	72.86	870.20	822.93
2014	18.32	65.32	224.4	1278.22	15.60	73.91	740.00	833.27
2015	17.76	70.02	401.26	1169.08	15.20	77.18	616.80	860.61
2016	17.85	68.66	259.57	1218.41	14.71	80.86	829.40	825.33
2017	17.59	68.51	307.26	1238.82	14.84	76.69	641.80	851.52
2018	17.6	68.06	684.02	1225.71	15.32	78.53	613.60	870.93
2019	17.79	66.59	427	1243.83	15.03	81.94	780.80	839.65
2020	18.09	72.95	731.94	1186.44	14.70	76.76	556.40	808.83
2021	17.495	75.40	494.72	1039.1	14.45	75.75	335.00	809.89
Mean	*17.52*	*68.70*	*488.37*	*1199.34*	*14.63*	*75.45*	*576.18*	*828.42*

Seeds were sown manually in plastic trays filled with commercial peat, placed into a growth chamber with a 16/8-h light/dark cycle, day/night temperatures of 25/22 °C, and 70–80% relative humidity and watered thrice per week with tap water. Forty days after sowing, seedlings of each species of uniform size and shape were transplanted into plastic pots (12 cm diameter) filled with 500 g of a mix of commercial peat (26% organic carbon, pH_{H2O} = 7.0, and EC = 0.6 dS m^{-1}) and perlite (80:20 *v*/*v*). Three seedlings were transplanted to each pot. The pots were transferred into the controlled environment of a greenhouse, placed over benches, and irrigated manually with tap water thrice per week. During the experimental period in the greenhouse, temperatures ranged between 21.3 ± 1.6 and 28.6 ± 1.8 °C and RH between 67.5 ± 9.9 and 92.6 ± 2.9%.

6.2.2. Experimental Design and Stress Treatments

Four weeks after transplanting, when the plantlets were fully established, the pots with individuals of each species were randomly divided into three groups and subjected to the following treatments: control (Ctrl, irrigation with tap water thrice per week), salt stress (SS, irrigation with a 700 mM NaCl aqueous solution, thrice per week), and water stress (WS, complete withholding of irrigation). Pots were placed in trays and were watered from the bottom, i.e., filling the trays, considering a volume of 0.13 L pot^{-1}. After one month of treatment, the stressed plants were allowed to recover during the following fifteen days through intensive pot washings with tap water in the salt stress treatment and through the restoring of the soil moisture level up to 80% in the drought-stress treatment. In this phase, pots were watered from the top (0.13 L pot^{-1} for Ctrl and 0.50 L pot^{-1} for SS and WS) and, only in the SS treatments, the drainage water was always discarded to remove the leached salt. The amount of water (L pot^{-1}) distributed per each treatment during the Stress and Recovery phases are shown in Table 2.

Table 2 Amount of water distributed per pot during the stress period (Stress) and the recovery period (Recovery) in the three treatments (Ctrl, control; SS, irrigation with 700 mM NaCl; WS, complete withholding of irrigation).

	Stress (L pot^{-1})	Recovery (L pot^{-1})	Total (L pot^{-1})
Ctrl	1.75	1	2.75
SS	1.75	4	5.75
WS	0	2	2

The three factors, plant species (PS, 3 levels), stress treatments (ST, 3 levels), and harvesting time (HT, 2 levels), were cross-combined, resulting in 18 treatments. Four completely randomised replicates were set up, totalling 72 pots. This number of replicates is quite commonly adopted in pot experiments on this topic [30,47-49].

The plants were harvested twice, the first half after the thirty days of stress treatments (T30) and the second half after the fifteen days of recovery (T45). Morphological parameters were determined on all individual plants (n = 12 per species and treatment). Samples of the aboveground biomass, i.e., of the leafless succulent green stems, were used for biochemical analysis; in this case, the shoots of the three plants grown in each pot were pooled (n = 4 per species and treatment, but each sample was a pool of three independent plants).

6.2.3. Plant growth

The three surveyed species are characterised by strongly reduced leaves, which are embedded to form articulated, photosynthetically active succulent stems appearing to be composed of jointed segments (Figure 1). The number of branches (excluding the main branch) and plant height were determined at the beginning of the treatments (T0), after fifteen (T15) or thirty (T30) days of the stress treatments and after 15 days of recovery; that is, 45 days from the beginning of the experiment (T45). At both harvests, 'Stress' and 'Recovery', the aboveground biomass of each plant was separated from the root and weighed (fresh weight, FW). Roots were cleaned with a brush and weighed. Portions of the shoots and the root material were oven-dried at 65 °C until a constant weight was reached (ca. 72 h) and were then weighed again (dry weight, DW) to determine the water content percentage according to the following formula:

$$\mathrm{WC}\ (\%) = \frac{\mathrm{FW} - \mathrm{DW}}{FW} \times 100 \quad (1)$$

Fresh shoot material was flash-frozen in liquid N_2 and stored at −75 °C, and dry material was stored at room temperature in tightly closed paper envelopes. Pot substrate was collected at each harvest time to determine moisture and electrical conductivity (EC) in the laboratory. Substrate moisture was calculated gravimetrically, as described above for the plant samples (Equation 1). For EC measurements, a 1:5 suspension of the dry substrate and deionised water was prepared and mixed for one hour at 600 rpm and 21 °C before being filtered. The EC was measured with a Crison 522 conductivity meter and expressed in dS m^{-1}.

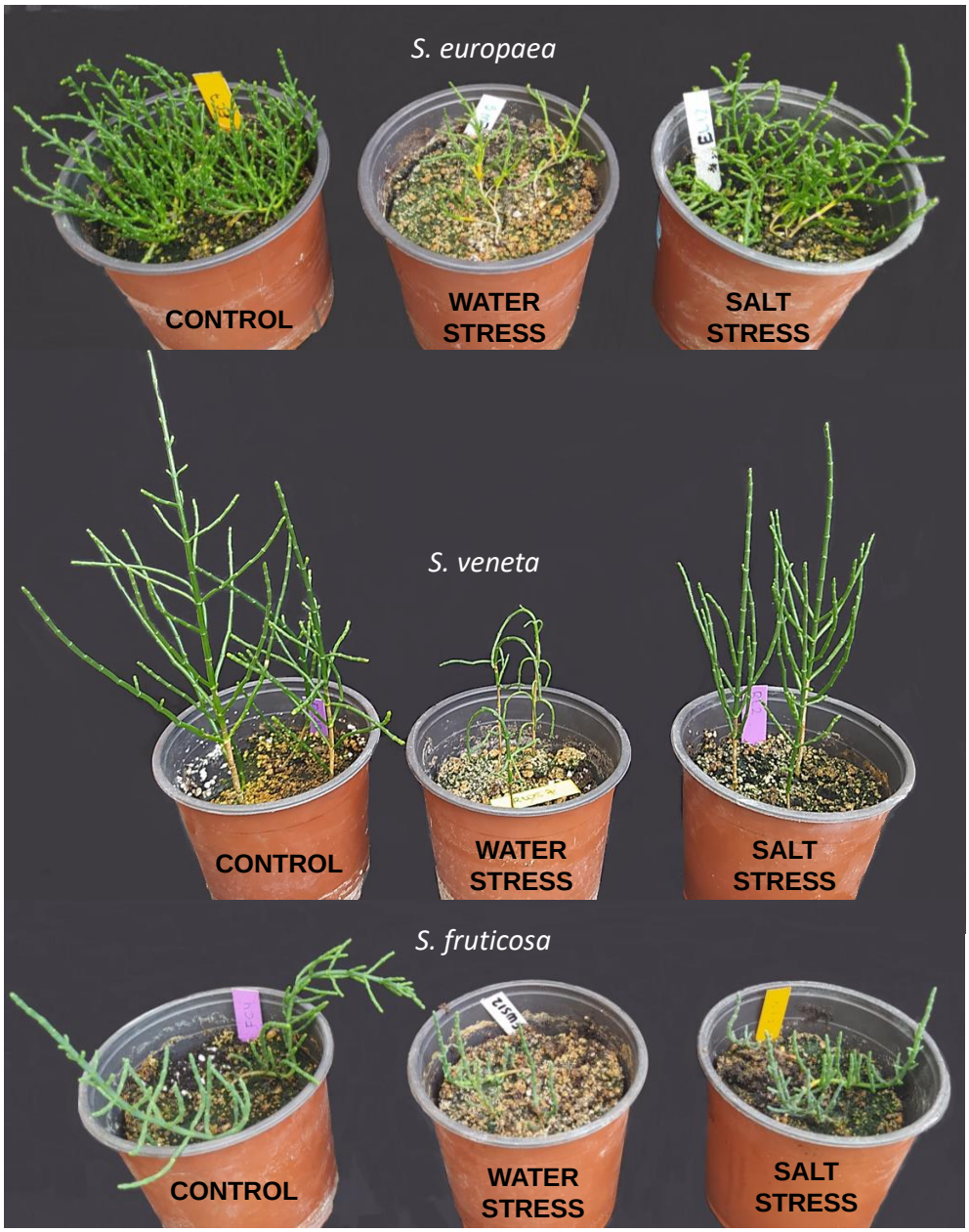

Figure 1. Picture of the three halophytes species after thirty days of stress treatments: control; water stress (complete withholding of irrigation); salt stress (watering with 700 mM NaCl).

6.2.4. Photosynthetic pigments

The concentrations (mg g^{-1} DW) of chlorophyll a (Chl. a), chlorophyll b (Chl. b), and carotenoids (Caro) in the plant tissues were measured spectrophotometrically, according to a previously described method [50]. Fresh ground shoot material (ca. 0.05 g) was extracted with 1 mL of ice-cold 80% acetone. The samples were mixed during 12 h in a shaker in the dark and then centrifuged at 13,300× *g* for 10 min at 4 °C. The supernatant absorbance was measured at 470, 646, and 663 nm, and the pigment concentrations were calculated, applying the equations described by Lichtenthaler and Wellburn [50].

6.2.5. Ion Quantification

The concentrations of Na^+, Cl^-, K^+, and Ca^{2+} were calculated separately for roots and shoots following the procedure described by Weimberg [51]. Two mL of Milli-Q water were added to ca 0.1 g of dry plant material, vortexed, and then mixed for 24 h in a shaker. The samples were then incubated in a water bath for 30 min at

95 °C, cooled on ice, and filtered through a 0.45 μm nylon filter. The cations were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), whereas the anions were measured using a chlorimeter (Sherwood, model 926, Cambridge, UK).

6.2.6. Quantification of osmolytes

The concentration of glycine betaine (GB) was determined as described by Grieve and Grattan [52], with some modifications [53]. Fresh shoot material (0.15 g) was shaken for 24 h at 4 °C with 1.5 mL Mili Q water and then centrifuged at 13,300× *g* for 10 min. The supernatant was mixed (1:1) with a 2N H_2SO_4 solution and stored in ice for 1 h. Then, 125 μL of the sample were supplemented with 50 μL of ice-cold KI-I_2 solution, which induces glycine betaine precipitation in the form of golden crystals. All the following steps were completed in the dark. The samples were maintained at 4 °C for 16 h and then centrifuged at 13,300× *g* for 45 min at 0 °C. The supernatant was carefully removed, and the glycine betaine crystals were dissolved into 1.4 mL of cold 1,2-dichloroethane; the tubes were kept for 2.5 h under dark and cold conditions, and, finally, their absorbance was recorded at 365 nm. Glycine betaine concentration was calculated against a GB standard calibration curve and expressed as μmol g^{-1} DW.

Proline (PRO) was quantified following the protocol of Bates et al. [54]. Fresh aboveground material (ca. 0.05 g) was extracted in 3% (*w*/*v*) aqueous sulpho-salicylic acid and subsequently supplemented with acid ninhydrin, incubated in a water bath for 1 h at 95 °C, cooled on ice, and then extracted with two volumes of toluene. The absorbance of the organic phase was read with a spectrophotometer at 520 nm, using toluene as a blank. A standard curve was obtained by running parallel assays with known PRO amounts. PRO concentration was expressed as μmol g^{-1} DW.

Total soluble sugars (TSS) were measured from ca. 0.05 g of ground fresh material extracted with 2 mL 80% (v/v) methanol, according to the method described by Dubois et al. [55]. After mixing in a shaker for 24 h, the samples were centrifuged at 13,300× *g* for 10 min; the supernatants, appropriately diluted with water, were mixed with 95% sulphuric acid and 5% phenol. After 20 min incubation at room temperature, the absorbance was measured at 490 nm. TSS concentration was expressed as equivalents of glucose, used as the standard (mg eq. glucose g^{-1} DW).

6.2.7. Determination of oxidative stress markers and antioxidant compounds

Malondialdehyde (MDA), total phenolic compounds (TPC), and total flavonoids (TF) were quantified in the same methanol extracts prepared for TSS measurements.

The method defined by Hodges et al. [56] was used for MDA quantification, with some modifications [57]. Extracts were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA)—or with 20% TCA without TBA for the controls—and then incubated at 95 °C for 20 min, cooled on ice, and centrifuged at 13,300× *g* for 10 min at 4 °C. The supernatant absorbance was measured at 532 nm. The non-specific absorbance at 600 and 440 nm was subtracted, and the MDA concentration was computed, applying the equations proposed by Taulavuori et al. [57]. MDA contents were expressed as nmol g^{-1} DW

Hydrogen peroxide content in plants was quantified as previously described [58]. Fresh plant material (0.05 g) was extracted with a 0.1% (*w*/*v*) trichloroacetic acid (TCA) solution. After centrifugation, the supernatant was mixed with one volume of 10 mM potassium phosphate buffer (pH 7.0) and two volumes of 1 M potassium iodide. The absorbance of the samples was determined at 390 nm. Reaction mixtures containing known concentrations of H_2O_2 were assayed in parallel to obtain a standard curve, and H_2O_2 concentrations were expressed as μmol g^{-1} DW.

TPC were measured by reaction with the Folin–Ciocalteu reagent, following the method previously [59]. The methanol extracts were mixed with Na_2CO_3, incubated at room temperature in the dark for 90 min, and the absorbance was read at 765 nm. Gallic acid (GA) was used as standard, and the measured TPC concentrations were expressed as GA equivalents (mg eq. GA g^{-1} DW).

TF were quantified by a previously described protocol [60], namely by sample incubation with $NaNO_2$, followed by a reaction with $AlCl_3$. After the reaction, the sample absorbance was determined at 510 nm, and TF contents were expressed as equivalents of the catechin standard (mg eq. C g^{-1} DW).

6.2.8. Statistical Analysis

The data of the measured traits within each plant species (PS) were subjected to two separated one-way ANOVAs for the respective stress treatments (ST) and harvesting times (HT). The Tukey's honestly significant difference (HSD) post hoc test at $p < 0.05$ was applied to indicate significant differences among levels in significant ANOVA sources. A two-way ANOVA was then performed to assess the interaction between stress treatment (ST) and harvesting time (HT). The two-way ANOVA results are reported in Table S4 of Supplementary Materials.

We investigated the relationships between the 22 traits measured within each halophyte species by computing the Pearson correlation coefficients (r) and then testing their significance with $\alpha = 0.05$. For each species, the correlation matrix is shown as a network diagram where each entity of the dataset represents a node, and highly correlated variables are clustered together. Each path represents a correlation between the two variables it joins. A blue path represents a positive correlation, and a red path represents a negative correlation. Only significant correlations ($p < 0.05$) are represented. The width and transparency of the line represent the strength of the correlation (wider and less transparent = stronger correlation).

Two principal component analyses were carried out on the data collected at the first (PCAstress) and second harvest time (PCArecovery) to summarise the performances outlined by the three genotypes under the Stress and Recovery periods with a multivariate approach.

The principal components (PCs) were obtained from centred and scaled quantitative variables through the diagonalisation of the correlation matrix and extraction of the associated eigenvectors and eigenvalues. All 22 measured traits were set as active quantitative variables, whereas the three halophyte species (S. *europaea*, *S. veneta*, and *S. fruticosa*) and the three treatments (Ctrl, SS, WS) were used as supplementary categorical variables, i.e., variables that were not used in the computation of PCs. The Pearson correlation coefficients were determined between the PCs and each quantitative variable (the 22 measured traits). The associated *p*-values were calculated to classify the variables according to their relevance (Table S2 of Supplementary Materials).

All the statistical analyses were performed with the R 6.3.6 statistical software, using Car [61] and Emmeans [62] packages for the analysis of variance and post hoc test, and the FactoMineR package for principal component analysis [63]. Charts were created with the ggplot2 [64] and corrr [65] R packages.

6.3. Results

6.3.1. Substrate Electric Conductivity and Moisture

During the stress period, the substrate electric conductivity (EC) increased significantly in the pots subjected to salt stress, reaching over 15 dS m^{-1} for all three halophytes, with a maximum of 21 dS m^{-1} in *S. fruticosa*, whereas the water stress treatment did not cause any change in the control EC values (Figure 2A). After 15 days of watering the pots with non-saline water ('recovery' treatment), the substrate EC in salt-treated pots decreased to control values (for *S. europaea* and *S. veneta*) or even slightly (but significantly) below the control for *S. fruticosa*. However, substrate salinity in the pots previously subjected to the withholding of irrigation remained similar to the controls after recovery (Figure 2A).

Contrary to the EC data, the substrate water content, with control values of about 65% for all three halophytes, was not affected by the salt treatment; however, soil moisture decreased significantly under water deficit conditions, down to between 25 and 30%, depending on the species (Figure 2B). After recovery from water stress, substrate moisture increased to reach values equal or even higher (in *S. veneta*) than the controls, whereas recovery from salt stress did not alter the soil water content when compared to the corresponding controls (Figure 2B).

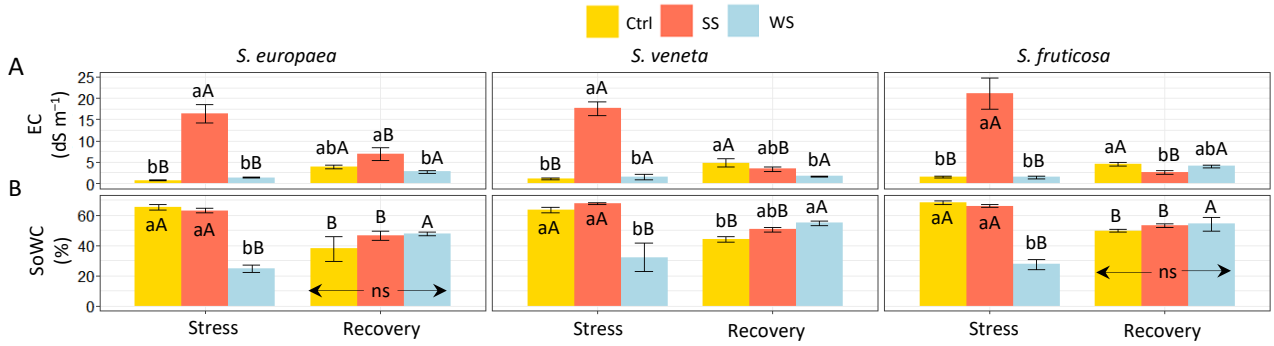

Figure 2. Effect of 30 days of stress treatments (Stress), followed by watering with non-saline water for 15 days (Recovery) on (**A**) Substrate electrical conductivity (soil EC) and (**B**) water content (soil WC). Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). For each species and sampling (Stress or Recovery), different lowercase letters over the bars indicate significant differences between treatments (Ctrl, SS, and WS) at $p \leq 0.05$. Different uppercase letters indicate significant differences between the two sampling times (Stress and Recovery) for each species and treatment, at $p \leq 0.05$. Vertical bars indicate standard error ($n = 4$).

6.3.2. Plant Growth

Plant height and the number of branches were measured in all plants at the beginning (T0) and every 15 days during the experiments; that is, after 15 and 30 days of water or salt stress and at the end of the 'recovery' treatment (Table 3). Both parameters increased significantly during the stress treatments in control and stressed plants. The salt treatment did not cause significant growth inhibition in any of the three species. In contrast, compared to the control, water deficit induced a significant plant height reduction in the two *Salicornia* species and also a reduction (down to 57% of the control) in the number of branches in *S. europaea*. However, this inhibitory effect was only observed after 30 days of withholding irrigation, not at day 15 of the treatment (Table 3). These data indicate a strong tolerance of the three species to salinity, even at very high salt concentrations (700 mM NaCl), and a slightly higher drought sensitivity of the two *Salicornia* species compared to *Sarcocornia fruticosa*.

Table 3. Plant height (cm) and number of branches in the three halophytes (SE, *S. europaea*; SV, *S. veneta*; SF, *S. fruticosa*) measured at the beginning (T0) and after 15 (T15), 30 (T30), or 45 (T45) days of starting the stress treatments. Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). The values shown are means ± SE ($n = 4$). For each species, different lowercase letters in a column indicate significant differences between the three treatments within the same sampling time, whereas different uppercase letters in each row indicate significant differences between sampling times for the same treatment, at $p \leq 0.05$.

		Plant Height (PH) (cm)				Number of Branches (No. B)			
		T0	Stress (T15)	Stress (T30)	Recovery (T45)	T0	Stress (T15)	Stress (T30)	Recovery (T45)
SE	Ctrl	5.8 ± 0.3 aC	8.7 ± 0.5 aB	12.9 ± 1.0 aA	12.3 ± 1.0 aA	5.7 ± 0.5 aC	10.2 ± 0.7 aB	18.1 ± 1.8 aA	23.8 ± 3.3 aA
	SS	5.7 ± 0.3 aC	8.5 ± 0.4 aB	11.4 ± 0.5 aA	10.5 ± 0.6 aA	6.3 ± 0.5 aC	11.0 ± 0.7 aB	18.2 ± 1.2 aA	20.8 ± 1.5 aA
	WS	5.3 ± 0.3 aC	7.6 ± 0.4 aB	7.0 ± 0.6 bA	10.4 ± 0.9 aA	6.0 ± 0.5 aB	12.0 ± 2.1 aAB	10.3 ± 1.5 bA	16.9 ± 2.4 aA
SV	Ctrl	9.8 ± 0.4 aC	14.7 ± 0.7 aB	21.6 ± 2.0 aA	22.6 ± 1.8 aA	2.1 ± 0.3 aC	6.9 ± 0.6 aB	11.8 ± 1.6 aA	13.0 ± 1.7 aA
	SS	10.2 ± 0.4 aC	15.8 ± 0.5 aB	20.6 ± 0.8 abA	19.9 ± 1.5 aA	1.5 ± 0.3 aC	8.0 ± 0.6 aB	10.1 ± 0.9 aA	15.0 ± 3.4 aA
	WS	9.6 ± 0.5 aC	15.1 ± 0.5 aB	16.1 ± 0.6 bA	19.6 ± 1.5 aA	1.5 ± 0.3 aC	7.3 ± 0.5 aB	9.5 ± 0.8 aB	9.3 ± 2.4 aA
SF	Ctrl	5.6 ± 0.3 aC	8.9 ± 0.5 aB	13.1 ± 1.3 aA	14.6 ± 1.3 aA	0.4 ± 0.2 aD	8.1 ± 1.0 aC	18.9 ± 3.0 aB	28.5 ± 1.5 aA
	SS	5.1 ± 0.4 aC	9.2 ± 0.4 aB	11.6 ± 0.7 aA	13.1 ± 0.6 aA	0.4 ± 0.2 aC	8.8 ± 1.0 aB	18.4 ± 2.3 aA	21.7 ± 1.7 bA
	WS	5.0 ± 0.3 aC	8.3 ± 0.5 aB	10.0 ± 0.9 aA	12.6 ± 0.7 aA	0.5 ± 0.2 aD	8.7 ± 1.0 aC	14.0 ± 2.4 aB	20.8 ± 2.5 bA

After 15 days of recovery, the plant height and the number of branches of *S. europaea* and *S. veneta* plants were statistically homogeneous in all treatments (control and water and salt stress); the same result was observed for plant height in *S. fruticosa*. The number of branches increased during recovery in the latter species but to a lesser extent in the previously stressed plants, which did not reach the control values (Table 3).

After the stress and recovery periods, plants were harvested to determine shoot fresh weight (FW) and water content percentage (WC) as the most reliable parameters to assess the treatment effects on plant growth. Salt stress did not affect the shoot FW or WC of the *Salicornia* species significantly, whereas *S. fruticosa* plants appeared to be slightly more affected, with a more accentuated (but still non-significant) reduction in the mean FW and a slight (but significant) reduction in WC (Figures 3A,B). On the other hand, water stress strongly reduced shoot FW in the three species (Figure 3A), partly due to plant dehydration, as it was accompanied by a small but significant WC decrease compared to the control plants (Figure 3B).

After recovery, the salt-stressed plants of the three halophytes maintained a shoot FW and WC similar to their corresponding controls. However, watering with non-saline water had distinct effects on plants previously subjected to water deficit, depending on the species. Thus, *S. europaea* plants showed a significant increase in FW upon recovery, but with values still well below those of the control plants and the complete rehydration of the shoots; in contrast, no significant effects were observed in *S. veneta*. Only in *S. fruticosa* shoot FW did not show any statistically significant differences from the control after recovery, although the mean value was lower (Figure 3). Therefore, confirming the measurements of other growth parameters, *S. fruticosa* appears to be more tolerant to drought than the *Salicornia* species and also shows better recovery from the water deficit treatment.

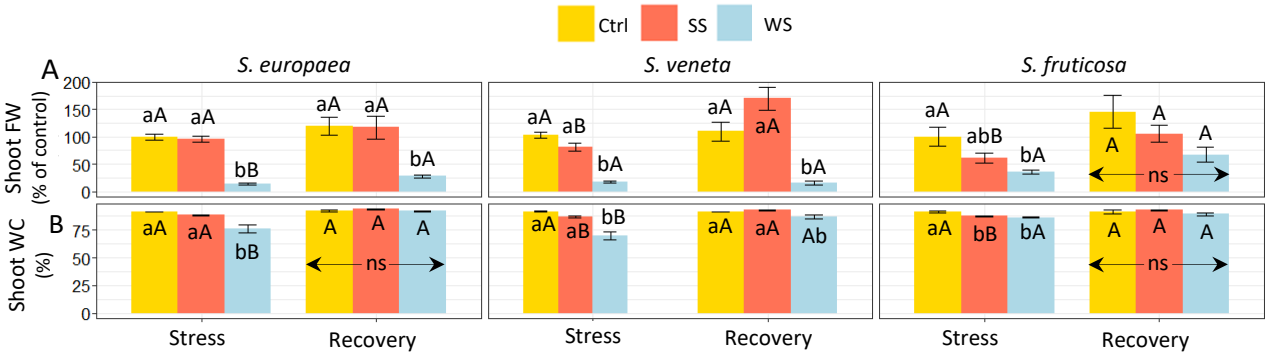

Figure 3. Effect of 30 days of stress treatments (Stress), followed by watering with non-saline water for 15 days (Recovery) on (**A**) shoot fresh weight (FW) and (**B**) shoot water content (SWC) in the three halophytes. Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). For each species and sampling (Stress or Recovery), different lowercase letters over the bars indicate significant differences between treatments (Ctrl, SS, and WS), whereas different uppercase letters indicate significant differences between the two samplings (Stress and Recovery) for each species and treatment, at $p \leq 0.05$. Vertical bars indicate standard error ($n = 4$). Values in (**A**) are shown as percentages of shoot FW of control plants (Ctrl, Stress), taken as 100%; the corresponding absolute values for *S. europaea*, *S. veneta*, and *S.fruticosa* were 13.3, 10.1, and 5.6 g plant^{-1}, respectively.

6.3.3. Photosynthetic Pigments

Mean values of photosynthetic pigment contents showed a decreasing trend in response to the salt treatment in plants of the two annual *Salicornia* species (Figure 4); however, the differences with the non-stressed plants were only significant for chlorophyll a (Chl. a) in *S. europaea* (Figure 4A) and carotenoids (Caro) in *S. veneta* (Figure 4C), whereas no variations in chlorophyll b (Chl. b)(Figure 4B) , the second most abundant chlorophyll in oxygenic photosynthetic organisms, were recorded. After irrigation with non-saline water, no significant differences with the controls were found for any pigment. In contrast, water deficit caused a significant reduction in the levels of the three pigments in both annual species; in all cases, mean pigment contents increased after the recovery treatment, reaching values not significantly different from the controls. On the other hand, in the perennial *S. fruticosa,* neither salt nor water stress induced any significant variation

in pigment concentrations, and the recovery treatment had no effect, except for a slight yet significant increase in Caro levels in salt-treated plants. However, it should be mentioned that the pigment levels in the *S. fruticosa* control plants were lower than those determined in *S. europaea* and *S. veneta* (Figure 4). These responses agree with the observed stress-induced changes in growth parameters, confirming the high salt tolerance of the three species, the relatively higher drought tolerance of *S. fruticosa* compared to the annual species, and the effectiveness of the recovery treatment.

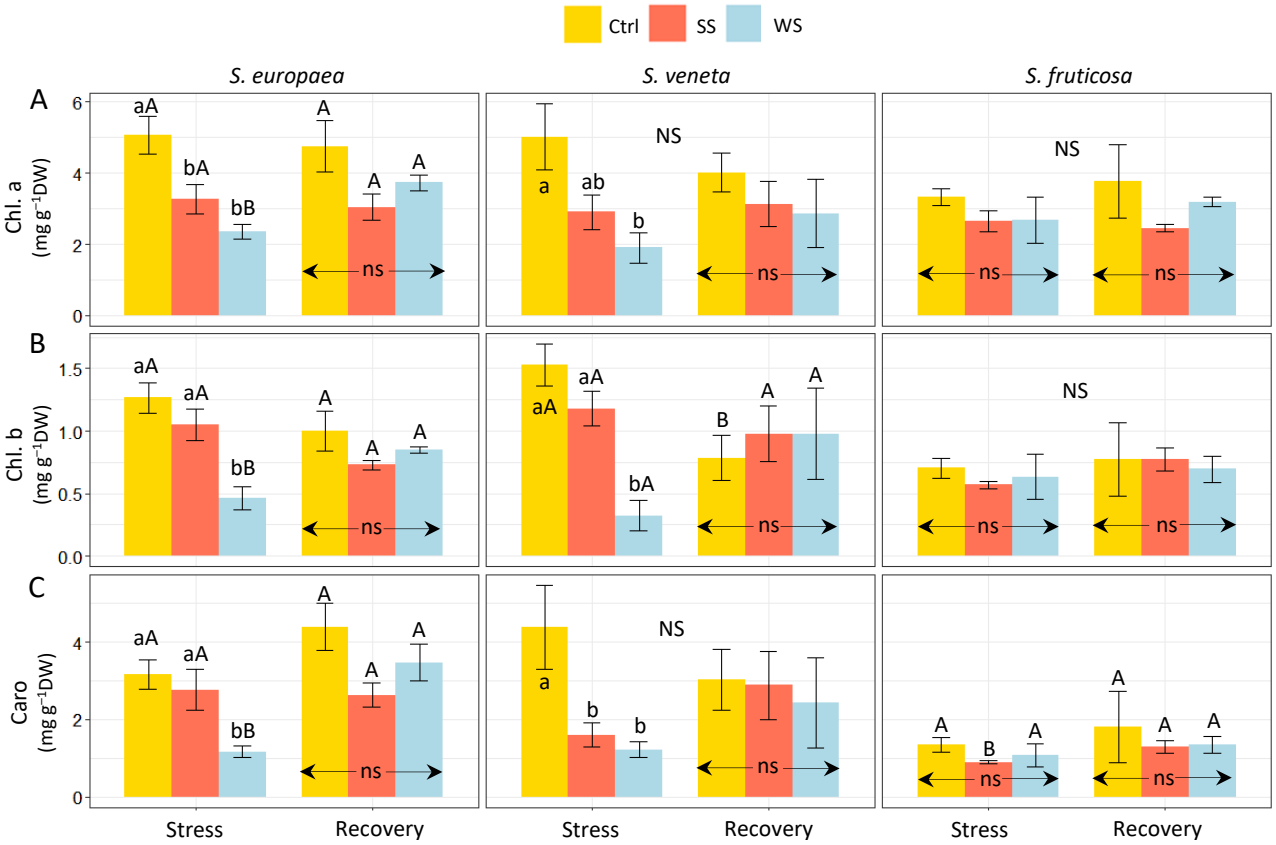

Figure 4. Effect of 30 days of stress treatments (Stress), followed by watering with non-saline water for 15 days (Recovery) on (**A**) chlorophyll a (Chl. a), (**B**) chlorophyll b (Chl. b), and (**C**) carotenoids (Caro) in the three halophytes. Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). For each species and sampling (Stress or Recovery), different lowercase letters over the bars indicate significant differences between treatments (Ctrl, SS, and WS), at $p \leq 0.05$; ns: non-significant. Different uppercase letters indicate significant differences between the two samplings (Stress and Recovery) for each species and treatment, at $p \leq 0.05$; NS: non-significant. Vertical bars indicate standard error ($n = 4$).

6.3.4. Ion Accumulation

Root and shoot Na^+ and Cl^- concentrations increased significantly in response to the salt stress treatment in the three halophytes, as expected, whereas water deficit did not have any effect on the ions levels. The recovery treatment reduced the contents of both ions in roots of salt-stressed plants down to control levels, except for Na^+ in *S. veneta*, which showed a still significant but less accentuated decrease. In contrast, no differences were observed in shoot Na^+ or Cl^- contents before and after recovery, except *for S. europaea*, in which Cl^- content increased slightly but significantly in the control. Under all tested conditions, the concentrations of both ions were substantially higher in shoots than in roots (Figure 5A,B).

Variations of K^+ concentrations showed different patterns, depending on the species and the treatments (Figure 5C). First, control levels in the roots of non-stressed plants differed substantially between species, being the highest in *S. veneta*—about 1.7-fold higher than in *S. europaea* and three-fold higher than in *S. fruticosa*, approximately. Shoot K^+ contents were similar to those in roots in *S. europaea*, whereas they were higher in shoots than in roots in *S. veneta* and *S. fruticosa*. The stress treatments did not cause changes in the root K^+

concentration, except for the significant decrease observed in salt-stressed *S. veneta* plants. At the shoot level, mean K^+ concentrations decreased upon salt treatment, although the difference with the control was non-significant in *S. europaea*. Under water stress, K^+ contents increased, decreased, and remained the same as in the controls in *S. europaea*, *S. veneta*, and *S. fruticosa*, respectively. After recovery, K^+ concentrations were generally lower than control values in the roots and shoots of salt-stressed plants and not significantly different from the controls in plants previously subjected to water stress, although some exceptions to this general behaviour were observed in *S. europaea* (Figure 5C).

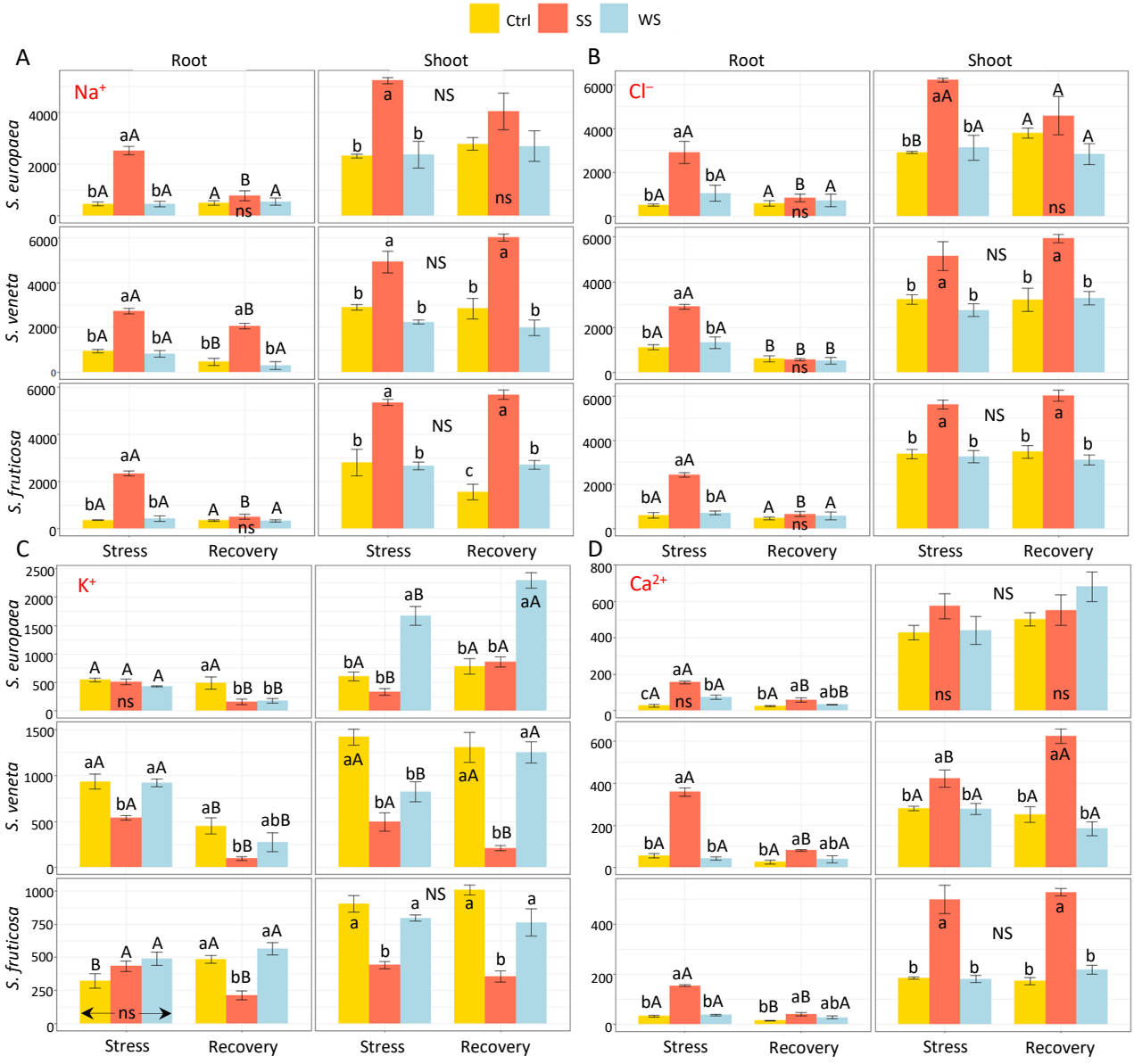

Figure 5. Effect of 30 days of stress treatments (Stress) followed by watering with non-saline water for 15 days (Recovery) on the root and shoot concentration (in µmol g^{-1} DW) of ions: (**A**) sodium (Na^+), (**B**) chloride (Cl^-), (**C**) potassium (K^+), and (**D**) calcium (Ca^{2+}) in the three halophytes. Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). For each species and sampling (Stress or Recovery), different lowercase letters over the bars indicate significant differences between treatments (Ctrl, SS, and WS), at $p \leq 0.05$; ns: non-significant. Different uppercase letters indicate significant differences between the two samplings (Stress and Recovery) for each species and treatment, at $p \leq 0.05$; NS: non-significant. Vertical bars indicate standard error ($n = 4$).

The patterns of Ca^{2+} variation in the roots of the three species were similar to those observed for Na^{+} and Cl^{-}, that is, a significant increase in response to salt stress and no effect of water stress except for an increase in *S. europaea* (Figure 5D). Shoot Ca^{2+} concentration significantly increased in the salt-treated plants of *S. veneta* and *S. fruticosa*, but not of *S. europaea*, with no effect of water stress. After the recovery period, root Ca^{2+} concentration in the salt-stressed plants decreased but remained significanlty higher than in control plants, and was statistically comparable with the water-stressed plants. In shoots, the Ca^{2+} concentration did not vary after recovery, except for an increase in the salt-treated plants of *S. veneta* (Figure 5D).

6.3.5. Osmolytes, Oxidative Stress Markers and Antioxidants

Common osmolytes, glycine betaine (GB), proline (PRO), and total soluble sugars (TSS) were determined and showed distinct accumulation patterns in the shoots of the selected species (Figure 6). Neither salt stress nor water deficit caused any significant change in GB contents in *S. europaea*; they augmented three-fold over control values in salt-stressed *S. veneta* and about 2.5-fold in *S. fruticosa* plants subjected to water stress. After the recovery period, the GB level increased significantly in non-stressed *S. europaea* and *S. veneta* plants and decreased in those of *S. fruticosa* that underwent the water deficit treatment. Nevertheless, no significant differences between treatments were found in the shoot GB contents of any of the three halophytes after recovery (Figure 6A).

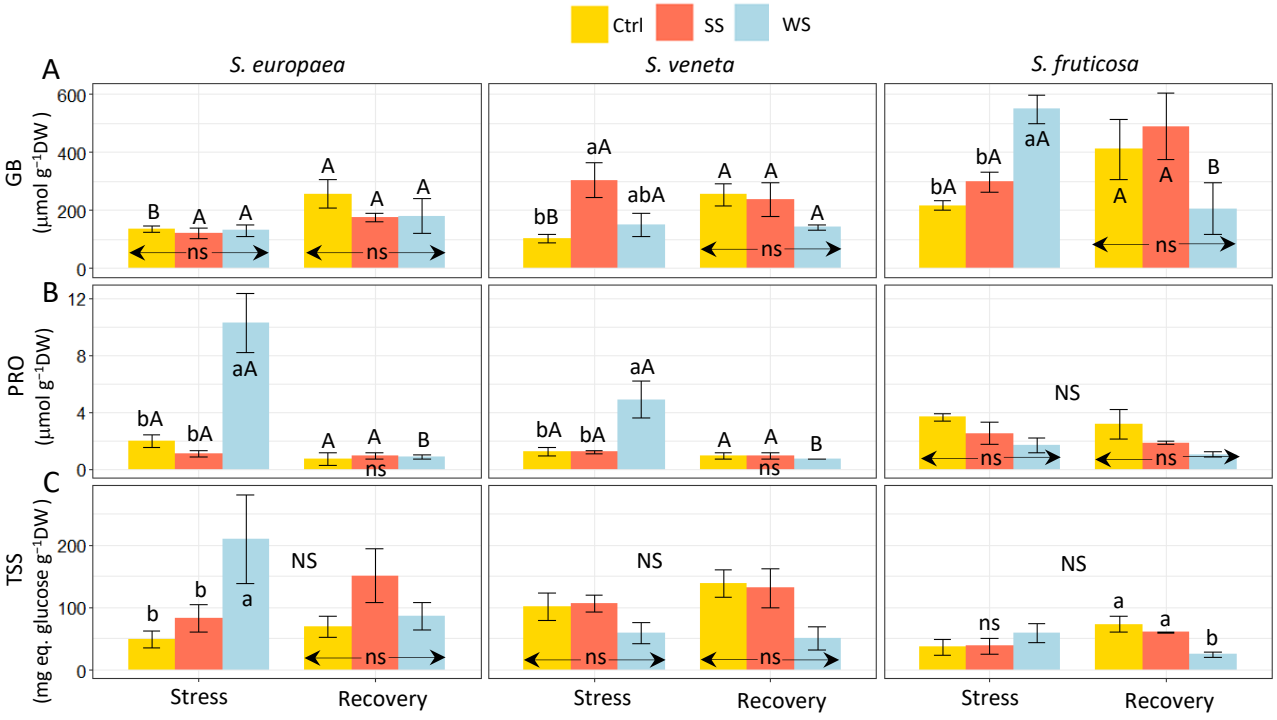

Figure 6. Effect of 30 days of stress treatments (Stress) followed by watering with non-saline water for 15 days (Recovery) on shoot concentration of (**A**) glycine betaine (GB), (**B**) proline (PRO), and (**C**) Total Soluble Sugars (TSS) in the three halophytes. Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). For each species and sampling (Stress or Recovery), different lowercase letters over the bars indicate significant differences between treatments (Ctrl, SS, and WS), at $p \leq 0.05$; ns: non-significant. Different uppercase letters indicate significant differences between the two samplings (Stress and Recovery) for each species and treatment, at $p \leq 0.05$; NS: non-significant. Vertical bars indicate standard error ($n = 4$).

PRO contents did not vary in any species in response to salt stress but increased in the water-stressed plants of *S. europaea* (about five-fold over the control) and, to a lesser extent, *S.veneta* (ca. four-fold). In these two *Salicornia* species, PRO levels decreased to control values after the recovery period, so that, in all cases, the differences between treatments became non-significant. In *S. fruticose*, no variation in PRO contents was

observed, for any of the samples, after the stress treatments and after recovery (Figure 6B). Under all experimental conditions, PRO concentrations in molar terms were much lower than those of GB in the three species. GB contents ranged between 100 and more than 500 μmol g^{-1} DW, whereas the maximum measured PRO level (in water-stressed *S. europaea* plants) was only ca. 10 μmol g^{-1} DW (Figure 6A,B).

Only the water-stressed *S. europaea* plants showed a significant increase in shoot TSS levels; all other differences between control and stressed plants in the stress and recovery treatments, or between the two samplings, were non-significant (Figure 6C).

To assess the possible generation of secondary oxidative stress in the plants subjected to salt or water stress treatments, the contents of two reliable biochemical markers, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), were determined in the shoots of all plants (Figure 7). No increase in MDA or H_2O_2 levels was detected in any of the samples from the stressed plants in relation to the non-stressed controls. MDA contents even decreased in some cases, namely under salt stress in *S. europaea* and under water stress in *S. veneta*. In contrast, no differences in H_2O_2 content between stressed and control plants were detected in the three species. A significant increase in MDA concentration was observed after the recovery period in the salt-stressed plants of *S. europaea* and *S. fruticosa* and in the water-stressed plants of *S. europaea* and *S. veneta*. On the other hand, H_2O_2 levels increased after recovery in the salt-treated plants of *S. veneta* and *S. fruticosa* (Figure 7).

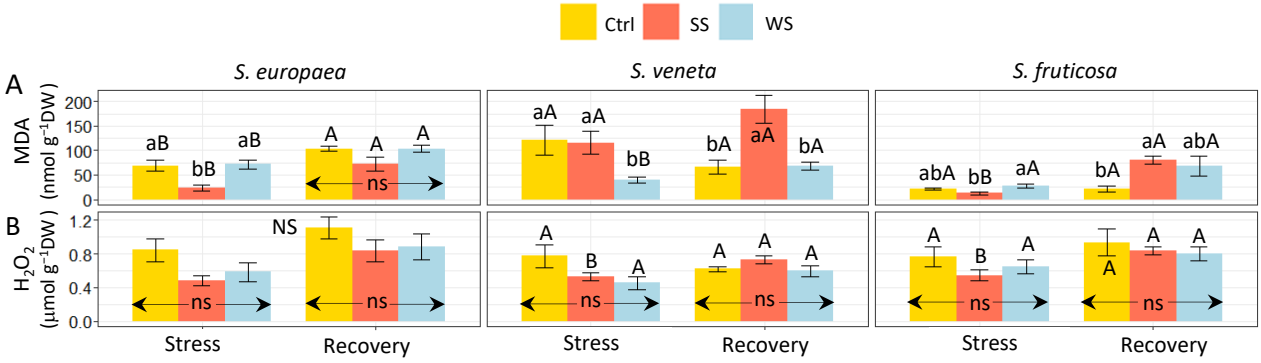

Figure 7. Effect of 30 days of stress treatments (Stress) followed by watering with non-saline water for 15 days (Recovery) on shoot concentration of (**A**) Malondialdehyde (MDA) and (**B**) hydrogen peroxide (H_2O_2) in the three halophytes. Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). For each species and sampling (Stress or Recovery), different lowercase letters over the bars indicate significant differences between treatments (Ctrl, SS, and WS), at $p \leq 0.05$; ns: non-significant. Different uppercase letters indicate significant differences between the two samplings (Stress and Recovery) for each species and treatment, at $p \leq 0.05$; NS: non-significant. Vertical bars indicate standard error ($n = 4$).

In agreement with the lack of a detectable generation of oxidative stress under high salinity and water deficit conditions, the activation of the synthesis of common antioxidant compounds, such as phenolic compounds (TPC) and, particularly, the subgroup of flavonoids (TF), was also not observed. Indeed, differences in TPC and TF contents between treatments during the stress and recovery periods were generally non-significant, except for the TF reduction in response to salt in *S. fruticosa*. Moreover, no differences were detected between samplings for each treatment (Figure 8).

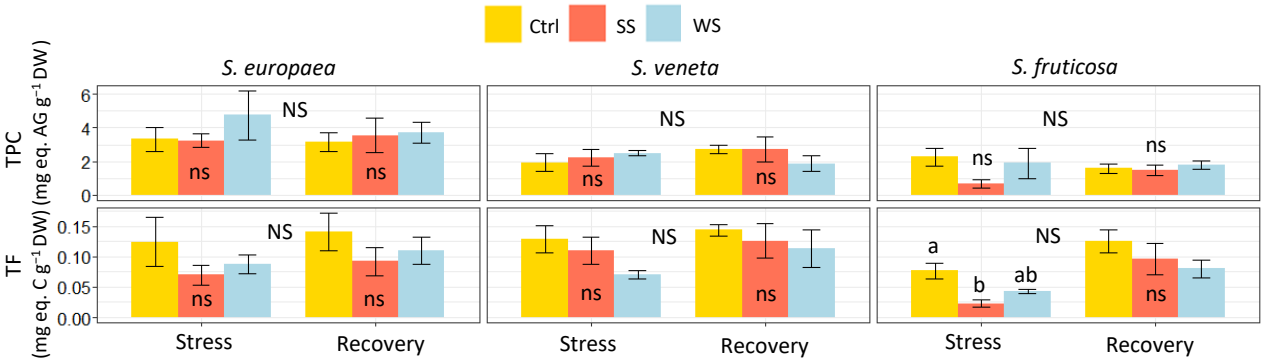

Figure 8. Effect of 30 days of stress treatments (Stress) followed by watering with non-saline water for 15 days (Recovery) on shoot concentration of (**A**) total phenolic compounds (TPC) and (**B**) total flavonoids (TF) in the three halophytes. Ctrl, control; SS, salt stress (watering with 700 mM NaCl); WS, water stress (complete withholding of irrigation). For each species and sampling (Stress or Recovery), different lowercase letters over the bars indicate significant differences between treatments (Ctrl, SS, and WS), at $p \leq 0.05$; ns: non-significant. Different uppercase letters indicate significant differences between the two samplings (Stress and Recovery) for each species and treatment, at $p \leq 0.05$. NS: non-significant. Vertical bars indicate standard error ($n = 4$).

6.3.6. Physiological Traits Relationships and Results of the Multivariate Analysis

In the three surveyed species, some common trait patterns could be observed (Figure 9). The pigments, namely Chl. a, Chl. B, and Caro, were positively correlated with each other in all three species, indicating their covariation. The potassium shoot concentration, K(s), instead, always resulted in being negatively correlated with Na(r), Na(s), and Cl(s). Plant FW was consistently positively correlated with the shoot water content (SWC), which was positively associated with Chl. a and Caro contents in the two annual plants. Furthermore, SWC in the two *Salicornia* species was negatively correlated with PRO, as the content of this osmolyte mostly increased under water stress, when the plant SWC was the lowest.

The near absence of significant correlations between TPC and other growth-related traits confirmed that salinity and water deficit, under our experimental conditions, did not generate a substantial degree of oxidative stress in the plants.

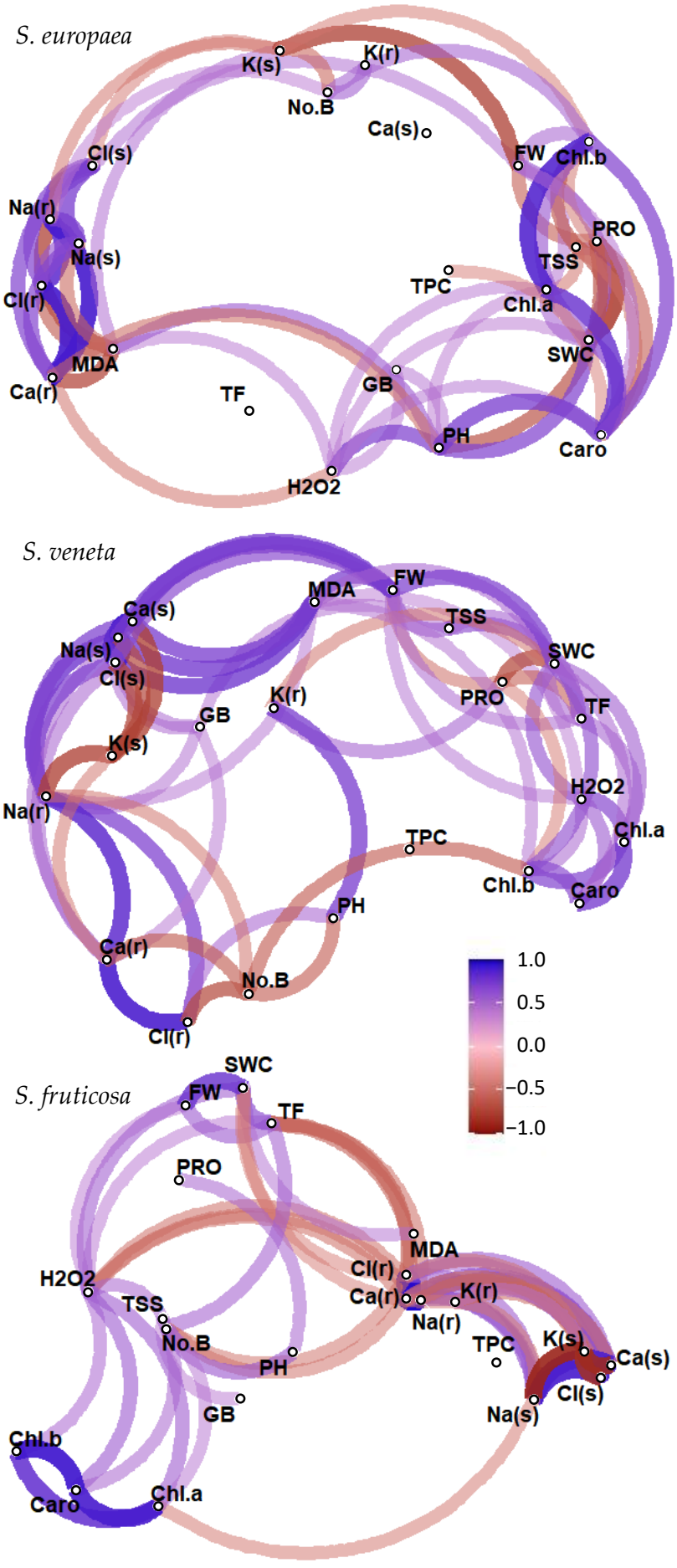
S. europaea
K(s)
K(r)
No.B
Ca(s)
FW
Chl.b
Cl(s)
Na(r)
PRO
Na(s)
TSS
TPC
Cl(r)
Chl.a
SWC
MDA
Ca(r)
TF
GB
PH
Caro
H2O2
S. veneta
MDA
FW
Ca(s)
TSS
Na(s)
SWC
Cl(s)
K(r)
PRO
TF
GB
K(s)
H2O2
Na(r)
TPC
Chl.a
Chl.b
Caro
PH
Ca(r)
No.B
Cl(r)
1.0
0.5
0.0
−0.5
−1.0
S. fruticosa
SWC
FW
TF
PRO
MDA
H2O2
Cl(r)
Ca(r)
K(r)
TSS
Na(r)
No.B
TPC
K(s)
Ca(s)
PH
Cl(s)
GB
Na(s)
Chl.b
Caro
Chl.a

Figure 9. Correlation network diagram showing significant correlations ($p < 0.05$) between the 22 measured traits within each halophyte species, based on the calculation of the Pearson correlation coefficients. Each measured trait represents a node, and highly correlated traits are clustered together. Each path represents a correlation between the two variables it joins. A blue path represents a positive correlation and a red path represents a negative correlation. Only significant correlations are represented. The width and transparency of the line represent the strength of the correlation (wider and less transparent = stronger correlation). Abbreviations: fresh weight (FW), shoot water content (SWC), plant height (PH), number of branches (No.B), chlorophyll a (Chl. a), chlorophyll b (Chl. b), carotenoids (Caro), root sodium concentration (Na(r)), shoot sodium concentration (Na(s)), root chloride concentration (Cl(r)), shoot chloride concentration (Cl(s)), root potassium concentration (K(r)), shoot potassium concentration (K(s)), root calcium concentration (Ca(r)), shoot calcium concentration (Ca(s)), glycine betaine (GB), proline (PRO), total soluble sugars (TSS), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total phenolic compounds (TPC), total flavonoids (TF).

Two principal component analyses (PCAs) were performed to further evaluate the relationships among traits after the stress (PCAstress) and recovery (PCArecovery) treatments and to quantify the strength and direction of correlations between the original traits and the extrapolated principal components (PCs). The first three PCs (eigenvalues are reported in Table S1 of the Supplementary Materials) explained 62% and 53% of the total variance in PCAstress and PCArecovery, respectively, and were used for PCA interpretation. The correlation circles and the biplots of the first two components, PC1 and PC2, and the variables measured after the 30 days of stress (PCAstress) and the 15 days of recovery (PCArecovery) are reported in Figure 10.

In PCAstress, PC1 accounted for the differences between the salt stress treatment, whose barycentre was located on the positive side of PC1, and the water stress treatment, whose barycentre was located on the negative side of PC1 (Figure 10B). PC1 was positively correlated with Na(r) (0.87), Na(s) (0.85), Cl(s) (0.82), Cl(r) (0.79), Ca(r) (0.75), and FW (0.62), and negatively correlated with K(s) (−0.63) and PRO (−0.56) (Figure 10A), meaning that the accumulation of Na, Cl, and Ca is the primary mechanism helping to sustain plant growth under salt stress, whereas PRO production and K(s) accumulation are the main mechanisms adopted under water stress.

PC2 showed the relationship between Na^+ and Cl^- accumulation, pigment production, and oxidative stress. PC2, indeed, presented the strongest positive correlations with Caro (0.84), Chl. a (0.83), Chl. b (0.75), and the highest negative correlations with Na(s) (−0.39) and Cl(s) (−0.39) (Figure 10A), meaning that the accumulation of these ions interfered with the production of pigments. Interestingly, the barycentres of the two annual species were located on the positive side of the PC2 axes, whereas the barycentre of *S. fruticosa* was located on the negative side (Figure 10B), indicating that pigment production was less affected by ion accumulation in this latter species.

PC3, finally, summarised the relationship between the plant species and the osmolytes. This third component was positively correlated with TSS (0.78), PRO (0.54), and TPC (0.42), and negatively correlated with GB (−0.51) (Table S2 of Supplementary Materials). *S. europaea* and *S. veneta* barycentres were placed on the positive side of the PC3 axis, whereas *S. fruticosa* was in the negative one (Table S3 of Supplementary Materials). This may suggest that the annual species rely on the production of sugars, proline, and phenolic compounds for osmotic adjustment under stress conditions, whereas the perennial species depends more on glycine betaine accumulation for its stress tolerance.

The PCArecovery outlined some evident changes: as in the PCAstress, the PC1 accounted for the different effects of the stress treatments, with the salt stress barycentre placed on the positive side of the PC1 axis and the water stress and control barycentres clustered on the negative side (Figure 10D), suggesting that, after recovery, water-stressed plants behaved similarly to control plants. PC1 was correlated positively with Na(r) (0.81), whose concentration decreased after recovery, especially in salt-treated plants, and negatively with K(r) (−0.55) (Figure 10C), whose concentration decreased after recovery, especially in the annual water-stressed plants.

The PC2 highlighted the differences between the annual *S. europaea* and the perennial *S: fruticosa*, with *S. veneta* showing an intermediate behaviour between the two other species. The barycentre of *S. europaea* was placed on the positive side of the PC2 axis (Figure 10D), which was positively correlated with PH (0.85), Caro (0.78), Chl. a (0.63), and Chl. b (0.44) (Figure 10C), whereas the barycentre of *S. fruticosa* was on the negative

side. This placement reflects the fact that the recovery of these traits was more pronounced in *S. europaea* than in *S. fruticosa*, since these traits were compromised more seriously in the annual than in the perennial species under water stress.

Finally, the third component differentiated the control treatment, standing on the positive PC3 side (Table S3 of Supplementary Materials), from the water stress treatment, standing on the negative PC3 side. PC3 was positively correlated with Chl. a (0.64) and Chl. b (0.57), as control plants showed the highest pigment content even at the recovery stage and was negatively correlated to K(s) (−0.35) (Table S2 of Supplementary Materials), which increased in water-stressed plants after the recovery, especially in the two annual halophytes.

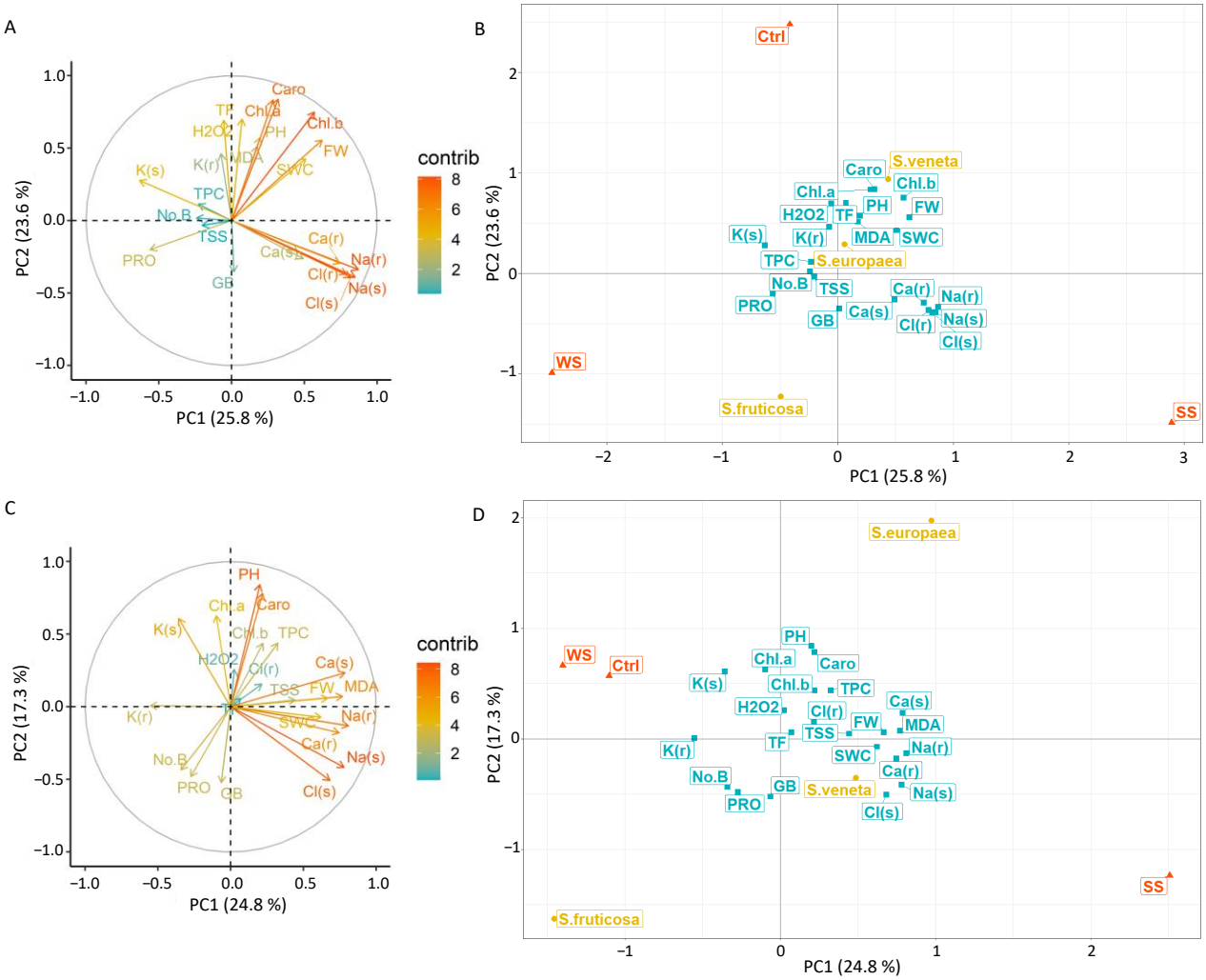

Figure 10. PCA correlation circles of the 22 measured parameters: (**A**) after 30 days of stress treatments (PCAstress) and (**C**) after 15 days of watering with non-saline water (PCArecovery). The increasing arrow lengths and shades of colour from light blue to red indicate the increasing contribution of variables to the definition of the first two principal components. PCA biplot of variables (**B**) after 30 days of stress treatments (Stress) and (**D**) after 15 days of watering with non-saline water (Recovery). Yellow circles show the barycentres of the three halophyte species (*S. europaea*, *S. veneta*, *S. fruticosa*), orange triangles show the barycentres of the three experimental treatments (Ctrl, control; SS, salt stress (watering with 700 mM NaCl water solution); WS, water stress (complete withholding of irrigation)), and the light blue squares show the quantitative variables, i.e., the measured traits (fresh weight (FW), shoot water content (SWC), plant height (PH), number of branches (No.B), chlorophyll a (Chl. a), chlorophyll b (Chl. b), carotenoids (Caro), root sodium concentration (Na(r)), shoot sodium concentration (Na(s)), root chloride concentration (Cl(r)), shoot chloride concentration (Cl(s)), root potassium concentration (K(r)), shoot potassium concentration (K(s)), root calcium concentration (Ca(r)), shoot calcium concentration (Ca(s)), glycine betaine (GB), proline (PRO), total soluble sugars (TSS), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total phenolic compounds (TPC), total flavonoids (TF).

6.4. Discussion

Cultivating drought- and salt-tolerant crops can build resilience to climate change and enhance farm productivity and livelihoods in drought- and salt-prone areas. Generally, salinity and drought regimes are not stable but fluctuate seasonally and geographically, depending on the climate and hydrological conditions of each specific environment. Thus, the extent to which a species can cope with these fluctuations is an important trait that can be selected for saline agriculture.

Salicornia europaea, S. veneta, and *Sarcocornia fruticosa* are three halophytic species already traded in the market as leafy vegetables and oil-seed crops, thanks to their high content of nutritional compounds with valuable health-related properties. The natural saline habitats of these species are especially sensitive to climate change effects, which will include more frequent, more intense, and longer drought periods and higher soil salinity levels, albeit with wide seasonal variations [66].

From a general overview of our results, all three species were shown to be remarkably tolerant to salinity but sensitive to water deficit, albeit to a lesser extent in *S. fruticosa*, which showed higher resistance to dehydration and greater ability to recover after drought exposition. Our findings are supported by the ecology and the evolutionary trends within this lineage of species. In the Mediterranean, the two genera grow in close sympatry but are separated ecologically [16]. *Salicornia* dominates inland or coastal lagoons which may remain flooded for longer periods after winter rains. By acquiring an annual life cycle, *Salicornia* species were able to adapt to more unstable habitats and to expand to colder northern areas [16]. European *Sarcorcornia* are frost sensitive and grow only in winter-mild Atlantic coasts or drier Mediterranean areas [14].

The surveyed *S. fruticosa* seeds were collected from a semiarid zone (La Albufera Natural Park, Valencia, Spain), with a mean annual temperature, precipitation, and evapotranspiration of 17.5 °C, 488 mm, and 1199 mm, respectively. On the other hand, the *S. europaea* and *S. veneta* seeds were sampled from a more humid area (Piallassa della Baiona, Ravenna, Italy), having mean annual temperature, precipitation, and evapotranspiration of 14.6 °C, 576 mm, and 828 mm, respectively. This difference in environmental conditions may be the primary reason for developing a more robust drought tolerance in *S. fruticosa*. However, the slower metabolism of perennial plants could represent an advantageous adaptive strategy for survival under stress conditions since it allows for the saving of water and resource consumption while enhancing the synthesis of protective compounds [67]. This may have contributed to the better performance of the perennial *S. fruticosa* under water stress with respect to the annual *S. europaea* and *S. veneta.*

Photosynthetic pigment contents in *S. fruticosa* were not affected by salinity or drought stress, whereas a reduction in pigment contents was recorded in *S. europaea* and *S. veneta*, being generally modest under salt stress but severe in response to water deficit. Here again, these differences could be a consequence of the better adaptation of *S. fruticosa* to semiarid conditions or dependent on its life cycle type. When exposed to stress, annual plants hasten the transition from the vegetative to the reproductive stage, activating a process of stress-induced senescence that shifts nutrient allocation to developing seeds [68,69]. The stress-induced senescence is regulated differently and occurs more gradually in the perennial plants, since they can also propagate vegetatively. When they experience stress, perennial plants prioritise biomass accumulation in roots, whose contribution to stress avoidance is fundamental, protect photosynthetic tissues to sustain C assimilation and boost the source strength, and enhance the conservation of meristematic tissues, which are essential for recovering after the stress period [70,71]. This basic distinction may also explain the different variations in pigment contents under stressful conditions between the perennial *S. fruticosa* and the two annual *S. europaea* and *S. veneta*. In any case, the two annual species were able to restore their pigment pools during the recovery phase.

Similar ion accumulation patterns were observed in all three species, with a consistent increase in Na^+ and Cl^- concentrations at the root and shoot level in response to high salinity. This response is in line with the finding that halophytes can take up and efficiently compartmentalise the ions naturally present in the growth media to conserve the water potential gradient and maintain water uptake [72]. The salt-treated plants retained their high content of Na^+ and Cl^- in the shoots notwithstanding the recovery treatment, since the transport of these ions, to be used as inorganic osmolytes, is energetically cheaper than the *de novo* synthesis of organic osmolytes [73]. It should also be pointed out that Na^+ and Cl^- content in shoots were very high, and much higher than in roots, in the absence of salt; that is, in the control and water stress treatments. This result

indicates the active transport of these ions to the aboveground organs, even at low external salinity, so that Na^+ and Cl^- can contribute to cellular osmotic balance also in non-stressed and water-stressed plants.

Salinity, however, caused a decrease in K^+ translocation to the shoots, likely related to the antagonism between K^+ and Na^+ ions, which are physicochemically similar [74]. This is evident in the PCAstress correlation circle, where the Na and Cl arrows are opposite to the K(s) arrow, implying that an increase in the former ions caused a decrease in the latter ion. The significant increase in K^+ shoot allocation under water stress suggested that this ion is a key osmoticum used to maintain water status in *Salicornia* and *Sarcocornia* spp. under water stress conditions. Indeed, water-stressed plants held a high K^+ shoot content even after recovery.

The significant increase in Ca^{2+} concentration under high salinity conditions in both below- and aboveground organs supports the notion that Ca, being involved in a diverse array of sensor proteins, plays a central role in orchestrating the whole-plant response to salt stress [75,76]. Indeed, Ca^{2+} content was positively correlated with Na^+ and Cl^- contents in the PCAstress correlation circle. The ability to preserve Ca uptake and retention under salinity seems to be a common feature of halophytes, since it was also reported in other salt-tolerant species such as *Sarcobatus vermiculatus*, *Climacoptera turcomanica*, *Salicornia persica*, *Halimocnemis pilifera*, *Petrosimonia glauca*, and *Atriplex verrucifera* [77].

To sum up, the effects of recovery on ion contents were relevant on roots, which are the organs more directly and dynamically in contact with the external environment, whereas ion remobilisation within shoots was not substantially affected by the recovery treatment.

Besides accumulating inorganic ions, glassworts species synthesise several organic osmolytes under osmotic stress, which contribute to cellular osmotic adjustment, free radical scavenging, and the activation of specific signalling pathways.

In both the stressed and non-stressed plants of the two genera, *Salicornia* and *Sarcocornia*, relatively high absolute values of GB were quantified, suggesting that GB accumulation is a constitutive defence mechanism against osmotic stress. Responses of these plants to abiotic stress probably rely more on changes in GB subcellular compartmentalisation, i.e., GB redistribution from the vacuole to the cytoplasm, rather than its *de novo* synthesis. There is indeed evidence for stress-induced changes in the intracellular localisation of compatible solutes in halophytes, for example, in *Limonium latifolium* [78]; however, data on these putative mechanisms are still scarce. Still, GB concentration can increase in response to stress, as observed under salinity in *S. veneta* and, mostly, in water-stressed *S. fruticosa* plants, suggesting that the higher drought tolerance of this latter species is partly due to a relatively higher GB accumulation.

Proline (PRO) is probably the most common compatible solute in plant species [79]. Nevertheless, no significant change in PRO concentration was detected in our experiments, except for the increase under water stress in *S. europaea* and *S. veneta*. However, the measured absolute PRO concentrations were too low to have any relevant osmotic effect when compared to GB or ion contents in the shoots. Still, PRO could have contributed to enhanced stress tolerance through its additional ability to scavenge ROS, directly stabilise proteins and other cellular structures, and provide cellular redox potential [80].

Comparing these outcomes, it appears that GB is the major organic osmolyte contributing to drought tolerance in *S. fruticosa*, whereas PRO plays a relatively more relevant role *in S. europaea* and *S. veneta*. Indeed, after recovery from water stress, a drop in GB concentration was observed in *S. fruticosa*, and PRO levels decreased significantly in *S. europaea* and *S. veneta*. These results are in agreement with the findings reported by Gil et al. [42], who measured high (>400 μmol g^{-1} DW) GB and very low (1–2 μmol g^{-1} DW) PRO concentrations in *S. fruticosa* under field conditions in the aforementioned semiarid La Albufera Natural Park, and with the results of Parida and Jha [81], who found PRO to be the main organic osmolyte accumulated in response to drought stress in *Salicornia brachiata*.

This supports the assumption that typical GB-accumulating species generally contain low PRO levels and vice versa [82], as already observed in many species, including both halophytes and glycophytes. For example, in the halophyte *Spartina alternifolia*, in the presence of 600 mM NaCl, GB contents were 10-fold higher than those of PRO (ca. 150 vs. 15 μmol g^{-1} FW, respectively) [83]. The differences were much more pronounced in another halophyte, *Halocnemum strobilaceum*, showing GB values > 200-fold greater than those of PRO (700 vs. 3 μmol g^{-1} DW) under 690 mM NaCl [84]. A similar pattern, although with much lower absolute values, was found in the glycophyte *Spinacia oleracea* in the presence of 170 mM NaCl, showing GB concentrations (3.25

μmol g^{-1} FW) about four-fold higher than those of PRO (0.78 μmol g^{-1} FW) [85]. Conversely, PRO appears to contribute relatively more to osmotic balance under drought conditions (200–400 μmol g^{-1} DW) than GB (40–60 μmol g^{-1} DW) in the genus *Capsicum* [86]. The halophyte *Juncus maritimus* also accumulated PRO rather than GB in response to salt stress (400 mM NaCl): ca. 130 vs. 25 μmol g^{-1} DW, respectively [87]. Similarly, a preferential accumulation of PRO over GB was observed in the halophyte *Limonium santapolense* under drought stress (ca. 120 vs. 23 μmol g^{-1} DW, respectively) [88].

The accumulation of the total soluble sugars (TSS) may enhance drought tolerance in *S. europaea*, since TSS levels increased in response to the water stress treatment; however, their contribution to *S. veneta* and *S. fruticosa* stress resistance was negligible. This result is in contrast to previous studies that have reported TSS accumulation as the primary mechanism for osmotic adjustment in *S. fruticosa* [20] and *Salicornia persica* [89]. However, as discussed by Gil et al. [90], sugar accumulation should be interpreted with caution. In fact, unlike other osmolytes occurring in plants at very low levels, unless stressful conditions stimulate their biosynthesis, soluble sugars are components of primary metabolism that play different functional roles unrelated to stress responses. This may be the reason why no significant changes in TSS contents were observed after stress recovery in any of the three studied species.

The fact that the stress treatments did not increase the levels of oxidative stress markers, i.e., MDA and H_2O_2, revealed that no oxidative stress was generated by salt or water stress in any of the three species. In some cases—salt stress in *S. europaea* and water stress in *S. veneta*—the contents of the oxidative stress markers, i.e., MDA and H_2O_2, even decreased with respect to the non-stressed controls. This response may be due to the increased activity of peroxidase, which is generally stored in the peroxisome and vacuoles, and plays an active role in reducing oxidative stress decreasing lipid peroxidation [91].

Consequently, we did not detect a significant accumulation of non-enzymatic, antioxidant compounds, i.e., total phenolic (TPC) or flavonoid (TF) compounds. This is reflected in the PCAstress correlation circle, in which the short and faded MDA, H_2O_2, TPC, and TF arrows denote a weak contribution of these traits to the variability of the whole dataset.

Taken together, these results suggest that the stress responses based on ion transport control and osmolyte accumulation were efficient enough to avoid or even reduce oxidative stress under our experimental conditions. However, we must note that the absence of oxidative stress may also result, at least in part, from efficient enzymatic ROS-detoxifying machinery, based on the activity of antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase, and peroxiredoxin [92], among others, which were not specifically addressed in this study.

6.5. Conclusions

The three investigated halophytes, the annual *S. europaea* and *S. veneta* and the perennial *S. fruticosa*, are highly tolerant to salinity but sensitive to water stress, although the latter species to a lesser extent. Salt tolerance seems to depend mainly on the salt-induced accumulation of ions (Na^+, Cl^- and Ca^{2+}) and the shoot biosynthesis of organic osmolytes, both contributing to osmotic adjustment under stress. Active transport of these ions to the aerial part of the plants and high concentrations of glycine betaine have also been detected in the control, non-stressed plants, indicating that these defence mechanisms against stress are at least partially constitutive.

The higher drought tolerance of *S. fruticosa*, compared to its annual counterparts, was reflected in a relatively lower reduction in shoot fresh weight and the absence of a decrease in photosynthetic pigment content under water deficit conditions and was attributed to the relatively higher accumulation of glycine betaine. *Sarcocornia fruticosa* also showed total recovery capacity after the water stress treatment, whereas the fresh weight of the water-stressed plants of *S. europaea* and *S. veneta* remained at values significantly lower than the controls after the recovery period.

Neither salinity nor drought stress generated oxidative stress. Consequently, the presence of stress response mechanisms based on the activation of antioxidant systems was not expected; indeed, no significant increase in the levels of antioxidant compounds was detected in any of the three halophytes. However, further studies should be carried out to assess the possible contribution of enzymatic antioxidant activities to the whole antioxidant network of these species.

The higher drought tolerance observed in *S. fruticosa* with respect to the two *Salicornia* species could be based on differences in the environmental conditions of the plants' natural habitats, as it is drier for *S. fruticosa*. However, a more gradual process of stress-induced senescence in the perennial *S. fruticosa* compared to the annual *S. europaea* and *S. veneta*, might have allowed water-stressed plants to preserve their pool of photosynthetic pigments and recover to control fresh weight after rewatering. Further studies will be required to confirm this hypothesis, including, for instance, the assessment of the responses to water deficit of annual and perennial plants growing in the same natural habitat.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Table S1. Eigen analysis of PCAstress and PCArecovery correlation matrix; Table S2. Correlation coefficients between the first three PCs (PC1, PC2, PC3) and the quantitative variables traits (fresh weight (FW), shoot water content (SWC), plant height (PH), number of branches (No.B), chlorophyll a (Chl. a), chlorophyll b (Chl. b), carotenoids (Caro), root sodium concentration (Na(r)), shoot sodium concentration (Na(s)), root chloride concentration (Cl(r)), shoot chloride concentration (Cl(s)), root potassium concentration (K(r)), shoot potassium concentration (K(s)), root calcium concentration (Ca(r)), shoot calcium concentration (Ca(s)), glycine betaine (GB), proline (PRO), total soluble sugars (TSS), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total phenolic compounds (TPC), total flavonoids (TF). The PCs were computed using 22 input data. Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$; Table S3. Coordinates of the barycentres of the supplementary categorical variables in PCAstress and PCArecovery biplots, respectively; Table S4. Two-way analysis of variance (ANOVA) of stress treatments (ST), harvesting time (HT), and their interactions (STxHT) for the three halophyte species, for the 22 measured traits (fresh weight (FW), shoot water content (SWC), plant height (PH), number of branches (No.B), chlorophyll a (Chl. a), chlorophyll b (Chl. b), carotenoids (Caro), root sodium concentration (Na(r)), shoot sodium concentration (Na(s)), root chloride concentration (Cl(r)), shoot chloride concentration (Cl(s)), root potassium concentration (K(r)), shoot potassium concentration (K(s)), root calcium concentration (Ca(r)), shoot calcium concentration (Ca(s)), glycine betaine (GB), proline (PRO), total soluble sugars (TSS), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total phenolic compounds (TPC), total flavonoids (TF). Significance codes: ^{ns}, $^{(+)}$, *, **, and *** mean, respectively, not significant and significant at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

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

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Data Availability Statement: Data is contained within the article or supplementary material.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 7: General Discussion and conclusion

This section recaps in a consistent way and with a broader viewpoint the results exposed in the previous chapters, integrating further considerations to comprehensively discuss the prospect of using halophytes as alternative crops in saline agriculture.

The accelerating trends of soil salinization and the shortage of freshwater are constraining crop production in many world areas. This is particularly relevant for less-developed, arid and semi-arid countries, in which problems of soil and water quality degradation are even more serious. In these circumstances, the use of modest-quality water to support cultivation on marginal soils is expanding rapidly. Saline water resources are more abundant than freshwater. Bringing these resources into sustainable productive use will offer opportunities to sustain food production while preventing freshwater depletion. However, this water cannot be directly applied to conventional crops without significant yield penalties. Its application also raises concerns about the risk of furtherly aggravating land salinization. Therefore, making long-term sustainable use of saline water resources requires ongoing research and monitoring.

The research work described in this thesis aimed at investigating, through a series of pot experiments, the response to saline water irrigation across a range of salt-tolerant plants potentially eligible as novel crop types for saline agriculture. These experiments gave the opportunity to observe how different plant species interact with saline water, and which morphological, physiological, and biochemical mechanisms they stage to cope with salinity.

Leaching, i.e. the practice of displacing salts from topsoil layers through freshwater irrigation, is considered the key element for a successful and long-time sustainable saline agriculture.

The experiment carried out on *Sorghum bicolor*, a multifunctional crop able to grow in areas with limited water supply, was intended to assess the effect of practicing salt leaching when irrigation water is saline itself. The results, illustrated in the second chapter, offer evidence that applying excess saline water for salt leaching, although involving a greater addition of salts to the soil, is more advantageous for plant growth than a regime of deficit irrigation aimed at containing salt addition into the growth media. These findings suggest that the application of higher volumes of water, although saline, fostered a reduction of the osmotic pressure exerted by salts, mitigating its detrimental effects on the plant homeostasis.

On the basis of these outcomes, it can be inferred that the beneficial effect of dilution due to the higher volume of applied water was greater than the detrimental effect due to the increase in salt content. However, the long-term net balance between these two opposite processes is difficult to predict, especially from an environmental point of view.

Furthermore, the study did not consider a series of other variables that can affect the effectiveness of leaching with saline water, such us the variability in soil texture and structure which, in turn, influence the infiltration and water-holding capacity, the ratio of saturation water content to field capacity, and the aeration conditions. The spatial variability of these soil properties is, by itself, a further difficulty to tackle in the field.

Moreover, it is necessary to consider that without an adequate drainage system, either natural or artificially constructed, leaching with saline water will inevitably result in a water table rise with consequent salinization of the rhizosphere and loss of aeration.

Provided that an appropriate system of irrigation and drainage has been installed to rationally manage salt flows within the soil-groundwater continuum, the second pillar for successful saline agriculture is the detection of suitable salt-tolerant species.

To select salt-tolerant genotypes, it is first necessary to establish an effective screening method. Germination and the early growth stages are the phenological phases most sensitive to salinity and, generally, varieties tolerating salinity at germination continue being resistant at later stages. Thus, evaluating the plant response to salinity at these stages represents a robust system to evaluate their tolerance potential.

However, many of the developed germination index are adversely affected by salinity showing inconsistent outputs when compared. For this reason, it is difficult to judge which are the most reliable ones.

Therefore, in the third chapter of this thesis eight commonly used germination indices were compared (Germination percentage (GP), germination energy (GE), germination value (GV), coefficient of germination

velocity (CVG), germination rate index (GRI), peak value (PV), mean germination time (MGT), and time to 50% germination (T_{50})), to evaluate which are the most appropriate for screening salt tolerance at germination stage, using *S. europaea* as a model crop.

The seeds were exposed to progressively higher levels of water salinity (control, 100, 200, 300 and 600 mM NaCl), and six out of eight applied indices exhibited consistency in detecting the germination response across the wide range of salt doses. In particular, the six indices detected a first drop in halophyte germination at 100 mM NaCl. Thereafter, no significant variation in germination was assessed up to 600 mM NaCl, a level after which a second and most severe germination drop was recorded.

The results hinted that low-medium salt stress (up to 400 mM NaCl) might break *S. europaea* seed dormancy and promote germination. At a higher salt concentration (600 mM NaCl), the germination was severely reduced but not completely impeded, confirming the high tolerance of *S. europaea* to salinity stress.

However, when screening plants for salt tolerance at the germination stage, it shall be taken into account that, among other adaptation strategies, halophytes can rely on the escape strategy, i.e. the ability to germinate when salinity is attenuated and the germination conditions are most favourable. This occurs after the rainy season, when the salts accumulated in the growth media are largely diluted.

Through this "escaping" mechanism, plants do not need to develop tolerance strategies to cope with salinity at the germination phase; therefore, even some of the more salt-tolerant halophytes may result salt sensitive at the germination stage. Thus, attention must be paid to avoid erroneous evaluations, attributing a low degree of tolerance to a species that is salt-tolerant as an adult but sensitive at the seedling stage.

Our model species presented an intermediate behaviour, since it showed maximum germination in the total absence of salinity, and a progressive, slight germination reduction with NaCl concentration increase.

Besides the escape strategy, a variety of different adaptation strategies have been developed by halophytes during their evolution under extreme habitats. Salt tolerance is given by a complex and multifaceted integration of mechanisms, and the diversity of successful forms and physiological types confounds generalizations. Thus, to amplify our understanding of the strategies involved and allow a better discernment, we restricted our field of observation to the Mediterranean basin and selected six distinguished halophytes diverging in habitus and life cycle, to be studied as model species.

The six species, *Artemisia absinthium*, *Artemisia vulgaris*, *Atriplex halimus*, *Chenopodium album*, *Salsola komarovii*, and *Sanguisorba minor,* were grown in pots and were irrigated with solutions at increasing NaCl concentration (control, 100, 200, 300 and 600 mM) for more than 4 months. Their reaction to the salinity treatments was assessed by measuring a series of parameters related to biomass accumulation, water homeostasis, and stomatal gas exchange.

As expected, the response across the species showed remarkable differences. *A. halimus* and *C. album* were shown to be the best adapted species to salinity, followed by the group of S. *komarovii* and *A. vulgaris*, whereas *S. minor* and *A. absinthium* emerged as the least able to adapt to increasing salinity levels.

What distinguished the former three halophytes species was their higher ability to modulate gas exchange under salinity. Indeed, in *A. halimus* and *C. album* the gas exchange was not impaired under low-intermediate levels of salinity, while in *S. komarovii* it was reduced, although to a lesser extent compared to the remaining halophytes. Furthermore, *A. halimus* showed the greatest lowering of leaf potential, probably obtained through active sodium compartmentalization into the vacuoles for osmotic adjustment, which allowed the plant to retain water in its tissue, while at the same time maintaining sufficient stomatal opening and subsequent gas exchange.

It is interesting to note that the three best performing species are all belonging to the Amaranthaceae family which, together with the Poaceae, are the two botanical families containing the greatest number of halophytic species [148]. Even today, research is investigating the possible evolutionary, biogeographic, and ecological reasons for the high incidence of salt-tolerant species within the Amaranthaceae family, although some authors consider this phenomenon related to structural anomalies frequently occurring within this family [149].

At increasing salinity, the larger decrease in stomatal conductance and, hence, in CO_2 uptake in the other three genotypes was probably responsible for the higher growth inhibition already at low salinity levels. Moreover, the two *Artemisia* species showed pronounced symptoms of dehydration already at low salinity

concentrations, most likely associated with the scarce capacity to lower the leaf water potential, which is a necessary adjustment to maintain water inside the tissues.

From this inter-genus comparative study, it emerged that control of stomatal conductance and the ability to lower water potential to sustain water retention are two important factors in the network of physiological attributes required for salt tolerance.

The marked tolerance to salinity demonstrated by *Atriplex halimus* with respect to the other species, could be related to its photosynthetic pathway, being a C4 plants.

It has been argued that the adaptation to harsh environments, such as arid or saline habitats, has pushed selection for C4 photosynthesis. Indeed, the potential for reduced transpiration rates in C_4 plants may be an advantage under salinity. The consequent higher water-use efficiency affords to reduce water (and salt) influx into the plant. This, in turn, lessens the amount of salts a plant has to exclude, compartmentalize, or secrete per unit of fixed carbon [150].

Moreover, it is worth noting that some C_4 plants require small amounts of Na^+ for growth [151] and do not thrive in its absence [152]. In some C_4 plants, sodium ions play a major rule in the osmotic adjustment [153] and are involved in the process of CO_2 concentration through the Na^+-coupled pyruvate transporters in chloroplasts [154]. However, the Na^+ requirement for growth in C_4 plants is not universal, as shown by some C_4 grasses, like maize and sugarcane, which do not benefit from the presence of this ion [152].

Starting from these considerations, a comparative study between the C4 *Atriplex halimus* and its C3 relative *Atriplex hortensis* was designed, with the aim of shedding light on the relation between salt tolerance and photosynthetic pathway.

In this experiment, the two species were watered with solutions at increasing NaCl content (ranging from 0 to 360 mM NaCl), and a series of morphological and physiological parameters were measured to assess their response.

The results met our expectations. Both species thrived under salinity, but the C4 *Atriplex halimus* performed slightly better than *A. hortensis,* thanks to the ability to maintain higher net photosynthesis despite the stronger stomatal conductance and transpiration drop under salinity. Additionally, *A. halimus* showed a greater lowering in leaf water potential, which allowed this plant to maintain a higher relative water content.

However, the photosynthetic pathway is not the only difference distinguishing these two species, since *A. halimus* is a perennial bush while *A. hortensis* is an annual plant.

Thus, we questioned how the length of the life cycle can affect the response to salinity in halophytic plants. On a side, annual plants must succeed to complete their life cycle within a year, even under adverse growing conditions. On the other side, perennial species must be able to survive across years even if the unfavourable conditions are prolonged and, sometimes, fluctuating. Owing to this, plant response to environmental stresses is generally different between annual and perennial species, as well as their recovery capacity.

Annual plants usually accelerate their transition to the reproductive stage when exposed to a source of stress, inducing nutrient remobilization from the vegetative organs to the fruits/seeds. Although this trait ensures the survivability of the species' next generation, it causes early senescence, greater loss of biomass, and, sometimes, even plant death [155,156]. Perennial plants have a different source–sink transition, allocating biomass and nutrients preferentially for vegetative growth. Indeed, they prepare to deal with stress by developing larger and deeper root systems [157], and by accumulating water-soluble carbohydrates in the meristematic tissues, that have the ability to remain alive, ensuring growth once the stressing episode has ended [158].

Hence, when exposed to stress, annual plants experience an early and sometimes total leaf senescence in favour of seed production. Compared to this, perennial plants decrease leaf elongation gradually, and induce senescence processes only in the oldest leaves [159], while in the younger leaves thylakoid membranes remain intact and chlorophyll loss is mild [160]. This phenomenon allows photosynthates assimilation to be maintained in young tissues, so that the plant can resume growth once the stress event ends.

To explore more in depth the relationship between life cycle length and the ability to adapt and recover from environmental stress, we set up an experiment by selecting three halophyte species belonging to the

Amaranthaceae subfamily of Salicornioideae, i.e. the two annual *Salicornia europaea* and *S. veneta*, and the perennial *Sarcocornia fruticosa*.

The plants were exposed to a month of sever salt and drought stress (irrigation with water solution at 700 mM NaCl and total withholding of irrigation, respectively) and then were allowed to recover during the following 15 days at full freshwater supply.

All three species resulted highly tolerant to salinity but sensitive to water stress, although the perennial *S. fruticosa* to a lesser extent. In all three species, the high salinity tolerance seemed to depend on efficient control of ions transport toward the aerial parts, as well as on the biosynthesis of organic osmolytes (glycine betaine, proline, soluble sugars) for intracellular osmotic adjustment. The greater drought tolerance of *S. fruticosa* with respect to the two *Salicornia* species, attributed to its higher constitutive level of glycine betaine, could depend on the fact that the surveyed *S. fruticosa* developed in a drier and warmer environment compared to the two annual species. However, we cannot exclude that the ability to modulate more gradually the process of stress-induced senescence in the perennial *S. fruticosa* with respect to the annual species may have contributed to its greater drought tolerance.

All the three species responded positively to the recovery treatment, restoring their biochemical values to levels as control. Nevertheless, the fresh weight of the water stressed plants of *S. europaea* and *S. veneta* remained significantly lower than control, confirming their greater susceptibility to water deficit conditions.

These observations might advise that the perennial *S. fruticosa* could be more resilient to dynamic environmental stress conditions and, hence, more suitable for cultivation of areas characterized by temporal variability in drought and soil salinity.

According to the Food and Agriculture Organization [161], mainstreaming the use of perennial crops into the current agronomic systems can contribute to stabilize fragile soils and prevent soil erosion, supplying at the same time a series of additional ecological and economic benefits. Indeed, respect to annual plants, perennial crops do not need to be sown every year, can provide higher ground cover and produce more extensive root systems, which make them more competitive against weeds and more effective in minimizing nutrient leaching and capturing water [162].

At the conclusion of this excursus, it should be evidenced that the outcomes described so far derive from pot experiments in a controlled environment, i.e. under conditions that cannot be assumed to be representative of the growing conditions in wild spaces nor at field level. Indeed, the response function to drought and salinity depends on soil type, duration of exposure and interaction with other sources of biotic and abiotic stresses e.g. waterlogging, high temperature, etc. Therefore, there is a dire need for research on halophyte domestication as premise for cultivation under field conditions. This could allow us to verify how the observed behaviours are affected by the interaction with other environmental factors, which, moreover, may be variable in time and space.

Investigating the strengths and threats of saline agriculture is crucial to evaluate its broader benefits and impacts, as well as to assist the implementation of guidelines aiding its sustainable establishment.

It is hoped that the insights provided with this research on some salt-tolerant species holding promise as future crops may contribute to build a stronger agricultural knowledge on saline agriculture, and trace an initial road map to support growers' confidence in the shrewd use of saline water for agricultural production.

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