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A MULTIDISCIPLINARY APPROACH TO UNDERSTAND THE EFFECTS OF SALINIZATION ON CROP-WEED INTERACTIONS

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Abstract

Soil salinization is increasingly affecting agro-ecosystems, contributing to the loss of arable land and reduction of crop yield. Due to climate change and the depletion of water resources, the agricultural land impacted by salinization is expanding worldwide. Sea-level rise and groundwater overexploitation, causing saltwater intrusion in coastal and inland aquifers, are among the main factors expected to exacerbate the negative effects of salinity. So far, most of the studies regarding salinization in agro-ecosystems have targeted either the control of soil salinity or the enhancement of crop stress tolerance. On the contrary, limited attention has been given to weed species, despite they represent one of the major constraints in plant production. Since weeds are known for their earlier emergence, faster growth rates and higher genetic plasticity compared to cultivated species, their adaptability to adverse environmental conditions might promote their spread under increasing salinity. Therefore, it is essential to investigate how morphological and physiological traits affect weed species and their interaction between weed and crop species. To assess the effects of the temperature-salinity interaction on germination and early growth stages, we carried out classic germination tests and growth tests on nutrient agar media at different combinations of salinity (0, 4, 8, 12, 16 dS/m) and temperature (12, 15, 18, 24 °C). To assess the effects of weed-crop competition, we arranged repeated hydroponic experiments with single- and mixed-species tanks, and greenhouse experiments with single- and mixed-species pots, in both cases comparing salt-treated and control plants. Plant height, chlorophyll content, fresh and dry weight, antioxidant enzyme activity, phenolic content, lipid peroxidation and proline content were used as indicators to evaluate the response to salt stress. In total, we analysed 4 crops (soybean, maize, rice, barley) and 11 weed species (*Abutilon theophrasti, Amaranthus retroflexus, Avena sterilis, Chenopodium album, Digitaria sanguinalis, Echinochloa crus-galli, Lolium rigidum, Oryza sativa var sylvatica, Portulaca oleracea, Setaria pumila, Setaria viridis*) collected in Italy and Greece. Results indicate that many weed species associated with spring-summer crops in the Mediterranean basin and other temperate and semi-arid regions show high adaptability and resilience to salt stress. Although germination and early growth stages are considered to be the most sensitive to salt for many plant species, we demonstrated that even weed ecotypes never exposed to salinity are capable of germinating and developing roots and shoots when exposed to moderate to strong salt stress. *A. theophrasti*, *C. album* and *S. pumila* appeared to be the most tolerant weed species at this stage, potentially representing an increased threat in semi-arid and

temperate regions affected by salinization. In these stages, the temperature played a key role: when far from the optimum, the effects of salt stress were accentuated in both winter and summer species. Considering also the weed-crop competition factor, *C. album* was confirmed to be the most tolerant among the analyzed species, resulting in decreased height, SPAD values and biomass production and increased lipid peroxidation in soybean grown with the weed. However, even weed species that appeared to be more sensitive, such as *A. retroflexus*, maintained their competition potential when grown with a crop, meaning that the presence of the crop did not exacerbate the effects of salinity in weed species. These results corroborate the hypothesis of increased weed competitiveness in salty environments. The comparison between winter and summer species suggested that whenever yield loss on summer crops becomes too extreme due to secondary salinization, winter crops, especially salt-tolerant ones such as barley, might be a cost-effective option also in terms of weed management.

Riassunto

La salinizzazione del suolo ha un impatto sempre maggiore sugli agroecosistemi, contribuendo alla perdita di aree coltivabili e alla riduzione della resa delle colture. A causa dei cambiamenti climatici e dello sfruttamento eccessivo delle risorse idriche, i terreni agricoli colpiti dalla salinizzazione si stanno espandendo in tutto il mondo. L'innalzamento del livello del mare e l'eccessivo sfruttamento delle acque sotterranee, che causano l'intrusione salina nelle falde acquifere costiere e interne, sono tra i principali fattori che potrebbero in futuro esacerbare gli effetti negativi della salinità. Finora, la maggior parte degli studi sulla salinizzazione negli agro-ecosistemi ha riguardato il controllo della salinità del suolo o il miglioramento della tolleranza allo stress delle colture. Al contrario, è stata prestata un'attenzione limitata alle specie infestanti, nonostante rappresentino uno dei maggiori vincoli nella produzione vegetale. Poiché le piante infestanti sono note per la loro comparsa precoce, tassi di crescita più rapidi e una maggiore plasticità genetica rispetto alle specie coltivate, la loro adattabilità a condizioni ambientali avverse potrebbe favorirne la diffusione in condizioni di salinità crescente. Pertanto, è essenziale indagare in che modo i tratti morfologici e fisiologici influenzano le specie infestanti e l'interazione tra piante infestanti e specie coltivate. Per valutare gli effetti dell'interazione temperatura-salinità sulla germinazione e sulle prime fasi di crescita, abbiamo effettuato i classici test di germinazione e di crescita su terreni nutritivi a base di agar a diverse combinazioni di salinità (0, 4, 8, 12, 16 dS/m) e temperatura (12, 15, 18, 24 °C). Per valutare gli effetti della competizione tra piante infestanti e colture, abbiamo organizzato ripetuti esperimenti idroponici con vasche a specie singola e specie miste, ed esperimenti in serra con vasi a specie singola e specie miste, in entrambi i casi confrontando piante trattate con sale e piante di controllo. Altezza della pianta, valori SPAD, peso fresco e secco, attività enzimatica antiossidante, contenuto fenolico, perossidazione lipidica e contenuto di prolina, sono stati utilizzati come indicatori per valutare la risposta allo stress salino. In totale sono state analizzate 4 colture (soia, mais, riso, orzo) e 11 specie infestanti (*Abutilon theophrasti*, *Amaranthus retroflexus*, *Avena sterilis*, *Chenopodium album*, *Digitaria sanguinalis*, *Echinochloa crus-galli*, *Lolium rigidum*, *Oryza sativa var sylvatica*, *Portulaca oleracea*, *Setaria pumila*, *Setaria viridis*) raccolte in Italia e Grecia. I risultati indicano che molte specie infestanti associate a colture primaverili-estive nel bacino del Mediterraneo e in altre regioni temperate e semiaride mostrano un'elevata adattabilità e resilienza allo stress salino. Sebbene la germinazione e le prime fasi di crescita siano considerate le più sensibili al sale per molte specie vegetali, abbiamo dimostrato che anche gli ecotipi di infestanti mai esposti alla salinità sono in grado di germinare e sviluppare radici e germogli se esposti a uno stress salino da moderato a forte. *A. theophrasti*, *C. album* e *S. pumila* sembrano essere le specie infestanti più tolleranti in questa fase, rappresentando potenzialmente

una minaccia maggiore nelle regioni semi-aride e temperate colpite dalla salinizzazione. In queste fasi la temperatura ha giocato un ruolo fondamentale: quando non ottimale, gli effetti dello stress salino sono stati accentuati sia in specie invernali che estive. Considerando anche il fattore competizione infestante-coltura, *C. album* si è confermato essere la più tollerante tra le specie analizzate, con conseguente riduzione dell'altezza, del contenuto di clorofilla e della produzione di biomassa e aumento della perossidazione lipidica nella soia cresciuta insieme ad esso. Tuttavia, anche le specie infestanti che sembravano essere più sensibili, come *A. retroflexus*, hanno mantenuto il loro potenziale competitivo quando cresciute con la coltura, poiché la presenza di essa non ha esacerbato gli effetti della salinità nelle infestanti. Questi risultati corroborano l'ipotesi di una maggiore competitività delle infestanti in ambienti salini. Il confronto tra specie invernali ed estive ha suggerito che ogni volta che la perdita di resa delle colture estive diventa troppo estrema a causa della salinizzazione secondaria, le colture invernali, specialmente quelle tolleranti al sale come l'orzo, potrebbero essere un'opzione economicamente conveniente anche in termini di gestione delle infestanti.

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List of Acronyms

Abutilon theophrasti – ABUTH*

Amaranthus retroflexus – AMARE*

Aminoquinolyl-N-hydroxysuccinimidyl carbamate – AQC

Ascorbate peroxidase – APX

Catalase – CAT

Chenopodium album – CHEAL*

Crude protein – CP

Digitaria sanguinalis – DIGSA*

Diode array detector – DAD

Dry weight – FW

Echinochloa crus-galli – ECHCG*

Ethylenediaminetetraacetic acid disodium salt – EDTA

Exchangeable sodium percentage – ESP

Fresh weight – FW

Guaiacol peroxidase – GPX

High-performance liquid chromatography – HPLC

Hydrogen peroxide – H_2O_2

Malondialdehyde – MDA

Mean germination time – MGT

Percentage of salt-treated over non treated plants – % nt

Photon flux density – PFD

Polyvinylpyrrolidone – PVP

Portulaca oleracea – POROL*

Reactive oxygen species – ROS

Reversed phase – RF

Setaria pumila – SETPU*

Setaris viridis – SETVI*

Thiobarbituric acid – TBA

Thiobarbituric acid reactive substances – TBARS

Trichloroacetic acid – TCA

* *Acronyms identifying weed species follow the EPPO Global Database coding system.*

I General background

1.1 Soil salinization in the current growing population and climate change framework

To meet the nutritional needs of a growing world population, an increase in food production of 87 to 100% will be needed by 2050 *(Alkharabsheh et al., 2021)*. Most likely, a large part of this will be provided by enhancing the production of staple crops, such as cereals and legumes, that already constitute a major portion of people's diets *(Long and Kromdijk, 2016; Rubiales and Mikic, 2015; Zaman et al., 2016)*. At present, complete global food security is far from being achieved, with estimates of over 800 million people still suffering from chronic hunger *(Mughal and Fontan Sers, 2020)*. Additionally, modern agricultural production is threatened by several abiotic stresses, defined as any environmental condition having a detrimental effect on the growth, survival, and reproduction of plants *(Boscaiu et al., 2008; Cramer et al., 2011)*. Major stress factors limiting plant growth and development are heat, drought, flooding, high light intensity, heavy metal stress and salinity *(Adnan et al., 2020; Seleiman et al., 2021; Zlatev, 2015)*. Soil salinization is currently one of the most critical abiotic stresses worldwide, affecting 20 to 30% of the world's cultivated land, particularly over 10% of the total irrigated land, and contributing to the loss of arable land at an annual rate of 10% *(Bhargava and Srivastava, 2020; Daliakopoulos et al., 2016; Jamil et al., 2011)*. On a global scale, these percentages are expected to grow due to the effects of climate change, combined with intensive farming and poor agricultural practices *(Hassani et al., 2021; Jamil et al., 2011; Taylor et al., 2013)*. The combination of a rapidly growing population with the harmful impacts of climate change, along with different biotic and abiotic stresses and arable land degradation, increase the current global food shortage and widen the gap between production and demand *(Alkharabsheh et al., 2021; Fraser et al., 2016; Seleiman et al., 2020)*.

1.2 Definition and main drivers of primary and secondary salinization

Salt-affected soils can be identified as saline, sodic, and saline–sodic soils *(Sparks, 2003)*. A soil is considered to be saline when the electrical conductivity (EC) of a soil extract from

a saturated paste is higher than 4 dS/m *(Zaman et al., 2018)*, although different plant species experience some detrimental effects at levels as low as 2 dS/m *(Abrol et al., 1988; Sparks, 2003; Talat, 2020)*. Sodic soils are characterized by high levels of exchangeable sodium, quantified as an exchangeable sodium percentage (ESP)>15, while saline–sodic soils have both an EC>4 dS/m and an ESP>15 *(Sparks, 2003)*. Typically, the most abundant ions in the soil solution are sodium (Na^+) and chloride (Cl^+), followed by sulphate (SO_4^{2-}), bicarbonate ($HCO3^-$), potassium (K^+), magnesium (Mg^{2+}), and nitrate ($NO3^-$). High concentrations of Na^+ in the soil solution can disrupt the balance favoring the monovalent cations over the divalent cations *(Alkharabsheh et al., 2021)*.

Soil salinization can be divided into primary and secondary salinization according to the factors causing this phenomenon. Primary salinization is reached through natural processes, mainly including physical or chemical weathering of parent material and following transport, geological deposits or groundwater *(Daliakopoulos et al., 2016)*. The soil can be rich in salts due to the prevalent minerals of the parent rock. Soil layers can also be enriched in salts due to geological events formations that lead to the increase in salt concentration in the groundwater, especially after groundwater salinity rises due to capillary effects or evapotranspiration *(Chari et al., 2013; Geeson et al., 2003)*. The final salinity might also depend on the aquifer conformation and hydraulic conductivity of the bedrock and soil and characteristics such as porosity, structure, texture, clay content, compaction rate, infiltration rate and potential salt content *(Daliakopoulos et al., 2016; van Beek and Tóth, 2012)*. Another way primary salinization can occur in soils is the pre-existence of saltwater enclosed within ancient marine deposits, uplifted due to geological activities *(Wendland et al., 2008)*. Besides historical marine waters, current sea level rises due to natural processes such as marine transgressions, storm flood events and tsunamis can lead to salinization and even seawater intrusion into coastal regions *(Raats, 2015; Trnka et al., 2013; van Weert et al., 2009)*. Secondary salinization is mediated by human activities, and derives mainly from irrigation with saline or brackish water, coming from saline surface water or groundwater, and poor irrigation practices, often paired with poor drainage conditions or excessive ponding *(Hassani et al., 2021; Jamil et al., 2011; Taylor et al., 2013)*. This is particularly

critical in regions with little rainfall and high evapotranspiration rates, where irrigated lands can become hotspots of salinization, especially if soil characteristics tend to retain salts from leaching. In this case, even water with moderate electrical conductivity can lead to salt accumulation *(Bhargava and Srivastava, 2020; Libutti and Monteleone, 2017; Maggio et al., 2011)*. Minor sources of secondary salinization include infiltration of water from canals, reservoirs and waterlogging *(Barros et al., 2012)* or effluents from wastewater treatment, industries and mining *(Lefebvre and Moletta, 2006; Moral et al., 2008)*.

Global warming is expected to intensify many processes leading to soil salinization, due to prolonged drought periods, high surface evaporation and sea-level rise resulting from the alterations of the hydrological cycle *(Hinkel et al., 2014; Kundzewicz et al., 2008; Sterling et al., 2012)*. The rise of sea level, in combination with groundwater overexploitation for irrigation purposes, is likely to exacerbate the saltwater intrusion in coastal and inland aquifers from neighboring saline aquifers *(Chen et al., 2004; Taylor et al., 2013)*. With the temperature rise, irrigation water will be more frequently needed, but a continuous addition of water followed by quick evaporation is likely to increase salt content in soils *(Haddeland et al., 2014)*. Due to all these reasons, the conditions leading to soil salinization typically occur in arid and semiarid regions, such as South and Southeast Asia *(Hakim et al., 2011; Shrivastava and Kumar, 2015)*, but also various Mediterranean regions *(Dazzi, 2010)*, that are quickly transitioning to a more arid climate. Current soil salinization hotspots worldwide include Pakistan, China, the United States, India, Argentina, Sudan and several countries in Central and Western Asia, and the Mediterranean coastline in Southern Europe and North Africa *(Daliakopoulos et al., 2016)*.

1.3 Detrimental effects of salt stress on plants

Besides leading to soil degradation, salinity is known to adversely affect plant basic functions and development. This is the main reason salt stress hampers crop yields and quality in salt-affected lands *(Shrivastava and Kumar, 2015; Nadjafi et al., 2017)*. In fact, high salinity levels in the soil solution impair water and essential nutrients uptake, and negatively affects plant growth and development and are expressed at the cellular,

biochemical and physiological levels *(Alkharabsheh et al., 2021)*. The main effects of salt stress are ion imbalance, oxidative stress and hyperosmotic stress *(Niu et al., 1995; Xing et al., 2013; Zhu, 2001)*. The first effect triggered by salinity is osmotic, with plants closing their stomata, decreasing their transpiration ability and losing cell turgor, which ultimately impairs cell functionality *(Zhao et al., 2020)*. The second effect, coming in response to the first one, is ionic. To increase the turgor pressure, plant cells tend to promote the mobilization of inorganic ions *(Iqbal, 2018)*. Because in saline conditions Na^+ ions are more abundant than usual, they can easily reach a toxic level for the plant cells, leading to a metabolic imbalance. In fact, due to its physicochemical properties similar to K^+, Na^+ can compete for binding sites involved in key metabolic processes, such as enzymatic reactions, protein synthesis and ribosome activity *(Almeida et al., 2017)*.

The metabolic imbalance can lead to oxidative stress and reactive oxygen species (ROS) overproduction *(Niu et al., 1995; Xing et al., 2013; Zhu, 2001)*. ROS are involved in many biological processes, but can also initiate cascade reactions that trigger oxidative stress, especially through lipid peroxidation and alteration of cell membranes by protein denaturation and DNA mutation *(Jbir-Koubaa et al., 2015).* For this reason, oxidative stress can disrupt membrane stability, cause ion leakage and ultimately cellular death. Among the outcomes of ROS overproduction, there is also a reduction in photosynthesis and CO_2 uptake, relative water content and an increase in plant photorespiration *(Alkharabsheh et al., 2021)*.

Different stages of plant development show different levels of tolerance to salinity, but the germination, seedling and reproductive stages are generally the most sensitive *(Alkharabsheh et al., 2021)*. Salt stress usually delays or even prevents seed germination *(Lokupitiya et al., 2020)*. Besides direct Na^+ and Cl^- toxicity to the seed embryos, a high concentration of salts in the soil solution can compromise seed imbibition, reducing the water uptake because of the decreased soil osmotic potential *(Debez et al., 2020; Mwando et al., 2020)*. After seed germination, salinity affects plant growth in two phases. At the start of the exposure to salt stress, there is a reduction in water uptake and growth rate because of

the high concentration of salts, which stops the expansion of root and shoot cells due to leads to osmotic stress *(Fricke et al., 2004; Munns et al., 2000)*. These symptoms are rapidly followed by physiological changes such as reduced enzyme activity, protein biosynthesis, and photosynthetic activity *(Alkharabsheh et al., 2021)*. If salt stress continues in the long run, salts can accumulate in the plant tissues and potentially reach toxic levels after days, weeks or months, depending on the species and salt stress level. In this phase, Na^+ can compete with and replace K^+ in binding sites involved in metabolic processes *(Benito et al., 2014; Shabala and Lew, 2002)*.

1.4 Plant adapting mechanisms

Although different species have different levels of tolerance to salinity, up until certain thresholds of salt stress, plants are able to activate different defensive mechanisms. For instance, in response to the osmotic stress, certain plants tend to accumulate soluble metabolites able to lower the cellular osmotic potential without affecting the normal metabolism *(Hasegawa et al., 2000)*. The most known osmolytes belong to four main chemical classes: onium compounds (e.g. glycine betaine), polyols/sugars (e.g. mannitol), amino acids (e.g. proline), and alkaloids (e.g. trigonelline). Some of these compounds are also believed to serve as osmoprotectants, meaning that they also stabilize proteins and lipid membranes or reduce ROS overproduction *(Phang et al., 2008)*. Another important strategy to cope with Na^+ toxicity and interference in metabolic reactions is keeping a high cytosolic K+/Na+ ratio in the cytoplasm *(Almeida et al., 2017)*.

1.5 The competitive advantage of weeds

While the morphological and physiological responses to salt stress of many crop species, such as wheat, rice, maize, soybean and barley, have been thoroughly studied *(Daei et al., 2009; Katerji et al., 1996; Phang et al., 2008; Tavakkoli et al., 2010; Zeng and Shannon, 2000),* the effects of salinity on weedy species, weed communities and weed-crop interactions have been so far poorly investigated, and generally limited on the germination and early growth stages of single weed species *(Cirillo et al., 2018)*. However, weed-crop competition is, up to these days, one of the major constraints in plant production, accounting

for great losses in crop yield *(Hamidzadeh Moghadam et al., 2021)*. Over thousands of years of co-existence in agro-ecosystems, weed species have become great competitors against crops and are able to rapidly adapt to new habitats *(Radosevich et al., 2007; Zimdahl, 2007)*.

The competitive ability of weeds is expressed in all forms of crop systems and with different agricultural management practices, regardless of soil properties, general climate conditions and nutrient availability *(Bir et al., 2014; Menalled et al., 2020)*. Many of the traits that give weed species a competitive advantage over crops, such as earlier emergence, faster growth and higher genetic resilience and plasticity *(Chen et al., 2015; Clements et al., 2004; Lu et al., 2016)* can also be expected to promote their adaptability to tolerate salt stress. Among the traits that have been proven to favor the tolerance of different weed species to salinity is their intraspecific variability, which allows the spread of weed ecotypes particularly suited for the environment where they are growing *(Eslami, 2011; Hamidzadeh Moghadam et al., 2021; Šoštarčić et al., 2018)*. Another example is seed heteromorphism, a phenomenon in which a single plant produces morphologically different seeds, that often show different tolerance to abiotic stresses, including salinity *(Yao et al., 2010)*.

Given that most crop species are considered to be glycophytes, whereas available data on weeds suggest that their spread and competition might be enhanced in saline environments *(Cirillo et al., 2018)*, it is crucially important to investigate the response of weed species to salinity at different stages, focusing both on morphological and physiological aspects. In the global warming scenario, where a temperature rise is expected, taking into account the interaction between temperature and salinity at the germination and early growth stages, which are generally very sensitive to temperature *(Leon et al., 2004; Wang et al., 2018)*, is also necessary. This is particularly relevant considering that the germination and seedling response to salinity depends on temperature in different crops and forage grasses *(AL-Shoaibi, 2020; Malik et al., 2022)*, but very little is known about weed species. Another fundamental step, almost unexplored *(Chauhan et al., 2013; Hakim et al., 2013; Korres et al., 2022)*, is trying to better understand how much the competition with different crops is going to change under salt stress. To fill this knowledge gap, different and multi-disciplinary

approaches are required, starting from controlled conditions that minimize other disturbing environmental factors, that would interfere with field conditions.

1.6 Specific objectives

The specific objectives of this research project were:

- To compare the effects of the salinity-temperature interaction on seed germination and early seedling development of different weed and crop summer species: soybean, maize, rice, *Echinochloa crus-galli* (L.) P. Beauv., *Chenopodium album* L., *Portulaca oleracea* L.
- To assess the effects of the salinity-temperature interaction on germination and early growth of five weed summer species: *Abutilon theophrasti* L., *Amaranthus retroflexus* L., *Digitaria sanguinalis* (L.) Scop., *Setaria pumila* (Poir.) Roem. & Schult and *Setaria viridis* L.
- To investigate the morphological and physiological responses of soybean and C. *album* seedlings to salinity in hydroponics, with and without the competition factor.
- To assess the effects of the salinity-temperature interaction on early growth of winter and summer weed-crop systems (barley, *Avena sterilis* L., *Lolium rigidum* Gaudin; rice, *E. crus-galli*; *Oryza sativa var sylvatica* L.).
- To compare the short-term responses to salinity at the morphological and physiological level of winter and summer weed-crop systems in hydroponics (barley + *L. rigidum*, rice + *O. sativa var sylvatica*), with and without the competition factor.
- To evaluate the effects of salinity and inter-specific competition on the growth and physiological parameters of soybean, *C. album* and *A. retroflexus* in controlled soil conditions.
- Ultimately, to gain new knowledge in order to build a baseline for weed management under salt stress.

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II Effects of the salinity-temperature interaction on seed germination and early seedling development: a comparative study of crop and weed species

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1. Introduction

Salt-affected soils (saline and sodic) are defined as the soils containing concentrations of soluble salts high enough to adversely affect the growth and productivity of most crops *(Shrivastava and Kumar, 2015; Nadjafi et al., 2017)*, and this effect can be accentuated with increasing temperature *(Liu et al., 2021)*. These soils hold an electrical conductivity (EC) above 4 dS/m, but the threshold should be lowered to 2 dS/m, due to the negative outcomes this level of EC has on different plant species *(Harris et al., 2010; Matthees et al., 2018; Talat, 2020)*. Salt-affected soils represent approximately 20 to 30% of total arable lands *(Bhargava and Srivastava, 2020; Jamil et al., 2011)*. This percentage is expected to increase in the future due to low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor agricultural practices *(Hassani et al., 2021; Jamil et al., 2011)*. The conditions leading to soil salinization are those that typically occur in arid and semiarid areas of South and Southeast Asia *(Hakim et al., 2011; Shrivastava and Kumar, 2015)*, and in various Mediterranean regions *(Dazzi, 2010)*. Also, the rise in mean sea level due to global climate change can lead to a secondary salinization phenomenon, resulting from irrigating crops with saline water *(Daliakopoulos et al., 2016; Hakim et al., 2011, 2010)*. Secondary salinization is predicted to affect 30% of arable land by the end of the next decade, and more than 50% by the end of the century *(Wang et al., 2003)*. Therefore, it is a matter of concern in agriculture, as it can impair crop production on a global scale and negatively impact the nutritional needs of a growing world population *(Zaman et al., 2016)*.

Salinity management in agroecosystems is currently concerned both with mitigating the sources of salt stress conditions to which crops may be exposed and with enhancing the salt

tolerance mechanisms of plants *(Cirillo et al., 2018)*. Salt stress can affect plants at the cellular, biochemical and physiological levels, potentially restricting germination, growth, and reproduction. In some plants, exposure to salinity can also give rise to genetic variations *(Carillo et al., 2011)*. Salt-induced damage in plants is primarily caused by hyperosmotic stress and ion imbalance due to increased accumulation of sodium (Na^+) and chloride (Cl^-) ions and concomitant reduction in potassium (K^+) and calcium (Ca^{2+}), resulting in oxidative stress and changes in protein conformation *(Khan and Panda, 2008; Shrivastava and Kumar, 2015)*. Salt-tolerant plants activate osmotic adjustments via increased production of osmolytes (e.g., proline, sugar alcohols) or efficiently regulate ion membrane transport so as to maintain cellular osmotic and turgor pressure. Osmoprotective and ion-detoxification strategies in these plants also consist in the removal of Na^+ from the cytosol and its compartmentation in the vacuoles, and in higher K^+ retention to maintain optimal K^+/Na^+ ratio *(Van Zelm et al., 2020)*.

The effect of salt stress on seed germination and early plant development can vary between crops and between varieties within the same species *(Hakim et al., 2010; Kumar, 2017)*. Resistance to salinity is therefore a critical trait for natural selection *(Amraee et al., 2019; Lema et al., 2019; Zhang et al., 2014)*. The research present in the literature is mostly focused on the combined effect of temperature and salinity on the growth and development of crop species *(AL-Shoaibi, 2020; Ramin, 2006)*. Indeed, temperature is major factor influencing seed germination and plant development *(Hat and Prueger, 2015)*, and different species have an optimal temperature under which seeds germinate best *(Liu et al., 2021)*. The general trend seems to indicate that crops can tolerate salinity up to a certain temperature after which germination starts declining, and seedling establishment and growth are altered *(AL-Shoaibi, 2020; Del Vecchio et al., 2021; Ramin, 2006)*. As for the weed species, the effects of salinity and temperature are often assayed separately. However, given the role of temperature in seed germination and seedling establishment, more studies should focus on the combined effects of salinity and temperature on these processes in weeds.

Weed species, in particular, are usually more tolerant to abiotic stresses than crops, but it is not possible to formulate general assumptions on their responses to salinity in combination with temperature *(Kheloufi et al., 2020; Wang et al., 2020)*. Thus, the responses of each individual weed species must be assayed. Furthermore, the comparison of crop and weed responses during germination with the same combinations of salinity and temperature is worth studying to improve knowledge of crop-weed interactions under a changing climate scenario.

Compared to their wild ancestors, modern crops appear to be more sensitive to salinity, probably due to a trade-off during the selection process in which the salinity tolerance trait was discarded in favor of higher productivity. Adverse effects of salinity have been described in major staple crops, such as maize (*Zea mays* L.), soybean (*Glycine max* (L.) Merr.), and rice (*Oryza sativa* L.) . Only a few crops have been reported to be more salt-tolerant than weeds, such as sorghum compared with *Striga hermonthica* (Delile) Benth. *(Hassan et al., 2010)*. Most studies indicate that weed species are more resilient than crops to salinity likely because of greater intraspecific genetic variability and more adaptive strategies developed during their evolution *(Cirillo et al., 2018)*. For example, *Chenopodium album* produces both black and brown seeds. Black seeds will preferentially be generated under salt stress due to their lower dormancy and higher tolerance to salinity than brown seeds *(Yao et al., 2010)*. Furthermore, *Watkinson et al. (1997)* suggested that the highly salt-tolerant weeds *C. album*, *Echinochloa crus-galli* and *Portulaca oleracea* could become more widespread in the Mediterranean area with increasing soil salinity levels, and possibly also more invasive. This is because the three weed species exhibit high adaptive potential and plasticity *(Guo et al., 2017; Hat and Prueger, 2015; Tanveer and Shah, 2017)*. *C. album* and *P.oleracea* can display high resilience to germination in saline conditions *(Tanveer and Shah, 2017; Yazici et al., 2007)*. *P.oleracea*, in particular, is defined as a halophyte *(Sdouga et al., 2019)*. On the other hand, *E. crus-galli* shows very different responses to salinity, probably due to its high intraspecific variability *(Chauhan et al., 2013; Serra et al., 2018; Wu et al., 2022)*. Different studies report that *P. oleracea*, *C. album* and *E. crus-galli* are already widespread weeds in different parts of the world. In Italy and the rest of the

Mediterranean area, they largely occur especially in summer crops such as maize and soybean, where they show high competitiveness with the crops. In addition, *E. crus-galli* is one of the main troublesome weeds in paddy soils, as it grows more vigorously than rice plants and competes better for nutrient resources.

The competition between crops and weeds could be very unbalanced in saline environments, starting from the seed germination stage *(Gołebiowska and Kieloch, 2016; Kim et al., 1992; Vidotto et al., 2016; Zanin et al., 1997)*. Furthermore, if weeds are more resilient than crops, as the data from the literature indicate, the competition between them could be exacerbated by suboptimal temperatures. To test this hypothesis, we conducted germination assays and growth tests of three weed species (*C. album*, *P. oleracea*, and *E. crus-galli*) and three staple crops (maize, soybean, and rice) in a saline environment using five different salinity levels in combination with three different temperatures.

2. Materials and methods

2.1 Seed collection and saline solution preparation

Mature seeds of three summer weed species, *Chenopodium album*, *Portulaca oleracea*, and *Echinochloa crus-galli,* common in the fields of soybean, rice and maize of Northern Italy, were collected from September to November 2020 at the experimental farm of the University of Padova (45°12' N, 11°58' E, Legnaro, north-eastern Italy). They were hand-harvested on warm dry days by shaking into paper bags to ensure that only mature seeds were collected. The seeds were then hand-cleaned and stored in paper bags at room temperature until the start of trials the following spring. For the crops, three major species were chosen: maize (cv. DKC5530), soybean (cv. P21T45), and rice (cv. Vialone Nano). The cultivars are commonly grown in the Po Valley, where the weeds were collected. At the time of the experiment, the sensitivity to salt stress of the soybean and maize cultivars were unknown. The Vialone Nano cultivar is classified as sensitive at the germination and seedling stage compared to other Italian rice cultivars *(Bertazzini et al., 2018; Formentin et al., 2018; Pesenti, 2019)*.

The saline solutions were prepared by adding pure sodium chloride (NaCl) to distilled water until the desired salinity level was reached, which was measured with an XS Instruments COND 80 electrical conductivity meter (Giorgio Bormac s.r.l, Carpi, Italy) at a sensitivity of 1 µS. Four different saline solutions were prepared: 4, 8, 12 and 16 dS/m. The control consisted of pure distilled water (0 dS/m).

2.2 Germination test

Germination tests for each species, treated with NaCl and untreated, were conducted in growth chambers (KW Apparecchi Scientifici s.r.l., Monteriggioni, Italy) with a 12h light/12h dark photoperiod, and at three different constant temperatures of 12 °C, 15 °C and 18 °C, simulating early spring conditions of the soil. Four biological replicates (1 replicate = 1 plate) were used for each species at each salinity/temperature combination. Therefore, 60 replicates were prepared for each species (5 salinity levels x 3 temperatures x 4 replicates). Each replicate consisted of 100 seeds in the case of the weeds, 50 in the case of the crops. The seeds were placed in 9 cm-diameter Petri dishes (14 cm-diameter for maize and soybean because of the larger seed size) lined with filter paper and moistened till filter paper was fully imbibed with saline solution (or distilled water for the controls), with 2 mL or 6 mL of liquid depending on the size of the Petri dish. The Petri dishes were set according to a randomized design inside the growth chamber. Their positions were exchanged every other day. After placing the seeds, the Petri dishes were sealed with parafilm and placed inside the growth chambers. Germination was monitored every 2–3 days, the seeds were considered germinated when a radicle of 1 mm or longer was developed, and was considered completed if all the seeds germinated or if 10 days elapsed without germination, as proposed by *Baskin and Baskin (2014)*. Upon completion, newly germinated seeds were counted and removed. Prior to the test described, preliminary germination tests were conducted for all species included in the experiment, during which *E. crus-galli* showed some degree of dormancy (data not shown). *E. crus-galli* is known for having dormancy that can be overcome by seed scarification with sulfuric acid *(Sung et al., 1987)*. Therefore, seeds of *E. crus-galli* were immersed in 98% sulfuric acid for 20 minutes and then thoroughly rinsed.

In order to prevent imbibition of the seeds with water, those meant for the trials with saline solutions were rinsed with those solutions after the acid scarification process.

2.3 Preparation of saline nutritive agar base and growth tests

To assess the effects of salinity and temperature on early seedling development, growth tests were conducted by sowing seeds in half-strength MS agar medium *(Murashige and Skoog, 1962)* without addition of sucrose and hormones, inside plastic containers 8 cm (height) x 9 cm (width) x 10 cm (length). NaCl was added to the MS medium until the required salinity level was reached: 4, 8, 12 or 16 dS/m measured with an XS Instruments COND 80 electrical conductivity meter (Giorgio Bormac s.r.l., Carpi, Italy). The containers were then closed with their original covers and autoclaved for 20 minutes at 120 °C, then left to cool down. This procedure was followed for both weeds and crops.

In line with the size of the containers and the size of the seeds, 20 crop seeds and 50 weed seeds were sown per container. Salinity and temperature levels were the same as in the germination tests, and four replicates were used for each species at each salinity/temperature combination. To prevent contamination of the agar medium, sowing took place inside a sterile environment under a laminar flow hood, and prior to sowing all seeds were sterilized for 30 seconds with 75% ethanol, followed by a 15-minute treatment with 15% (v/v) sodium hypochlorite (NaClO). The seeds were then washed in distilled water for 5 x 5 minutes. Once the sowing was completed, the containers were placed inside the growth chambers. In accordance with their different growing speeds, the growth of the crop species was measured after two weeks, the weed species after five weeks. After the established growth period, the plants were carefully removed from the containers, and their stem and root elongation were measured with a digital caliper (TESA Technology, Renens, Switzerland).

2.4 Statistical analysis

For the germination tests, mean germination time (MGT) was calculated using the formula proposed by *Ellis and Roberts (1980)* and in accordance with *Borsai et al. (2018)*:

$$MGT = \Sigma(nD) / \Sigma n$$

where D is the number of days since the start of the test, and n is the number of newly germinated seeds at day D. The effects of the three factors (species, salinity and temperature) on the germination percentage and on MGT were assessed with a factorial analysis of variance (ANOVA) after a Bartlett homogeneity test. Mean differences were analyzed with a Fisher's LSD test ($\alpha = 0.05$). All data analyses were conducted with TIBCO Statistica 14.0.0 software.

3. Results

3.1 Seed germination

The ANOVA revealed a significant effect of all factors and their interactions on seed germination (**Table 1**).

Table 1. *Effects of species, salinity, temperature, and their interactions on germination*

Factors	p-value
Species	0.000
Salinity	0.000
Temperature	0.000
Species x Salinity	0.000
Species x Temperature	0.000
Salinity x Temperature	0.002
Spec x Sal x Temp	0.000

Spec=species, Sal=salinity, Temp=temperature.

As a general rule, the germination percentage decreased with increasing salinity at each temperature (**Figure 1**). With respect to the effect of the salinity-temperature interaction on seed germination, the germination percentage increased with increasing temperatures at almost every salinity level. An exception was for seeds germination with the highest salinity level (16 dS/m), at which an increase in germination was observed at 18 °C, but not at 12 °C and 15 °C (**Figure A 1**). Crop species had a relatively even and high germination percentage (**Table A 1**), with maize and rice performing best at every temperature. However, maize seeds exhibited the highest germination percentage with significant reduction only above 12 dS/m, while low salinity levels appeared to have a positive effect on germination. Soybean was the most sensitive crop to salinity, showing a notable decline in germination even at the lowest salinity level (4 dS/m) at any temperature. When soybean seeds were sown in the

low-saline medium (4 dS/m), the negative effect of increasing temperature on germination was evident, with reductions of 28% at 15°C and 45% at 18 °C. In contrast to the pattern observed for crops, the germination percentages of weed species were highly variable as a function of salinity and temperature (**Table A 2**). At low temperatures in the absence of NaCl, *E. crus-galli* had a higher germination percentage than the other two weeds. However, differences in germination between the weed species were less pronounced with increasing temperature. *E. crus-galli* seeds germinated well with low salinity, less so at salinity levels of 8 dS/m or higher. A similar trend was observed for *P. oleracea*, with seed germination inhibited not only by salinity but also by low temperatures. Specifically, there was no variation in germination percentage between the seeds in the low-salinity medium (4 dS/m) and the seeds in the control medium at 12 °C. However, variations in germination did occur at higher temperatures (15 °C and 18 °C). The germination percentage of *C. album* was generally low (maximum 68% at 18 °C at 12dS/m salinity level), this species showed little or no reduction with increasing salinity, indicating that the seeds of this species were less sensitive to salinity during the germination process.

If we look at salinity alone, its effect on the germination of each species across all temperature ranges is even more evident (**Figure 2**). Comparing weed and crop species, differences in their levels of salinity tolerance were evident, even though there were already differences between the species in their germination percentages without salt stress. The most tolerant species to salinity were maize among the crops, and *C. album* among the weeds, while soybean and *E. crus-galli* were the most sensitive.

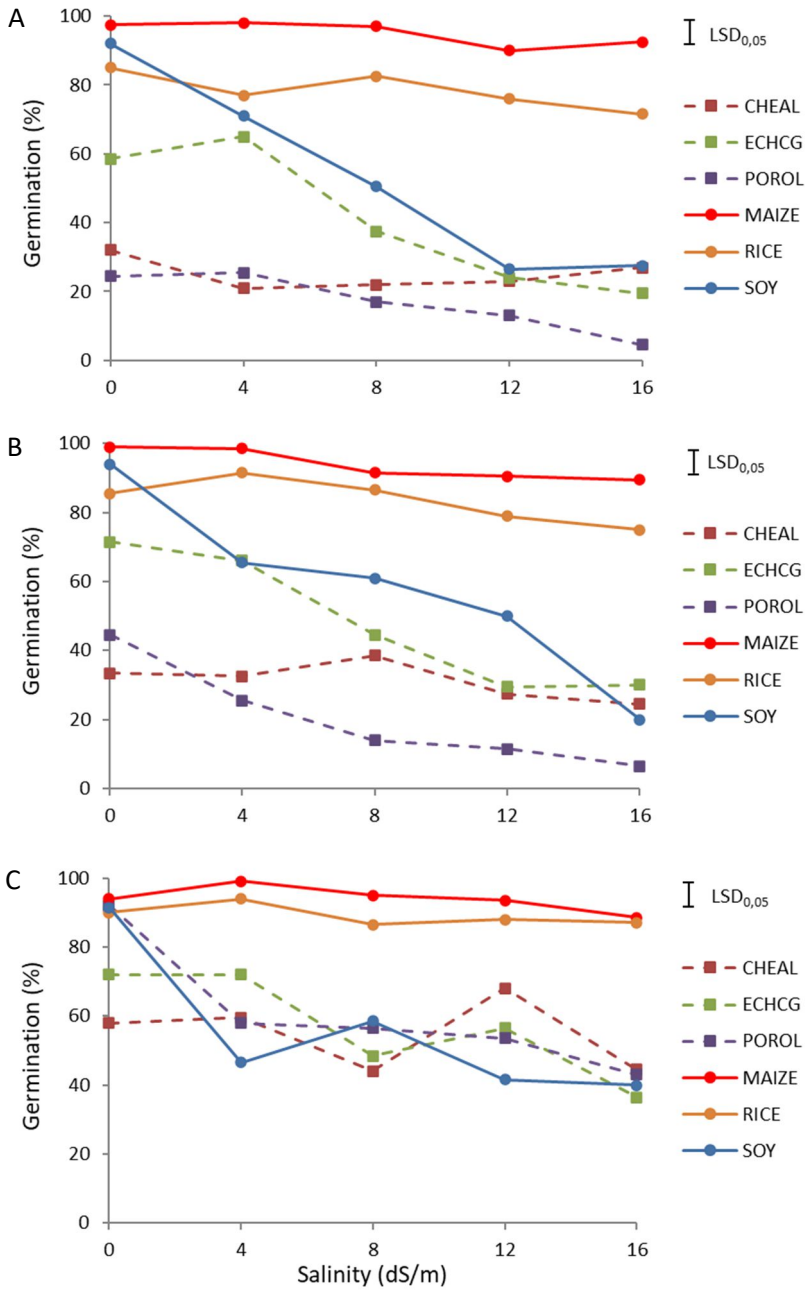

Figure 1. *Germination percentages of the three weed species CHEAL (C. album), ECHCG (E. crus-galli), and POROL (P. oleracea), and the three crop species MAIZE (Z. mays), RICE (O. sativa), and SOY (G. max) at different salinity levels and temperatures of 12 °C (A), 15 °C (B) and 18 °C (C).*

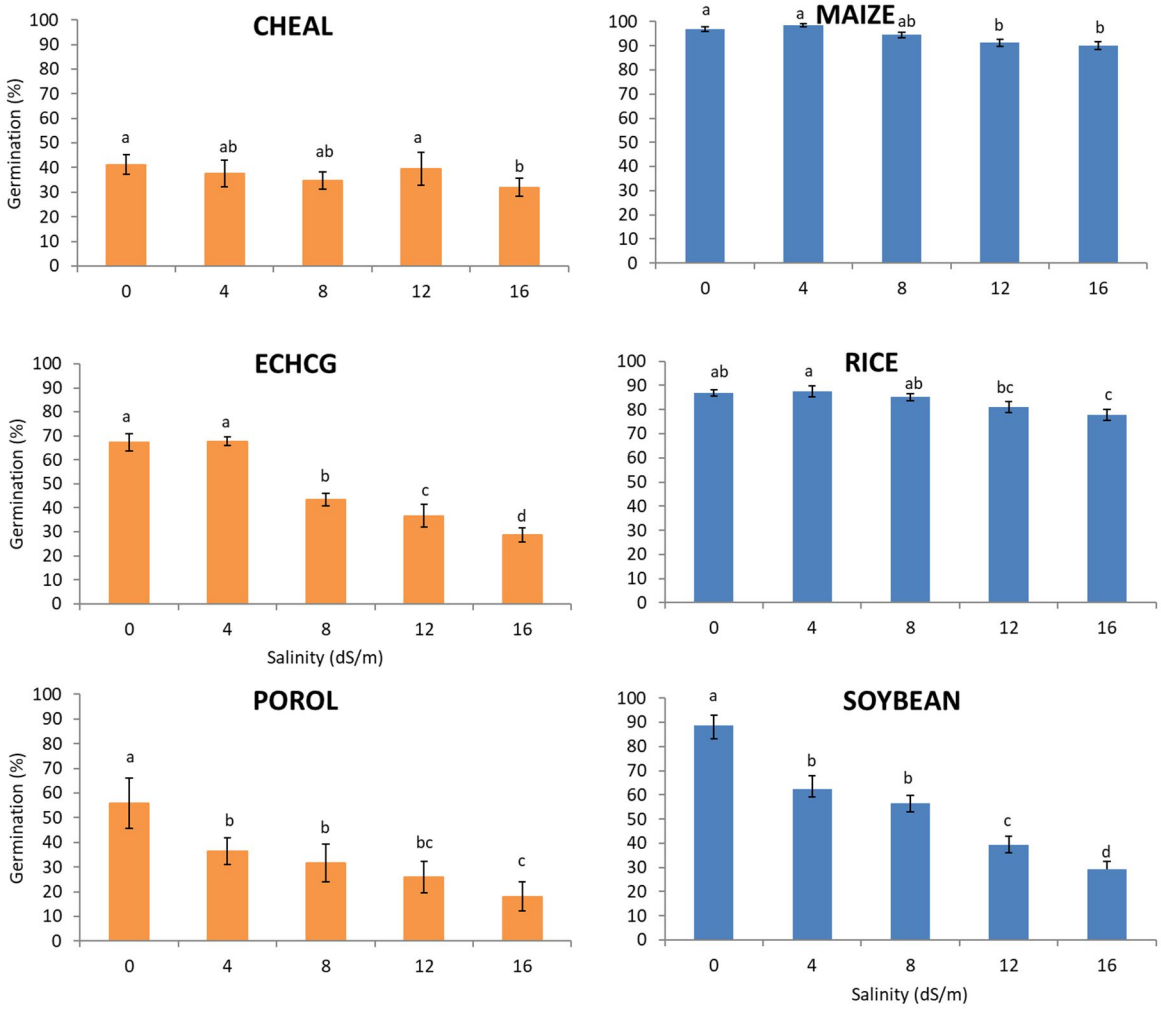

Figure 2. *Germination percentages of the three weed species CHEAL (C. album), ECHCG (E. crus-galli), and POROL (P. oleracea), and the three crop species MAIZE (Z. mays), RICE (O. sativa), and SOYBEAN (G. max) at different salinity levels. Letters indicate significant differences (α = 0.05).*

3.2 Mean germination time

The results obtained from the ANOVA performed on the mean germination time (MGT) of the six species showed a significant effect of all factors and their interactions (**Table 2**).

Table 2. *Effects of species, salinity, and temperature, and their interactions on mean germination time (MGT).*

Factors	p-value
Species	0.000
Salinity	0.000
Temperature	0.000
Species x Salinity	0.032
Species x Temperature	0.000
Salinity x Temperature	0.002
Spec x Sal x Temp	0.003

Spec=species, Sal=salinity, Temp=temperature.

The MGT of all species generally increased with increasing salinity, and this effect was more pronounced at the lower temperatures (12 °C and 15 °C), particularly in soybean and *E. crus-galli* (**Figure 3**). In the control conditions (0 dS/m), the MGT was lower for crops than for weeds. Among the crops, maize and rice had a lower MGT than soybean, and were less affected by increased salinity in terms of delayed germination, especially at lower temperatures. For example, at 12 °C, the MGT for soybean at 0 dS/m was 9 days, but rose to 16 days at 16 dS/m. On the other hand, at the same temperature the MGT for rice was 9 days at 0 dS/m, rising to 13 days at 16 ds/m, and for maize it was 10 days at 0 dS/m rising to 12 days at 16 dS/m, maize results, however, showed high variability, as can be seen in **Table A 3**. Among the weeds, *P. oleracea* seeds germinated the fastest at every temperature and salinity level, with an MGT ranging from 7 days at 12 °C to 4 days at 18 °C (values at 0 dS/m). *E. crus-galli* seeds were in general the slowest to germinate, with a MGT ranging from 15 days at 12 °C to 8 days at 18 °C (values at 0 dS/m), and was also the most sensitive weed species to increased salinity, especially at low temperature, with an MGT at 12 °C of 15 days at 0 ds/m, and 21 days at 16 dS/m. Overall, our results show that MGT decreased with increasing temperature, regardless of the salinity level (**Table A 4**).

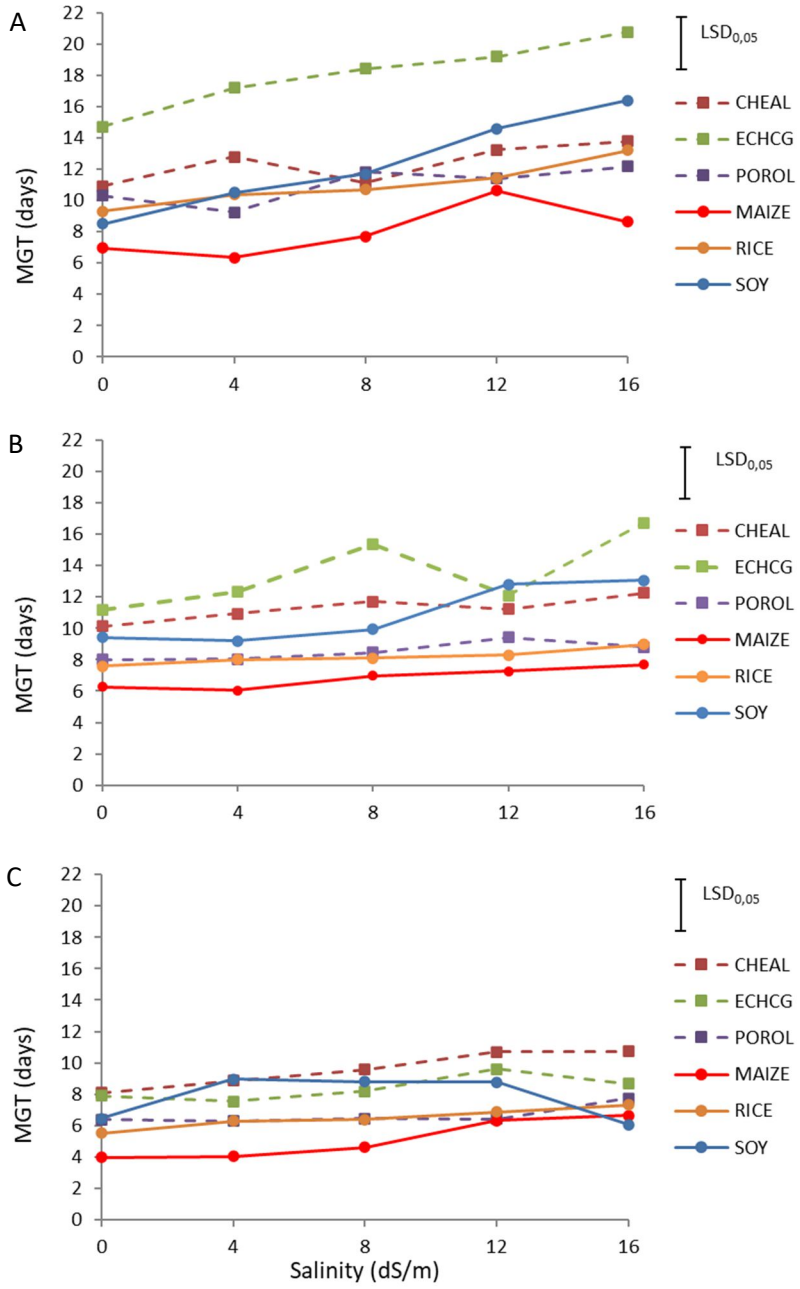

Figure 3. *Mean germination time (MGT) of the three weed species CHEAL (C. album), ECHCG (Echinochloa crus-galli), and POROL (P. oleracea), and the three crop species MAIZE (Z. mays), RICE (O. sativa), and SOY (G. max) at different salinity levels and different temperatures: 12 °C (A), 15 °C (B), and 18 °C (C).*

3.3 Growth tests

Growth test results indicated that the six species tested in this study were able to develop at different salinity and temperature levels. However, successful germination does not always imply successful early-stage development (**Figure 4** and **Figure 5**). For example, *C. album* was the least affected by salinity in terms of germination, but its stem growth was reduced with increased salinity (**Figure 4**), an effect that was exacerbated by a combination of high

salinity (16 dS/m) and high temperature (18 °C), when no stem elongation was observed. Root elongation followed a similar trend, except that it improved at the low levels of salinity, especially at 12 °C. The stem growth of *E. crus-galli*, the weed most sensitive to salt stress in terms of germination, decreased with increased salinity, but not with temperature. The root elongation of this species followed a trend similar to stem growth, except that at low salinity levels and 15 °C root development was better than at 0 dS/m. The stem growth of *P. oleracea*, on the other hand, was stimulated by low temperature (12°C), at low salinity (4 dS/m), while only slightly altered by 15°C and 18°C. Increasing the salinity level determined the inhibition of stem growth, which was sharper at low temperature (**Figure 4**). Root elongation was stimulated by all temperatures under low salinity. At 15°C and 18°C, the increase of root elongation was observed under salinity levels up to 12 dS/m and 8 dS/m, respectively.

Regarding the crops, the stem length of maize seedlings was reduced by medium and high salinity, particularly at 12°C and 18°C (**Figure 5**). Root elongation showed the same trend, although it was less affected by salt stress than stem growth, and was less reduced at high temperatures. Stem elongation of rice seedlings decreased with rising salinity, but was barely affected by temperature. Like maize, the reduction in stem growth was not very pronounced, especially when compared to some of the weed species. Nevertheless, root elongation was more affected by temperature, especially at 12°C and 15°C, even at low salinity levels. The stem and root growth of soybean was consistent with germination, confirming this species as the least tolerant of the crops tested in this study to salinity (**Figure 5**). The growth of the soybean stem was highly dependent on temperature: it was completely inhibited at the lowest temperature (12°C), and significantly reduced at higher temperature with increasing salinity. In contrast, the roots of soybean were able to develop at 12 °C and were less inhibited by salinity.

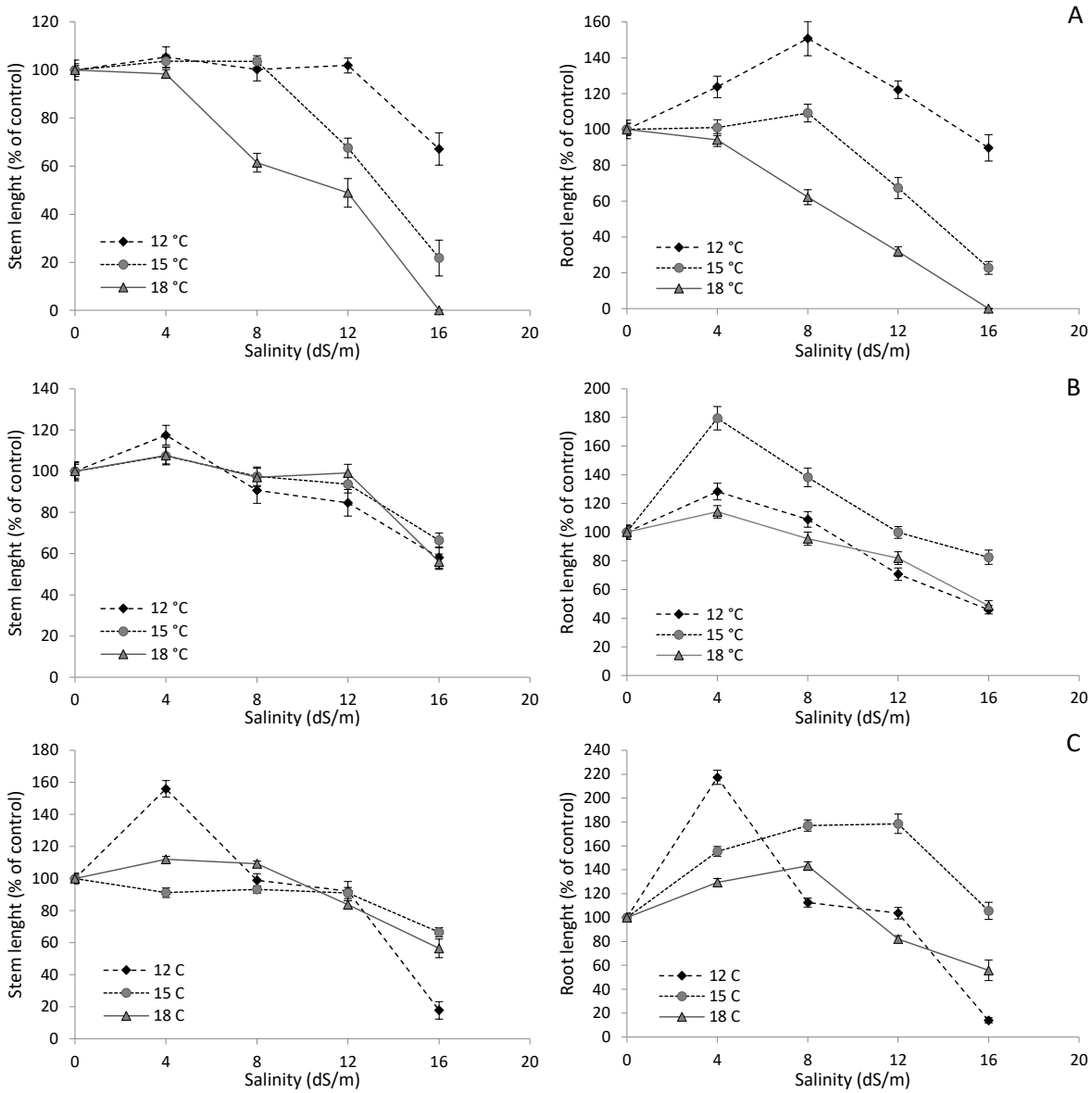

Figure 4. *Stem and root lengths expressed as percentage over the control of C. album (A), E. crus-galli (B) and P. oleracea (C) at different salinity levels and temperatures (root and stem control length can be seen in the* ***Table A5****).*

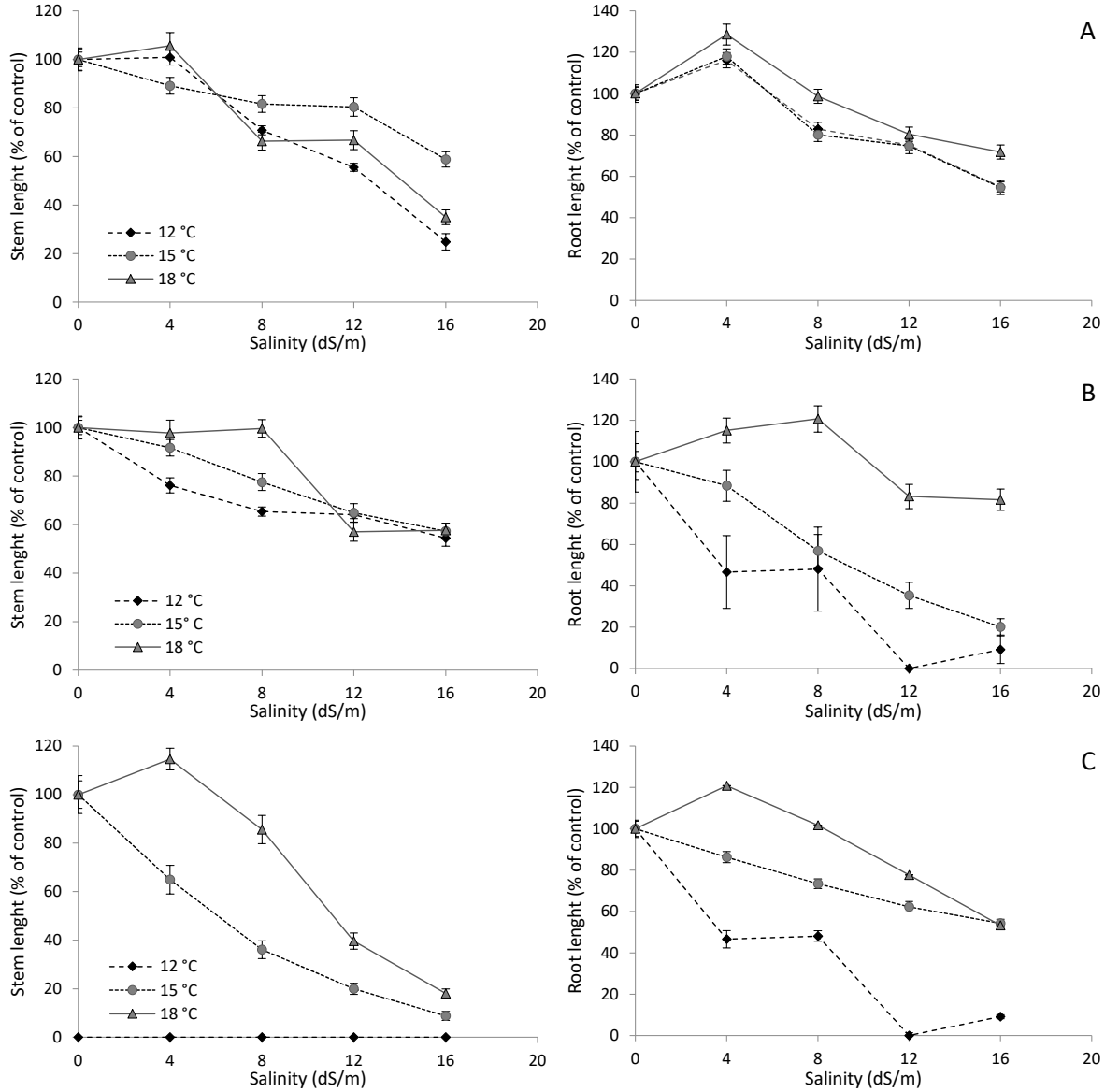

Figure 5. *Stem and root lengths expressed as percentage over the control of maize (Z. mays, A), rice (O. sativa, B) and soybean (G. max, C) at different salinity levels and temperatures (root and stem control length can be seen in the* ***Table A5****).*

4. Discussion

This study shows that the effect of salt stress on seed germination and early seedling development depend on the species, the degree of salinity, the temperature, and the combination of salinity with temperature. Soybean was found to be the least tolerant of the crops to salinity. This is consistent with literature classifying soybean as a moderately salt-sensitive species with its yield reduced by soil salinity above 5 dS/m *(Phang et al., 2008)*. Our results concerning germination are in agreement with those of *Essa (2002)*, who reported a significant reduction in germination rates for soybean seeds exposed to salt levels

of 4.5 dS/m or higher. However, we observed that high salinity had a greater effect on the MGT of this species, contrary to what was reported by *Hosseini et al. (2002)*, who found no significant increase in MGT with NaCl concentrations below 16 dS/m. This discrepancy may be due to the fact that germination was tested at different temperatures in the two studies: *Hosseini et al. (2002)* used an experimental temperature close to the optimum, whereas the temperatures we applied were closer to the base temperature. It should be noted, however, that the effect of salt stress on MGT was much less severe at 18 °C than at lower temperatures. Unlike soybean, maize appeared to be relatively tolerant to salinity at the germination stage. This result is in contrast to others reported in the literature for this species. Indeed, many authors consider maize moderately sensitive to salt as productivity and growth parameters are often significantly reduced as a function of increased salinity *(Farooq et al., 2015; Khayatnezhad and Gholamin, 2011; Ouda et al., 2008)*. The high tolerance of maize seeds to salinity in our study could be due to the particular cultivar used, its sensitivity to salt being unknown at the time of the experiment. Maize is highly polymorphic and is considered to have the highest genetic variability among crops *(Akram et al., 2010; Fernie, 2021)*, with its various cultivars differing in salt tolerance at the germination stage *(Khayatnezhad and Gholamin, 2011; Khodarahmpour et al., 2011)*. The fact that seed germination was not delayed with increased salinity supports the hypothesis that our cultivar was probably salt-tolerant. Rice was found to be relatively tolerant to salinity. The capacity to tolerate salt by rice is generally achieved through two principal mechanisms, i.e. ion exclusion preventing the excess accumulation of Na^+ and Cl^- in leaves, and osmotic tolerance via sequestration of Na^+ in the vacuole, synthesis of osmolytes and production of antioxidant enzymes *(Munns and Tester, 2008; Reddy et al., 2017)*. Our results are consistent with those reported by other authors who observed rice tolerance to salt at concentrations up to 10 dS/m *(Ologundudu et al., 2014)* or 16 dS/m *(Heenan et al., 1988; Khan et al., 1997)* at the germination phase, with no substantial reduction in the germination percentage and speed. As in the case of maize, many authors have reported varying degrees of salt tolerance in different rice varieties, with some of the most common cultivars being extremely salt-sensitive *(Abbas et al., 2012; De Leon et al., 2015; Hakim et al., 2010; Jayabalan et al.,*

2022). Although our cultivar (Vialone Nano) is considered to be salt-sensitive, literature data are too limited to allow any comparison *(Bertazzini et al., 2018; Formentin et al., 2018; Pesenti, 2019)*. In the case of salt-sensitive rice varieties, transplanting aged seedlings could be a possible option to alleviate the salinity at the seedling stage and reduce competition with salt-tolerant weeds.

Among weeds, *C. album* has been definitely confirmed as a salt-tolerant species *(Ghirardelli et al., 2021)*, especially with respect to seed germination *(Tanveer and Shah, 2017)*. Our findings are consistent with those of *Eslami (2011)*, who found *C. album* to be salt tolerant up to 20 dS/m at the germination stage, with no steep increase in MGT and a germination percentage of 40% even at a salinity of 30 dS/m. However, high salinity levels exerted a negative effect on plant growth in the early stages of plant development, especially at lower temperature. Interestingly, different results can be obtained when salinity is applied once the seedling stage has been established. In a recent study by *Ghirardelli et al. (2021)*, *C. album* was reported to be highly tolerant to 150 mM NaCl (above 10 dS/m), when the salt was applied to 10 cm high-plants. In this case, the weed resilience to salt was mainly due to elevated initial K^+ concentration and abundant K^+ delivery to the shoot, high accumulation of phenolics and proline, high antioxidant activity and low lipid peroxidation in the weed. One possible hypothesis is that at a later stage of development the weed might be more efficient in activating the mechanisms involved in salinity stress tolerance. *Portulaca oleracea* is considered to be either a halophyte or a moderately tolerant species, depending on the scientific source *(Nasir et al., 2010; Teixeira and Carvalho, 2009)*. In our study, however, we observed a significant reduction in the germination percentage of this species, which could be due to the experimental temperatures, lower than the optimum, and/or to the specific traits of the ecotype used, given that *P. oleracea* has considerable morphological and physiological plasticity *(Feng et al., 2015)*. Therefore, its capacity to tolerate high-salt concentrations cannot be generalized to the species, rather to the ecotype. Our results indicate *E. crus-galli* as the most sensitive weed to salinity, which is in contrast to the findings of *Hakim et al. (2011)* and *Chauhan et al. (2013)*, who classified this species as salt tolerant. It must be noted that in the study by *Chauhan et al. (2013) E. crus-galli* was not

subjected to NaCl since the seed stage, but 10 days after sowing, i.e. when 50% of the seedlings reached 5 cm height. Thus, it is possible that the weed at this stage was more efficient in activating salt-tolerance mechanisms. In addition, *Serra et al. (2018)* found substantial variation in the response of different ecotypes of *E. crus-galli* from Italy to salinity, although for all, germination was almost unaffected up to 250 mM NaCl (above 20 dS/m). The ecotype we used was able to tolerate salt stress up to 4 dS/m, but seed germination was significantly affected at and above 8 dS/m. This could be due to the experimental temperatures used in our study, perhaps all suboptimal for the weed, as suggested by the decreasing gap between salinity levels with increasing temperature.

The effects of salinity and temperature at the early growth stage were on the whole consistent with the effects on seed germination, with a few exceptions. Those species that appeared to be salt tolerant at the germination stage (i.e. maize, rice, and *C. album*) showed significantly reduced root and stem lengths with increasing salinity. This discrepancy can be explained by the fact that many plant species exhibit different responses to salt stress depending on the growth stage, among which are the activation of salt-stress responsive genes, the induction of Na^+ and Cl^- efflux root transporters, the accumulation of osmolytes and Na^+ vacuolar sequestration *(Mbarki et al., 2020; Reddy et al., 2017)*. Rice and maize are considered to be more vulnerable to salinity at the early growth stages *(Akram et al., 2010; Hakim et al., 2010)* than at the germination stage *(Bertazzini et al., 2018; Khayatnezhad and Gholamin, 2011)*. In the case of *C. album*, temperature also played a key role: at higher temperatures a greater percentage of seeds germinated in all treatments, but salt stress inhibited further growth, resulting in a large number of slowly developing seedlings. Soybean at the seedling stage was even more sensitive to salinity than at the germination stage, confirming the findings of *Hosseini et al. (2002)*, who reported that the early growth stages of this species are affected by lower salinity levels. Like soybean, *P. oleracea* and *E. crus-galli* were rather sensitive to salt at the seedling stage, in agreement with *Franco et al. (2015)*, *Hakim et al. (2011)*, and *Fogliatto et al. (2021)*. Despite the general trend of root length reduction, root growth in all the weed species was slightly stimulated at low salinity levels (4-8 dS/m). Such a phenomenon is known as the hormetic effect *(Calabrese, 2013)*, previously observed in

numerous weed species, including *P. oleracea* and *E. crus-galli (Fogliatto et al., 2021; Sdouga et al., 2019)*. Although many sources in the literature report salt-tolerance traits in maize and rice, these crops are generally more sensitive to salinity than most of the weeds associated with them, including *C. album*, *P. oleracea* and *E. crus-galli (Cirillo et al., 2018; Fogliatto et al., 2019; Serra et al., 2018)*. Maize and rice seem to perform better in controlled conditions, but the interspecific variability and genetic plasticity of weeds could make them more competitive in the field, where many environmental factors interact. Our study shows that temperature plays an important role in how salinity stress affects germination and early seedling growth, and highlights that the effect of the temperature-salinity interaction is species-specific, which is expected given the high intraspecific variability. We also found that rising salinity levels had a negative effect on both germination and seedling growth, although in most cases this effect was somewhat mitigated by an increase in temperature. The most detrimental combination was high salinity and low temperature. The weed species tested had a high degree of salt tolerance either as a reduction in the low germination percentage (*C. album*) or in shoot growth and root elongation (*E. crus-galli, P. oleracea*). This finding suggests that these weed species could be important competitors with crops grown on saline or salt-affected soils, especially at higher temperatures, intensifying their negative effects on crop development. On the other hand, two of the crop species tested, maize and rice, also showed tolerance to high salinity levels at the germination and growth stages. In view of this, one of the ways to suppress or reduce crop-weed competition in saline soils might be to grow salinity-tolerant crop cultivars, given that crops usually tend to germinate at a homogeneous rate, grow fast, and create large canopies that shade the weeds. This can be seen in the case of maize, the germination and growth of which seem to be stimulated by low salinity concentrations.

5. Conclusions

Our results suggest that under increasing salinity the competitiveness between weeds and crops could be relevant based on the effects recorded at the seed germination stage and early development. The competitiveness can become even more stringent at high temperatures. Therefore, it is important to control weeds at the early stage of crop development. In view

of the increase of salinity and temperature levels due to climate changes and of scarce water resources for irrigation in arid and semi-arid lands, breeding efforts are needed to generate salinity-tolerant cultivars incorporating weed-competitive traits, which is particularly important for crops that are highly salt-sensitive, such as soybean. The salt-tolerant cultivars can be used in weed pre- and post- management control in combination with other strategies. More studies should investigate: 1) the germination and seedling response of other weed species that could infest crops grown on saline soils; 2) the competition between crops and different weed species in the further stages of development. In this regard, soil experiments in controlled saline conditions could confirm our hypothesis of increased weed competitiveness in saline environments. In addition, studies concerning the influence of salinity on soil seedbank might give us further insights into possible development of future weed infestation.

Appendix

Table A 1. *Germination percentage of the three crop species MAIZE (Zea mays), RICE (Oryza sativa), SOY (Glycine max) at different salinity levels and different temperatures.*

Temperature		12°C		15°C		18°C	
Species	Salinity dS/m	Germination %	Std.Err	Germination %	Std.Err	Germination %	Std.Err
MAIZE	0	97.5	0.96	99	1.00	94	1.63
MAIZE	4	98	0.82	98.5	0.96	99	1.00
MAIZE	8	97	1.29	91.5	2.75	95	0.58
MAIZE	12	90	3.46	90.5	2.22	93.5	2.22
MAIZE	16	92.5	2.99	89.5	1.26	88.5	4.11
RICE	0	85	3.11	85.5	1.50	90	1.41
RICE	4	77	1.29	91.5	0.50	94	1.63
RICE	8	82.5	3.59	86.5	1.26	86.5	2.22
RICE	12	76	3.92	79	3.87	88	0.82
RICE	16	71.5	3.20	75	2.38	87	1.29
SOY	0	92	1.63	94	2.58	91.5	2.06
SOY	4	71	7.94	65.5	6.34	46.5	7.93
SOY	8	50.5	5.56	61	4.65	58.5	6.34
SOY	12	26.5	4.92	50	0.82	41.5	5.50
SOY	16	27.5	2.50	20	4.40	40	4.55

Table A 2. *Germination percentage of the three weed species CHEAL (Chenopodium album), ECHCG (Echinochloa crus-galli), POROL (Portulaca oleracea) at different salinity levels and different temperatures.*

Temperature		12°C		15°C		18°C	
Species	Salinity dS/m	Germination %	Std.Err	Germination %	Std.Err	Germination %	Std.Err
CHEAL	0	32	3.56	33.5	3.59	58	3.37
CHEAL	4	21	4.65	32.5	2.87	59.5	5.38
CHEAL	8	22	1.83	38.5	4.50	44	4.97
CHEAL	12	23	2.38	27.5	3.10	68	6.78
CHEAL	16	27	5.20	24.5	3.10	44.5	4.79
ECHCG	0	58.5	5.91	71.5	7.04	72	3.74
ECHCG	4	65	2.65	66	2.94	72	3.56
ECHCG	8	37.5	5.74	44.5	3.10	48.5	3.30
ECHCG	12	24	6.38	29.5	2.63	56.5	3.30
ECHCG	16	19.5	4.11	30	5.60	36.5	0.96
POROL	0	24.5	5.19	44.5	3.59	92	5.23
POROL	4	25.5	7.89	25.5	2.50	58	4.97
POROL	8	17	5.07	14	2.31	56.5	3.59
POROL	12	13	4.43	11.5	3.86	53.5	5.19
POROL	16	4.5	1.71	6.5	2.22	43	7.59

Table A 3. *MGT of the three crop species MAIZE (Zea mays), RICE (Oryze sativa), SOY (Glicine max). On different salinity levels and different temperatures.*

Temperature		12°C		15°C		18°C	
Species	Salinity dS/m	MGT	Std. Err	MGT	Std. Err	MGT	Std. Err
MAIZE	0	10	0.55	8	0.08	6	0.30
MAIZE	4	9	0.36	8	0.27	6	0.37
MAIZE	8	12	0.73	8	0.25	6	0.35
MAIZE	12	11	0.95	9	0.42	6	0.33
MAIZE	16	12	4.12	9	3.27	8	0.26
RICE	0	9	0.60	8	0.14	6	0.14
RICE	4	10	0.39	8	0.29	6	0.10
RICE	8	11	0.49	8	0.08	6	0.06
RICE	12	11	0.44	8	0.35	7	0.13
RICE	16	13	0.48	9	0.32	7	0.08
SOY	0	9	0.22	9	0.44	6	0.21
SOY	4	11	0.79	9	0.52	9	0.62
SOY	8	12	0.63	10	0.24	9	0.75
SOY	12	15	0.56	13	0.52	9	0.18
SOY	16	16	0.12	13	0.84	6	0.22

Table A 4. *MGT of the three weed species CHEAL (Chenopodium album), ECHCG (Echinochloa crus-galli), POROL (Portulaca oleracea). On different salinity levels and different temperatures.*

Temperature		12°C		15°C		18°C	
Species	Salinity dS/m	MGT	Std. Err	MGT	Std. Err	MGT	Std. Err
CHEAL	0	11	0.43	10	0.11	8	0.24
CHEAL	4	13	0.34	11	0.25	9	0.25
CHEAL	8	11	0.52	12	0.32	10	0.43
CHEAL	12	13	0.33	11	0.83	11	0.20
CHEAL	16	14	0.22	12	0.69	11	0.16
ECHCG	0	15	1.56	11	0.17	8	0.19
ECHCG	4	17	0.79	12	0.53	8	0.13
ECHCG	8	18	1.12	15	1.18	8	0.14
ECHCG	12	19	2.95	12	1.08	10	0.27
ECHCG	16	21	1.17	17	1.46	9	0.06
POROL	0	7	0.09	6	0.09	4	0.11
POROL	4	6	0.04	6	0.16	4	0.07
POROL	8	8	0.01	7	0.06	5	0.21
POROL	12	11	0.09	7	0.06	6	0.15
POROL	16	9	0.34	8	0.26	7	0.15

Table A 5. *Stem and root length of control (0 dS/m) of the three crop species [*MAIZE (*Zea mays*), RICE (*Oryza sativa*), SOY (*Glycine max*)*]and weed species [CHEAL (Chenopodium album), ECHCG (Echinochloa crus-galli), POROL (Portulaca oleracea)] used in the trial*

Crops	Temperature (°C)	Stem length (mm)	± err.st	Root length (mm)	± err.st
Maize	12	13.5	0.40	16.6	0.71
Maize	15	24.6	1.16	23.8	0.76
Maize	18	38.9	1.71	24.4	0.77
Rice	12	4.9	0.29	2.2	0.32
Rice	15	8.2	0.25	9.4	0.81
Rice	18	24.5	1.88	24.1	1.19
Soybean	12	0.0	0.00	52.8	2.20
Soybean	15	46.6	3.65	29.1	1.07
Soybean	18	79.6	4.51	33.5	1.99
Weed species	**Temperature (°C)**	**Stem length (mm)**	**± err.st**	**Root length (mm)**	**± err.st**
CHEAL	12	36.4	1.50	8.3	0.43
CHEAL	15	40.8	1.08	11.1	0.39
CHEAL	18	39.6	0.74	14.3	0.45
ECHCG	12	30.5	1.02	14.9	0.53
ECHCG	15	39.1	1.60	12.9	0.64
ECHCG	18	51.4	2.39	16.2	0.78
POROL	12	5.4	0.17	2.8	0.11
POROL	15	9.2	0.30	3.9	0.09
POROL	18	9.2	0.18	5.9	0.16

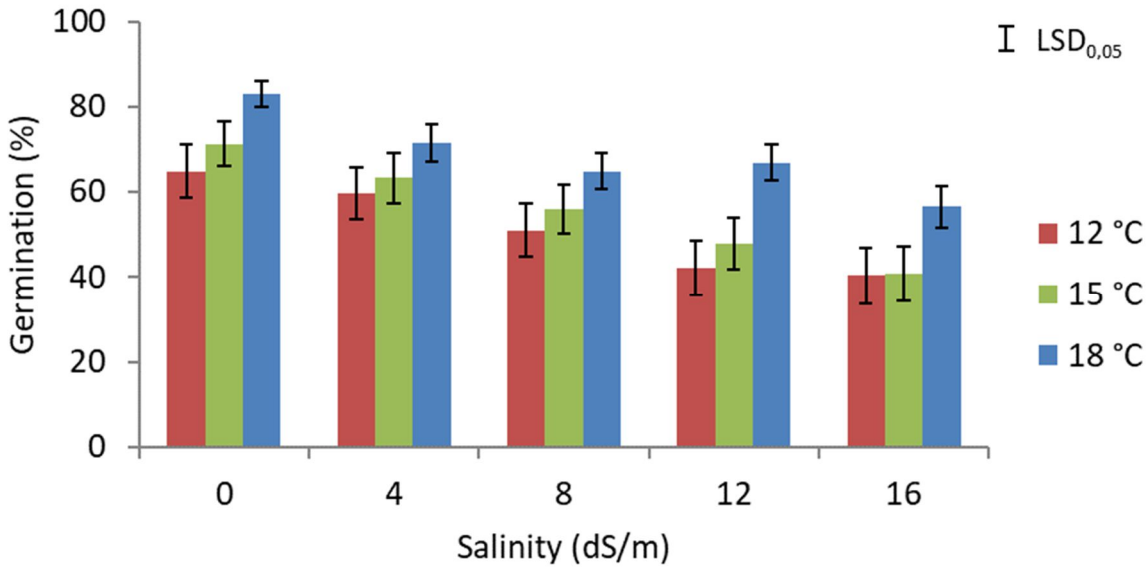

Figure A 1. *Cumulative germination percentages of all species tested at different salinity levels and temperatures. Bars indicate the standard errors.*

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III Effect of salinity and temperature interaction on germination and early growth of five weed species

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1. Introduction

Soil salinization is a critical environmental issue, increasingly affecting the agro-ecosystems and contributing to the loss of arable land and reduction of crop yield. Besides primary salinization due to the development of salts through natural processes, nowadays the phenomenon mainly results from climate change and the depletion of natural resources *(Daliakopoulos et al., 2016)*. Sea-level rise and groundwater overexploitation, causing saltwater intrusion in coastal and inland aquifers *(Taylor et al., 2013)*, are among the main factors expected to exacerbate the negative effects of salinity. As a consequence, the cultivated lands impacted by salinization, corresponding to 20 to 30% of the total, are expanding worldwide at an annual rate of 10% *(Bhargava and Srivastava, 2020; Jamil et al., 2011)*. Drought, high surface evaporation and sea-level rise, combined with poor agricultural practices are among the main factors contributing to secondary salinization *(Hassani et al., 2021; Jamil et al., 2011)*. This phenomenon is seriously affecting the arid and semi-arid regions but is now expanding towards temperate regions, including the Mediterranean *(Daliakopoulos et al., 2016; Dazzi, 2010; Hakim et al., 2011; Shrivastava and Kumar, 2015)*. Here the prevalent phenomenon is secondary salinization, associated with seawater intrusion in coastal areas *(Libutti and Monteleone, 2017)*.

Salinity can affect plants at the cellular, biochemical and physiological levels. Major toxic effects include ion imbalance, leading to the accumulation of Na^+ and Cl^- ions and depletion of K^+ and Ca^{2+} in plant tissues *(Khan and Panda, 2008)*, decreased water uptake, and oxidative stress *(Niu et al., 1995; Xing et al., 2013; Zhu, 2001)*, leading to hyperosmotic stress *(Zhu, 2001)*. For these reasons, many studies have targeted the effects of salt stress on crops like wheat, rice, maize and soybean *(Daei et al., 2009; Katerji et al., 1996; Phang et al., 2008; Zeng and Shannon, 2000)* and the enhancement of crop stress tolerance *(Arzani*

and Ashraf, 2016; Egamberdieva et al., 2019). Conversely, despite their impact on agro-environments, limited attention has been given to weed species *(Cirillo et al., 2018)*. Since weeds generally have earlier emergence, faster growth rates, higher genetic plasticity and higher resilience than crop species *(Chen et al., 2015; Clements et al., 2004; Lu et al., 2016)*, their adaptability to adverse environmental conditions might promote their spread under increasing salinity. However, not many studies dissect their responses to salt stress at different growth stages, including morphological and physiological parameters, and very few address weed-crop competition *(Cirillo et al., 2018)*. The majority of studies on the response of weed species to salt stress are focused on germination or early growth stages *(Chauhan and Johnson, 2009; Hao et al., 2017; Khan et al., 2022; Wu et al., 2018)*. Despite these stages being hugely affected by temperature, often this parameter is observed separately, without investigating the interaction with salinity. Considering the great intra-specific variability of many weed species, the response to salinity and temperature interaction effects should be tested for every species and may differ between ecotypes *(Eslami, 2011; Kheloufi et al., 2020; Wang et al., 2020)*.

In contexts where soil salinity is associated with secondary salinization and saltwater intrusion, the most critical time is the spring-summer growing season, when high evapotranspiration rates and reduced rainfall promote the accumulation of salts in the upper soil layers *(Maggio et al., 2011)*. Therefore, weed species infesting spring-summer crops should be the first target of studies regarding salt stress.

Abutilon theophrasti L., *Amaranthus retroflexus* L., *Digitaria sanguinalis* (L.) Scop., *Setaria pumila* (Poir.) Roem. & Schult and *Setaria viridis* L. are common weeds associated with spring-summer crops and widely distributed in temperate and subtropical regions *(Amini et al., 2015; Cirillo et al., 2018; Holm et al., 1997; McDonald et al., 2004; Mei et al., 2013)*. Their response to salinity varies from tolerant, like *A. theophrasti* and *S. pumila*, to moderately tolerant, like *A. retroflexus* and sensitive like *D. sanguinalis*. In fact, *Sadeghloo et al. (2013)* observed that *A. theophrasti* seeds could germinate at concentrations up to 350 mM, similar to *Amini et al. (2015),* who observed the ability of *S. pumila* seeds to germinate

at NaCl concentrations up to 320 mM. Conversely, *S. viridis* germination is completely inhibited at 160 mM NaCl. *A. retroflexus* has also been described as a moderately tolerant species, with the ability to germinate at 160 mM NaCl *(Hao et al., 2017)* and grow in a wide range of environments, especially in semiarid habitats *(Sharma et al., 2021)*. *D. sanguinalis* is generally classified as a glycophite, particularly sensitive at the seedling stage, but halophyte-like adaptive strategies have been reported in the species *(Zhang et al., 2013)*.

While some studies are available on the effects of salinity on these species, mainly at the germination stage, to our knowledge, there are no studies focused on the combined effect of salinity and temperature. However, given the adaptability of these species to salinity, it is worth investigating in depth their seed ecology and early growth to understand whether they might become more competitive over the crops. To better understand weed response to salinity and temperature interactions, we conducted germination assays and growth tests on five weed spring-summer species in a saline environment using four different salinity levels in combination with four different temperatures.

2. Materials and Methods

2.1 Seed collection and saline solution preparation

Germination tests were carried out on five weed species: *A. theophrasti*, *A. retroflexus*, *D. sanguinalis*, *S. pumila* and *S. viridis*. Species were selected considering their abundance and spread in the agro-ecosystems of the Po Valley and their emergence season. Summer species were preferred over winter species, assuming that weed-crop competition under salinity stress is more likely to happen in the spring-summer growing season. Mature seeds were collected from September to October 2018 at the experimental farm of the University of Padova (45°20′53″ N 11°57′05″ E, Legnaro, Italy), with a soil electrical conductivity (EC) of 0.3 dS/m. The seeds were then hand-cleaned and stored at 4 °C until the start of the trials.

The saline solutions for germination tests were prepared by adding pure NaCl to deionized water until the desired salinity level was reached, measured with an XS Instruments COND 80 EC meter (Giorgio Bormac s.r.l, Carpi, Italy) at a sensitivity of 1 µS. Four different saline

solutions were prepared: 4, 8, 12 and 16 dS/m. The control consisted of pure deionized water (0 dS/m).

2.2 Germination tests

Seeds of the five weed species were placed on 9 cm-diameter petri dishes lined with adsorbent filter paper. Two mL of saline solution (or deionized water in the case of the control) were applied to each Petri dish.

Petri dishes were then sealed with parafilm and kept in growth chambers (KW Apparecchi Scientifici s.r.l., Monteriggioni, Italy) at four constant temperatures (12, 15, 18 and 24 °C) with a 12 h light/ 12 h dark photoperiod. Four replicates of 100 seeds each (50 seeds in the case of *A. theophrasti*, because of the bigger seed size) were prepared for every combination of salinity and temperature, for a total of 80 Petri dishes per species. The Petri dishes were set according to a randomized design inside the growth chamber. Their positions were exchanged every other day.

Seed germination was monitored every 2-3 days, and new seedlings were removed from the Petri dishes. The seeds were considered germinated if a radicle of 1 mm or longer was present. The experiment was considered completed if all the seeds germinated or if 10 days elapsed without germination, as proposed by *Baskin and Baskin (2014)*. The percentage of germination and the germination dates were recorded for each species at different temperatures and salinity levels.

2.2 Growth experiment

Seedlings of the five weed species were grown in plastic containers of 8 cm (height) x 9 cm (width) x 10 cm (length) on half-strength MS agar medium *(Murashige and Skoog, 1962)*, containing no sucrose and no hormones, following the protocol of *Nikolić et al. (2023)*. Treatments consisted of five levels of salt stress (0, 4 8, 12 and 16 dS/m), obtained by dissolving pure NaCl in the solution used to prepare the medium and checked with an XS Instruments COND 80 electrical conductivity meter (Giorgio Bormac s.r.l., Carpi, Italy). The boxes were then autoclave-sterilized for 20 minutes at 120 °C and left to cool down. The seeds were surface-sterilized with 75 % (v/v) ethanol solution for 30 seconds, followed

by 20 % (w/v) sodium hypochlorite for 10 minutes and then rinsed four times with deionized water and once with NaCl solution (the EC was 4 8, 12 or16 dS/m depending on the treatment, while pure deionized water was used for the control), then transferred to the boxes with sterile tweezers. In order to work in sterile conditions, both the preparation of the seeds and their placement on the agar media were done in a laminar flow cabinet. The boxes were kept in growth chambers at four constant temperatures (12, 15, 18 and 24 °C). Four replicates were prepared for every combination of salinity and temperature, for a total of 40 boxes per species. Each box contained 50 seeds. The growth of the seedlings was measured after five weeks. The seedlings were removed from the agar medium and the length of each root and shoot was measured with a digital caliper (TESA Technology, Renens, Switzerland). The percentage of germination and the average root and shoot elongation were recorded to compare the six species at different temperatures and salinity levels.

2.4 Statistical analysis

For the germination tests, mean germination time (MGT) was calculated using the formula proposed by *Ellis and Roberts (1980)* and in agreement with *Borsai et al. (2018)*:

$$MGT = \Sigma(nD) / \Sigma n$$

where D is the number of days since the start of the test, and n is the number of newly germinated seeds at day D.

The effects of two factors (salinity and temperature) and their interaction on germination percentage, MGT, root and shoot length were assessed with a factorial analysis of variance (ANOVA). In the case of growth tests, root and shoot lengths were expressed as a percentage of treated over non-treated plants (% nt). The tests were followed by pair-wise post hoc analyses (Tukey test) to determine which means differed significantly at $p < 0.05$. The homogeneity of variances was confirmed by the Levene test. Whenever the homogeneity of variances of the original dataset was not confirmed, the data underwent an arcsine, squared root or logarithmic transformation in order to satisfy this ANOVA requirement. Although in the graphs significant letters are reported on the original data, we specified in the figure captions if any transformations were applied. All data analyses were conducted on R 4.2.0 (2022).

3. Results

3.1 Germination percentages

The ANOVA revealed a significant effect of all factors and their interactions on seed germination, except for *S. viridis*, with no significant interaction between salinity and competition (**Table 1**).

Table 1. *Effects of salinity, temperature, and their interactions on germination percentage.*

Factors	ABUTH p-value	AMARE p-value	DIGSA p-value	SETPU p-value	SETVI p-value
Salinity	0.011	0.000	0.000	0.000	0.000
Temperature	0.014	0.000	0.000	0.000	0.000
Salinity x Temperature	0.046	0.000	0.000	0.000	0.124

ABUTH=A. theophrasti; AMARE=A. retroflexus; DIGSA=D. sanguinalis; SETPU=S. pumila; SETVI=S. viridis

As a general rule, the germination percentage decreased with the increase of salinity at every temperature (**Figure 1** and **Table A 1**). However, the germination percentage of *A. theophrasti* was over 70% in all combinations of temperatures and treatments, without significant differences between salt-treated seeds and control seeds (**Figure 1 A**). *A. retroflexus* seeds incubated at 12 °C did not germinate when exposed to salinity, except for some seeds (0.25% of the total) in the control petri dishes. At 15 °C and 18 °C, there was minimal germination at all salinity levels, while at 24 °C the germination gradually decreased with increasing salinity levels until 16 dS/m, where only 0.25% of the seeds germinated (**Figure 1 B**). Similarly, *D. sanguinalis* did not have any germinated seeds at 12 °C, and little germination at 15 °C, mainly at 0 dS/m and 4 dS/m, while at 18 and 24 °C the germination decreased with increasing salinity levels, reaching 6.5% and 0.75% respectively (**Figure 1 C**). Both *S. pumila* and *S. viridis* seeds germinated even at low temperatures, but while germination percentages in *S. pumila* were generally similar within each salinity level at all temperatures above 12 °C, in *S. viridis* they were gradually lower at each temperature, with the highest values being those of seeds germinated at 24 °C (**Figure 1 D**, **E**).

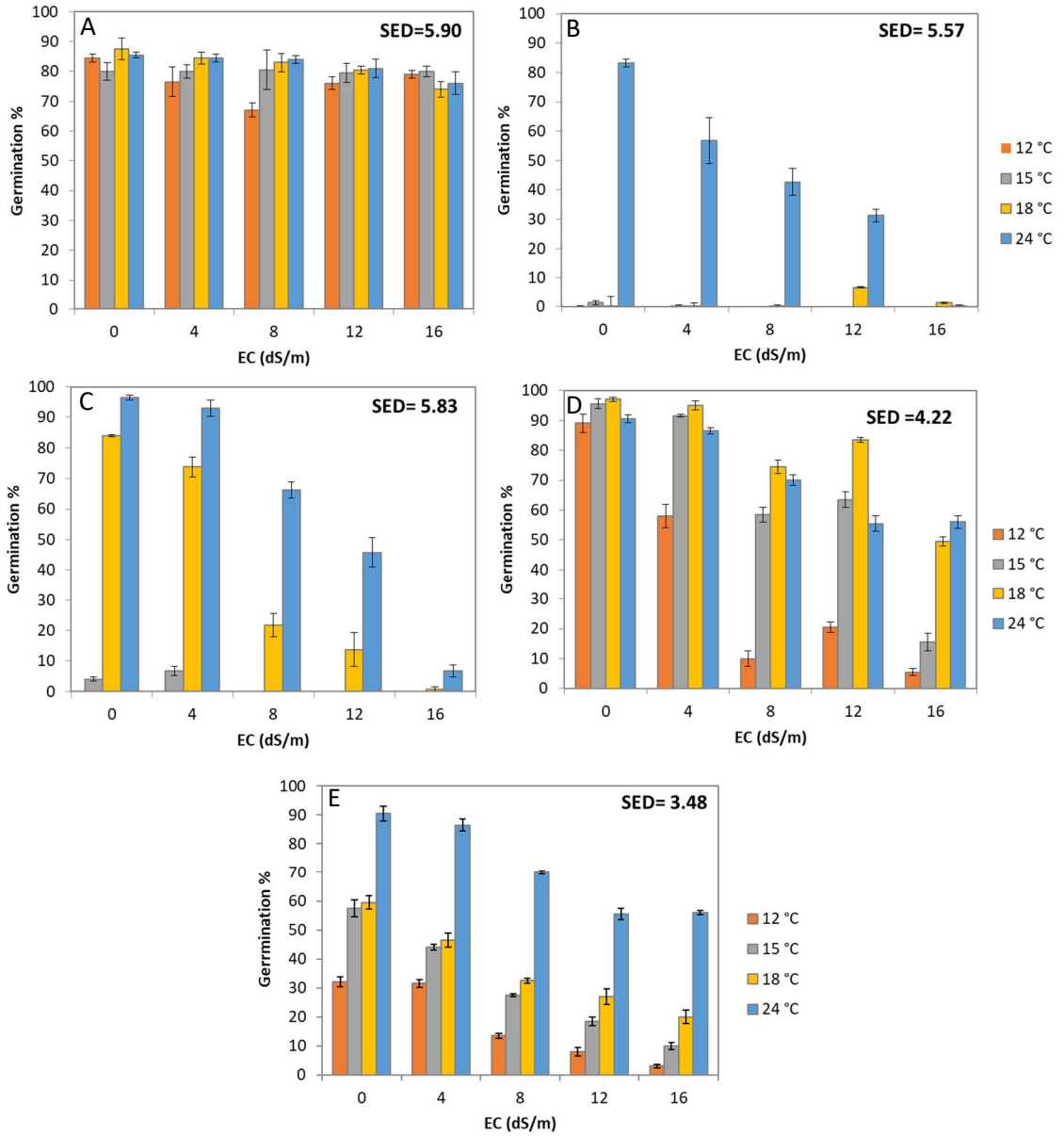

Figure 1. *Germination percentages of (A) A. theophrasti, (B) A. retroflexus, (C) D. sanguinalis, (D) S. pumila and (E) S. viridis at different salinity levels (0–16 dS/m) and temperatures (12, 15, 18, 24 °C). Vertical bars indicate the standard error. The ANOVA on A. retroflexus was performed on transformed data (logarithmic transformation).*

3.1 Mean germination time

The ANOVA revealed a significant effect of all factors and their interactions on seed germination, except for *S. pumila*, with no significant interaction between salinity and competition (**Table 2**).

Table 2. *Effects of salinity, temperature, and their interactions on mean germination time.*

Factors	ABUTH p-value	AMARE p-value	DIGSA p-value	SETPU p-value	SETVI p-value
Salinity	0.000	0.036	0.000	0.000	0.000
Temperature	0.000	0.000	0.000	0.000	0.000
Salinity x Temperature	0.000	0.000	0.000	0.127	0.000

ABUTH=A. theophrasti; AMARE=A. retroflexus; DIGSA=D. sanguinalis; SETPU=S. pumila; SETVI=S. viridis

The MGT generally remained the same or increased slightly at the increase of salinity levels, but was generally reduced at high temperatures (**Figure 2** and **Table A 2**). In the case of *A. theophrasti*, MGT only increased at the highest salinity level (16 dS/m), especially at 12°C, while the other salinity levels did not significantly differ from the control (**Figure 2 A**). In the case of *A. retroflexus*, only control seeds germinated at all temperatures, therefore the decreasing MGT with increasing temperatures is only visible here. At 24 °C, MGT was similar at all salinity levels but generally lower than MGT at 18 °C (**Figure 2 B**). *D. sanguinalis* was the species with the highest MGT at 18 °C, where an increase was observed at high salinity levels (12–16 dS/m). On the contrary, at 24 °C all the MGTs were similar (**Figure 2 C**). Both *S. pumila* and *S. viridis* had no significant differences in salinity levels (only a slight increase at 12 and 16 dS/m in the case of *S. pumila*), but the increase of temperatures always shortened the MGT (**Figure 2 D**, **E**).

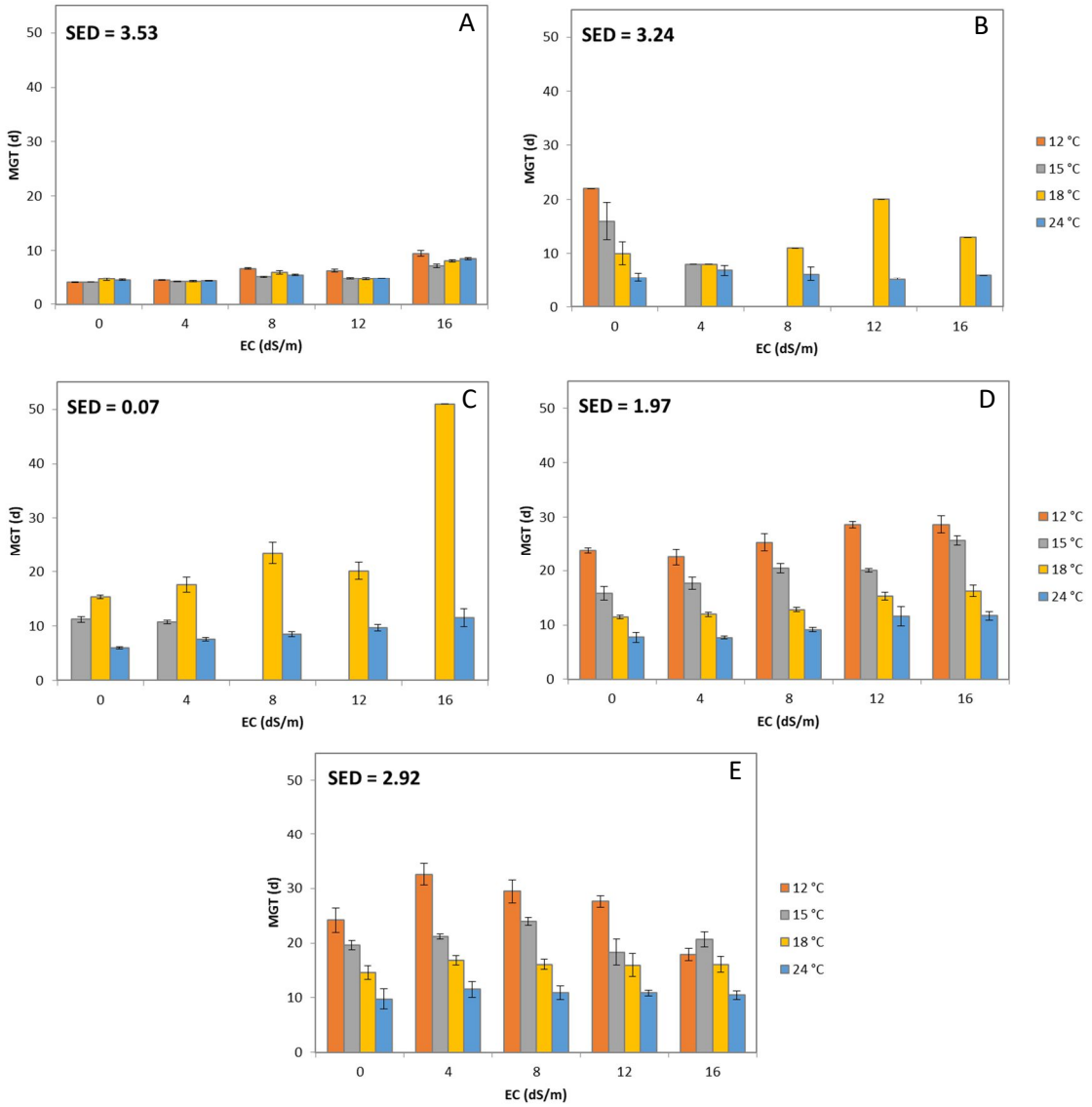

Figure 2. *Mean germination time (MGT) of (A) A. theophrasti, (B) A. retroflexus, (C) D. sanguinalis, (D) S. pumila and (E) S. viridis at different salinity levels (0 –16 dS/m) and temperatures (12, 15, 18, 24 °C). Vertical bars indicate the standard error. The ANOVA on D. sanguinalis was performed on transformed data (squared root transformation).*

3.1 Growth tests

The ANOVA revealed a significant effect of all factors and their interactions on root and shoot length expressed as a percentage over the control (**Table 3**).

Table 3. *Effects of salinity, temperature, and their interactions on root and shoot length expressed as a percentage over the control.*

Factors	ABUTH		AMARE		DIGSA	
	Shoot p-value	**Root** p-value	**Shoot** p-value	**Root** p-value	**Shoot** p-value	**Root** p-value
Salinity	0.000	0.000	0.000	0.000	0.000	0.000
Temperature	0.000	0.000	0.000	0.000	0.000	0.000
Salinity x Temperature	0.000	0.000	0.000	0.000	0.000	0.000

Factors	SETPU		SETVI	
	Shoot p-value	**Root** p-value	**Shoot** p-value	**Root** p-value
Salinity	0.000	0.000	0.000	0.000
Temperature	0.000	0.000	0.176	0.000
Salinity x Temperature	0.000	0.000	0.032	0.000

ABUTH=A. theophrasti; AMARE=A. retroflexus; DIGSA=D. sanguinalis; SETPU=S. pumila; SETVI=S. viridis

Growth test results indicate that not all the five species assayed in this study were able to develop shoots and roots at high salinity levels and low temperatures. The general trend was a reduction in shoot and root length with increasing salinity levels. However, most species at low salinity levels (4–8 dS/m) have higher root and shoot lengths compared to the control, especially at high salinity levels (**Figure 3** and **Figure 4**). At 18 and 24 °C, both root and shoot lengths of *A. theophrasti* treated with 4 and 8-dS/m-salt solution were higher than the control, while at 12 C° the values were higher than the control only at 4 dS/m. At rising salinity levels, root and shoot lengths were gradually reduced except for those of seedlings grown at 12 C°, which did not germinate at salinity levels higher than 4 dS/m (**Figure 3 A**). After five weeks of incubation, seeds of *A. retroflexus* and *D. sanguinalis* incubated at 12 and 15 °C were not germinated at any salinity level higher than the control. Shoot lengths of *A. retroflexus* were higher or comparable to the control until 12 dS/m, while a growth reduction was observed at 16 dS/m. Root growth was not enhanced by salinity and remained similar to or lower than the control up until 12 dS/m, and slightly decreased at 16 dS/m (**Figure 3 B**). Conversely, the shoot length of *D. sanguinalis* seedlings was equal to or lower than the control at all temperatures, with a significant decrease at 12 dS/m, where no seeds germinated at 15 °C. At 24 °C, root and shoot lengths were higher than the control up until 12 dS/m, except for the 16 dS/m level. Up until 8 dS/m, all root lengths were similar (**Figure 3 C**). *S. pumila* grown at 12 °C showed a significant reduction in shoot length at all salinity

levels, reaching 20% of the control at 16 dS/m, while the shoot length of seedlings grown at 15 °C was higher than the control at all salinity levels up until 12 dS/m. Seeds germinated at 18 and 24 ° had values similar to the control at all salinity levels. Root lengths of salt-treated seeds were generally lower than the control at all salinity levels except for seedlings grown at 12 °C and 4 dS/m and those grown at 15 °C (**Figure 4 A**). *S. viridis* shoot and root lengths were generally similar or lower than the control at all salinity levels, except for shoot lengths at 4 ds/m. At 12 °C, the seeds exposed to 8 dS/m only developed roots, while at 12 and 16 dS/m no germination occurred (**Figure 4 B**).

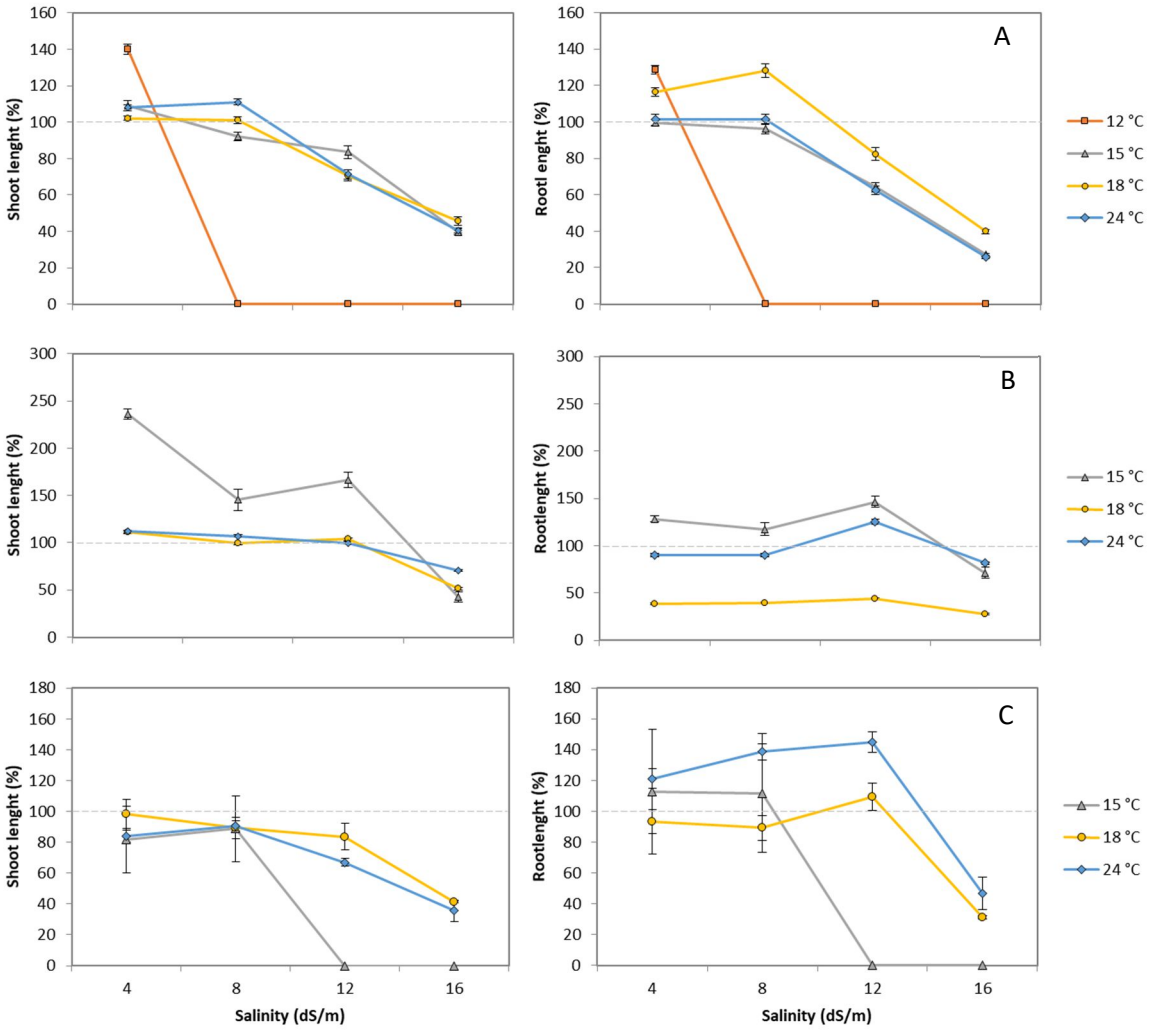

Figure 3. *Shoot and root lengths expressed as percentage over the control of (A) A. theophrasti (B) A. retroflexus; (C) D. sanguinalis at different salinity levels and temperatures. Vertical bars indicate standard error. The ANOVA on A. retroflexus and D. sanguinalis was performed on transformed data (arcsine and logarithmic transformation, respectively).*

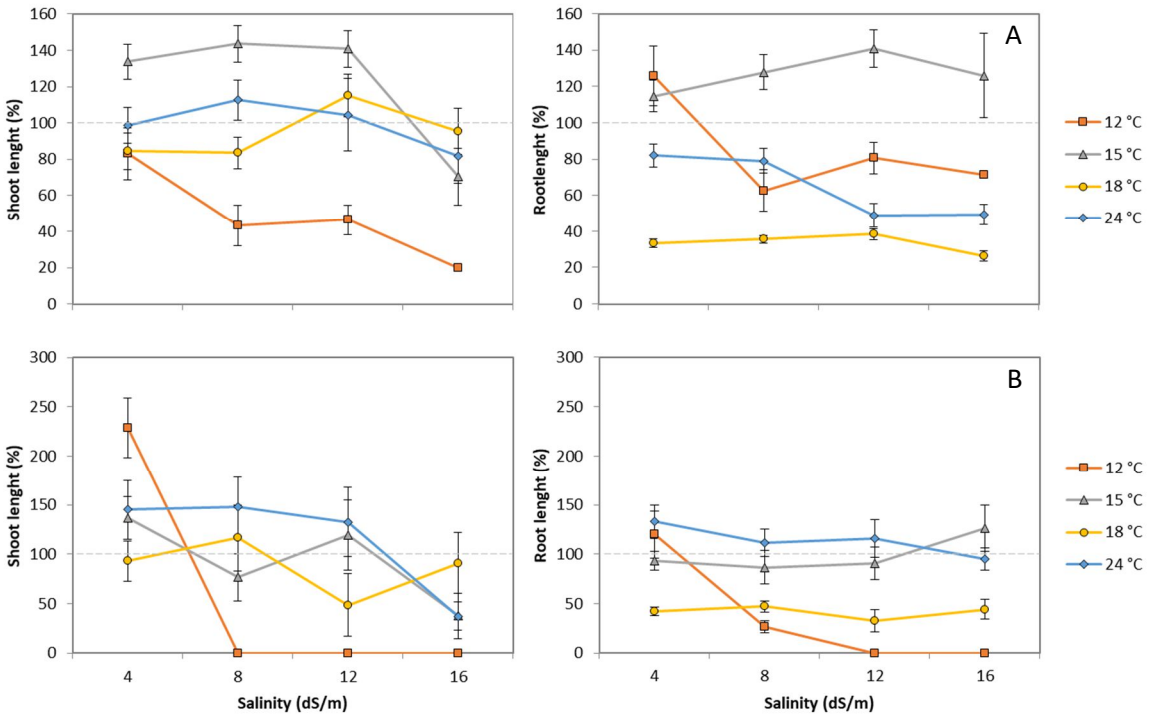

***Figure 4**. Shoot and root lengths expressed as percentage over the control of (A) S. pumila (B) S. viridis at different salinity levels and temperatures. Vertical bars indicate standard error. The ANOVA on S. viridis was performed on transformed data (arcsine transformation).*

4. Discussion

It is well known that germination and early growth are often the most sensitive stages to salt stress, but the level of sensitivity at each stage might vary depending on the species *(Bertazzini et al., 2018; Khayatnezhad and Gholamin, 2011)*. This study shows that in the five analyzed weed species, *A. theophrasti*, *A. retroflexus*, *D. sanguinalis*, *S. pumila* and *S. viridis*, the effects of salt stress on seed germination and early growth stages depend on the degree of salinity, and temperature. Each species had different responses and tolerance at the germination and early growth stages, but they were all able to germinate and develop roots and shoots at mild and medium salinity levels, even though the ecotypes used in the trials had never been exposed to salty soils before.

A. theophrasti was the most tolerant species at the germination stage, with very high germination percentages and low MGT at all salinity levels and temperatures, including 12 °C. This finding is in agreement with the observations of *Sadeghloo et al. (2013)*, that recorded seed germination up until 250 mM NaCl (approximately corresponding to an EC

of 21 dS/m), although at 110 mM NaCl, germination was inhibited in 50% of the seeds. However, *Xiong et al. (2018)* reported that no germination occurred at NaCl concentrations equal to or higher than 150 mM NaCl (approximately 16 dS/m) in seeds from Chinese populations, suggesting a difference in tolerance between different ecotypes, as also suggested by *Zhang et al. (2011)*. Growth tests showed that *A. theophrasti* was more sensitive at the early growth stages, with a significant reduction in root and shoot lengths at high salinity levels, and complete germination inhibition at 12 °C with medium to high salinity levels. To our knowledge, all the previous studies assessing the salt tolerance of *A. theophrasti* were focused on germination, therefore it is not possible to compare our results with similar experiments. However, *A. theophrasti* is known to tolerate a variety of environmental conditions, including drought and a wide range of temperatures *(Leon et al., 2004; Sadeghloo et al., 2013).* The fact that the inhibitory effect was evident only at the lowest temperature is in line with the typical behavior of the species. In fact, *A. theophrasti* can germinate at temperatures up to 40 °C and generally prefers higher temperatures rather than lower temperatures, with complete germination inhibition around 4–8 °C *(Leon et al., 2004; Xiong et al., 2018)*. Overall, our results suggest that *A. theophrasti* seeds can easily germinate and survive at moderate salinity levels, in various climatic conditions. Conversely, *A. retroflexus* appeared to be more sensitive at the germination stage than the seedling stage, although in both cases, the degree of tolerance was dependent on the temperature of incubation. Germination percentages were high only at 24 °C, and decreased to almost zero at 16 dS/m, while MGT was relatively short (less than 10 d), but significantly increased at 18 °C. This behavior is in line with the studies of *Hao et al. (2017)* and *Khan et al. (2022)*, who observed the ability of *A. retroflexus* seeds to germinate at 150 mM NaCl (approximately corresponding to 16 dS/m), with no significant decrease in germination percentage up until 100 mM. However, both experiments were carried out at optimum temperatures (30/25˚C and 35/25 °C, respectively). The inhibitory effect of low temperatures observed in the present study is consistent with the typical behavior of *A. retroflexus*, a thermophile species with a maximum germination capability between 25 and 40 °C *(Cristaudo et al., 2014)*. Excluding the seeds incubated at 12 °C, in the growth tests, *A.*

retroflexus showed a slight increase in shoot length compared with the control, as observed also in *D. sanguinalis*, *S. pumila* and *S. viridis* shoots and roots. This behavior is known as the hormetic effect *(Calabrese, 2013)*, and has been observed in other weed species *(Fogliatto et al., 2021; Nikolić et al., 2023; Sdouga et al., 2019)*. *A. retroflexus* showed minimum differences between salinity levels up until 12 dS/m, and a decrease in shoot length at 16 dS/m, in agreement with *Sharma et al. (2021),* which observed a reduction in plant height and root length in plants exposed to 150 and 300 mM NaCl. The results confirm that *A. retroflexus* is moderately tolerant to salinity, and might become increasingly aggressive in salt-affected soils of hot semiarid environments, especially given the high water use efficiency and seed production ability of the species *(Sharma et al., 2021)*. *D. sanguinalis* appeared to be the most salt-sensitive species, being affected both at the germination and the seedling stage. Also in this case, temperature greatly affected the performance of the seeds. Only seeds incubated at 24 and 18 °C had relatively high germination percentages at 4 and 8 dS/m, which significantly decreased at higher salinity levels, especially at 18 °C. Also MGT was very long (up to 50 d) for seeds germinated at 18 °C. This is in line with the observations of *Zhang et al. (2012)*, who observed delayed germination at low temperatures, no germination at 15 °C under saline conditions (50–250 mM NaCl), and reduced germination at 20 and 25 °C under high saline conditions. Similar trends were observed in our growth tests, where plants significantly decreased shoot length at high salinity levels, in agreement with *Zhang et al. (2013)*. The low tolerance of temperatures equal to or lower than 15 °C, is also confirmed by *Wang et al. (2018)* and *Zhang et al. (2013)*. However, root length at 18 and 24 °C was increased compared to the control, showing the abovementioned hormetic effect, except for seeds incubated at 16 dS/m. Based on our results, *D. sanguinalis* is less likely to become an increased threat in salt-affected soils, especially in temperate regions where the germination occurs in the spring at relatively low temperatures. Even though *D. sanguinalis* is classified as a glycophyte, Chinese ecotypes have been reported to germinate equally from 0 to 160 Mm NaCl, and different studies have observed its presence in salty soils *(Šerá, 2008; Wang et al., 2018)*, confirming the ability of the species to develop proper coping mechanisms against salinity. *S. viridis* and *S. pumila* appeared to be salt-

tolerant both at the germination and growth stages, but the former appeared to be more affected at low temperatures than the latter. In fact, the germination percentages of *S. pumila* at 15, 18 and 24 °C did not differ much within each salinity level, except for the highest level (16 dS/m). Conversely, *S. viridis* had much lower germination percentages at 12, 15 and 18 °C at all salinity levels, although the MGT of salt-treated seeds generally did not differ from the control. This is in agreement with *Amini et al. (2015)*, who compared two populations of *S. viridis* and *S. pumila*, and found that the increase in temperature from 15 ° to 30 °C increased seed germination of *S. viridis*, but decreased seed germination of *S. pumila*, that showed the maximum germination percentage at 20 °C. The *S. viridis* ecotype tested in the present study appeared to be more salt-tolerant than the ones observed by *Guo et al. (2011)* and *Amini et al. (2015)*, whose seeds did not germinate above 100 mM NaCl. Conversely, *S. pumila* germination is more in line with previously published studies, reporting the ability of the species to germinate at 300 mM *(Amini et al., 2015)*. Despite this discrepancy, our data confirm that *S. pumila* is more tolerant to high salinity levels than *S. viridis*, especially at low temperatures (12–15 °C). This was observed in the growth tests, where *S. viridis* germination was completely inhibited at 12 °C in salt-treated seeds, except at the lowest salinity level (4 dS/m), where the shoot length at all temperatures showed the hormetic effect. Conversely, *S. pumila* grown at 15 °C showed an increase in root and shoot length at all salinity levels except the highest level (16 dS/m), and none of the other temperatures had a dramatic decrease in root and shoot lengths. Overall, our results suggest that *S. pumila* is more likely to expand in salt-affected soils than *S. viridis*, especially in temperate regions characterized by lower spring temperatures.

5. Conclusions

This study shows that even weed ecotypes previously unexposed to salinity are capable of germinating and developing roots and shoots under salinity stress. It also confirms that temperature plays an important role in how salt stress affects the germination and growth of weed species, with species-specific temperature-salinity interaction. As a general rule, rising salinity levels have a detrimental effect on both germination and early growth, usually mitigated by increased temperatures. However, our results showed that good salt tolerance

at the germination stage does not always imply good tolerance at the early growth stages *A. theophrasti* showed very high salt tolerance at the germination stage but was more sensitive at the first growth stages, while *A. retroflexus* appeared to be more tolerant at the early growth stages. *D. sanguinalis* was quite sensitive both at the germination and early growth stages, while *S. pumila* and *S. viridis* appeared to be salt tolerant both at the germination and seedling stages, although the latter was more affected at high salinity levels and low temperatures. *A. retroflexus* and *D. sanguinalis* were the most sensitive species to low temperatures, followed by *S. viridis*. Overall, *A. theophrasti* and *S. pumila* appeared to be the most tolerant species, potentially representing an increased threat in semi-arid and temperate regions affected by salinization. However, given the high intra-specific variability of weed species, further studies are needed to evaluate the response of different ecotypes, as well as different species. Our results represent a useful baseline for the study of weed species in saline environments.

Appendix

Table A 1. *Germination percentage of Abutilon theophrasti, Amaranthus retroflexus, Digitaria sanguinalis, Setaria pumila and Setaria viridis at different salinity levels and different temperatures.*

T (°C)	Salinity (dS/m)	ABUTH		AMARE		DIGSA		SETPU		SETVI	
		mean	st. dev	mean	st. dev	mean	st. dev	mean	st. dev	mean	st. dev
12	0	84.50	2.52	0.25	0.50	0.00	-	44.50	6.03	16.00	3.37
12	4	76.50	9.98	0	-	0	-	29.00	7.70	15.75	2.87
12	8	67.00	4.76	0	-	0	-	5.00	5.35	6.75	1.71
12	12	76.00	4.32	0	-	0	-	10.25	3.59	4.00	2.94
12	16	79.00	2.58	0	-	0	-	2.75	2.22	1.50	1.29
15	0	80.00	5.89	1.25	1.26	4.00	1.41	47.75	3.30	28.75	5.74
15	4	80.00	4.32	0.25	0.50	6.75	2.87	45.75	0.96	22.00	2.00
15	8	80.50	13.20	0	-	0	-	29.25	4.99	13.75	0.96
15	12	79.50	6.40	0	-	0	-	31.75	5.19	9.25	2.99
15	16	80.00	3.65	0	-	0	-	7.75	5.85	5.00	2.45
18	0	87.50	7.19	6.50	6.45	84.00	0.82	48.50	1.29	29.75	4.43
18	4	84.50	4.12	1.25	1.89	73.75	6.65	47.50	3.11	23.25	4.86
18	8	83.00	6.22	0.25	0.50	21.75	7.63	37.25	4.35	16.25	1.71
18	12	80.50	2.52	0.25	0.50	13.75	11.03	41.75	1.71	13.50	5.32
18	16	74.00	5.16	0.25	0.50	0.75	1.50	24.75	3.20	10.00	4.55
24	0	85.50	1.91	83.25	2.75	96.50	1.73	45.25	2.50	25.00	4.97
24	4	84.50	2.52	56.75	15.54	93.00	5.35	43.25	2.22	26.25	4.11
24	8	84.00	2.83	42.75	9.22	66.25	5.32	35.00	3.65	18.00	1.41
24	12	81.00	6.00	31.25	4.50	45.75	9.71	27.75	5.38	16.25	3.86
24	16	76.00	7.48	0.25	0.50	6.75	4.03	28.00	4.32	7.00	1.63

ABUTH=A. theophrasti; AMARE=A. retroflexus; DIGSA=D. sanguinalis; SETPU=S. pumila; SETVI=S. viridis

Table A 2. *Mean germination time of Abutilon theophrasti, Amaranthus retroflexus, Digitaria sanguinalis, Setaria pumila and Setaria viridis at different salinity levels and different temperatures.*

T (°C)	Salinity (dS/m)	ABUTH		AMARE		DIGSA		SETPU		SETVI	
		mean	st. dev	mean	st. dev	mean	st. dev	mean	st. dev	mean	st. dev
12	0	4.07	0.27	22.00	0.06	0	-	23.87	0.94	24.18	4.58
12	4	4.37	0.22	0	-	0	-	22.51	3.11	32.57	3.93
12	8	6.70	0.63	0	-	0	-	25.31	3.16	29.46	4.19
12	12	6.30	1.03	0	-	0	-	28.61	1.18	27.60	2.05
12	16	9.48	2.28	0	-	0	-	28.65	3.16	17.89	2.31
15	0	4.07	0.12	16.00	6.93	11.25	1.08	15.78	2.55	19.57	1.69
15	4	4.17	0.27	8.00	0.04	10.74	0.78	17.66	2.16	21.22	0.95
15	8	5.19	0.53	0	-	0	-	20.42	1.79	23.96	1.51
15	12	4.85	0.76	0	-	0	-	20.03	0.72	18.36	4.72
15	16	7.24	1.35	0	-	0	-	25.71	1.71	20.67	2.82
18	0	4.62	1.25	10.02	4.25	15.34	0.57	11.46	0.66	14.66	2.52
18	4	4.20	0.45	8.00	0.06	17.62	2.84	11.97	0.79	16.86	1.61
18	8	5.97	1.35	11.00	0.07	23.54	4.00	12.83	0.79	16.11	1.82
18	12	4.74	1.18	20.00	0.06	20.16	3.16	15.34	1.43	15.95	4.23
18	16	8.10	0.95	13.00	0.05	51.00	0.00	16.26	2.14	16.07	2.91
24	0	4.46	0.45	5.52	1.58	6.02	0.39	7.70	1.81	9.74	3.69
24	4	4.27	0.41	6.91	1.79	7.55	0.65	7.68	0.59	11.52	2.90
24	8	5.50	0.61	6.20	2.61	8.48	1.01	9.15	0.86	10.89	2.52
24	12	4.84	0.35	5.14	0.57	9.66	1.20	11.60	3.51	10.86	1.07
24	16	8.47	0.83	6.00	0.00	11.48	3.28	11.67	1.59	10.46	1.56

ABUTH=A. theophrasti; AMARE=A. retroflexus; DIGSA=D. sanguinalis; SETPU=S. pumila; SETVI=S. viridis

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IV Short-term responses to salinity of soybean and *Chenopodium album* grown in single and mixed-species hydroponic systems

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1. Introduction

Soil salinization, a condition characterized by a high concentration of soluble salts, among which NaCl is the most soluble and widespread *(Almeida et al., 2017)*, is increasingly affecting agroecosystems, thus contributing to the loss of arable land and adversely impacting crop yields *(Corwin, 2021)*. Due to climate changes and depletion of natural resources, the agricultural land injured by salinization, currently accounting for 20% to 30 % of the world's cultivated areas, is expanding globally at an annual rate of 10% *(Jamil et al., 2011)*. Sea-level rise and groundwater overexploitation responsible for saltwater intrusion in coastal and inland aquifers *(Taylor et al., 2013)*, are among the main factors expected to exacerbate the negative effects of salinity.

High soil salinity impairs seed germination, root length, plant height, leaf size and productivity of many cultivated species, including staple crops like wheat, rice, maize and soybean *(Daei et al., 2009; Katerji et al., 1996; Phang et al., 2008; Zeng and Shannon, 2000)***.** Major toxic effects of salinity on the plant at the cellular level include ion imbalance, which affects plant metabolism by increasing the accumulation of Na^+ and Cl^- ions while depleting K^+ and Ca^{2+} in tissues *(Khan and Panda, 2008)*, and hyperosmotic stress *(Zhu, 2001),* which is primarily due to decreased water foraging and reactive oxygen species (ROS) overproduction *(Niu et al., 1995; Xing et al., 2013; Zhu, 2001)*. ROS are involved in many biological processes, such as growth and development, cell cycle and programmed cell death *(Garg and Manchanda, 2009)*, but can also initiate cascade reactions that induce oxidative stress, especially through lipid peroxidation and alteration of cell membranes by protein denaturation and DNA mutation *(Jbir-Koubaa et al., 2015).* Enzymes such as ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and catalase (CAT), along with non-enzymatic antioxidants like ascorbate and glutathione, and the osmolyte proline, are involved in plant defense mechanisms against ROS *(Apel and Hirt, 2004; McKersie and Leshem, 1994)* and can be used as markers to define the salt stress status of the plant.

The physiological responses to salt stress have been thoroughly studied in soybean (*Glycine max* (L.) Merr.), which is a salt-sensitive glycophyte *(Phang et al., 2008)*. Soil salinization affects important agronomic traits of this crop, such as the growth rate, nodulation, seed quality and quantity *(Phang et al., 2008)*, which result in severe yield reduction (up to 40%) *(Chang et al., 1994)*. In contrast, weed responses to salinity have been overlooked so far, as well as weed-crop interactions *(Cirillo et al., 2018)*. Because invasive weeds exhibit earlier emergence, faster growth rates and higher genetic resilience and plasticity over cultivated species under hostile environmental conditions *(Chen et al., 2015; Clements et al., 2004; Lu et al., 2016)*, their spread might be favored with increasing salinity in arable lands.

Chenopodium album L. is one of the most widespread weeds associated with spring and summer crops (e.g. soybean, maize, sugar beet) and displays a plethora of traits, including allelopathic potential, high seed production and longevity, that qualify it as a fearsome weed *(Cirillo et al., 2018)*. It is also recognized as a high-salt tolerant species owning typical halophytic traits, such as seed dimorphism, sodium exclusion, potassium retention, high production of osmolytes and antioxidants (Tanveer and Shah, 2017), and generally exhibits greater plasticity in response to changing environment *(Kraehmer and Baur, 2013)*. Thus, this weed can grow within a wide range of climates and soil conditions (pH*,* soil type, fertility*)* and is likely to spread in agroecosystems increasingly affected by climate change.

To our knowledge, only a few and updated studies exist that appraise the interactions of *C. album* with staple crops in saline and non-saline environments. Also, most of these studies consider the interactive effects of species only at the seed and early emergence level, while only a few of them evaluated the competition along all the plant life cycle *(Sartorato et al., 1996; Shurtleff and Coble, 1985)*. On this account, the current work aims to investigate the responses of soybean and *C. album* seedlings to salinity (NaCl) in hydroponics, according to single-species and co-cultivation (mixed) set-ups. We assayed the plant biomass and changes in protein and elemental content, as well as the intensity of oxidative stress-related markers (antioxidant capacity, antioxidant enzyme activity, lipid peroxidation, content of total phenolic compounds and the osmolyte proline). Hydroponics was chosen over soil because it is a simplified system that allows studying specific stressors while minimizing variations in measured traits apart from those due to applied treatments, thereby avoiding the stochastic factors that typically affect *in-field* experiments. Furthermore, the understanding of weed-crop interactions in a controlled environment will provide a solid knowledge basis for further studies performed in greenhouse and open field conditions.

2. Materials and Methods

2.1. Plant growth conditions and experimental design

C. album seeds were harvested from a soybean field at the University of Padova experimental farm (45° 20' 53" N 11° 57' 05" E, Legnaro, Italy), with a soil electrical conductivity of 0.3 dS/m. Seeds were cleaned and kept at 4 °C until the start of the experiment. The weight of 1000 seeds was 0.540 ± 0.001 g, and 90% of the seeds were black. The soybean cultivar (cv. PD1T45) used in this study was salt-sensitive, as in preliminary tests conducted in hydroponics plants manifested stress signs (e.g., reduced turgor, stunted growth) at low salt concentration.

Soybean and *C. album* seeds were allowed to germinate in silty loam soil inside a growth chamber set at 25/20 °C, with a lighting period of 14 hours, relative humidity of 70/85% and at a photon flux density (PFD) of 280 mol $m^{-2}s^{-1}$, until *C. album* seedlings reached the height of 10 cm. For each species, equal-size plants were carefully washed with double distilled water to remove the majority of soil particles from their radicle and then transferred to a hydroponic set-up consisting of sixteen 5-L tanks filled with half-strength Hoagland's solution *(Hoagland and Arnon, 1950)*, at a density of 6 plants per tank. After 3 days of acclimation, plants were divided as follows: soybean plants (-/+ NaCl), *C. album* plants (-/+ NaCl), 3 soybean plants plus 3 *C. album* plants (-/+NaCl). Plants subjected to salt stress were supplied with 100 mM NaCl. The salt stress treatment was determined based on preliminary experiments where NaCl concentrations ranging from 25 mM to 150 mM were tested on plants. In these experiments, soybean was found to be excessively affected by NaCl concentrations over 100 mM, by showing extensive necrosis of leaf tissues after 7 days, while 100 mM NaCl was the concentration at which the effects of salt on plant growth became clearly manifest. The duration of the experiment was limited to one week in order to observe short-time effects of various treatments on soybean physiological responses. For each salt treatment, either minus or plus NaCl, two tanks were set for the individual species, and four tanks for the mixed species. The tanks with both soybean and *C. album* were twice those with single-species in order to obtain the same number of biological replicates per treatment (**Figure 1**). The whole trial was repeated a second time for data confirmation, with the same number of tanks and plant density in each tank. Individual treatments and relative acronyms are reported in **Table 1**. After 1 week since then, plants were collected. The fresh (FW) and dry (DW) weight of leaves and roots of individual plants (six per treatment) were measured. For dry weight determination, the plant material was oven-dried at 70°C for 48 h.

The same dry material was used for elemental quantification, while the remaining plants were immediately frozen in liquid nitrogen and stored at -80 °C until further biochemical analyses. In this case, assays were conducted on three samples per treatment. Each sample consisted of a bunch of two plants. Protein concentration in leaves was determined using the Bradford method *(Bradford, 1976)*.

Table 1. Treatment applied and corresponding acronyms in the figures.

Progressive number	Treatment	Acronym
1 [1]	Soybean No NaCl	S
2	Soybean plus 100 mM NaCl	S+NaCl
3 [2]	Soybean and *C. album* No NaCl	S+C
4	Soybean and *C. album* plus 100 mM NaCl	S+C+NaCl
5	*C. album* No NaCl	C
6	*C. album* plus 100 mM NaCl	C+NaCl
7 [2]	*C. album* and Soybean No NaCl	C+S
8	*C. album* and Soybean plus 100 mM NaCl	C+S+NaCl

[1] From treatment 1 to 4, soybean plants were analyzed, from 5 to 7 *C. album* plants were analyzed.
[2] Treatments 3 and 7 were performed and compared to evaluate possible allelopathic interferences between soybean and *C. album* plants when grown together (in the same tank).

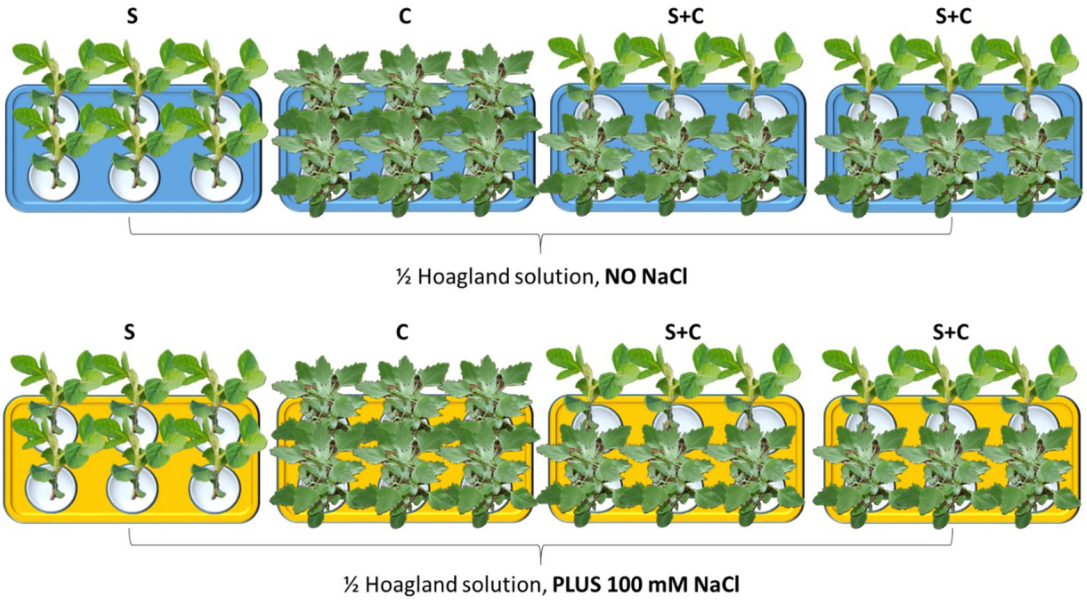

Figure 1. *Experimental design of the hydroponic set-up. S = Soybean; C = C. album.*

2.2. Soluble protein quantification

Frozen samples (200 mg) were ground in a mortar with liquid nitrogen and extracted with phosphate buffer (pH 7.8) containing polyvinylpyrrolidone (PVP) (10 g L^{-1}) in the ratio of 1:10. Samples were centrifuged for 20 min at 13,000 g at 4°C. The supernatant was collected and the extract (50µL) was used for the protein assay. Protein content was quantified using a UV/VIS spectrophotometer (Eppendorf Biophotometer® Basic D30, US) by comparing the values measured at $\lambda = 595$ nm with those provided by a reference calibration curve prepared using bovine serum albumin (BSA) at different dilutions. Data were expressed as milligrams of protein per gram of fresh weight.

2.3. Elemental content quantification

Nitrogen (N) contents were determined in dried plant material using a CNS elemental analyzer (Vario MACRO CNS, Hanau, Germany). The quantification of Na and K in leaves and roots was performed after an acid-digestion procedure. Digestion reactions were carried out inside closed Teflon vessels of 100 mL volume using 500 mg dry plant material in 9 mL HNO_3 and H_2O_2 30% (7:2) in a microwave (Millestone Start-D 1200W). Mineralized samples were then diluted in 25 mL ultrapure water and each element was assayed via Inductively Coupled Plasma Atomic Emission Spectroscopy (Optima 2000 DV, Perkin Elmer Instruments, Germany). Data were expressed as milligrams per kilo (ppm) of dry weight.

2.4. Determination of total antioxidant activity and phenol content

The total antioxidant activity in leaves and roots was evaluated by measuring the ferric-reducing antioxidant power (FRAP). The assay was based on the methodology of *Benzie and Strain (Benzie and Strain, 1996)*. Ten grams of plant material (leaves and roots) were homogenized in 20 mL of high-performance liquid chromatography (HPLC) grade methanol using an Ultra-Turrax tissue homogenizer (Takmar, Cincinnati, OH, United States) at a moderate speed (setting of 60) for 30 s. The FRAP reagent was freshly prepared, containing 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate buffer at pH 3.6. One hundred microliters of the methanol extract were added to 1,900 µL of FRAP reagent and accurately mixed. After leaving the mixture at 20ºC for 4 min, the absorbance was determined at 593 nm. Calibration was against a standard curve (0–1,200 mg mL^{-1} ferrous ion) obtained by the addition of freshly prepared ammonium ferrous sulfate. FRAP values were calculated as micrograms per milliliter ferrous ion (ferric-reducing power) and are presented as milligrams per kilogram of Fe^{2+} Eq (ferrous ion equivalents).

The concentration of total phenols in leaves and roots was determined according to the Folin-Ciocalteu (FC) assay with gallic acid as calibration standard, using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Columbia, MD, United States). The FC assay was performed placing 200 µL of plant extract (obtained as described above for the total antioxidant activity) into a 10 mL PP tube. This procedure was followed by the addition of 1 mL of the FC reagent. The mixture was vortexed for 20 to 30 s. 800 µL of sodium carbonate solution (20% w/v) were added to the mixture 5 min after the addition of the FC reagent. This was recorded as time zero; the mixture was then vortexed for 20 to 30 s after the addition of sodium carbonate. After 2 h at room temperature, the absorbance of the colored reaction product was measured at λ= 765 nm. The concentration of total phenols in the extracts was calculated from a standard calibration curve obtained with different concentrations of gallic acid, ranging from 0 to 600 mg mL^{-1}. Results were expressed as milligrams of gallic acid equivalent per kilogram of FW *(Nicoletto et al., 2013)*.

2.5. Antioxidant enzyme activity

The analysis of enzyme activity was performed in frozen leaves (200 mg) ground in a mortar with liquid nitrogen and extracted with 50 mM phosphate buffer (pH 7.8) containing PVP (10 g L^{-1}) and Triton X-100 (250 µL), in the ratio 1:10 (w/v). Guaiacol peroxidase activity was determined by measuring the oxidation of guaiacol in the presence of H_2O_2 (extinction coefficient, 26.6 mM cm^{-1}) at λ = 470 nm over a 3 min interval. The reaction mixture contained 50 µL of 20 mM guaiacol, 2.9 mL of 0.036% H_2O_2 (v/v), and 50 µL of enzyme extract. For APX, the activity was determined following the decrease of ascorbate (extinction coefficient 2.8 mM cm^{-1}) and measuring the change in absorbance at λ=290 nm over a 3 min interval. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA)-Na_2, 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 50 µL of enzyme extract *(Nakano and Asada, 1981)*. Results were expressed as enzymatic units per milligram of protein. CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient, 39.4 mM cm^{-1}) at λ = 240 nm over a 2 min interval. The reaction mixture contained 2.9 mL of 0.036% H_2O_2 (w/w) and 100 µL of enzyme extract. The reaction was initiated by adding the enzyme extract. Results were expressed as enzyme units per milligrams of protein.

2.6. Lipid peroxidation

For malondialdehyde (MDA) assay, frozen leaf tissues (150 mg) were ground in liquid nitrogen and added with phosphate buffer (pH 7); butylated hydroxytoluene (BHT) to

prevent sample autoxidation and to minimize formation of artifacts. Extracts were further centrifuged at 20,000 g at 4°C for 20 min, and 200 µL of each supernatant was added with 1.3 mL of 0.3% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA). Tubes were placed in a heat block for 30 min at 95°C. Then, they were cooled in ice and centrifuged at 15,000 g at 4°C for 10 min. The absorbance was read at 532 and 600 nm. The OD_{600} must be subtracted from the OD_{532} value (correction for turbidity). Extinction coefficient = 155 mM cm^{-1}. Data were expressed as TBARS (thiobarbituric acid reactive substances).

2.7. Proline quantification

Proline content in leaves and roots was determined by reversed-phase (RP)-HPLC followed by UV detection. Each sample was prepared by placing 100 mg of plant material in a 6 × 50 mm borosilicate glass tube. HCl (7.5 mL, 6 M) was added to the sample, which was then heated at 105 °C for 24 h. After hydrolysis, the sample was neutralized to pH 9 using 8N NaOH and brought up to 100 mL with water. The solution was then filtered through a syringe filter of 0.45µm to conduct the derivatization procedure. Ten µL of extract were then mixed with 70 µL of 0.2 M borate buffer (pH 9.0), followed by 20 µL of aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) dissolved in acetonitrile. The mixture was incubated for one minute at room temperature, then for 10 min at 55 °C. The resulting AQC-derivatized mixture was diluted by adding 900 µL of borate buffer.

The chromatographic analysis was performed using an Agilent Infinity 1260 liquid chromatograph with binary pump (Agilent Technologies, Santa Clara, CA, United States), equipped with a CORTECS C18 column (2.7 µm, 2.1 x 150 mm). The mobile phase was 0.1 % formic acid (v/v) in deionized water and the flow rate was 0.4 mL/min. The injection volume was 5 µL. Proline was detected with a diode array detector (DAD) and its concentration was determined based on a standard curve and the results were expressed in nanomoles of proline per gram of fresh weight.

2.8. Statistical analysis

The parameters evaluated were compared within soybean treatments (progressive numbers: 1–4) and within *C. album* treatments (progressive numbers: 5–8) (i.e. plants grown in single-species or mixed tanks, with or without the addition of NaCl, **Table 1**). In addition, the interaction between salt stress and interspecific competition was compared between soybean and *C. album*.

To assess differences among treatments (salinity and competition), one-way analysis of variance (ANOVA) was performed separately for soybean and *C. album*, using TIBCO 13.6.0 Statistica (2019). The test was followed by pair-wise post hoc analyses (Student-Newman-Keuls test) to determine which means differed significantly at $p < 0.05$ (±SD). The homogeneity of variances was confirmed by the Levene test. The number of biological replicates varied depending on the analysis performed, as reported in the figure legends. A factorial ANOVA was performed on TIBCO 13.6.0 Statistica (2019) to assess the combined influence of species and competition on all the parameters, expressed as percentage of treated over non treated plants (% nt).

3. Results

3.1. Effect of NaCl, plant-competition and combination of both on plant biomass

The fresh leaf and root biomass of soybean plants subjected to salinity stress were significantly reduced compared to the relative NaCl-untreated controls (minus and plus *C. album*) (**Figure 2 A, C**). In the absence of NaCl, a slight decrease of soybean leaf and root fresh biomass was observed when plants were held in the mixed group. NaCl impaired the leaf and root dry biomass of soybean plants grown separate from *C. album* (**Figure 2 B, D**). The leaf dry biomass was also reduced by the crop co-existence with the weed, but the decrease was not significant in this case (**Figure 2 B**). In contrast, the root dry biomass of soybean was impaired when plants were grown with *C. album*, without any further negative effect due to NaCl (**Figure 2 D**). With respect to *C. album*, no appreciable differences in leaf and root biomass were evident depending on the growth set-up (single or mixed), NaCl application, or the combination of both these two factors (**Figure 2 A**–**D**).

When data of fresh and dry biomass of salt-treated plants were computed over untreated plants (% nt), a significant difference between species was determined for fresh leaf matter only, with *C. album* displaying higher mean values (**Figure 3 A**, **Table 2A**). Even though no differences were detected either between species or between single-species and mixed growth set-up, the interaction between species and competition was however significant in terms of dry root biomass. In fact, the % nt of *C. album* dry biomass increased with competition, as opposed to soybean (**Figure 3 B**).

The difference between species was also significant for the plant water content (**Figure 4 A, B**). In particular, higher leaf DW/FW ratios in soybean were indicative of lower water content in leaves (**Figure 4 A**). With respect to root DW/FW ratios, the competition and

interaction factors were significant. In fact, the root water content of soybean was lower (i.e. higher DW/FW ratios) than *C. album* in mixed-species tanks and higher than *C. album* in single-species set-up, but both species showed a lower root water content (i.e. higher DW/FW ratios) in the presence of competition (**Figure 4 B**).

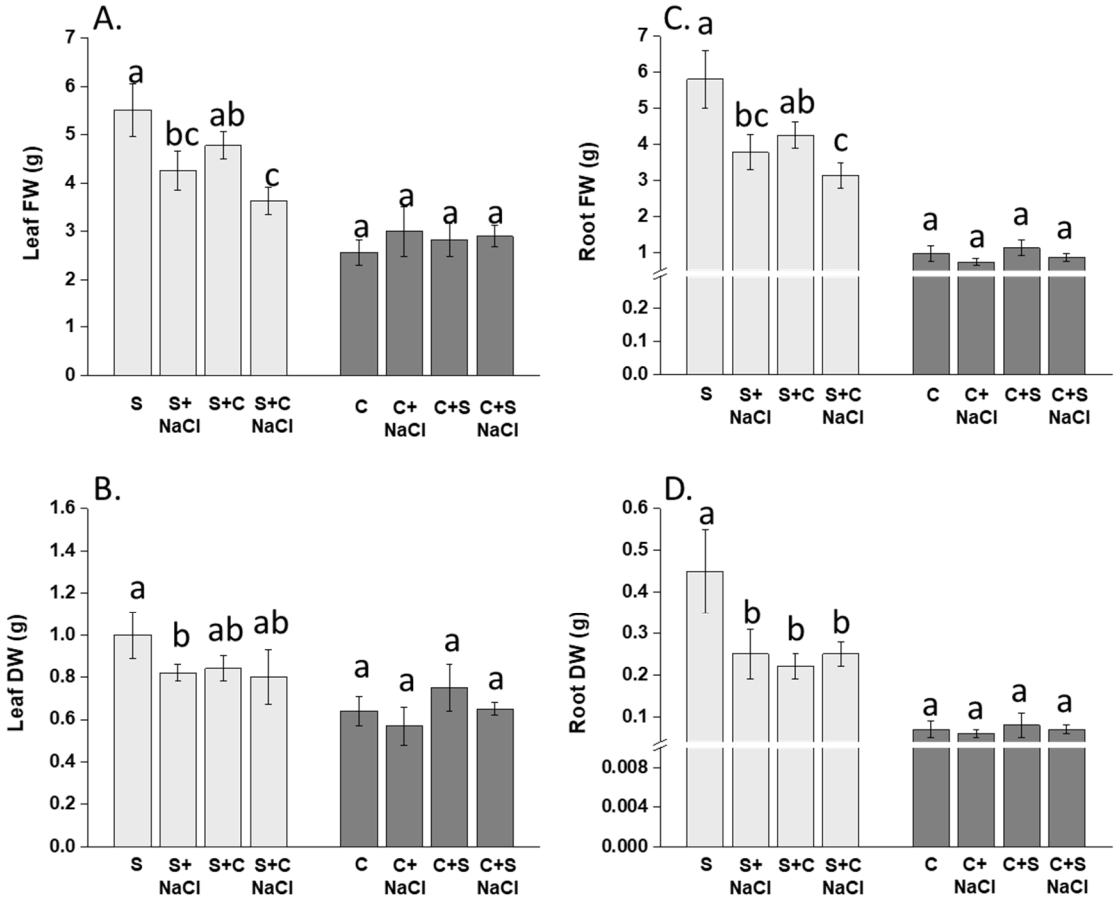

Figure 2. *(A) Leaf fresh weight (FW) of soybean and C. album, treated and non-treated with NaCl. (B) Leaf dry weight (DW) of soybean and C. album, treated and non-treated with NaCl. (C) Root fresh weight (FW) of soybean and C. album treated and non-treated with NaCl. (D) Root dry weight (DW) of soybean and C. album, treated and non-treated with NaCl. Different letters within each group of bars indicate significant differences at $p<0.05$, n=6. S = soybean; C = C. album. The experiment was replicated twice and only data from one representative experiment are shown.*

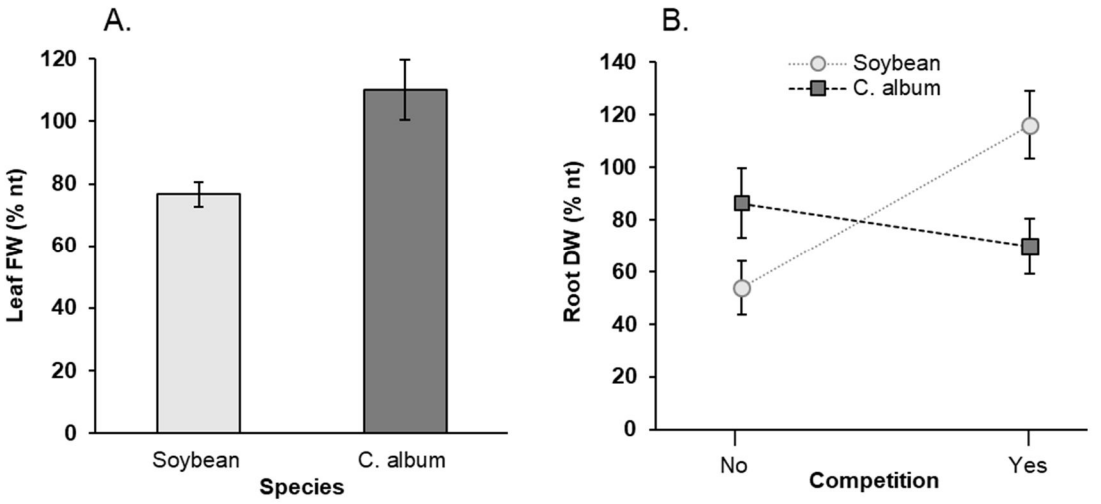

Figure 3. *(A) Average leaf fresh weight (FW), expressed as percentage of salt-treated samples over non-treated samples (% nt). (B) Species-competition interaction for average root dry weight (DW) of soybean and C. album (% nt). Values on the left are referred to plants grown in single-species set-up (no competition). Values on the right are referred to plants grown in mixed-species set-up (competition between soybean and C. album). Vertical bars denote the standard error. The experiment was replicated twice and only data from one representative experiment are shown.*

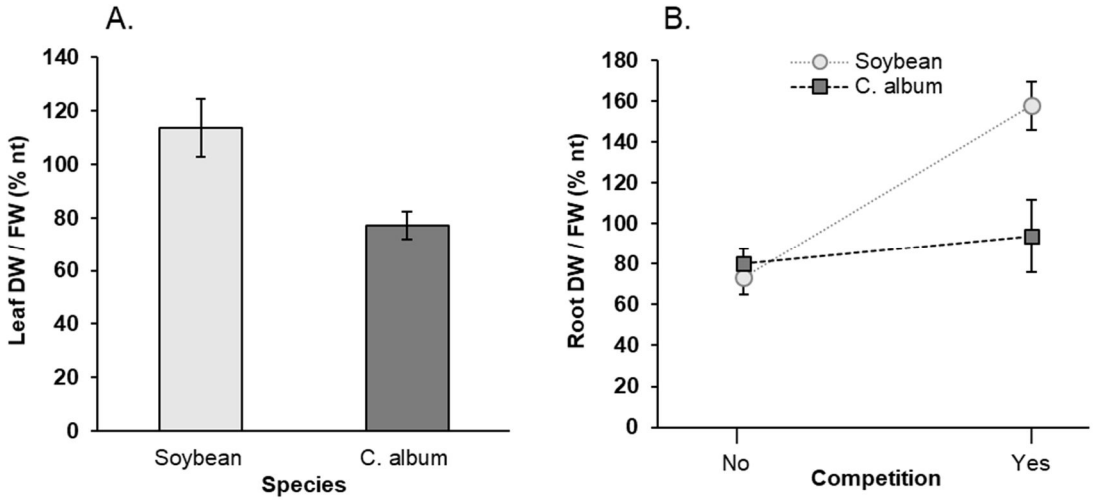

Figure 4. *(A) Leaf dry weight (DW)/fresh weight (FW) ratios, expressed as percentage of salt-treated samples over non-treated samples (% nt). (B) Species-competition interaction for dry weight (DW)/fresh weight (FW) ratios of soybean and C. album roots (% nt). Values on the left are referred to plants grown in single-species tanks (no competition). Values on the right are referred to plants grown in mixed-species tanks (competition between soybean and C. album). Vertical bars denote the standard error. The experiment was replicated twice and only data from one representative experiment are shown.*

3.2. Effect of NaCl, plant-competition and combination of both on the content of N and soluble proteins

No relevant changes in N contents were observed in soybean and *C. album* plants, irrespective of NaCl treatment and/or the growth set-up (**Figure 5 A**). Feeding plants with NaCl caused a decrease in protein accumulation in both species (**Figure 5 B**). However, such a reduction was particularly pronounced (about 50% relative to the control) for soybean plants settled in the mixed set-up. With respect to *C. album*, the co-existence with soybean did not cause a further decline of protein accumulation compared to the salinity stress condition alone (**Table 2 B**).

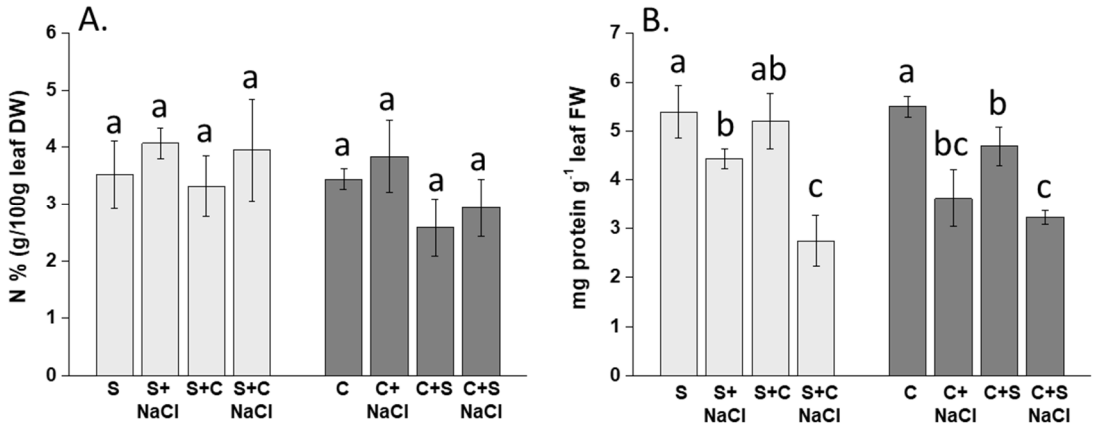

Figure 5. *(A) Percentage of N in dried leaves of soybean and C. album, treated and non-treated with NaCl. (B) Protein content per gram of leaf fresh weight (FW) of soybean and C. album, treated and non-treated with NaCl. Different letters within each group of bars indicate significant differences at p<0.05, n=3. S = soybean; C = C. album. The experiment was replicated twice and only data from one representative experiment are shown.*

3.3. Effect of NaCl, plant-competition and combination of both on Na+ and K+ accumulation

C. album plants exhibited a very high capacity to accumulate Na^+ in leaves, while root Na^+ concentration was similar as in soybean (**Figure 6 A, B**). Consequently, the translocation factor (TF) of Na^+ was about two-fold higher in *C. album* than in soybean (**Figure 6 C**). Furthermore, in the absence of NaCl, *C. album* plants contained from 2 to 3 fold more Na^+ than soybean. The co-cultivation set-up did not significantly modify Na^+ accumulation by both species.

The distribution of K^+ also differed between soybean and *C. album* (**Figure 6 D, E**). Soybean plants contained less K^+ in their leaves compared to *C. album* under no salt treatment, and no significant variation was evident when NaCl was applied (**Figure 6 D**). Conversely, *C. album* plants contained very high K^+ concentrations in leaves, which were though severely

decreased by NaCl application, regardless of the growth set-up. Soybean plants contained more K^+ in roots compared to *C. album*, but NaCl caused the reduction of K^+ accumulation in both species when co-cultivated (**Figure 6 E**). Consequently, the TF of K^+ was greater in *C. album* than in soybean but declined when plants received NaCl (**Figure 6 F**). Interestingly, the cohabitation of both species improved the capacity of *C. album* to maintain K^+ in roots and leaves when plants were NaCl-untreated (**Table 3**). The increase of Na^+ accumulation and concomitant decrease of K^+ content in *C. album* plants accounted for the about 2-fold higher Na^+/K^+ ratios determined in this species compared to soybean.

Data expressed as % nt confirmed the existence of relevant differences between soybean and *C. album* in terms of Na^+ and K^+ contents in roots and leaves (**Table 2 C**). With respect to K^+ root content (% nt), a significant difference was also found between single-species and mixed set-up, and for the interaction between species and competition (**Table 2 C**). In the presence of competition, the decrease in K^+ root content was more pronounced in *C. album* than in soybean.

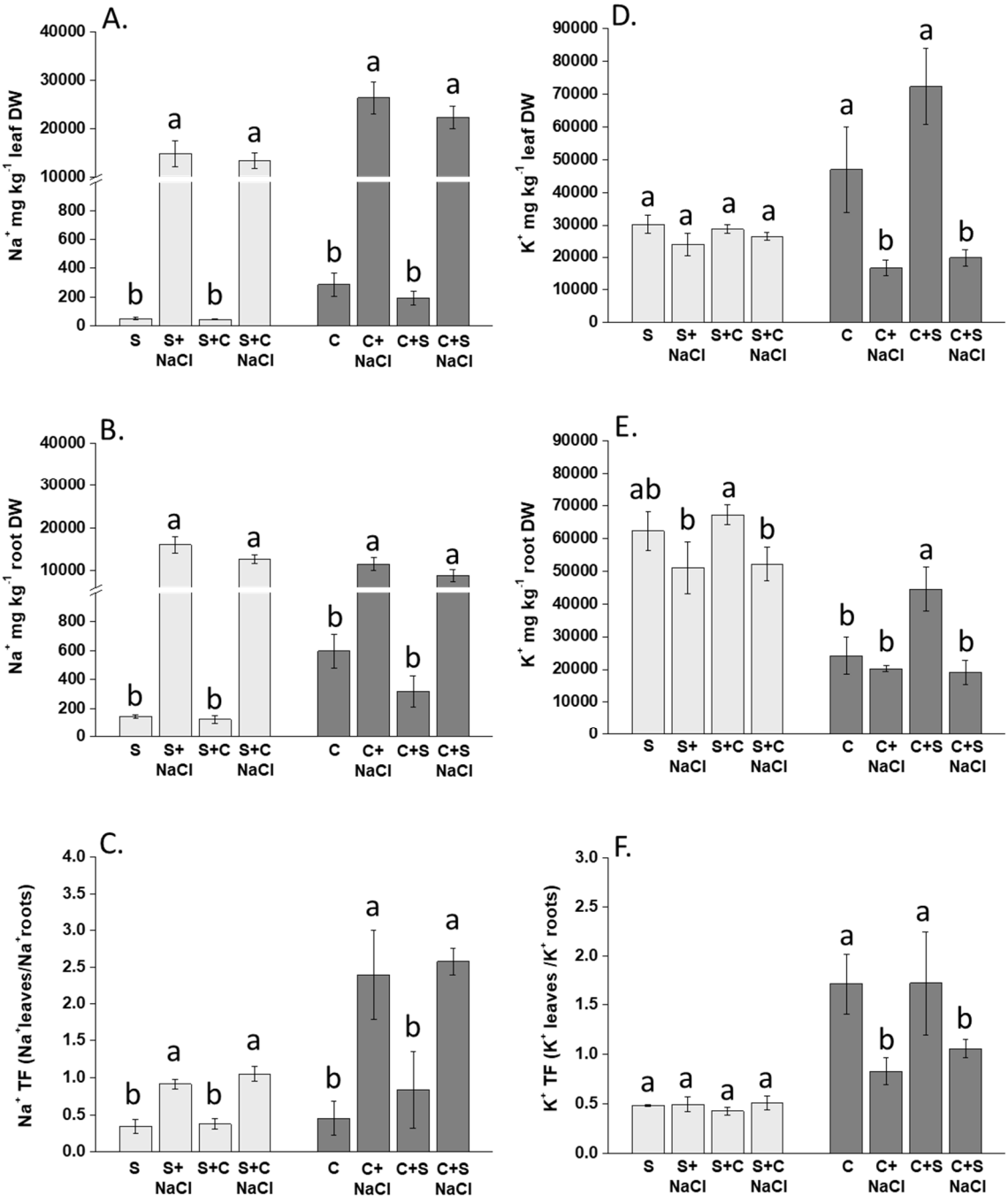

Figure 6. *Na^+ content in leaves (A) and roots (B) of soybean and C. album, treated and non-treated with NaCl. (C) Na^+ translocation factor (TF) of soybean and C. album, treated and non-treated with NaCl. K^+ content in leaves (D) and roots (E) of soybean and C. album, treated and non-treated with NaCl. (F) K^+ translocation factor (TF) of soybean and C. album, treated and non-treated with NaCl. Different letters within each group of bars indicate significant differences at $p<0.05$, $n=3$. S = soybean; C = C. album. The experiment was replicated twice and only data from one representative experiment are shown.*

Table 2. *ANOVA significance for the effect of species (soybean and C. album), competition (single-species tanks or mixed-species tanks) and their interaction on the percentage of salt-treated samples over non-treated samples.* ***(A)*** *Fresh weight (FW), dry weight (DW) and DW/FW ratio of leaves and roots.* ***(B)*** *Content of N and soluble proteins in leaves.* ***(C)*** *Na^+ and K^+ content in leaves and roots, Na and K translocation factor (Na leaves/Na roots, K leaves/K roots).* ***(D)*** *Phenolic compounds in leaves and roots and antioxidant capacity via FRAP in leaves and roots.* ***(E)*** *Antioxidant enzyme activity (guaiacol peroxidase (GPX), ascorbate peroxidase (APX), catalase (CAT) and lipid peroxidation via malondialdehyde (MDA) assay in leaves.* ***(F)*** *Proline content in leaves and roots.*

A	**leaves**	**roots**	**leaves**	**roots**	**leaves**	**roots**
Factors	**FW**	**FW**	**DW**	**DW**	**DW/FW**	**DW/FW**
Species	0.005	ns	ns	ns	0.007	0.028
Competition	ns	ns	ns	ns	ns	0.001
Species x Competition	ns	ns	ns	0.003	ns	0.008

B	**leaves**	**leaves**
	N tot	**Proteins**
Species	ns	ns
Competition	ns	ns
Species x Competition	ns	0.038

C	**leaves**	**leaves**	**roots**	**roots**	**Na leaves/**	**K leaves/**
	Na	**K**	**Na**	**K**	**Na roots**	**K roots**
Species	0.000	0.000	0.000	0.045	0.047	0.000
Competition	ns	ns	ns	0.011	ns	0.043
Species x Competition	ns	ns	ns	0.020	ns	ns

D	**leaves**	**roots**	**leaves**	**roots**
	Phenols	**Phenols**	**FRAP**	**FRAP**
Species	ns	ns	0.010	ns
Competition	ns	ns	ns	ns
Species x Competition	ns	0.012	0.012	0.003

E	**leaves**	**leaves**	**leaves**	**leaves**
	MDA	**GPX**	**APX**	**CAT**
Species	ns	0.038	0.017	0.047
Competition	0.031	ns	0.004	0.023
Species x Competition	ns	ns	0.008	0.016

F	**leaves**	**roots**
	Proline	**Proline**
Species	0.024	0.000
Competition	0.028	0.000
Species x Competition	0.008	0.000

Table 3. *Na+/K+ ratios in leaves and root of soybean and C. album, treated and non-treated with NaCl. Letters along columns indicate significant differences within both species groups at p < 0.05, n=3 (+ SE). The experiment was replicated twice and only data from one representative experiment are shown.*

Treatment	**Na^+/K^+**	
	leaves	**roots**
Soybean No NaCl	0.002 ± 0.000d	0.002 ± 0.000d
Soybean plus 100 mM NaCl	0.618 ± 0.028b	0.324 ± 0.029b
Soybean and *C. album* No NaCl	0.002 ± 0.000d	0.002 ± 0.000d
Soybean and *C. album* plus 100 mM NaCl	0.506 ± 0.038c	0.249 ± 0.043b
C. album No NaCl	0.008 ± 0.004d	0.028 ± 0.012c
C. album plus 100 mM NaCl	1.601 ± 0.177a	0.569 ± 0.076a
C. album and Soybean No NaCl	0.003 ± 0.000d	0.007 ± 0.002d
C. album and Soybean plus 100 mM NaCl	1.156 ± 0.205a	0.483 ± 0.117ab

3.4. Effect of NaCl, plant-competition and combination of both on the content of phenolic compounds and plant antioxidant capacity (FRAP)

The leaf and root content of phenols was appreciably increased by NaCl in soybean plants belonging to the single-species group (**Figure 7 A, B**). Such an effect was also evident in *C. album* plants cultivated in the mixed set-up. The trend of the plant antioxidant activity, which is reported as FRAP, was similar to that described for phenols (**Figure 7 C, D**).

In terms of % nt, phenols in leaves were not significantly different according to the species and the growth set-up (**Table 2 D**). However, the interaction between species and competition was significant for phenols in roots and FRAP in leaves and roots. FRAP in leaves was also different between species (**Table 2 D**).

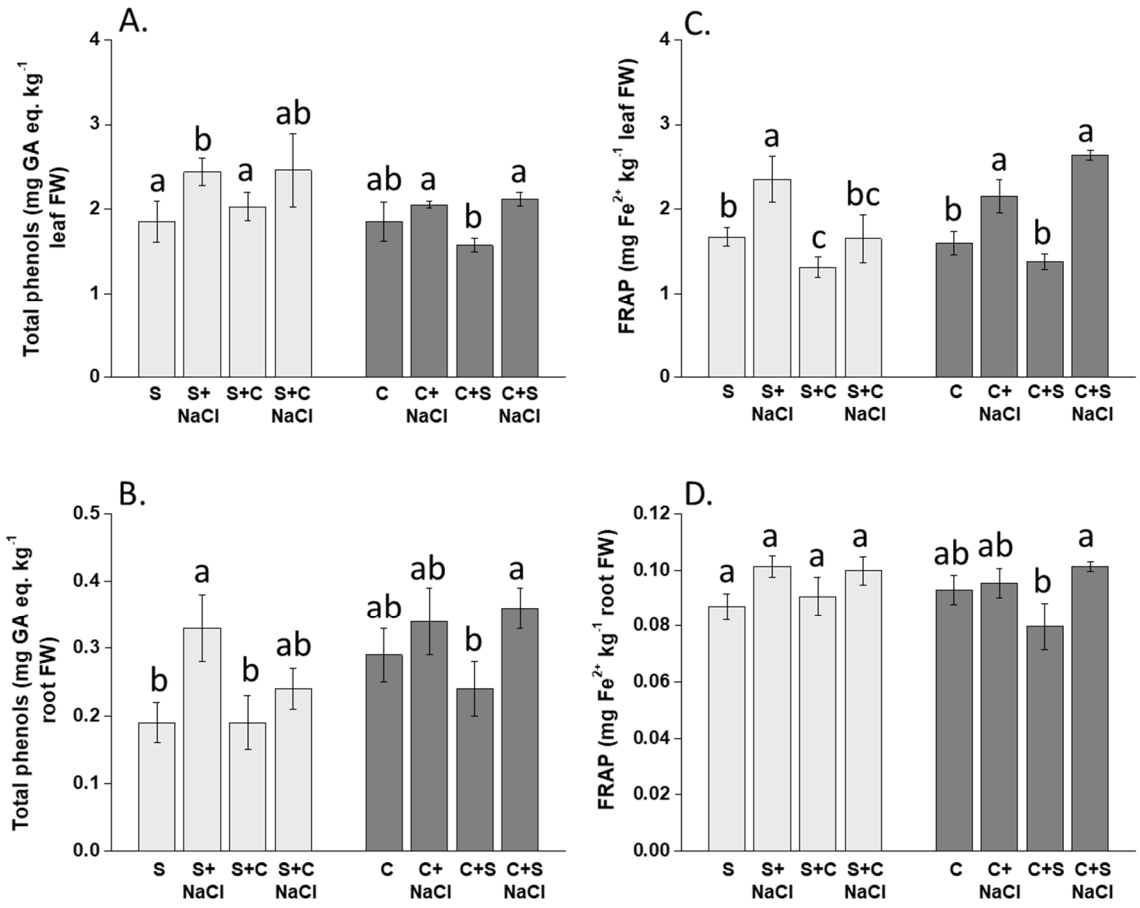

Figure 7. *Total phenols in leaves (A) and roots (B) of soybean and C. album, treated and non-treated with NaCl. Ferric-reducing antioxidant power (FRAP), expressed as milligrams per kilogram of ferrous ion equivalent, in leaves (C) and roots (D) of soybean and C. album, treated and non-treated with NaCl. Different letters within each group of bars indicate significant differences at p<0.05, n=3. S = soybean; C = C. album. The experiment was replicated twice and only data from one representative experiment are shown.*

3.5. Effect of NaCl, plant-competition and combination of both on antioxidant enzyme activity (GPX, APX, CAT) and lipid peroxidation

The activity of antioxidant enzymes was generally more pronounced in soybean than in *C. album*. In more detail, NaCl application increased the activity of GPX and CAT enzymes in soybean (**Figure 8 A**, **B**), while APX activity was enhanced by either NaCl or co-cultivation with *C. album* (**Figure 8 C**). The increase in activity of antioxidant enzymes due to NaCl was also observed in *C. album* plants of the single-species set-up (**Figure 8 A**–**C**), while CAT and APX activities were higher in *C. album* co-cultivated with soybean, either with or without NaCl, than in plants of the single-species group and NaCl-untreated (**Figure 8 B, C**).

Lipid peroxidation was lower in *C. album* compared to soybean plants. However, in both species, lipid peroxidation was significantly intensified by NaCl in the single-species set-

ups (**Figure 8 D**). Increased lipid peroxidation was also observed in *C. album* co-cultivated with soybean, without receiving NaCl.

With reference to % nt, a significant difference between the two species was found for APX, GPX and CAT activity (**Table 2 E**). CAT (**Figure 9 A**) and APX (**Figure 8 B**) also showed a relevant difference between single-species and mixed set-up and a significant interaction between species and competition factors (**Table 2 E**). In the case of lipid peroxidation, a substantial difference in % nt was recorded only for the competition factor (**Table 2 E**).

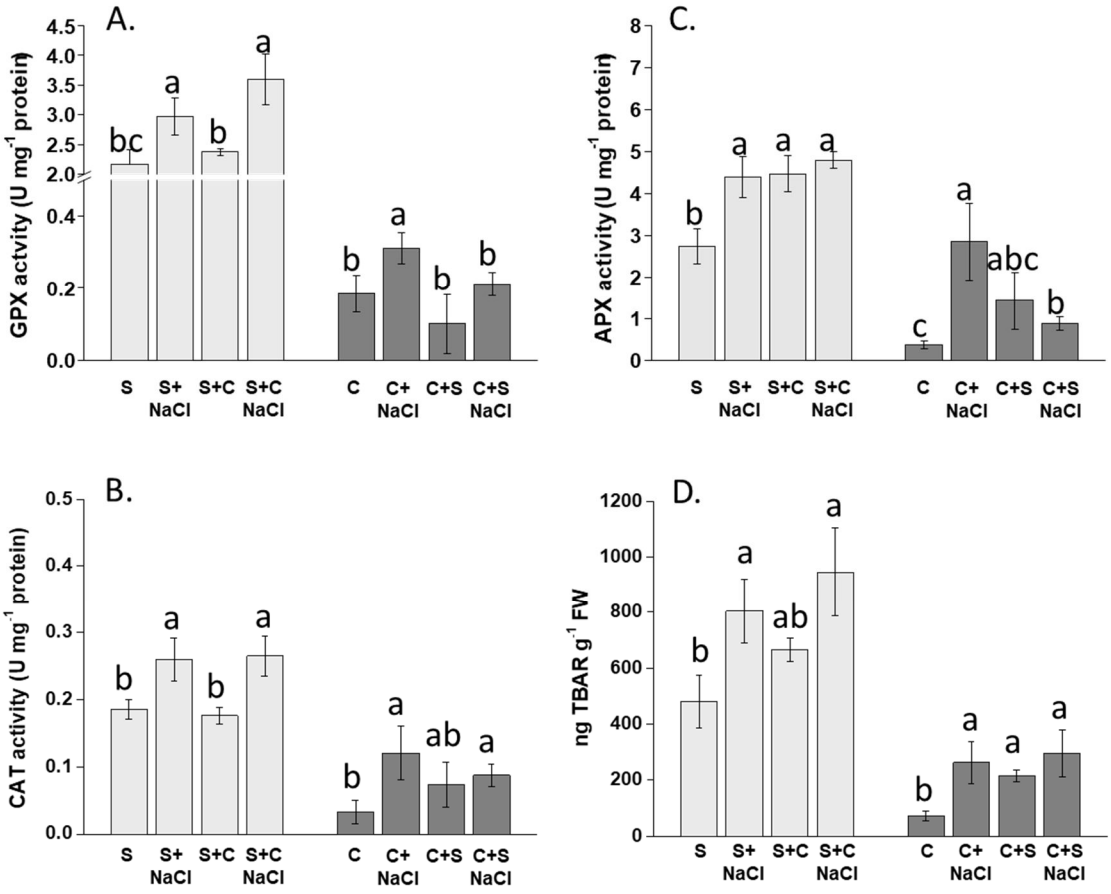

Figure 8. *(A) Guaiacol peroxidase (GPX) activity in leaves of soybean and C. album, treated and non-treated with NaCl. (B) Catalase (CAT) activity in leaves of soybean and C. album, treated and non-treated with NaCl. (C) Ascorbate peroxidase (APX) activity in leaves of soybean and C. album, treated and non-treated with NaCl. (D) Lipid peroxidation, expressed as thiobarbituric acid reactive substances (TBARS) in leaves of soybean and C. album, treated and non-treated with NaCl. Different letters within each group of bars indicate significant differences at $p<0.05$, $n=3$. S = soybean; C = C. album. The experiment was replicated twice and only data from one representative experiment are shown.*

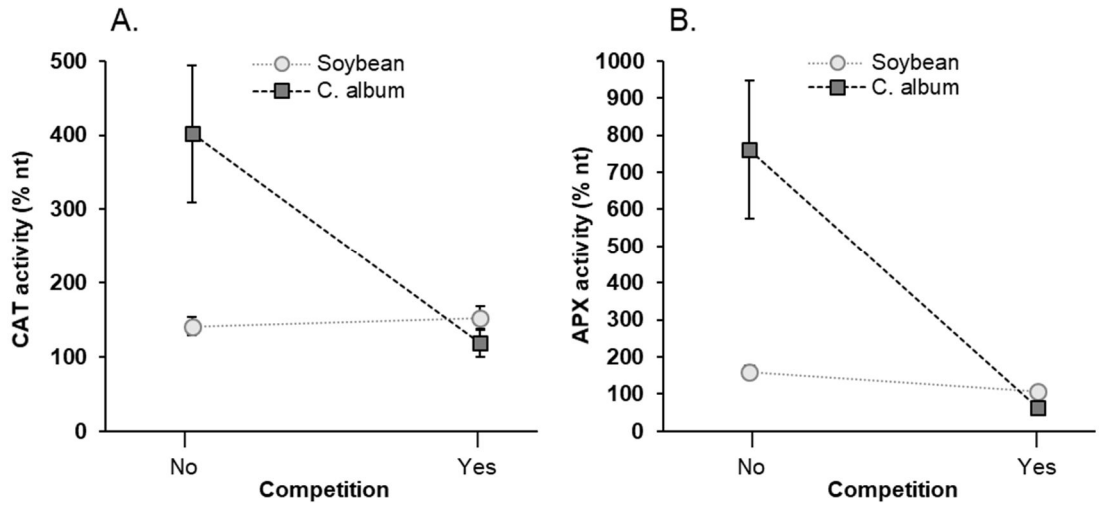

Figure 9. *Species-competition interaction for average catalase (CAT) activity (A) and average ascorbate peroxidase (APX) activity (B) in fresh leaves of soybean and C. album, expressed as percentage of salt-treated samples over non-treated samples (% nt). Values on the left are referred to plants grown in single species tanks (no competition). Values on the right are referred to plants grown in mixed-species tanks (competition between soybean and C. album). Vertical bars denote the standard error.*

3.6. Effect of NaCl, plant-competition and combination of both on proline accumulation

The addition of NaCl caused the accumulation of proline in leaves of soybean, which was however significant only when plants were grown without *C. album*. Conversely, *C. album* plants subjected to NaCl treatment contained more proline in leaves when co-cultivated with soybean (**Figure 10 A**). Root proline content was significantly increased by NaCl in both species, regardless of the growth set-up. The most pronounced effect was evident in *C. album* plants treated with NaCl in the single-species arrangement. Combining *C. album* and soybean in the absence of NaCl also caused the raise, although moderate, in root proline content compared to the individual species growth set-up (**Figure 10 B**).

In terms of % nt, a significant difference was found for species and competition, and the interaction between species and competition (**Table 2 F**), with opposite behaviour for leaves and roots. The leaf proline relative content (+NaCl/-NaCl) was higher in soybean than in *C. album* when plants were grown in the single set-up, while lower in the presence of competition. The opposite trend was observed for root proline content (**Figure 11 A, B**).

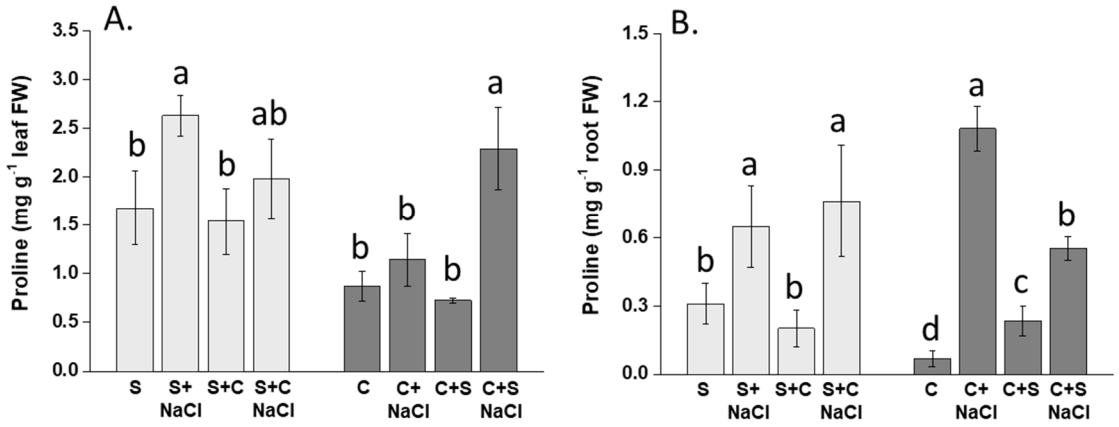

Figure 10. *Proline content in leaves (A) and roots (B) of soybean and C. album, treated and non-treated with NaCl. Different letters within each group of bars indicate significant differences at $p<0.05$, n=3. S = soybean; C = C. album, FW = fresh weight. The experiment was replicated twice and only data from one representative experiment are shown.*

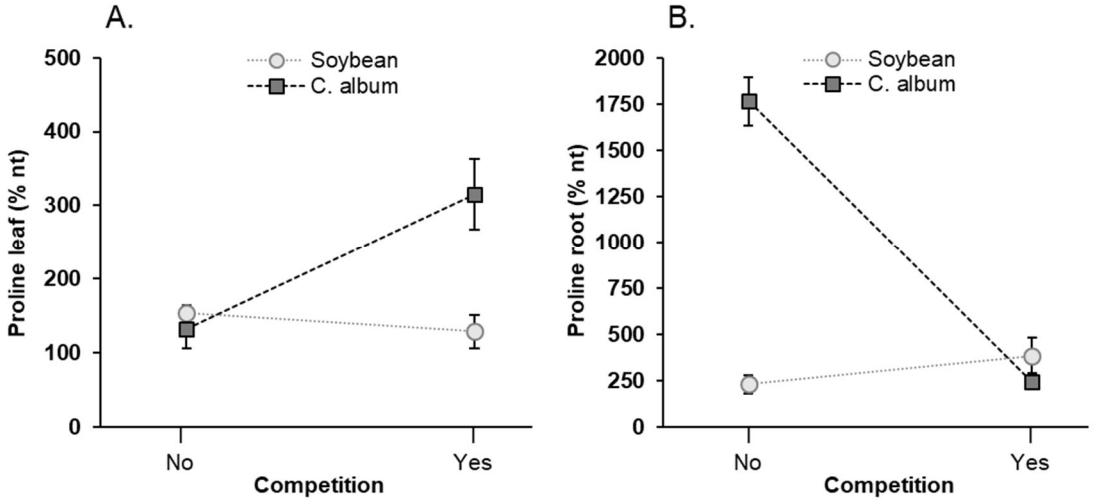

Figure 11. *(A) Species-competition interaction for average proline content in fresh leaves of soybean and C. album, expressed as percentage of salt-treated samples over non-treated samples (% nt). (B) Species-competition interaction for average proline content in fresh roots of soybean and C. album (% nt). Values on the left are referred to plants grown in single-species tanks (no competition). Values on the right are referred to plants grown in mixed-species tanks (competition between soybean and C. album). Vertical bars denote the standard error.*

4. Discussion

This study aims to evaluate whether salinity impacts crop and weed competition, which in turn might affect the crop resilience to salt stress and require adjustments of weed management strategies in the global warming framework. To better understand how crops and weeds possibly disturb each other while responding to NaCl, we chose the hydroponic set-up as a simplified system for plant growth.

Our results indicate that soybean plants suffered from salt stress, as revealed by the impairment of leaf and root biomass. This is consistent with the literature that recognizes

soybean as a salt-sensitive glycophyte (Phang et al., 2008). We also observed a decrease in root dry biomass of soybean plants grown with the weed. It is known that C. *album* is a very strong competitor of soybean, especially in the case of early weed emergence *(Sartorato et al., 1996; Shurtleff and Coble, 1985)*, and its interference with crops was previously postulated to depend on various factors, including nutrient competition and allelopathy. In this study, the reduction in root dry biomass of NaCl-untreated soybean plants of the mixed set-up was not due to competition with *C. album* for nutrient foraging, because the amount of nutrients in tissues was similar to that measured in soybean plants settled in the single group. Possibly, the weed released substances through exudates that impaired the development of the neighboring crop. Indeed, *C. album* was formerly found to reduce the growth of other crops like rapeseed *(Rezaie and Yarnia, 2005)*, sunflower, tomato *(Reinhardt et al., 1994)*, and rice *(Alam et al., 1997)* through the release of allelochemicals, known to consist mainly of phenolic acids (e.g. ferulic acid) *(Mallik et al., 1994)*. Also, *Namvar et al. (2009)* reported the inhibitory effect of *C. album* aqueous extracts obtained from leaves, roots and the whole plant on soybean growth, which was further exacerbated by combining the extracts with NaCl. The synergy of *C. album* and salt stress in determining significant reduction of soybean growth was however not observed during the period of treatment assayed in our study, which was aimed at evaluating short-term responses. We do not rule out that extending the period of treatment may lead to a more pronounced reduction of soybean growth under this condition, especially considering that plants collected at the end of the experiment showed a substantial decrease in the content of proteins.

C. *album* confirmed to be a salt-tolerant species, being NaCl unable to affect its biomass and water conservation. Like other halophytes, *C. album* tolerance to NaCl had been ascribed to various mechanisms, which appear to depend on the intensity of salt stress *(Flowers and Colmer, 2008; Tanveer and Shah, 2017)*. *Yao et al. (2010)*, for instance, observed a preferential uptake of K^+ over Na^+ in *C. album* plants treated with mild NaCl stress, and the increase of K^+/Na^+ ratio in the cytoplasm and Na^+ sequestration in vacuoles under severe NaCl stress. This last process is thought to be crucial in determining the tolerance to salt stress not only of weeds, but also of crops *(Wu et al., 2019)*. Other reports highlight the extraordinary capacity of *C. album* to accumulate Na^+ in leaves, a process that is also termed "craving for salt" *(Osmond et al., 1980)*. In our study, *C. album* displayed a greater capacity of Na^+ accumulation and root-to-shoot delivery than soybean, which justified the 2-fold higher Na^+/K^+ ratios in its tissues, while K^+ accumulation in leaves was conversely reduced

by NaCl. K^+ loss from plants is a common phenomenon under salinity stress and the capacity of plants to counteract salt-induced harms depends on K^+ availability and K^+ retention in tissues *(Wu et al., 2018)*. K^+ losses, however, are generally more pronounced in salt-sensitive than tolerant plant varieties *(Chen et al., 2007; Wu et al., 2018).* Our results seem thereby to be at odds with the current literature regarding *C. album*, but it must be noted that K^+ content was very high in the weed untreated with NaCl. Elevated initial levels of K^+ possibly counteracted the early negative effects of Na^+ accumulation in *C. album*, thus aiding the weed to maintain the osmotic balance and better acclimate to the adverse salt condition, even though K^+ was later partly lost. This hypothesis is plausible considering that the salt-sensitive soybean plants contained less K^+ in their leaves, but no K^+ losses were evident due to NaCl. In addition, the TF for K^+ was always higher in *C. album* than in soybean, regardless of salinity, which suggested the better ability of *C. album* to control long-distance K^+ transport, either by more efficient xylem loading and delivery to the shoot or minimizing the extent of K^+ recirculation in the phloem *(Wu et al., 2018)*. Unlike *C. album*, soybean plants accumulated Na^+ equally between roots and leaves, while K^+ was preferentially retained in the roots. Previous studies report that salt-sensitive species may even increase the overall root K^+ content compared to salt-untreated plants *(Ai-Rawahy et al., 1992; Bulut and Akıncı, 2010; Hamada and El-Enany, 1994)*. The restricted K^+ translocation to the aerial parts, along with the low leaf K^+ accumulation, were both likely responsible for the limited capacity of soybean plants to tolerate NaCl, which was manifest in the decline of plant growth. Although the weed and the crop did not interfere with the capacity of each other to accumulate Na^+ and K^+ in leaf tissues, soybean promoted K^+ accumulation in the root of *C. album* unless salinity was applied, and K^+ was significantly lost from the roots of both species when co-cultivated under salt stress.

NaCl decreased protein accumulation in both species. This outcome has been reported in many crops *(Debouba et al., 2006; Tester and Davenport, 2003)*, but becomes particularly significant for soybean, which is a relevant protein crop and its protein content is indissolubly linked to its nutritional value. Sharing the same set-up with C. *album* made this effect even worse. Under salt stress, *C. album* did not subtract N from soybean for N uptake, as the capacity of the crop to accumulate N in leaves was unchanged. Thus, the effect of *C. album* was apparently on the process of N assimilation into proteins rather than N uptake. It must also be noted that soybean increased the production of N compounds like antioxidant enzymes, phenolics and the osmolyte proline under salt stress, generally without differences

between plants of the single and mixed set-ups, which may suggest substantial use of N resources to support the antioxidant machinery of the plant.

Leaves of C. *album* contained less proteins under salinity possibly because of K^+ losses and was not influenced by the co-existence with soybean. C. *album* has documented capacity to tolerate severe salt stress by producing numerous compatible solutes that contrast osmotic imbalance and promote cell turgor maintenance, similar to salt-stress resistant plants *(Hasegawa et al., 2000)*. Proline is one major organic osmolyte *(Liu et al., 2017; Osman et al., 2020)* and its concentration was dramatically increased in the weed, as in the crop, under salinity. The increase in proline generally reflects the osmoregulatory role of this compound *(Heuer, 2010)*. In leaves of C. *album*, this effect was more pronounced when plants were grown with soybean and concurred with the increase in accumulation of antioxidant phenol compounds. The opposite was observed for soybean. Therefore, the weed and the crop influenced reciprocally for the production of proline and phenols, with different outcomes in leaves and roots, which may depend on the osmotic status of the plant organ.

Soybean plants showed enhanced activity of antioxidant enzymes under salinity, which was consistent with the lipid peroxidation trend, as having higher antioxidant activity is a strategy that protects plants from cellular injuries caused by ROS *(Haq et al., 2013)*. In *C. album*, the activity of antioxidant enzymes was stimulated by NaCl only in the single growth set-up, but overall it was lower than in soybean because lipid peroxidation intensity was concurrently very low. The observation that the leaf antioxidant activity in the weed was increased by salt stress and was comparable to that of soybean opens the hypothesis that other antioxidant mechanisms and ROS-scavenging molecules other than those explored in this study may be involved in the elevated tolerance of C. *album* to NaCl.

5. Conclusions

This study confirms the low and high salt tolerance of soybean and *C. album* plants, respectively, by dissecting different intensities of individual responses. The presence of *C. album* in the same growth system with soybean repressed the crop growth and protein accumulation, but neither affected its N nutrition, nor its capacity to accumulate Na^+. Unlike other investigations conducted in halophytic plants, we found significant K^+ losses in *C. album* after one week of NaCl application, which was quite unexpected. Perhaps, high initial levels of K^+ in the weed and greater root to shoot K^+ translocation accounted for its acclimation and resilience to early salinity stress. The presence of the crop along with salinity

triggered the activation of antioxidative defences and osmotic balance adjustment mechanisms in the weed. However, the effect was not intense enough to hamper the weed growth and induce oxidative stress in its tissues.

We conclude that *C. album* is salt-resilient irrespective of the co-cultivation with soybean, and its occurrence along with salinity has a strong, early negative effect on the content of proteins in the crop. Thus, although under such a condition *C. album* did not impair soybean growth and nutrition more than salinity alone, it interfered with N assimilation processes in the crop. A reduced content in proteins in soybean is expected to result in biomass losses that would become more evident in the longer period. These results are particularly relevant to salt-sensitive cultivars, like the one used in the present work. While these studies were conducted in hydroponics to evaluate interactive effects between *C. album* and soybean without the interference of soil within a short-term period, further experiments carried out in pots will be useful to evaluate them at the soil-plant level and in the long term. In a climate-change scenario characterized by increasing salinization, we may expect *C. album* to exhibit even greater competitiveness. Possible sustainable strategies to mitigate soybean losses due to competition with *C. album* might go in two directions: 1) at the genetic level, by selecting soybean varieties more tolerant to salt stress and/or allelochemicals released by the weed; 2) at the agronomic level, by sowing soybean in correspondence with the highest probability of precipitation, so that salt can be partly leached from the soil by rainfall, or by applying the false seedbed technique to remove *C. album* seedlings from the topsoil before sowing soybean.

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V Comparing short-term responses to salinity of winter and summer weed-crop systems

1. Introduction

Soil salinization is a major abiotic stress affecting agroecosystems, contributing to the loss of arable land at an annual rate of 10% and reducing crop productivity and quality in 20 to 30% of the world's cultivated land *(Bhargava and Srivastava, 2020; Jamil et al., 2011)*. These percentages are expected to increase in the future due to the effects of global warming, such as drought, high surface evaporation, sea-level rise, combined with poor agricultural practices *(Hassani et al., 2021; Jamil et al., 2011)*. Saline soils can be found in arid, semiarid and temperate regions, including the Mediterranean basin *(Daliakopoulos et al., 2016; Dazzi, 2010; Hakim et al., 2011; Shrivastava and Kumar, 2015)*. In most Mediterranean coastal areas, where agriculture is highly dependent on irrigation, secondary soil salinization is widely spread *(Libutti and Monteleone, 2017)*, mainly due to saltwater intrusion in coastal and inland aquifers as a result of groundwater overexploitation *(Daliakopoulos et al., 2016)*. In this context, the most critical period is the spring-summer growing season, when high evapotranspiration rates and reduced rainfall concur to accumulate salts in the upper soil layers. Salinization of the root zone evolves throughout the growing season and the effects of salt stress depend on the coincidence between toxic salt levels and sensitive phenological stages *(Maggio et al., 2011)*. The toxic effects of salinity on plants can be expressed at the cellular, biochemical and physiological levels. Among the major effects are ion imbalance, resulting in the accumulation of Na^+ and Cl^- ions and depletion of K^+ and Ca^{2+} in plant tissues *(Khan and Panda, 2008)*, decreased water uptake, and oxidative stress with overproduction of reactive oxygen species (ROS) *(Niu et al., 1995; Xing et al., 2013; Zhu, 2001)*, associated with hyperosmotic stress *(Zhu, 2001)*. Negative effects have been observed on seed germination, morphology and productivity of staple crops like wheat, rice, maize and soybean *(Daei et al., 2009; Katerji et al., 1996; Phang et al., 2008; Zeng and Shannon, 2000)*. Weed species are generally more tolerant to abiotic stresses due to higher genetic variability and resilience *(Chen et al., 2015; Clements et al., 2004; Lu et al., 2016)*. However, not many studies dissect their responses to salt stress at different growth stages, including physiological parameters, and very few consider how weed-crop competition changes under salt stress *(Cirillo et al., 2018)*.

Among crops, rice is known to be salt-sensitive, especially at the emergence and early growth stages. Although the plant can germinate in moderately saline conditions, a salinity level of 2 dS/m can already affect seedling growth in some varieties *(Bertazzini et al., 2018; Zeng and Shannon, 2000)*. Another sensitive stage is the reproductive stage when salt stress can hamper grain formation *(Bertazzini et al., 2018; Dramalis et al., 2020)*. Weeds are estimated to cause an annual 9.5% yield loss in rice fields globally, but the magnitude depends on the infesting species and ecology, and also on soil conditions, including salinity *(Hakim et al., 2013)*. Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] and weedy rice (*Oryza sativa* L.) are two of the main weeds infesting rice fields in the Mediterranean region and worldwide *(Vidotto et al., 2020)*. *E. crus-galli* is generally considered to be more salt-tolerant than rice and weedy rice *(Fogliatto et al., 2021; Hakim et al., 2011)*, but to our knowledge, no studies have specifically considered the combined effect of salt stress and inter-specific competition between rice and these weed species.

On the opposite side of the spectrum, barley is considered to be one of the most salt-tolerant crops in the *Poaceae* family, being able to tolerate a salinity level of up to 10 dS/m without showing significant signs of osmotic stress *(Tavakkoli et al., 2010)*. Because of its adaptability to abiotic stresses, such as salinity, drought and low temperatures, barley is often cultivated in marginal environments, even if many differences exist among varieties *(Katerji et al., 2006; Kotzamani et al., 2021)*. Although barley is considered to be more competitive against weeds compared to other crops *(Paynter and Hills, 2009)*, its yield is seriously affected by weeds infesting winter crops. In Mediterranean climates, two of the most common and troublesome species infesting barley are *Avena sterilis* L. *(Castellanos-Frías et al., 2014)* and *Lolium rigidum* Gaudin *(D'amico et al., 2021; Fernandez-Quintanilla et al., 2000)*. *A. sterilis* is widespread across Europe, India, North America and Australia *(Alshallash, 2018; Mahajan and Chauhan, 2021)* and different studies have reported 20–80% yield losses in barley due to competition with this species *(Ruiz et al., 2008)*. *Alshallash (2018)* observed the ability of *A. sterilis* to germinate at moderate salt stress levels (100 mM NaCl). *L. rigidum* is originally from the Middle East but is nowadays spread across the Mediterranean, the Indian subcontinent, North and South America, South Africa and Australia *(Castellanos-Frías et al., 2015)*. Yield losses in barley due to *L. rigidum* have been estimated to reach up to 80%, with variations across seasons and infestation levels *(Izquierdo i Figarola et al., 2003)*. Different studies showed the ability of *L. rigidum* to tolerate moderate to high salinity levels (up to 200 mM NaCl, *Rahman and Asaduzzaman (2019); Thompson et al.(2021)*). As for rice and its weed species, to our knowledge, no studies have

addressed the combined effect of salt stress and inter-specific competition between barley and its weed species.

In both scenarios, weed competitiveness in salty environments might be attenuated or exacerbated depending on the seasonal dynamics of soil salinization and the different tolerance levels of weeds and crops. In lands severely affected by salinity, where yield loss becomes too extreme in the summer due to secondary salinization, salt-tolerant winter crops might be preferred over summer crops.

To test this hypothesis and better understand weed response to salt stress after the germination stage, we conducted growth tests and hydroponic experiments on winter species (barley, *A. sterilis*, *L. rigidum*) and summer species (rice, *E. crus-galli*, weedy rice) in a saline environment. In the case of hydroponic set-ups, weed-crop competition was also considered in combination with salinity.

2. Materials and Methods

2.1 Growth experiment

The experiment consisted of multiple growth tests carried out on three winter species, barley*, A. sterilis, L. rigidum* and tree summer species, rice, weedy rice and *E. crus-galli*, to assess how salinity affects the first growth stages of seedlings at different temperatures. *A. sterilis* seeds were collected in Aliatros (38°23'11.3"N 23°06'23.4"E, Central Greece, Greece). *L. rigidum* seeds were collected in Kilikis (40°59'26.2"N 22°53'28.7"E, Central Macedonia, Greece). Weedy rice and *E. crus-galli* were collected in Chalastra (40°38'12.5"N 22°44'05.3"E, Central Macedonia, Greece) in 2019. Barley and rice cultivars were Finola and Ronaldo, respectively.

Seedlings were grown in plastic containers of 8 cm (height) x 9 cm (width) x 10 cm (length) on half-strength MS agar medium *(Murashige and Skoog, 1962)*, containing no sucrose and no hormones, following the protocol of *Nikolić et al. (2023)*. Treatments consisted of five levels of salt stress (0, 4, 8, 12 and 16 dS/m), obtained by dissolving pure NaCl in the solution used to prepare the medium and checked with a CT 600 electrical conductivity meter (BOECO, Hamburg, Germany). The boxes were then autoclave-sterilized for 20 minutes at 120 °C and left to cool down. The seeds were surface-sterilized with 75 % (v/v) ethanol solution for 30 seconds, followed by 20 % (w/v) sodium hypochlorite for 10 minutes and then rinsed four times with deionized water and once with NaCl solution (pure deionized water was used for the control), then transferred to the boxes with sterile tweezers. Before

this step, seeds of *E. crus-galli* were immersed in 98% sulfuric acid for 20 minutes and then thoroughly rinsed. In order to prevent imbibition of the seeds with water, those meant for the trials with saline solutions were rinsed with the corresponding solutions after the acid scarification process. In order to work in sterile conditions, both the seed preparation and their placement on the agar media were done in a laminar flow cabinet. The boxes were kept in growth chambers (Memmert GmbH, Schwabach, Germany) at two constant temperatures: 12 and 24 °C. Four replicates were prepared for every combination of salinity and temperature, for a total of 40 boxes per species. Each box contained 20 seeds in the case of barley, *A. sterilis*, rice and weedy rice and 50 seeds in the case of *L. rigidum* and *E. crus-galli*, according to the different seed sizes. In accordance with their different growing speeds, the growth of barley, rice and weedy rice was measured after two weeks, and that of *L. rigidum* and *E. crus-galli* after five weeks. The seedlings were removed from the agar medium and the length of each root and shoot was measured with a digital caliper (TESA Technology, Renens, Switzerland). The percentage of germination and the average root and shoot elongation were recorded to compare the six species at different temperatures and salinity levels.

2.2 Hydroponic experiments

The experiments aimed to investigate the response of barley + *L. rigidum* and rice + weedy rice to salt stress in hydroponic conditions during winter and summer periods, respectively. The experiments were carried out in a greenhouse at the Benaki Phytopathological Institute (Attica, Greece) with constant light and temperature settings (17 °C and 8 h lighting period in the winter, 24 °C and 14 h lighting period in the summer). All the seeds (the same ecotypes and cultivars employed for the growth test) were sown in silty loam soil and seedlings were allowed to grow until they reached a height of 10 cm. Because of the different growth rates, crops were sown only after the first weed seedlings had emerged. Seedlings of each species were then transferred to hydroponics, in a set-up consisting of eight tanks filled with 20 L of half-strength Hoagland's solution *(Hoagland and Arnon, 1950)*, at a density of 12 plants per tank. Two of the tanks contained only crop plants (barley in the winter, rice in the summer), two contained only weed plants (*L. rigidum* in the winter and weedy rice in the summer), and the remaining contained 6 crop and 6 weed plants according to the season. The number of tanks with both crop and weed was twice of those with the single species to obtain the same number of biological replicates per treatment (**Figure 1**). After three days of acclimation, half of the tanks were added with 100 mM NaCl. Salt treatment lasted for 25

days in the winter and 18 days in the summer, and the experiments ended before root systems would become entangled.

At the end of the experiment, 8 out of 12 plants were collected and immediately processed to quantify biomass. The remaining plants were frozen in liquid nitrogen and stored at -80 °C for the following assessment of elemental content and oxidative-stress parameters on leaves (lipid peroxidation and H_2O_2). The same parameters were compared among four crop treatments and four weed treatments (i.e. plants grown in single-species or mixed tanks, with or without the addition of NaCl). The whole trial was repeated a second time for data confirmation, with the same setup.

Figure 1. *Hydroponic experimental setup for evaluating weed and crop responses to salt stress.*

2.3 Hydrogen peroxide (H_2O_2) and lipid peroxidation

Hydrogen peroxide (H_2O_2) leaf content was calculated as described by *Junglee et al. (2014)*. 500 mg of fresh plant tissue were homogenized in 10 mL of 0.5 % v/v trichloroacetic acid (TCA) for 30 s each in an ice bath. Centrifugation was done at 12000 x g for 15 min at 4 °C. 1 mL of supernatant was incubated with 10 mM potassium phosphate buffer (0.5 mL) and 1 mM KI (0.5 mL) for 30 minutes in the dark. The absorbance of each of the samples was recorded at 390 nm against a standard hydrogen peroxide curve. Hydrogen peroxide was used as standard.

Malondialdehyde (MDA) leaf content was assessed following the method described by *Heath and Packer (1968)* with some modifications. Frozen leaf tissues (500 mg) were homogenized in 10 mL of 0.5 % v/v TCA for 30 s each in an ice bath, followed by filtration and centrifugation at 12000 × *g* at 4 °C for 15 min. Then, 200 µL of each supernatant was mixed with 4 mL of 0.5 % thiobarbituric acid (TBA) water solution. Tubes were then heated at 95°C for 30 minutes. The absorbance was read at 532 nm and 600 nm. The OD_{600} was

then subtracted from the OD_{532} value to achieve an extinction coefficient of 155 mM cm^{-1}. Data are expressed as μmol/g fresh weight (FW) of MDA.

2.4 Statistical analysis

The parameters evaluated in the hydroponic experiments were compared within each species (i.e. plants grown in single-species or mixed tanks, with or without the addition of NaCl). To assess differences among treatments (salinity and competition), one-way analysis of variance (ANOVA) was performed separately for each species. The test was followed by pair-wise post hoc analyses (Tukey test) to determine which means differed significantly at $p < 0.05$. The homogeneity of variances was confirmed by the Levene test. The number of biological replicates varied depending on the analysis performed, as reported in the figure legends. Whenever the homogeneity of variances of the original dataset was not confirmed, the data underwent a squared root or logarithmic transformation in order to satisfy this ANOVA requirement. Although in the graphs significant letters are reported on the original data, we specified in the figure captions if any transformations were applied. A factorial ANOVA was performed to assess the combined influence of species and competition on plant height and biomass, expressed as percentage of treated over non treated plants (% nt). The tests were followed by pair-wise post hoc analyses (Tukey test) to determine which means differed significantly at $p < 0.05$. The homogeneity of variances was confirmed by the Levene test. All data analyses were conducted on R 4.2.0 (2022). MDA and H_2O_2 (% nt) were not included in the factorial ANOVA as they did not satisfy the ANOVA assumptions.

3. Results

3.1 Growth tests

Growth test results indicate that the four species assayed in this study were able to develop shoots and roots at all salinity levels and both temperatures. For winter species, the general trend was a reduction in both root and shoot length at increased salinity levels (**Figure 2**). This was particularly evident in *A. sterilis*, especially at 24 °C and at the highest salinity level (16 dS/m), where shoot and root lengths were almost down to zero (**Figure 2B**). If *A. sterilis* seemed to be the winter species most affected by salinity, barley was the least affected, with only a slight decrease in root and shoot length with the increase in salinity levels, apart from shoot length at 16 dS/m and 12 °C (**Figure 2A**). *L. rigidum* was not significantly affected by salinity in terms of shoot length, but root length decreased from 8 to 16 dS/m, with values down to 20% of the control (**Figure 2C**).

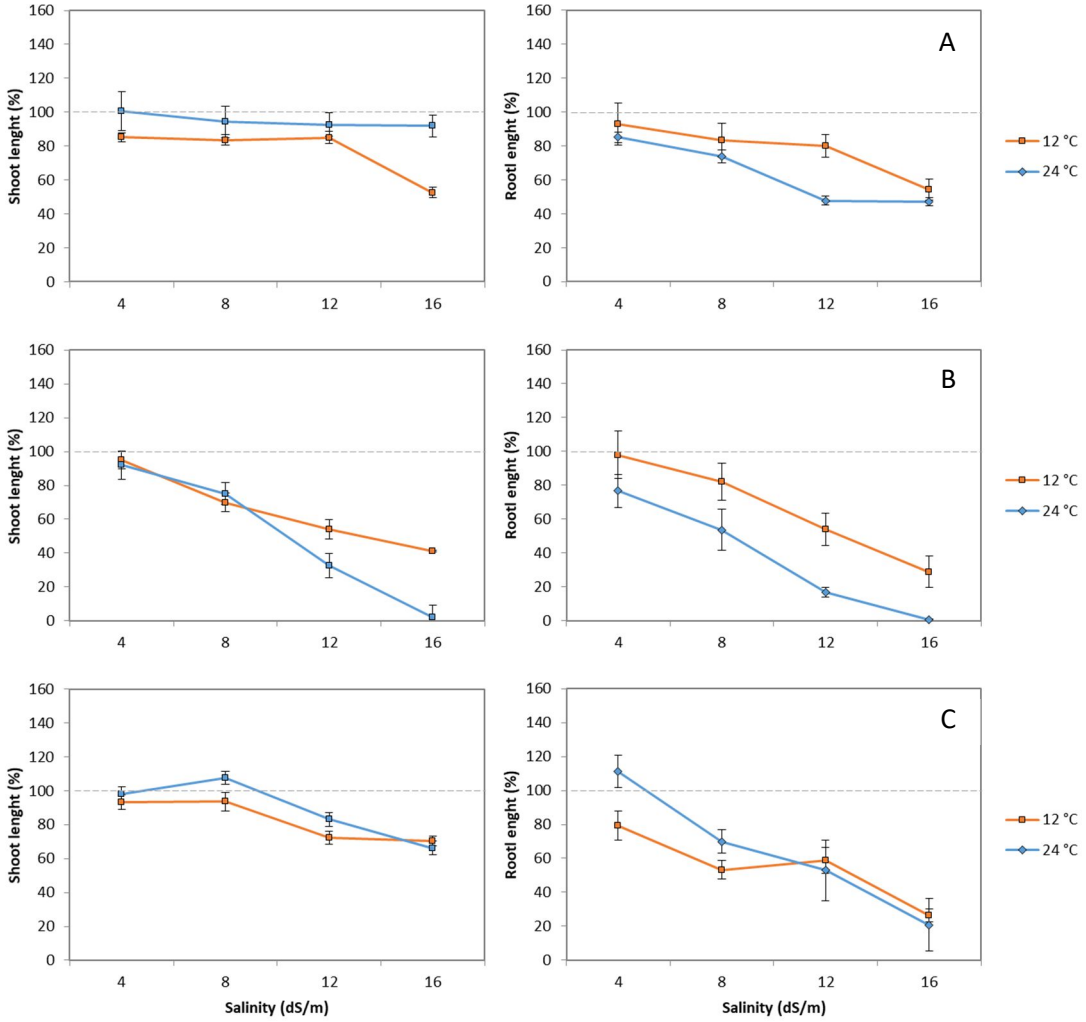

***Figure 2**. Shoot and root lengths expressed as percentage over the control of winter species at different salinity levels and temperatures. (A) Barley; (B) A. sterilis; (C) L. rigidum. Vertical bars indicate the standard error.*

For summer species, the trend was less consistent (**Figure 3**). Rice shoot and root length did not appear to decrease with the increase of salinity levels, but at 12 °C all salt treatments showed a significant reduction in shoot length compared to the control (**Figure 3A**). The least affected species appeared to be *E. crus-galli*, which did not show any significant growth reduction at high salinity levels, and at lower salinity levels (4 and 8 dS/m) even showed higher shoot lengths compared to the control (**Figure 3C**). The most sensitive species was weedy rice, which showed a drop in root and shoot length at 12 °C and high salinity levels (12 and 16 dS/m). However, at low salinity levels also in this case we observed roots and shoots longer than the control (**Figure 3B**).

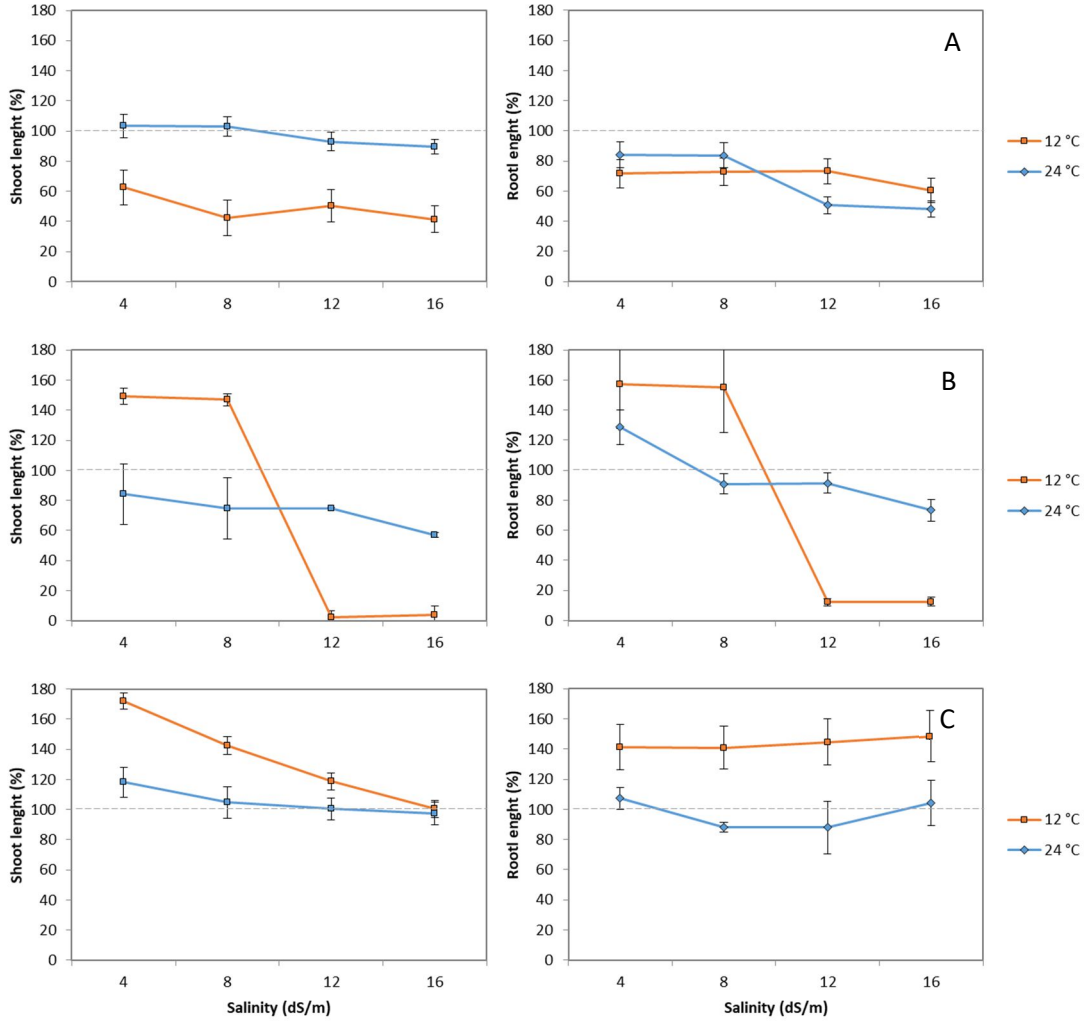

Figure 3. *Shoot and root lengths expressed as percentage over the control of summer species at different salinity levels and temperatures. (A) Rice; (B) weedy rice (Oryza sativa var sylvatica); (C) E. crus-galli. Vertical bars indicate the standard error.*

3.2 Hydroponic experiments

3.2.1 Plant heights

At the end of the summer trial, the height of rice was significantly lower in NaCl-treated tanks, but no difference was found between single- and mixed-species tanks (**Figure 4 A**). Conversely, weedy rice height was lower only in salt-treated plants grown in mixed-species tanks compared to untreated plants (**Figure 4 B**). At the end of the winter trial, both barley and *L. rigidum* heights were reduced in salt-treated plants, while no difference was observed between single- and mixed-species tanks (**Figure 4 C, D**).

When data of final heights were computed over untreated plants (% nt) in summer species, no differences were detected either between species or between single- and mixed- species set-up. However, the interaction between species and competition was significant. In fact, the % nt of weedy rice heights slightly decreased with competition, while that of rice slightly

increased (**Table 1 A, Figure 5 A)**. Data expressed as % nt confirmed the existence of relevant differences between barley and *L. rigidum* in terms of plant height. A significant difference was also found between single-species and mixed set-up, and for the interaction between species and competition. In the presence of competition, the % nt of plant *L. rigidum* heights decreased, while that of barley slightly increased (**Table 1 B, Figure 5 B)**.

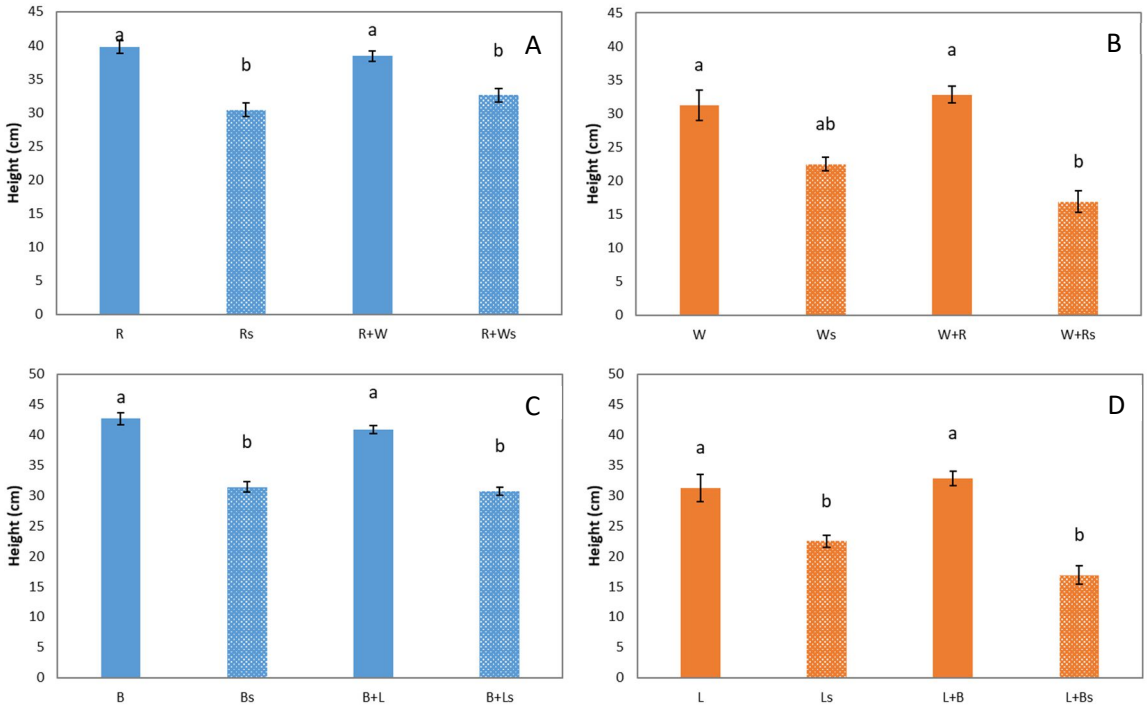

Figure 4*. Average plant height of rice (A) and weedy rice (B) at the end of the trial. Average plant height of barley (C) and L. rigidum (D) at the end of the trial. Different letters within each group of bars indicate significant differences at p<0.05, n=4. R=rice; W=weed; B=barley; L=L. rigidum; s=salt treatment. The experiment was replicated twice and only data from one representative experiment are shown.*

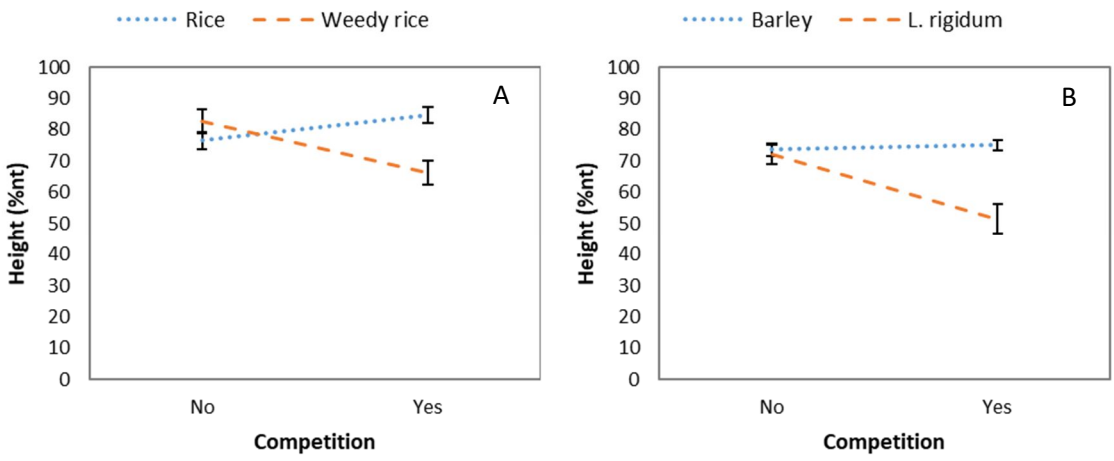

Figure 5. *Species-competition interaction for average plant height (% nt) of (A) summer species (rice and weedy rice) and (B) winter species (barley and L. rigidum) at the end of the trial. Values on the left are referred to plants grown in a single-species set-up (no competition). Values on the right are referred to plants grown in a mixed-species set-up. Vertical bars denote the standard error.*

3.2.2 Biomass quantification

In the summer trial, total FW of rice was significantly lower in NaCl-treated tanks, but no difference was found between single- and mixed-species tanks (**Figure 6 A**). Weedy rice biomass was also reduced in the presence of salt treatment in both single- and mixed-species tanks, compared to the control grown with rice, but not compared with the control grown in single-species tanks (**Figure 6 B**). The same trend was observed in stem and leaves FW in both species. However, in the case of root biomass only rice was affected by salinity, while no significant difference was observed in weedy rice (**Figure 6 C–F**). The different responses of rice and weedy rice were more accentuated in dry weight (DW): in the case of rice, both total, stem and leaves and root DW were significantly reduced with salt treatment, while weedy rice did not show any significant difference among treatments (**Figure 7**).

Data expressed as % nt did not show any relevant differences in total FW and stem and leaves FW between rice and weedy rice or between single- and mixed-species set-ups. Also the interaction between species and competition was not significant. A significant difference in terms of root FW was found between the two species and between single- and mixed-species set-ups, but the interaction between species and competition was not significant (**Table 1 A**, **Figure A 1)**. Conversely, total DW and stem and leaves DW showed relevant differences between rice and weedy rice, between single- and mixed-species set-ups, and also significant species x competition interactions. Root DW did not show any significant differences between species, but did show significant differences between single- and mixed-species set-ups and a significant interaction between factors (**Table 1 A)**. In the presence of competition, the decrease in all the DW parameters was more pronounced in weedy rice than in rice (**Figure 8**).

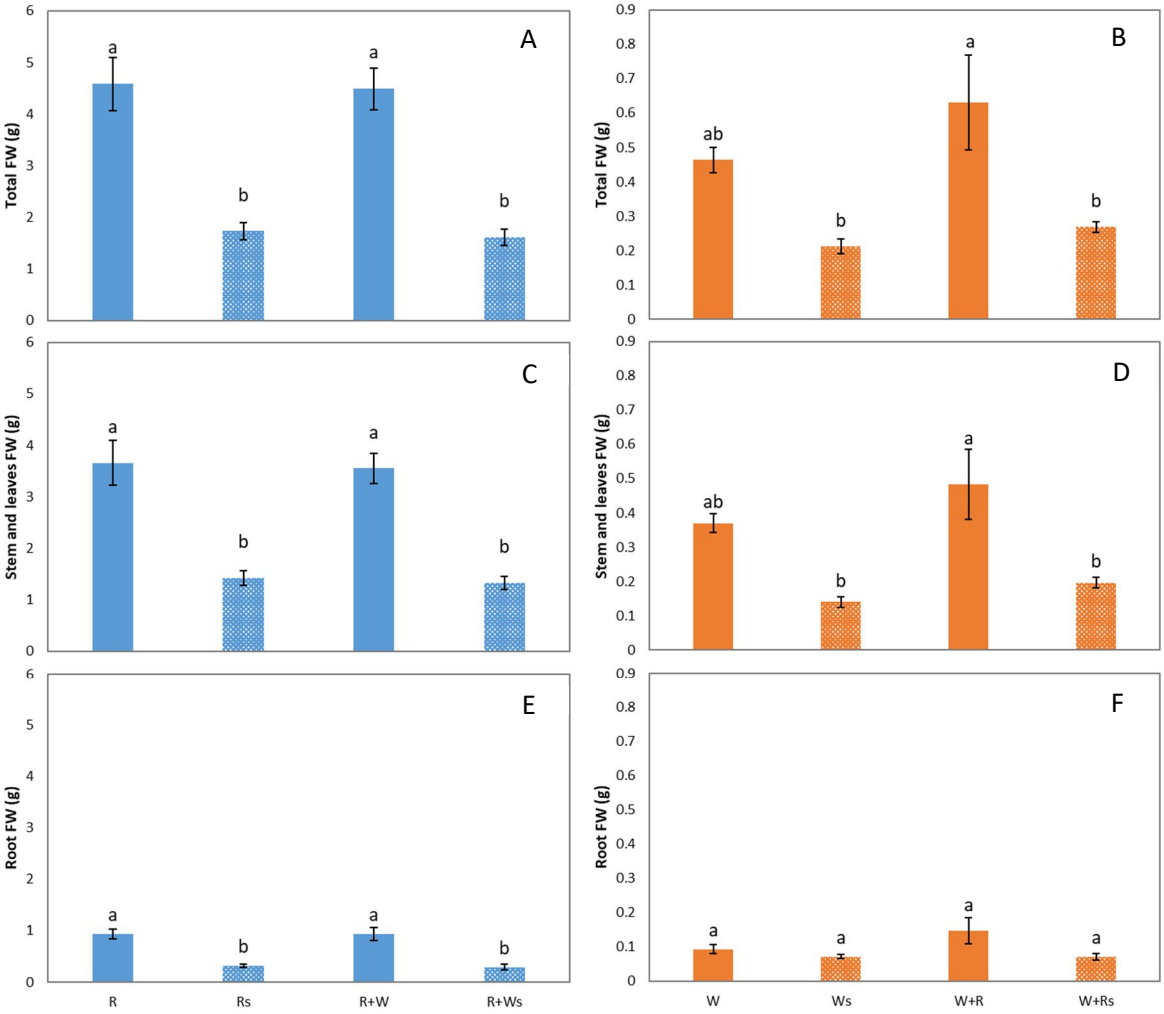

Figure 6. *Total fresh weight (FW) of rice (A) and weedy rice (B). Stem and leaves fresh weight of rice (C) and weedy rice (D). Root fresh weight of rice (E) and weedy rice (F). Different letters within each group of bars indicate significant differences at $p<0.05$, $n=8$. Tukey test on rice total FW and root FW was performed on transformed data (logarithmic transformation). R=rice; W= weedy rice; s=salt treatment. The experiment was replicated twice and only data from one representative experiment are shown.*

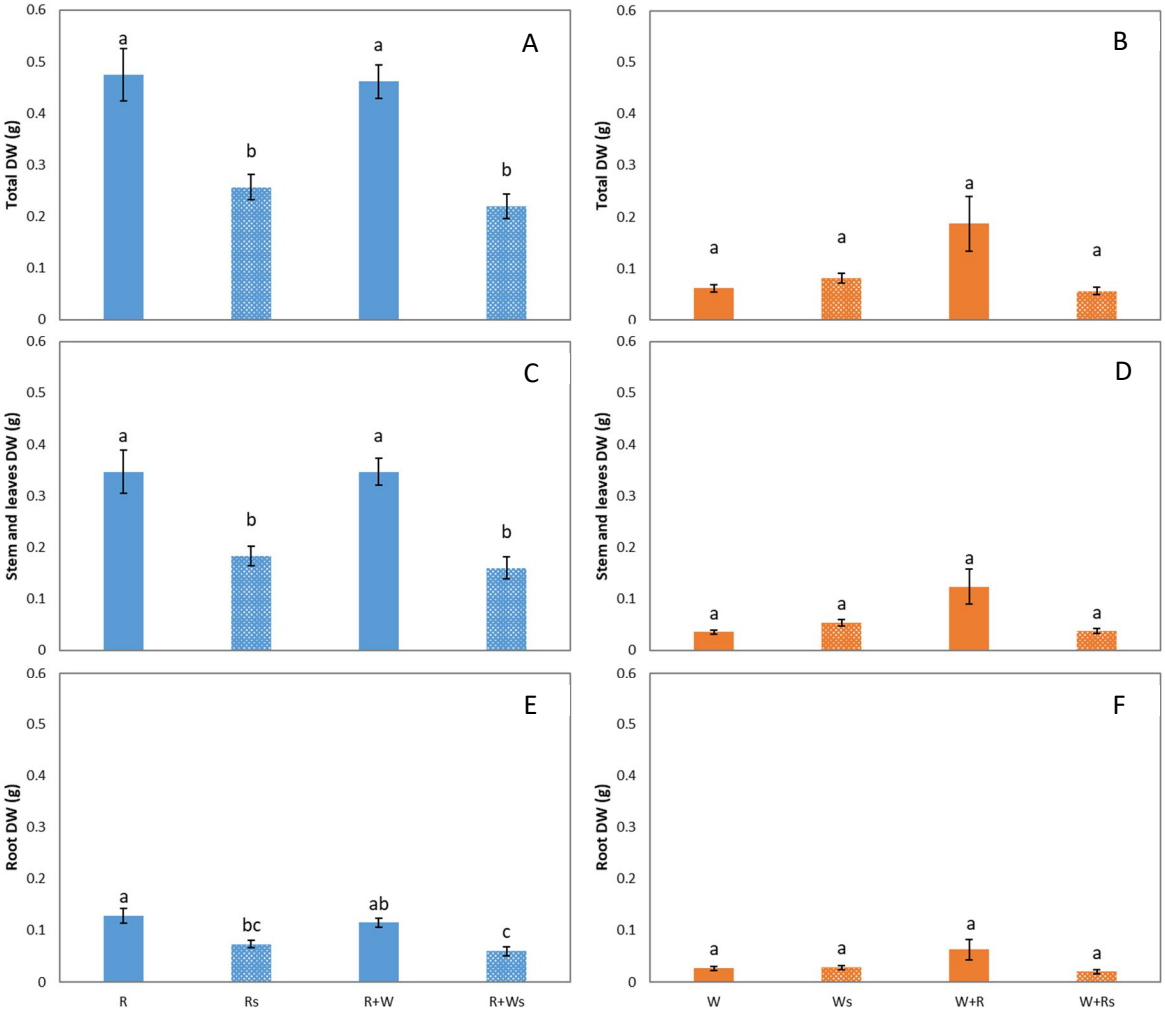

Figure 7. *Total dry weight (DW) of rice (A) and weedy rice (B). Stem and leaves dry weight of rice (C) and weedy rice (D). Root dry weight of rice (E) and weedy rice (F). Different letters within each group of bars indicate significant differences at $p<0.05$, n=8. Tukey test on weedy rice was performed on transformed data (logarithmic transformation). R=rice; W= weedy rice; s=salt treatment. The experiment was replicated twice and only data from one representative experiment are shown.*

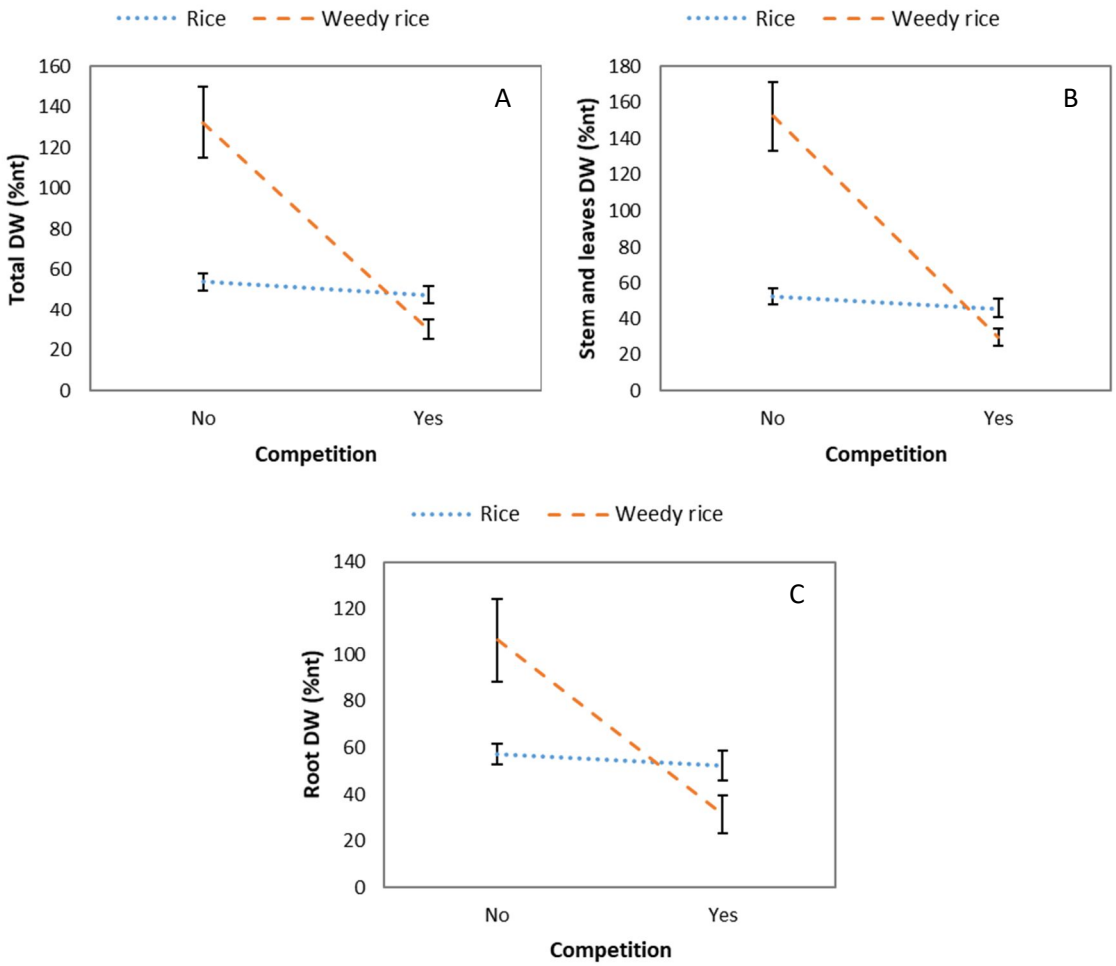

Figure 8. *Species-competition interaction for (A) total DW (% nt), (B) stem and leaves DW (%nt) and (C) root DW of summer species (rice and weedy rice). Values on the left are referred to plants grown in a single-species set-up (no competition). Values on the right are referred to plants grown in a mixed-species set-up. Vertical bars denote the standard error.*

In the winter trial, both barley and *L. rigidum* showed a reduction in total and stem and leaves FW when salt treatment was applied, but no difference was observed among single- and mixed-species tanks (**Figure 9 A–D**). In the case of root, the only plants that showed a significant reduction in FW were those grown in mixed-species tanks in both species (**Figure 9 E, F**). Total DW was reduced in both species when salt treatment was applied (**Figure 10 A, B**). Stem and leaves DW was reduced in both species especially when salt-treatment was combined with competition (**Figure 10 C, D**). Root DW of salt-treated plants did not significantly differ from control plants grown in single-species tanks but was significantly lower than plants grown in mixed-species tanks (**Figure 10 E, F**).

Data expressed as % nt showed relevant differences in total FW both between barley and *L. rigidum* and between single- and mixed-species set-ups. Also the interaction between species and competition was significant. A significant difference in terms of root FW was found between the two species but not between single- and mixed-species set-ups, and the

interaction between factors was not significant. Conversely, a significant difference in terms of stem and leaves FW was found between single- and mixed-species set-ups, but not between species, and the interaction between factors was not significant (**Table 1 B**, **Figure A 2)**. Total and root DW showed no relevant differences between species, between single- and mixed-species set-ups, and no significant interactions. Stem and leaves DW did show significant differences between species, but did not show significant differences between single- and mixed-species set-ups. The interaction between factors was not significant (**Table 1 B**, **Figure A 3)**.

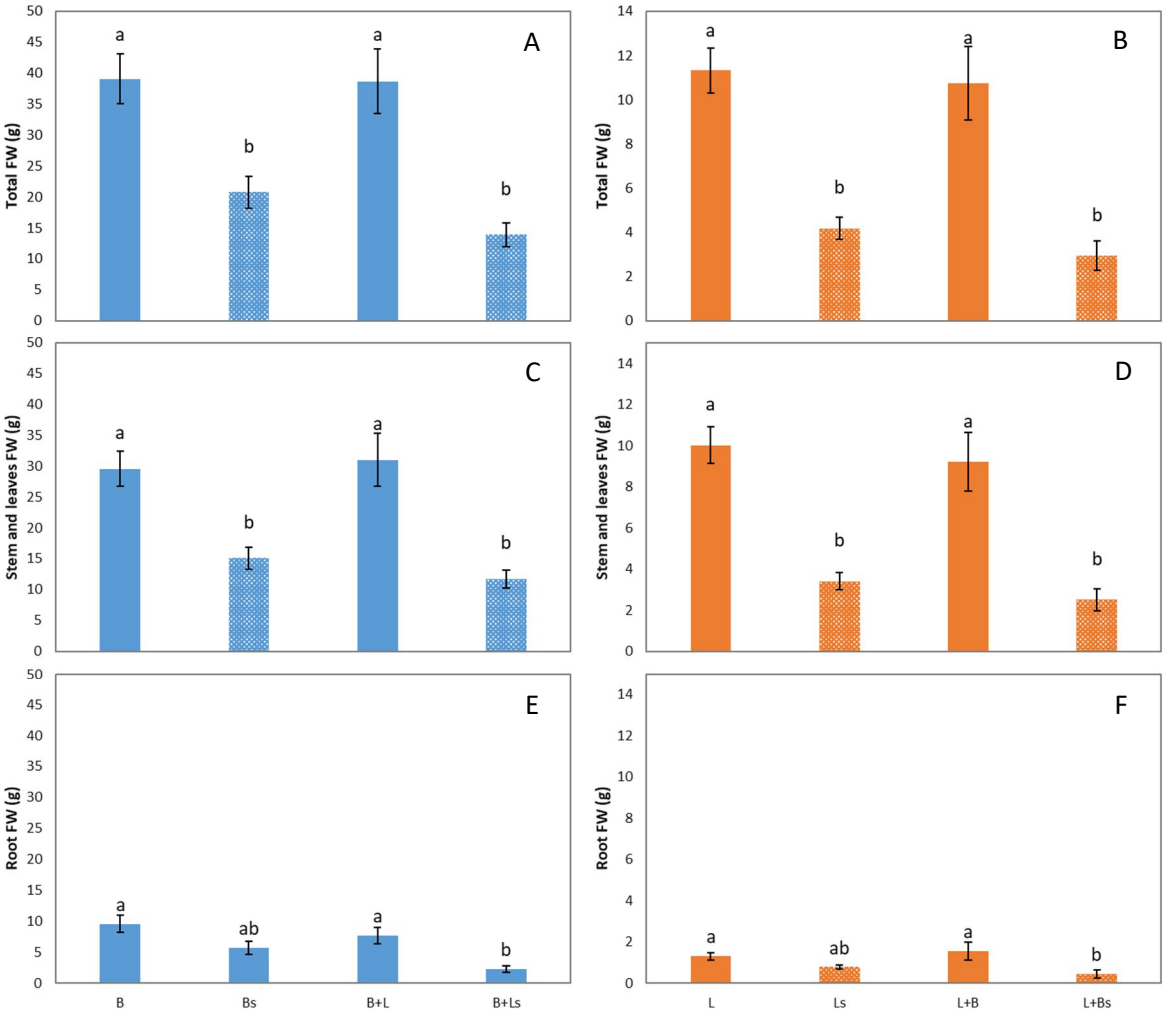

Figure 9. *Total fresh weight (FW) of barley (A) and L. rigidum (B). Stem and leaves fresh weight of barley (C) and L. rigidum (D). Root fresh weight of barley (E) and L. rigidum (F). Different letters within each group of bars indicate significant differences at p<0.05, n=8. Tukey test on L. rigidum total FW and stem and leaves FW was performed on transformed data (squared root transformation). B=barley; L=Lolium rigidum; s=salt treatment. The experiment was replicated twice and only data from one representative experiment are shown.*

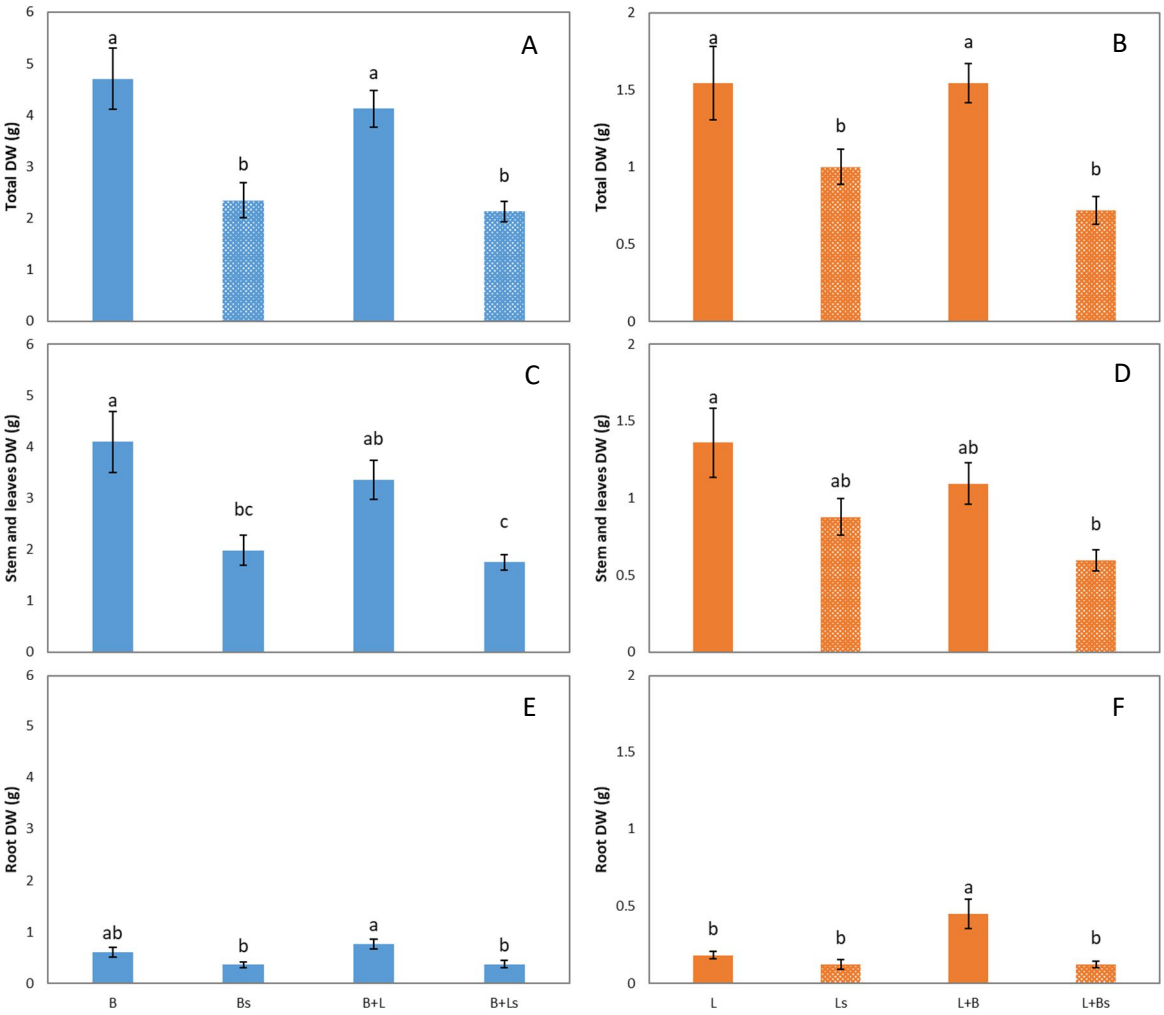

Figure 10. *Total dry weight (DW) of barley (A) and L. rigidum (B). Stem and leaves dry weight of barley (C) and L. rigidum (D). Root dry weight of barley (E) and L. rigidum (F). Different letters within each group of bars indicate significant differences at p<0.05, n=8. B=barley; L=L. rigidum; s=salt treatment. The experiment was replicated twice and only data from one representative experiment are shown.*

Table 1. *ANOVA significance for the effect of species, competition (single-species tanks or mixed-species tanks) and their interaction on the percentage of salt-treated samples over non-treated samples. (A) Summer species. (B) Winter species*

A	**Height**	**Total FW**	**Stem & leaves FW**	**Root FW**	**Total DW**	**Stem & leaves DW**	**Root DW**
Factors	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**
Species	0.072	0.095	0.767	0.000	0.006	0.001	0.220
Competition	0.230	0.557	0.885	0.028	0.000	0.000	0.002
Species x Competition	0.001	0.885	0.607	0.066	0.000	0.000	0.006

B	**Height**	**Total FW**	**Stem & leaves FW**	**Root FW**	**Total DW**	**Stem & leaves DW**	**Root DW**
Factors	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**
Species	0.002	0.034	0.975	0.014	0.431	0.667	0.185
Competition	0.005	0.024	0.003	0.067	0.206	0.042	0.651
Species x Competition	0.002	0.509	0.970	0.548	0.128	0.209	0.317

3.2.3 Hydrogen peroxide and lipid peroxidation

Hydrogen peroxide (H_2O_2) content showed wide variations within each treatment in both species, resulting in no significant differences. The only exception was salt-treated weedy rice grown in single-species tanks, which showed significantly lower H_2O_2 content compared to the other treatments (**Figure 11 A, B, E, F**). MDA content in summer species generally increased when salt treatment was applied. However, in the case of rice only plants grown in competition with the weeds had significantly higher MDA content than untreated plants (**Figure 11 C**). The trend was similar in weedy rice, where salt-treated plants grown in competition with the weeds had significantly higher MDA content than control plants grown in single-species tanks (**Figure 11 D**). Winter species did not show a consistent increase in MDA content due to salt stress, as barley salt-treated plants had values similar to those of control plants grown in mixed species tanks (**Figure 11 G**). Conversely, MDA content in salt-treated *L. rigidum* was significantly higher in mixed-species tanks than in single-species tanks (**Figure 11 H**).

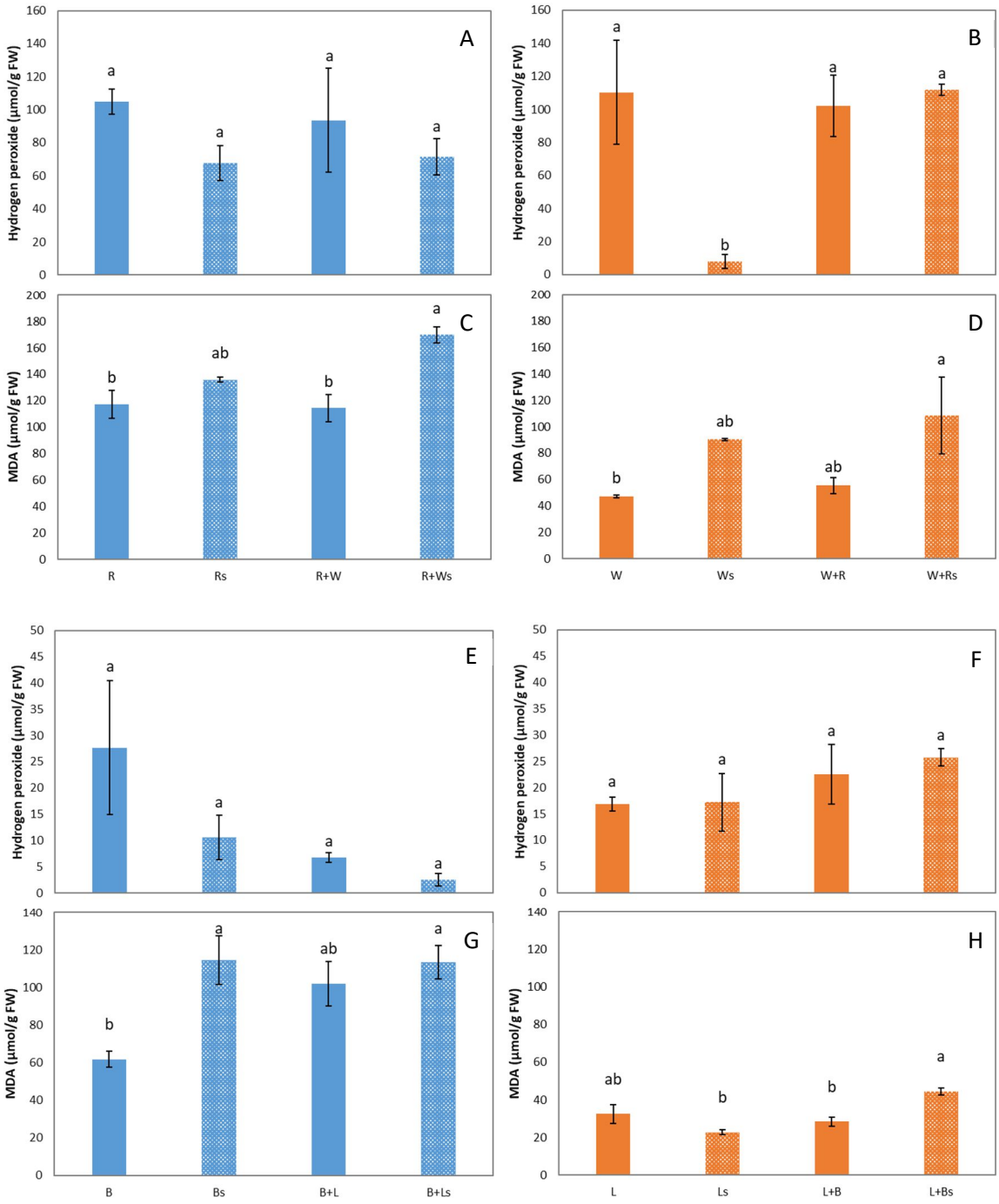

Figure 11. *Hydrogen peroxide content of rice (A) and weedy rice (B). Malondialdehyde (MDA) content of rice (C) and weedy rice (D). Hydrogen peroxide content of barley (E) and L. rigidum (F). Malondialdehyde (MDA) content of barley (G) and L. rigidum (H). Different letters within each group of bars indicate significant differences at $p<0.05$, $n=4$. Tukey test on weedy rice and barley hydrogen peroxide content was performed on transformed data (squared root transformation). R=rice; W=weed; B=barley; L=L. rigidum; s=salt treatment. The experiment was replicated twice and only data from one representative experiment are shown.*

4. Discussion

This study aimed to evaluate the effects of salinity on the early growth stages of crop and weed species and investigate the role of crop-weed competition on rice and barley, as prototypes of winter and summer crops, respectively. The hydroponic set-up served as a

simplified system for plant growth to better understand how crops and weeds interact under salt stress, without the many interfering factors of soil environments.

The growth tests indicated that out of the three summer species, *E. crus-galli* was the most tolerant at the early developmental stages, while weedy rice was seriously affected by salinity levels higher than 4 dS/m, especially at 24° C. The rice cultivar we used showed a relatively high tolerance to increasing salinity stresses at 24 °C, but shoot growth was reduced at 12 °C and root growth was always lower than the control at all temperatures. The results are in agreement with the observations of *Fogliatto et al. (2021)*, who recorded a higher tolerance of *E. crus-galli* compared to weedy rice and rice at early seedling stages. However, plant height and biomass of *E. crus-galli* were significantly reduced at 10 dS/m, and weedy rice was completely inhibited at 6 dS/m, thus showing a much lower tolerance to salinity than the Greek ecotypes tested in the present study. Our data are more consistent with those of *Hakim et al. (2011)* and *Chauhan et al. (2013)*, who observed the ability of *E. crus-galli* and weedy rice to develop roots and shoots up to 24 dS/m, with better performances in *E. crus-galli* than weedy rice. The results also reflect our previous study *(Nikolić et al., 2023)*, where a slight growth stimulation was observed in *E. crus-galli* at low salinity levels (4–8 dS/m), a phenomenon known as the hormetic effect *(Calabrese, 2013)*, and previously observed in this species *(Fogliatto et al., 2021)*. Both *Hakim et al. (2011)* and *Fogliatto et al. (2021)* pointed out that root lengths were more affected than shoot lengths at increasing salinity levels, as observed in the present study. The response of rice is also in line with observations conducted by *Hakim et al. (2010)* and *Abbas et al. (2012)*, who tested seed germination and seedling growth in twelve and six rice varieties respectively, with different tolerance to salinity. Given that our cultivar did show only a slight decrease in root and shoot length, this means it is fairly tolerant at the early growth stages compared to other cultivars, including the Italian "Vialone Nano" used in our previous experiments *(Nikolić et al., 2023)*.

The growth tests conducted on winter species indicated barley as the most tolerant species, followed by *L. rigidum*, which was more affected in terms of root growth reduction, and *A. sterilis*, which was severely affected at high salinity levels (12–16 dS/m). This finding is in line with the observations of *Alshallash (2016)*, *Rahman and Asaduzzaman (2019)* and *Thompson et al. (2021)*, who found signs of tolerance at moderate levels of salt (100–131 mM NaCl) at the germination stage in both species. Apparently, this threshold is lower than what we observed in our experiments. However, since our results are not referred to the

germination stage, it is not possible to determine whether these ecotypes are more tolerant than the ones found in literature or if the plants are less tolerant at the germination stage than the early growth stages. To our knowledge, all the available studies assessing the effect of salinity on these species are focused on germination, however some studies investigating the early growth stages are available for species belonging to the same genera. For instance, *Dinari et al. (2013)* and *Azim et al. (2016)* observed a significant reduction in root and shoot length of *Avena fatua* L. seedlings at 150 and 100 mM respectively, but the seeds were able to germinate up to 300 mM. *Chen et al. (2017)* observed the ability of *Lolium multiflorum* Lam. seedlings to tolerate salt stress up to 170 mM. As for barley, the results confirmed the salt tolerance of the species and specifically of the cultivar Finola, used in our experiments. This is consistent with the findings of *Katerji et al. (2006)*, who observed no significant differences in plant height among six varieties exposed to 5 and 10 dS/m. However, the tolerance to salinity strictly depends on the cultivar, as demonstrated by *Tolera Angessa et al. (2017)*, who found up to 85% fresh weight decrease in seedlings exposed to 150 mM.

Keeping in mind the growth test results, it is evident that in the hydroponic experiments the summer and winter pairs (rice + weedy rice and barley + *L. rigidum*) consisted of a sensitive crop and weed species in the first case and a tolerant crop and weed species in the second case. Although that might have decreased the competition pressure in rice and increased it in barley, the summer experiment was the one where the weed, *O. sativa var. sylvatica* in this case, was significantly less affected than the crop. In fact, both root FW and total, root, stem and leaves DW, showed no significant difference among treatments and no competition effect. In addition, plant heights of salt-treated single-species tanks were not significantly lower than untreated plants, and MDA content in salt-treated single-species tanks was not significantly lower than untreated plants. Overall, rice was more affected by weedy rice, with a significant decrease in height and biomass, with significantly lower root DW and higher MDA content when weedy rice was present in addition to salt stress. This is in line with the findings of *Hakim et al. (2013)*, that observed a decrease in plant height, till number and DW of rice grown in a greenhouse competing with different weeds (*Ehinochloa colona* L., *Cyperus iria* L. and *Jussia linifolia* Vahl) and exposed to salt stress, pointing out that the duration of the critical period of crop-weed competition increased with the increase of salinity levels.

Conversely, in the winter experiment, the response of barley to salinity was generally very similar to *L. rigidum* and not exacerbated by competition. Plant heights, total FW and DW,

stem and leaves FW in both species were only decreased by salinity and not by competition. Root FW loss was significant only with the combination of salt stress and competition in both species. Only stem and leaves DW showed a difference between species, with barley being affected by salinity both in single- and mixed-species tanks. However, this was counterbalanced in *L. rigidum* by a significantly higher MDA content in salt-treated plants grown in competition with barley, corresponding to higher lipid peroxidation in plant tissues, which is one of the main symptoms ascribed to oxidative damage *(Khan and Panda, 2008)*. Despite *L. rigidum* being such a competitive and adaptable weed *(Izquierdo i Figarola et al., 2003)*, that proved to tolerate moderate levels of salt stress, barley was scarcely affected by its presence. Barley, on the other hand, is well-known for producing allelochemicals able to reduce germination, emergence and growth of weeds including *A. sterilis* and *L. retroflexus*, as well as *Phalaris paradoxa* L., *Alopecurus myosuroides* Huds., *Bromus diandrus* Roth., and *Sinapis arvensis* L. *(Bouhaouel et al., 2015; Farhoudi et al., 2012; Kotzamani et al., 2021; Vasilakoglou et al., 2009)*. However, the allelopathic potential is dependent on the cultivar *(Vasilakoglou et al., 2009)*, which might explain why no consistent barley competition effects were observed in *L. rigidum* in the present study. Other possible reasons might be the hydroponic setup, that tends to dilute the substances released in the nutrient solution and the fact that seedlings were originally sown separately and then transplanted into the tanks.

In the studied summer and winter species, H_2O_2 leaf content did not show any significant differences among treatments, apart from salt-treated weedy rice in single-species tanks. However, H_2O_2 leaf content is generally found to increase salt-stressed plants and is used as an indicator of oxidative stress *(Sang et al., 2005)*. The lack of H_2O_2 content increase in salt-treated plants might be due to the low sample number (4 replicates for each species and treatment) and the high variability within each treatment. Therefore, in this case, H_2O_2 leaf content was not a good marker of oxidative stress.

5. Conclusions

This study confirms the salt sensitivity of rice and the tolerance of *E. crus-galli* and weedy rice, dissecting different intensities of individual responses, with weedy rice being less tolerant than *E. crus-galli*. It also confirms the salt tolerance of barley and sheds new light on the response of *L. rigidum* and *A. sterilis* to salt stress at the early growth stages. When grown in hydroponic conditions, weedy rice appeared more tolerant than rice, while barley and *L. rigidum* had similar responses. Although the present study was carried out in

controlled conditions and in a short timeframe, the results represent a useful baseline for agricultural systems in saline environments.

Considering the expansion of soil salinization in the Mediterranean basin, especially due to secondary salinization in the summer season, careful land management has to be considered. In lands severely affected by salinization, winter crops, especially those less affected by salt stress, might be preferred over summer crops. Barley, especially cultivars with high allelopathic potential, would be an appropriate crop able to grow in saline soils and potentially outcompete even salt-tolerant weed species. Further experiments will be useful to evaluate the response of more crop-weed pairs, including long-term pot experiments, where the soil-plant interactions are also present.

Appendix

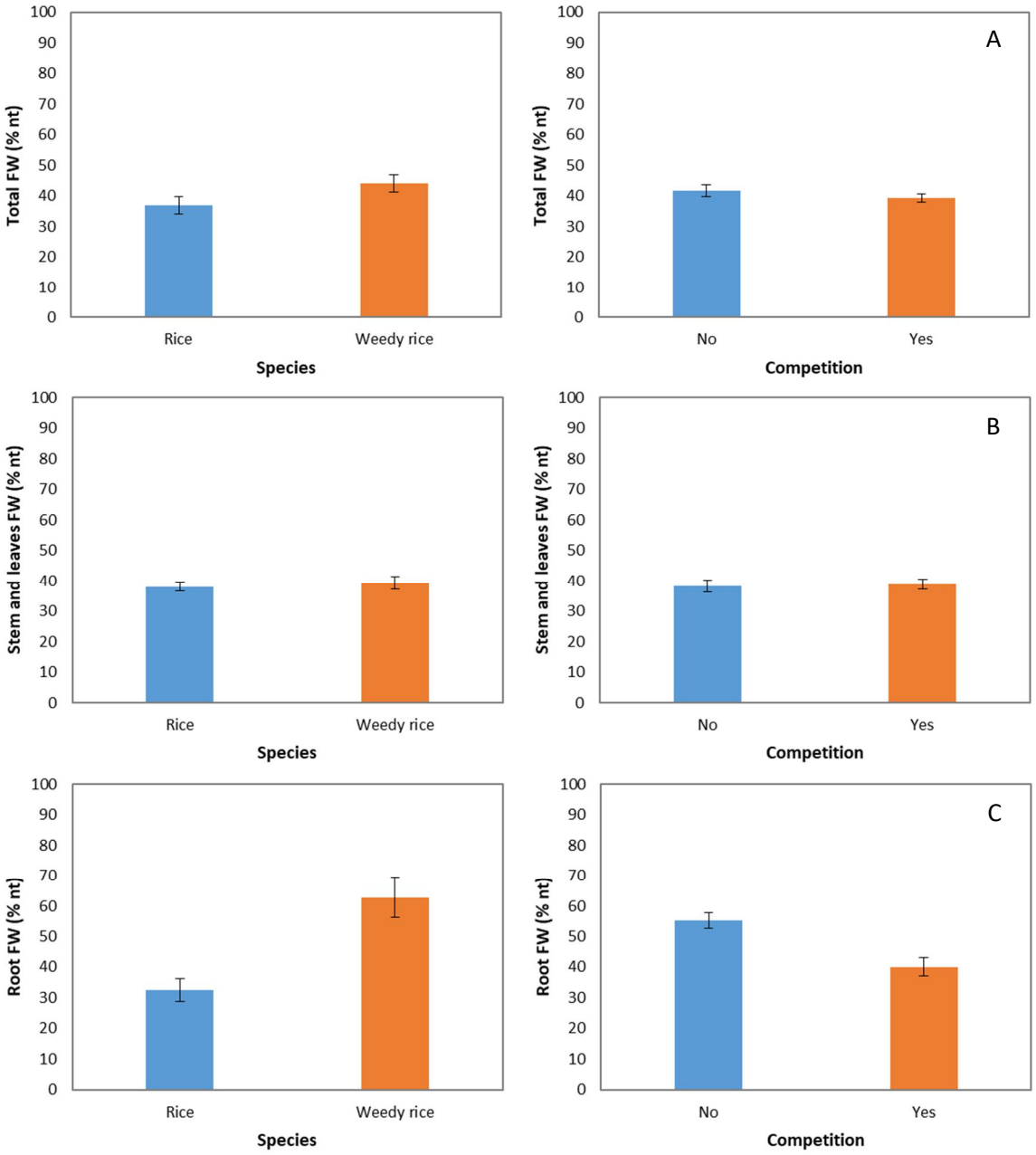

Figure A 1 *(A) Total fresh weight (FW), (B) stem and leaves FW and (C) root FW of summer species, expressed as percentage of salt-treated samples over non-treated samples (% nt). The graphs on the left compare the two species (rice and weedy rice). The graphs on the right, compare single- and mixed-species setups (competition factor). Vertical bars denote the standard error.*

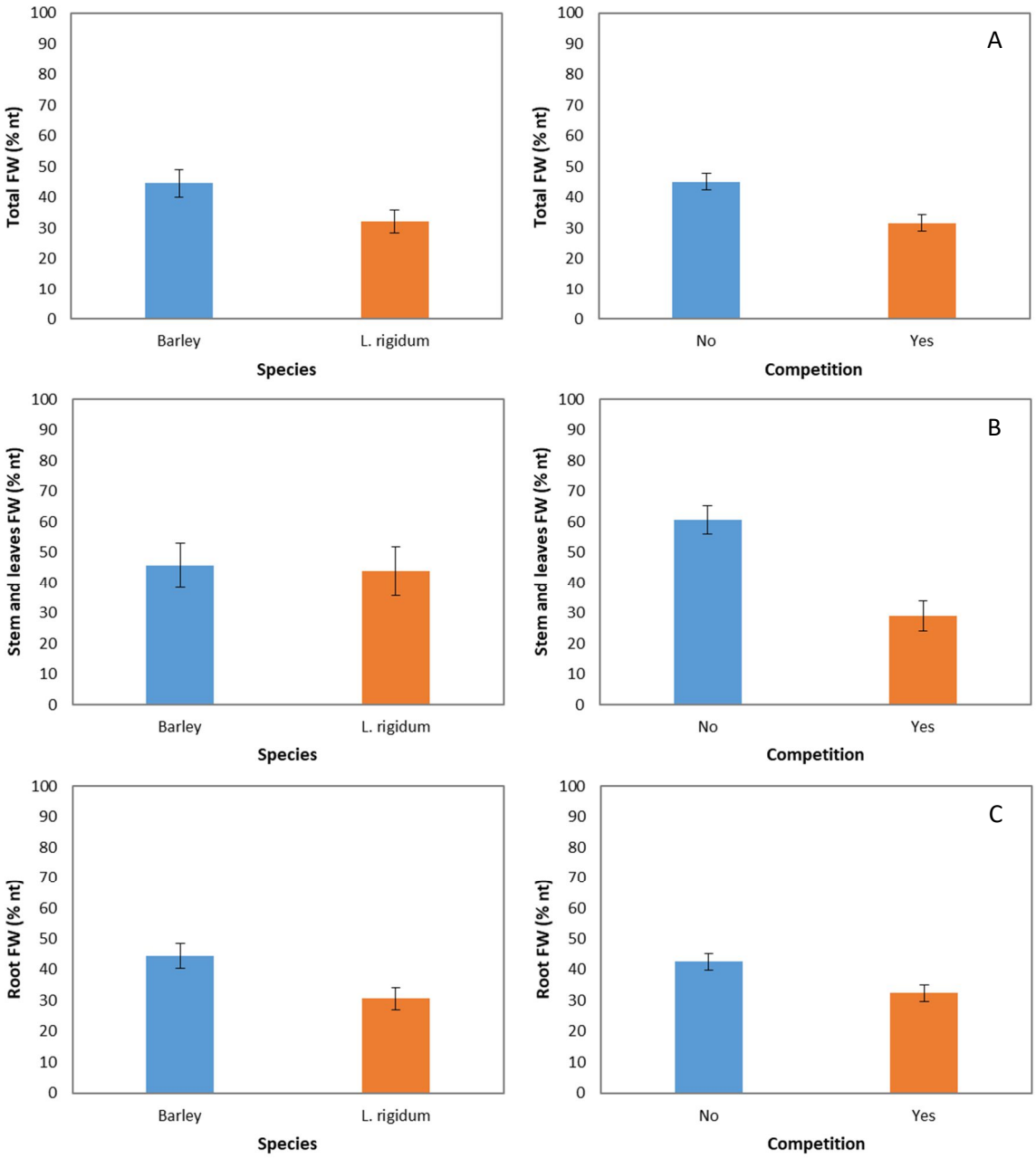

Figure A 2 *(A) Total fresh weight (FW), (B) stem and leaves FW and (C) root FW of winter species, expressed as percentage of salt-treated samples over non-treated samples (% nt). The graphs on the left compare the two species (barley and L. rigidum). The graphs on the right, compare single- and mixed-species setups (competition factor). Vertical bars denote the standard error.*

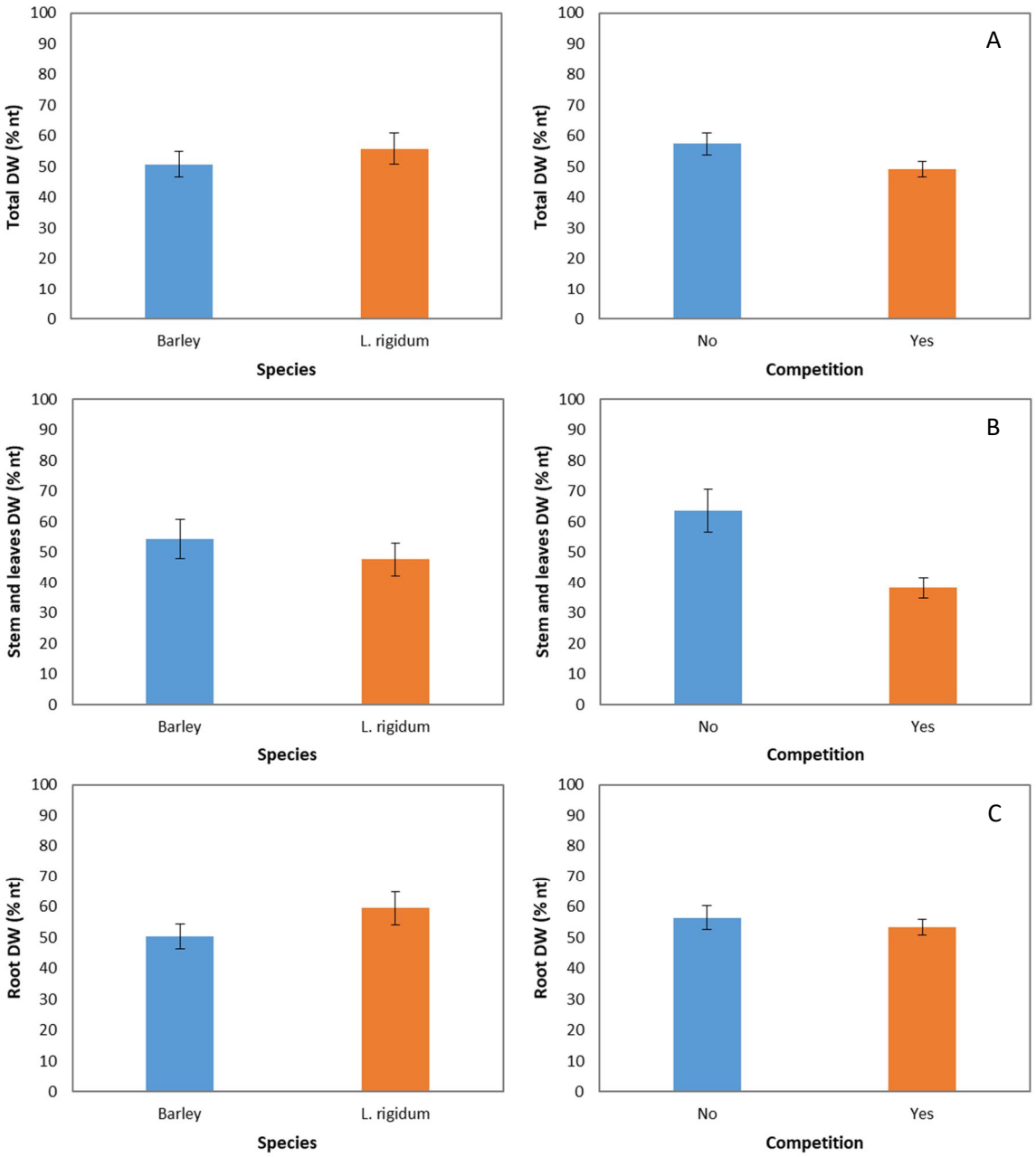

Figure A 3 *(A) Total dry weight (DW), (B) stem and leaves DW and (C) root DW of winter species, expressed as percentage of salt-treated samples over non-treated samples (% nt). The graphs on the left compare the two species (barley and L. rigidum). The graphs on the right, compare single- and mixed-species setups (competition factor). Vertical bars denote the standard error.*

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VI Effects of salinity and inter-specific competition on the growth of soybean (*Glycine max*), *Chenopodium album* and *Amaranthus retroflexus*

1. Introduction

Soil salinization, characterized by high concentrations of soluble salts in soils, primarily NaCl *(Almeida et al., 2017)*, is a pressing environmental problem affecting many agroecosystems. The agricultural land affected by salinization, estimated to be between 20 and 30% of the world's cultivated land *(Bhargava and Srivastava, 2020; Jamil et al., 2011)* is expanding worldwide due to global warming, depletion of natural resources and poor agricultural practices *(Hassani et al., 2021; Jamil et al., 2011)*. In addition to primary salinization, developed through natural processes, the rise of sea level and groundwater overexploitation are leading to saltwater intrusion in coastal and inland aquifers, the main cause of secondary salinization, caused by human actions *(Daliakopoulos et al., 2016)*. These phenomena, typically found in arid and semiarid regions *(Hakim et al., 2011; Shrivastava and Kumar, 2015)*, are now affecting temperate areas, including the Mediterranean Basin *(Daliakopoulos et al., 2016)*. Although saline soils have an electrical conductivity (EC) equal to or higher than 4 dS/m, several plant species are already damaged by EC levels as low as 2 dS/m *(Abrol et al., 1988; Sparks, 2003; Talat, 2020)*. Soil salinity can decrease crop yields of previously productive cropland due to its ability to impair seed germination and plant growth of many cultivated species, among which staple crops like rice, wheat, maize and soybean *(Daei et al., 2009; Katerji et al., 1996; Phang et al., 2008; Zeng and Shannon, 2000)*. The main toxic effects of salinity on plant cells are ion imbalance, inducing the accumulation of Na^+ and Cl^- ions and depletion of K^+ and Ca^{2+} ions in tissues *(Khan and Panda, 2008)*, and hyperosmotic stress *(Zhu, 2001),* associated with decreased water uptake and overproduction of reactive oxygen species (ROS) *(Niu et al., 1995; Xing et al., 2013; Zhu, 2001)*. ROS can initiate chain reactions involved in oxidative stress processes, leading to lipid peroxidation and oxidative damage to proteins and nucleic acids *(Jbir-Koubaa et al., 2015).* Plant coping mechanisms against ROS involve oxidoreductase enzymes and the osmolyte proline *(Apel and Hirt, 2004; McKersie and Leshem, 1994)*, which can be used as a proxy for the plant-stress status.

The morphological and physiological responses of soybean (*Glycine max* (L.) Merr.) to salinity have been widely studied, and include effects on the growth rate, nodulation, seed

production and photosynthetic activity *(He et al., 2016; Phang et al., 2008)*. These traits classify soybean as a salt-sensitive glycophyte *(Phang et al., 2008)*.

Conversely, the effects of salinity on weedy species and weed-crop interactions have been poorly investigated so far *(Cirillo et al., 2018)*. Since weeds are known to show earlier emergence, faster growth and higher genetic resilience and plasticity than cultivated species *(Chen et al., 2015; Clements et al., 2004; Lu et al., 2016)*, their adaptability to adverse environmental conditions might favor their spread under increasing salinization.
Chenopodium album L. and *Amaranthus retroflexus* L. are common weeds naturalized to the temperate regions of all continents, associated with spring and summer crops, including soybean *(Cirillo et al., 2018; Holm et al., 1997),* and thanks to their adaptability they are likely to spread in agroecosystems increasingly affected by climate change.

C. *album* is classified as a salt-tolerant species displaying halophytic traits, including seed dimorphism, sodium exclusion and potassium retention, high osmolyte and antioxidant production *(Tanveer and Shah, 2017)*. Although *A. retroflexus* is not a halophyte, it is highly tolerant to abiotic stresses and displays high water use efficiency, seed production potential and the ability to germinate in a wide range of environments, especially in semiarid habitats, that are more likely to be affected by salinization *(Bhargava and Srivastava, 2020; Khan et al., 2022; Sharma et al., 2021)*. Multiple authors reported that the germination of *A. retroflexus* is not sensitive to salt stress, with better performances than other Amaranth species *(Hao et al., 2017; Sharma et al., 2021)*.

To our knowledge, very few studies have assessed the interaction of *C. album or A. retroflexus* with cultivated species under salt stress *(Ghirardelli et al., 2021; Vecchio and Casini, 1983)*. Therefore, the current work aims to investigate the response of soybean, *C. album* and *A. retroflexus* to salt stress in greenhouse conditions, and how it changes when interspecific competition is present. Plant height, photosynthetic activity (SPAD), biomass, Na, K and protein content, and parameters related to osmotic and oxidative stress (lipid peroxidation, proline content) were assessed in leaves and roots of the three species. The greenhouse setup was chosen as a bridge between hydroponic and field experiments because it allows to introduce the soil variable while minimizing other environmental factors, such as atmospheric conditions.

2. Material and methods

2.1 Experimental setup

The greenhouse setup consisted of two levels of salinity (0 and 100 mM NaCl), three combinations of species (soybean, weed, soybean + weed) and 3 replicates for each combination of species and salt treatment, arranged in a randomized block design of 42 pots (**Figure 1**). Plants were grown at a density of 6 plants per pot: 6 soybean or weed plants in the case of single-species pots, 3 crop plants + 3 weed plants in the case of mixed-species pots. The pots with both soybean and *C. album* or *A. retroflexus* were twice those with single species in order to obtain the same number of biological replicates per treatment. Therefore, each block contained 7 pots (volume 50 L, diameter 40 cm), one for each species grown alone and two for each combination of soybean + weed (*C. album* and *A. retroflexus*). Pots were filled with a mixture of sand (55% v/v), peat moss (35% v/v) and perlite (10 % v/v), to minimize ion exchange that could alter electrical conductivity measurements.

Seeds of *C. album* and *A. retroflexus* were collected from September to November 2017 at the Experimental Farm of the University of Padova (Legnaro, north-east Italy, 45°12'N, 11°58'E, 6 m above sea level) with a soil electrical conductivity of 0.3 dS/m. Seeds were stored at 4 °C until the start of the experiment. The soybean cultivar (cv. PD1T45), commonly grown in the area of weed seed collection, was salt-sensitive, as demonstrated by previous experiments *(Ghirardelli et al., 2021)*. To reach the same final number of seedlings per pot, 18 seeds and 9 seeds of soybean were planted in single-species and mixed-species pots respectively, while *C. album* and *A. retroflexus* seeds were approximately 50 per pot. Seedlings were thinned at the emergence stage to obtain a homogeneous layout within the pots. After ten days of irrigation with regular water, salt-treated pots were continuously irrigated with the same volume of 100 mM NaCl solution every other day until reaching a soil EC of 4 dS/m. The increase in electrical conductivity was monitored with an EC tester (Hanna Instruments, Woonsocket, US) once a week.

2.2 Plant sampling

Once the final number of plants per pot was reached, plant height was measured weekly, and photosynthetic activity was monitored with a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies, US), that was used to estimate chlorophyll content *(Castelli et al., 1996)*. At the end of the trial, leaves of 2 out of 6 plants per treatment were sampled and immediately frozen in liquid nitrogen and stored at -80 °C until further biochemical analyses (lipid peroxidation). The remaining plants were separated and thoroughly cleaned to determine the

fresh weight (FW) of roots and aerial parts. In the case of soybean, also the number and size of root nodules were recorded in all plants. For dry weight (DW) measurement, the plant material was oven-dried at 65°C for 48 h. The dry material was then ground and prepared for protein content, proline and elemental quantification.

The first trial started on 25th March 2021 and ended when signs of senescence processes were visible on salt-treated plants, on 8th June 2021. The second and third trials lasted from 25th September to 8th December 2021 and from 30th March to 9th June 2022, respectively. In all cases, the greenhouse light system was set with a 14 h light/10 h dark photoperiod. Temperatures inside the greenhouse were monitored with two HOBO Pendant Temperature 64K data loggers (Onset, Cape Cod, MA, United States) and ranged between 16 and 33 °C in the first trial, 13 and 22 °C in the second trial, 17 and 31 °C in the third trial.

Figure 1. *Experimental setup for the greenhouse trials.*

2.3 Biochemical analyses

Elemental quantification and protein content

The quantification of Na^+ and K^+ in leaves was conducted after an acid-digestion procedure, carried out inside closed Teflon vessels of 100 mL volume. For each sample, 500 mg of dry plant material were dissolved in 7 mL HNO_3 67% and 2 mL H_2O_2 30% in a microwave (Millestone ETHOS EASY 1600W, Bergamo, Italy). Mineralized samples were then diluted in 25 mL ultrapure water and each element was quantified via Inductively Coupled Plasma Atomic Emission Spectroscopy (SPECTRO ARCOS, SPECTRO Analytical Instruments GmbH, Kleve, Germany). Calibration standards were matched with 1% ethanol absolute (Prolabo VWR International PBI S.r.l. Milano, Italy). The elements to be determined were added from single-element solutions (Inorganic Ventures, Christiansburg, VA, USA). The

concentrations range of the calibration solutions was between 0 and 100 mg/L for both Na and K. Data were expressed as milligrams per kilo (ppm) of dry weight.

Crude protein (CP) content in leaves was quantified with a Kjeldahl protein auto-analyzer (Kjeltec 8400, FOSS, Hilleroed, Denmark) using the block digestion method with a copper catalyst and steam distillation into boric acid. Protein content was calculated by using the formula:

Protein (mg/100g) = Nitrogen x 6.25.

Lipid peroxidation

Malondialdehyde (MDA) leaf content was assessed following the method described by *Heath and Packer (1968)* with some modifications. Frozen leaf tissues (500 mg) were homogenized in 10 mL of 0.5 % v/v trichloroacetic acid (TCA) for 30 s each, followed by centrifugation at 3,000 × *g* at 4 °C for 10 min. Then, 200 µL of each supernatant was mixed with 4 mL of 0.5 % thiobarbituric acid (TBA) water solution. Tubes were then heated at 95°C for 30 minutes. The absorbance was read at 532 nm and 600 nm. The OD_{600} was then subtracted from the OD_{532} value to achieve an extinction coefficient of 155 mM cm^{-1}. Data are expressed as µmol/g FW of MDA.

Proline

Proline content in leaves was determined by reversed-phase high-performance liquid chromatography (RP)-HPLC followed by UV detection. Each sample was prepared by placing 100 mg of plant material in a 6 × 50 mm borosilicate glass tube. HCl (7.5 mL, 6 M) was added to the sample, then heated at 105 °C for 24 h. After hydrolysis, the sample was neutralized to pH 9 using 8M NaOH and adjusted to 100 mL with water. The solution was then filtered at 0.22 µm to conduct the derivatization procedure according to the manufacturer's instructions (Waters, AccQTag Ultra Derivatization Kit). Then, 10 µL of extract was mixed with 70 µL of 0.2 M borate buffer (pH 9.0), followed by 20 µL of aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) dissolved in acetonitrile. The mixture was incubated for 1 min at room temperature, then for 10 min at 55 °C. The resulting AQC-derivatized mixture was diluted by adding 900 µL of borate buffer.

The chromatographic analysis was performed on an Agilent Infinity 1260 liquid chromatograph with a binary pump (Agilent Technologies, Santa Clara, CA, United States), equipped with a CORTECS C18 column (2.7 µm, 2.1 × 150 mm). The mobile phase was 0.1% formic acid (v/v) in deionized water, the flow rate was 0.4 mL/min and the injection volume was 5 µL. Proline was detected with a diode array detector (DAD), and its

concentration was determined based on a standard curve. Results are expressed in nanomoles of proline per gram of fresh weight.

2.4 Statistical analysis

All the parameters were compared among the three species through a one-way analysis of variance (ANOVA) in randomized blocks on data expressed as percentage of salt-treated over non treated plants (% nt). A one-way ANOVA in randomized blocks was also performed separately on soybean, *C. album* and *A. retroflexus* to assess differences among treatments (no salinity and competition, salinity only, competition only, salinity and competition), using R 4.2.0 (2022). Mean differences were analyzed with pair-wise post hoc analyses (Tukey test) at $p < 0.05$. The homogeneity of variances was verified by the Levene test. Whenever the homogeneity of variances of the original dataset was not confirmed, the data underwent a squared root or logarithmic transformation in order to satisfy this ANOVA requirement. Although in the graphs significant letters are reported on the original data, we specified in the figure captions if any transformations were applied. The number of biological replicates varied depending on the analysis performed, as reported in the figure legends.

3. Results

Although the experiments were conducted in a greenhouse with a proper photoperiod and sheltered walls, some differences were observed between the spring (first and third) trials and autumn (second) trial. For this reason, all data from each trial are present separately and not pooled.

3.1 Plant height

Comparing the three species, *C. album* plants had the biggest final height (%nt) in all the trials. In the second and third trial, plants grown with soybean had significantly higher values than those of all the other species and treatments, and, in the second and third trials, even higher than the average control plants (> 100%). *A. retroflexus* height percentages were lower than the average control plants (< 100%) in all the trials. In the second and third trial, the plants grown in single-species pots had values lower than the plants grown with soybean, and comparable to those of *C. album* grow alone, while in the first trial, plants grown both in single- and mixed-species pots had values comparable to those of *C. album*. Also soybean height percentages were always lower than the average control plants (< 100%) in all the trials, but only plants grown in single-species pots always had values comparable to *A. retroflexus* grown in mixed-species pots and *C. album* grown in single-species pots, while

the percentage heights of plants grown with *C. album* were always significantly lower. In the second and third trial, plants grown with *A. retroflexus* also had significantly lower percentages (**Figure 2 A**, **C**, **E**). Looking at each species separately, final heights were significantly lower in salt-treated soybean and *A. retroflexus* compared to the control, while no difference was found in *C. album* (**Figure A 1**). Soybean heights were particularly lower when the effect of competition was added to salt treatment, especially in the case of soybean + *C. album* in the first and third trials and soybean + *A. retroflexus* in the second trial (**Figure A 1 C**, **F**, **I**). Conversely, *A. retroflexus* appeared to be more affected by salt stress than competition, as no significant difference was found between single- and mixed-species pots irrigated with salt water (**Figure A 1 A**, **D**, **G**).

3.2 SPAD values

Comparing the three species, all the final SPAD values (%nt) were lower than the average of salt-untreated plants (< 100%). *C. album* plants had the highest percentage SPAD value in all the trials, without significant differences between single- and mixed-species pots. Both *A. retroflexus* and soybean always had significantly lower percentage values, with the exception of soybean grown in single-species pots in the third trial. In the case of *A. retroflexus*, the percentage SPAD values in plants grown with soybean were higher than those grown in single-species pots. Conversely, in the case of soybean the values were significantly lower when plants were grown with *A. retroflexus* or *C. album* (**Figure 2 B**, **E**, **F**).

Looking at each species separately, salt treatments significantly lowered SPAD values in soybean, *A. retroflexus* and *C. album* compared to the control. In the case of soybean, the effect of competition further reduced the SPAD indices in all trials. Salt-treated *A. retroflexus* and *C. album* did not show any differences when grown with soybean (**Figure A 2**).

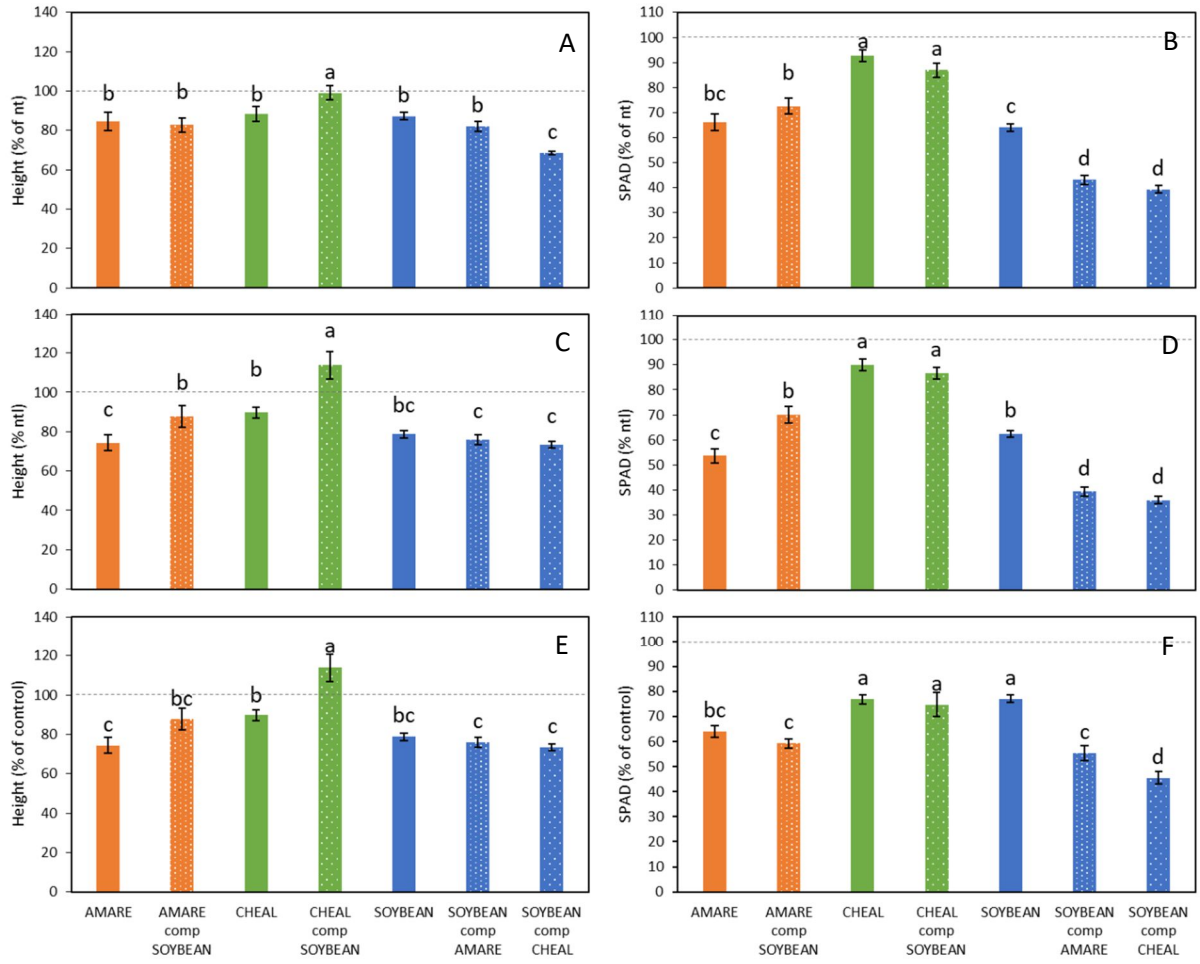

Figure 2. *Final height and SPAD of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max) expressed as percentage of salt-treated samples over non-treated samples (% nt), at the end of the first (A-B), second (C-D) and third trial (E-F). Different letters within each group of bars indicate significant differences at p<0.05, n=9. Tukey test on the height values of the 2nd and 3rd trial and on the SPAD values of the 1st and 2nd trial was performed on transformed data (logarithmic and arcsine transformation, respectively). AMARE=A. retroflexus; CHEAL=C. album; comp=competition.*

3.3 Biomass quantification

Comparing the three species, *C. album* plants had the biggest FW and DW (%nt) in all the trials, with values even higher than the average control plants (> 100%). No significant differences were found between plants grown in single- and mixed-species pots. *A. retroflexus* FW and DW percentages were lower than the average control plants (< 100%) in all the trials, and significantly lower than *C. album*. No significant differences were found between plants grown in single- and mixed-species pots, with the exception of the third trial, were plants grown in single-species pots had values lower than the plants grown with soybean. Also soybean FW and DW values were always lower than the average control plants (< 100%) in all the trials, and significantly lower than *C. album*. Plants grown in single-species pots generally had values comparable to those of *A. retroflexus* grown with soybean, while the percentage FW and DW plants grown with *C. album* were always

significantly lower than single-species pots. In the second trial, plants grown with *A. retroflexus* also had FW and DW percentages lower than single-species pots, while in the third trial the same was observed only in the DW (**Figure 3**).

Looking at each species separately, the total FW of soybean plants exposed to salinity was significantly lower than relative NaCl-untreated controls, with a significant reduction in the case of mixed-species posts in all trials (**Figure A 3**). The effect of competition on biomass was particularly evident in the first and third trials. Similar trends were observed in root FW and leaves and stem FW, except for root FWs in the first trial (**Figure A 4**), where there was no significant difference among salt-treated plants, and leaves and stem FW in the second trial (**Figure A 2**), where the only significant difference was between single-species pots (with and without NaCl) and mixed-species pots with salt treatment. The effect of *C. album* competition was more pronounced in the third than in the second trial. Regarding total DW, the combined effect of salt and competition led to significant reductions in the first and third trials, while in the second trial the only treatments with higher values were control soybean grown with *A. retroflexus* and in single-species pots (**Figure A 6**). Similarly, root DW (**Figure A 7**) and leaves and stem DW (**Figure A 8**) were significantly reduced in salt-treated plants of the first and third trials, especially when grown in competition with weeds. In this regard, root biomass was significantly lower in plants grown with *C. album*, while the presence of *A. retroflexus* did not cause any biomass reduction compared to the single-species setup. In the second trial, results were more unclear, but control soybean grown with *A. retroflexus* appeared to have slightly higher root biomass values than any salt treatment and even in control plants grown with *C. album* (**Figure A 7**), differences were not significant. In the second and third trials, the total FW of *A. retroflexus* salt-treated plants was significantly lower in single species-pot, while in the first trial the only significant reduction was in mixed-species pots (**Figure A 3 A**, **D**, **G**). A similar trend was recorded in leaves and stem FW (**Figure A 5**), while root FWs did not show any significant difference (**Figure A 4**). The effect of salt on total DW was more pronounced in all trials, although no significant difference was found between single- and mixed-species pots (**Figure A 6**). The same behavior was observed in leaves and stem DW of the third trial (**Figure A 8**). In the case of root DWs, control plants grown with soybean also showed lower biomass compared to single-species pots, except for the third trial (**Figure A 7**). *C. album* plants did not show any significant biomass difference between treatments, except for the third trial, when leaves and stem FW was higher in salt-treated plants grown with soybean than in control plants, with and without competition (**Figure A 2**).

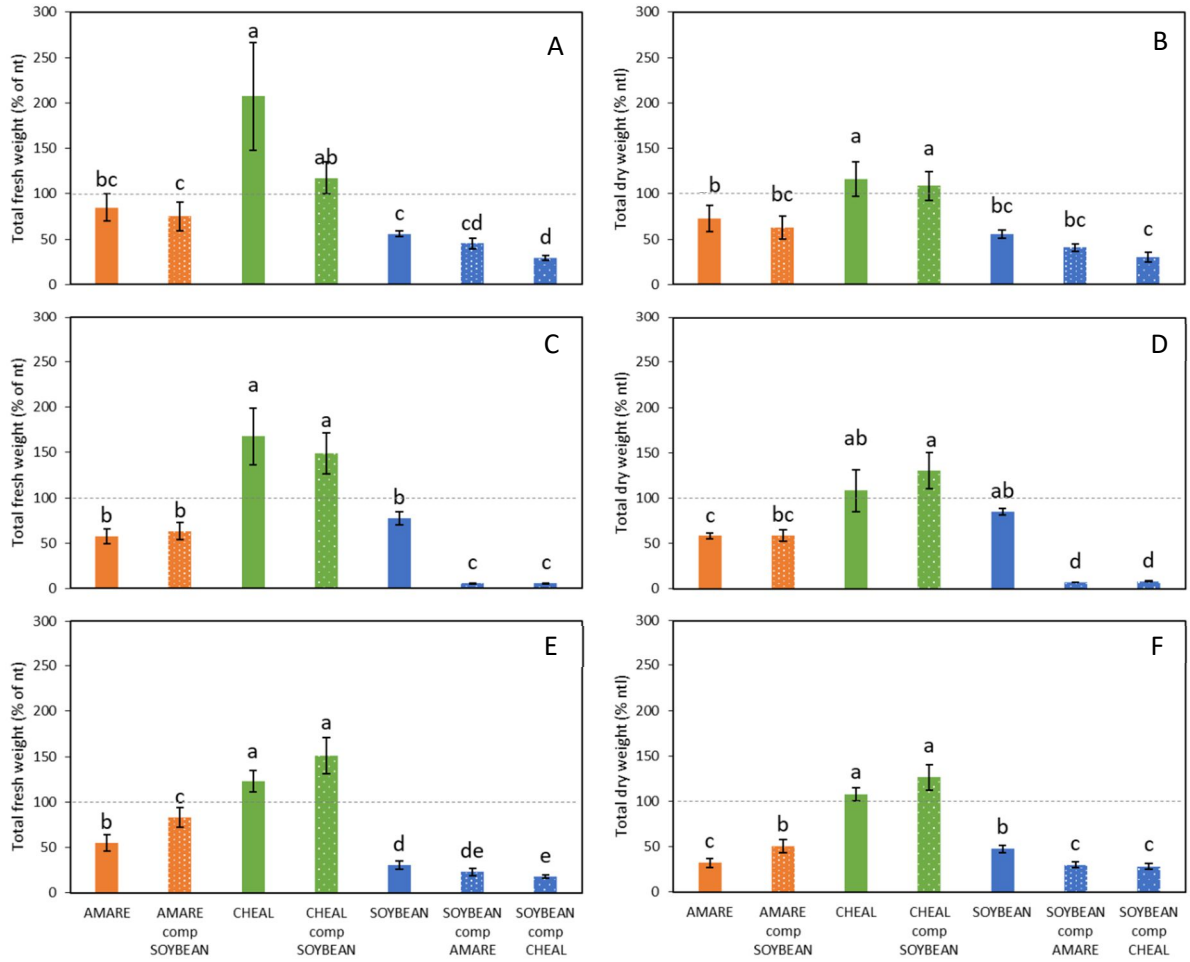

Figure 3. *Total fresh weight and dry weight of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max) expressed as percentage of salt-treated samples over non-treated samples (% nt), at the end of the first (A-B), second (C-D) and third trial (E-F). Different letters within each group of bars indicate significant differences at p<0.05, n=6. Tukey test on the FW and DW values of the 1st, 2nd and 3rd trial was performed on transformed data (logarithmic and arcsine transformation). AMARE=A. retroflexus; CHEAL=C. album; comp=competition.*

3.4 Soybean nodulation

In both the first and third trials (spring 2021 and 2022 trials), salt stress severely affected the development of roots and root nodules in soybean (**Table 1**). When salt treatment was applied, none of the plants presented more than 10 active nodules, except for soybean grown in single-species pots, where 16.7% of the plants had more than 10 nodules in the 2022 trial (**Table 1 C**). Plants grown with *C. album* Zwere the most affected, with zero nodules in 61.1 and 66.7 % of the samples in 2021 and 2022 respectively. The diameter of existing nodules was lower than 0.5 cm in more than two-thirds of the salt-treated samples (100% in soybean + *A. retroflexus* from the 2022 trial), and only soybean grown in single-species pots had nodules bigger than 1 cm. Conversely, all of the control plants had more than 10 nodules, except for soybean grown with *A. retroflexus* in 2021 (**Table 1 A**). Ninety-five to 100 % of the nodules had diameters higher than 0.5 cm, and frequently higher than 1 cm. None of

the second-trial (autumn 2021) plants presented more than ten nodules, all smaller than 0.5 cm. Salt-treated plants showed no sign of nodule formation (**Table 1 B**).

Table 1. *Frequency and prevalent size of root nodules within each treatment of soybean in the first (A), second (B) and third trial (C) expressed as a percentage of samples per class of abundance and diameter. A=A. retroflexus; C= C. album; S=soybean, contr=control.*

A

1st trial	**N of nodules**					**⌀ (cm)**			
Percentage	0	0<n<10	10<n<20	20<n<50	n>50	0	0<n<0.5	0.5<n<1	n>1
S contr	0.00	0.00	5.56	77.78	16.67	0.00	5.56	50.00	44.44
S+A contr	5.56	5.56	11.11	50.00	27.78	5.56	0.00	44.44	50.00
S+C contr	0.00	0.00	11.11	77.78	11.11	0.00	0.00	22.22	77.78
S salt	22.22	77.78	0.00	0.00	0.00	22.22	50.00	22.22	5.56
S+A salt	27.78	72.22	0.00	0.00	0.00	27.78	27.78	44.44	0.00
S+C salt	61.11	38.89	0.00	0.00	0.00	61.11	27.78	11.11	0.00

B

2nd trial	**N of nodules**					**⌀ (cm)**			
Percentage	0	0<n<10	10<n<20	20<n<50	n>50	0	0<n<0.5	0.5<n<1	n>1
S contr	61.11	38.89	0.00	0.00	0.00	61.11	38.89	0.00	0.00
S+A contr	77.78	22.22	0.00	0.00	0.00	77.78	22.22	0.00	0.00
S+C contr	88.89	11.11	0.00	0.00	0.00	88.89	11.11	0.00	0.00
S salt	100.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
S+A salt	100.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
S+C salt	100.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00

C

3rd trial	**N of nodules**					**⌀ (cm)**			
Percentage	0	0<n<10	10<n<20	20<n<50	n>50	0	0<n<0.5	0.5<n<1	n>1
S contr	0.00	0.00	5.56	94.44	0.00	0.00	0.00	72.22	27.78
S+A contr	0.00	0.00	0.00	94.44	5.56	0.00	5.56	61.11	33.33
S+C contr	0.00	0.00	38.89	61.11	0.00	0.00	5.56	83.33	11.11
S salt	16.67	66.67	16.67	0.00	0.00	16.67	61.11	22.22	0.00
S+A salt	38.89	61.11	0.00	0.00	0.00	38.89	61.11	0.00	0.00
S+C salt	66.67	33.33	0.00	0.00	0.00	66.67	22.22	11.11	0.00

3.5 Elemental quantification

Comparing the three species, K^+ content (%nt) did not show consistent patterns between the trials. In the first and third trial, all the values were higher than those of the control plants (> 100%), while in the second trial only soybean grown alone and with *C. album*, as well as *A. retroflexus* grown with soybean, exceeded the control. In the first trial, soybean grown in single-species pots had K^+ percentages higher than those of *C. album*, soybean grown with *C. album*, and *A. retroflexus* grown with soybean. In the second trial, soybean grown in single-species pots had K^+ percentages higher than those of *C. album*, soybean grown with

A. retroflexus, and *A. retroflexus* grow in single-species pots. No significant differences were found between species and treatments in the third trial (**Figure 4 A**, **C**, **E**). Like K^+, also Na^+ content (%nt) did not show consistent patterns between the trials, besides the fact that the values of all the species and treatments were much higher than those of the control (> 100%). Na^+ percentage values of soybean plants grown with *C. album* were generally the highest. However, in the first trial they were only statistically different from those of soybean grown with *C. album* and in single-species pots. In the second trial they were significantly higher than *A. retroflexus* and soybean grown in single-species pots and *C. album* grown with and without competition. In the second trial they were significantly higher than *A. retroflexus* grown with soybean, pots and *C. album* grown with and without competition, and soybean grown with *A. retroflexus*. *C. album* showed no differences between single and mixed-species pots, while *A. retroflexus* only did in the second trial (**Figure 4 B**, **D**, **F**).

Looking at each species separately, we can see that in salt-treated soybean leaves, K^+ content was significantly higher than the control in the first trial. In the third trial, only soybean grown without competition had significantly higher K^+ content, while no differences were recorded in the second trial (**Figure A 9 C**, **F**, **I**). The effect of salinity was much more evident in Na content, that was higher in salt-treated plants of all trials, especially in those grown with *C. album* during spring trials (**Figure A 10 C**, **F**, **I**). *C. album* leaves did not show any significant difference in K^+ content, except for the plants grown in single-species pots in the third, with higher K^+ content than the other treatments (**Figure A 9 B**, **E**, **H**). Similarly, *A. retroflexus* showed higher K content only in single-species pots of the first trial (**Figure A 9 A**, **D**, **G**). Conversely, Na^+ content was higher in salt-treated plants of all the trials, but the increment compared to the control was much higher in *A. retroflexus* than in *C. album* (**Figure A 10 A**, **B**, **D**, **E**, **G**, **H**).

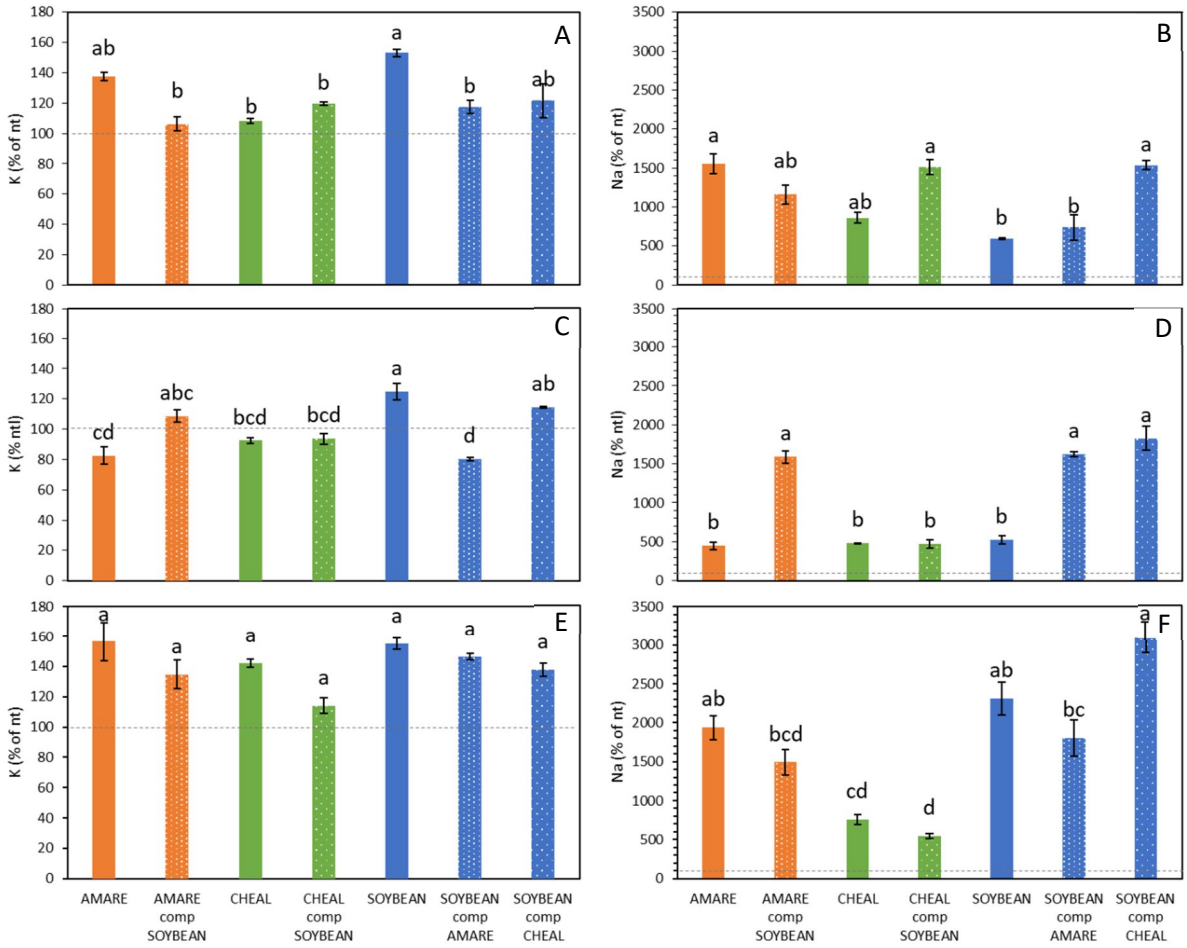

Figure 4. *Potassium and sodium leaf content of of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max) expressed as percentage of salt-treated samples over non-treated samples (% nt), at the end of the first (A-B), second (C-D) and third trial (E-F). Different letters within each group of bars indicate significant differences at p<0.05, n=6. AMARE=A. retroflexus; CHEAL=C. album; comp=competition.*

3.6 Crude protein content

Comparing the three species, CP content (%nt) of *A. retroflexus* was higher than those of *C. album* and soybean both in the first and third trial, while in the second trial *C. album* grown with soybean had the highest value, although statistically comparable to that of *A. retroflexus* grown with soybean and that of soybean grown with *C. album*. In the first trial, all the soybean and *C. album* values were similar, and lower than those of *A. retroflexus*, while in the third trial the percentage value of soybean grown in single-species pots was higher than soybean grown in mixed-species pots and *C. album* with or without competition (**Figure 5**).

Looking at each species separately, we can see that protein content in soybean leaves was lower in mixed-species pots exposed to salt stress in both the first and second trials, while no significant difference was found in mixed-species pots of the third trial (**Figure A 11 C**, **F**, **I**). *A. retroflexus* showed higher protein content in salt-treated plants of the first and third trial, while no significant difference was found in the second trial (**Figure A 11 A**, **D**, **G**).

Also *C. album* showed no difference between the treatments, except for the salt-treatment plants grown in competition with soybean in the second trial (**Figure A 11 E**).

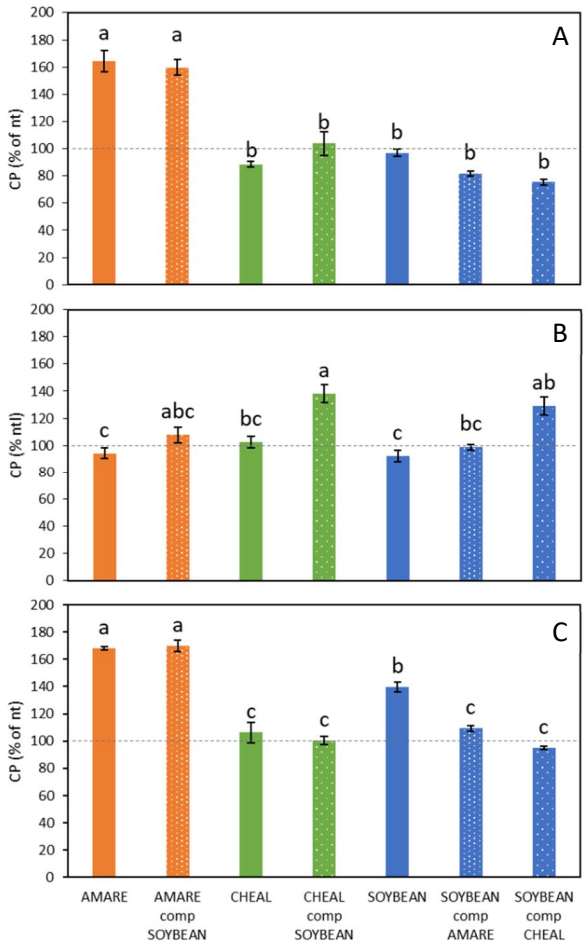

Figure 5. *Crude protein (CP) leaf content of of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max) expressed as percentage of salt-treated samples over non-treated samples (% nt), at the end of the first (A), second (B) and third trial (C). Different letters within each group of bars indicate significant differences at p<0.05, n=6. AMARE=A. retroflexus; CHEAL=C. album; comp=competition.*

3.7 Lipid peroxidation

Comparing the three species, MDA content (%nt) was generally higher in soybean and lower in *A. retroflexus*. However, the only significant differences between *A. retroflexus* (with and without competition) and soybean grown with *A. retroflexus* in the first trial, between *A. retroflexus* grown with soybean and soybean grown with *A. retroflexus* in the second trial, and between soybean (grown alone and with *A. retroflexus*) and the other species, with and without competition, in the third trial (**Figure 6 A**, **C**, **E**).

Looking at each species separately, leaves of salt-treated soybean plants generally had a higher MDA content than the corresponding untreated plants, especially in the case of mixed-species pots and soybean + *A. retroflexus* pots of the first and second trials, while no significant differences were found in the autumn trial (**Figure A 12 C**, **F**, **I**). Similarly, neither *A. retroflexus* nor *C. album* showed significant differences between the treatments (**Figure A 12 A–B**, **D–E**, **G–H**).

3.8 Proline content

Comparing the three species, proline content (%nt) was generally similar in all treatments. More specifically, *A. retroflexus* and *C. album* showed no difference between plants grown with and without soybean. However, in the second trial the proline percentage of soybean grown in mixed-species pots was statistically higher than the other species and also than soybean grown in mixed-species pots. In the third trial, the same soybean treatments were statistically higher than *C. album* (**Figure 6 B**, **D**, **F**).

Leaves of salt-treated soybean plants generally had a higher proline content than control plants, however, due to high variations within treatments, not many significant differences were found (**Figure A 13 C**, **F**, **I**). In the first trial, no difference was observed between treatments. In the second trial, untreated plants grown in competition with *A. retroflexus* had significantly lower proline content than salt-treated plants grown alone and in competition with *C. album*. In the third trial, both untreated soybean plants grown with *A. retroflexus* and *C. album* had significantly lower proline content than the corresponding salt-treated plants, with lower values in the case of competition with *C. album*. In both the second and third trials, *A. retroflexus* grown in mixed-species tanks had lower proline content in salt-treated plants, while no difference was observed in the first trial (**Figure A 13 A**, **D**, **G**). *C. album* showed no difference between treatments, with and without competition (**Figure A 13 B**, **E**, **H**).

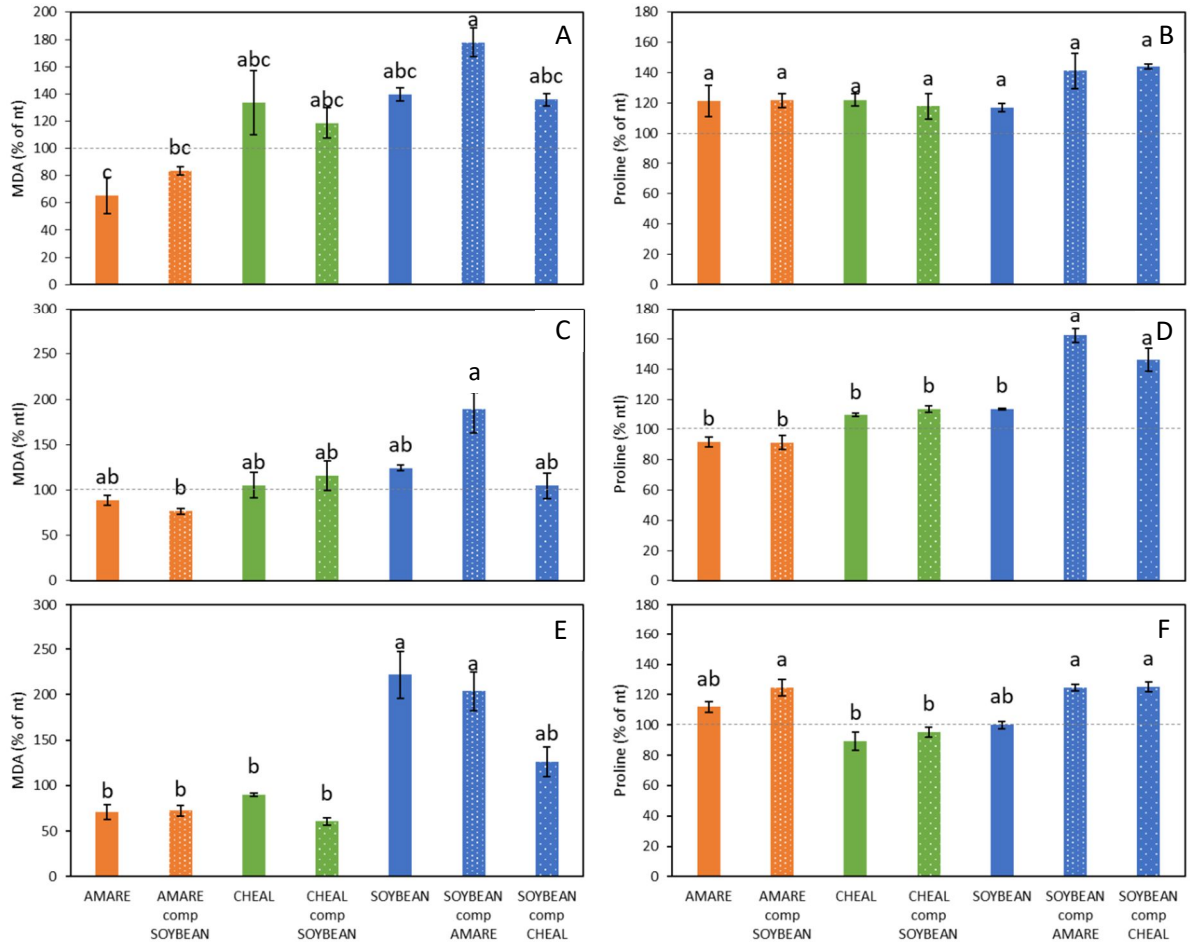

Figure 6. *Malondialdehyde (MDA) and proline leaf content of of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max) expressed as percentage of salt-treated samples over non-treated samples (% nt), at the end of the first (A-B), second (C-D) and third trial (E-F). Different letters within each group of bars indicate significant differences at p<0.05, n=6. AMARE=A. retroflexus; CHEAL=C. album; comp=competition.*

4. Discussion

Plant heights, SPAD measurements and biomass revealed that, out of the three analyzed species, soybean was the most affected by salt stress. This result is consistent with many studies reporting a decrease in plant height, photosynthetic activity and biomass production in different salt-treated soybean cultivars *(Amirjani, 2010; Essa, 2002; He et al., 2016; Tunçturk et al., 2008; Ullah et al., 2019)*. Our findings confirm the categorization of soybean as a salt-sensitive glycophyte *(Aghaleh and Niknam, 2009; Phang et al., 2008)*. The fact that growth parameters and SPAD, an index correlated with chlorophyll content, in salt-treated soybean were often lower in mixed-species pots, confirmed the competition potential of *A. retroflexus* and *C. album* as weed species in saline environments. Interestingly, no reduction was recorded in control pots with *A. retroflexus* and *C. album*, despite being strong competitors of soybean, accounting for considerable yield reduction in field conditions *(Crook and Renner, 1990; Gawęda et al., 2020)*. Compared to previous experiments conducted on soybean and *C. album* in hydroponics, where biomass reduction was seen even on NaCl-untreated soybean *(Ghirardelli et al., 2021)*, the greenhouse setup might have mitigated both nutrient competition and weed allelopathic effects. In fact, plants were gradually thinned in the first growth stages, considered the critical period for weed control in soybean *(Green-Tracewicz et al., 2012)*. In these stages, salt irrigation was not performed, therefore the interaction with salt stress was not present. In addition, the trials ended before the completion of the phenological cycle, thus preventing the competition for space and nutrients in later stages. Both *C. album* and *A. retroflexus* are well-known for their allelopathic effects on multiple crops, such as soybean *(Bhowmik and Doll, 1982; Namvar et al., 2009)*, rapeseed *(Rezaie and Yarnia, 2009)*, sunflower, tomato *(Reinhardt et al., 1994)*, rice *(Alam et al., 1997)*, maize cucumber, alfalfa, common bean and wheat *(Salehi-Lisar et al., 2015; Shahrokhi et al., 2012)*, therefore it is possible that the weeds released exudates affecting soybean. However, the soil medium might have attenuated the allelopathic interactions, that became apparent only when abiotic stress was also present. The synergistic effect of competition and salt stress in impairing soybean growth is in accordance with the studies of *Namvar et al. (2009)*, who found that the inhibitory effect of aqueous extracts from *C. album* plant tissues on soybean growth was intensified by combining the treatment with salt stress.

The number and size of root nodules in soybean also seem to reflect the combined effect of salinity and competition on root development. It is well known that the abundance and size of root nodules are essential to the nutritional status of the plant, due to the role of symbiotic

N-fixating bacteria living inside the nodules *(Phang et al., 2008)*. Many studies have observed the reduction of soybean nodulation, nitrogen fixation, and the final number and biomass of nodules in salt-treated plants *(Phang et al., 2008; Singleton and Bohlool, 1984; Song et al., 2017)*, in accordance with our results. Because of their allelochemical activities, both *C. album* and *A. retroflexus* have been reported to inhibit nodulation of soybean *(Mallik et al., 1994; Samaee et al., 2013)*, but to our knowledge, no studies are available on the combined effect of competition and salinity on root nodulation. It is therefore interesting to notice the extremely low number of root nodules in mixed-species pots of the first and third trials. However, in the second trial, no root nodulation occurred on any salt-treated plants. This finding might be explained by the fact that the trial took place in autumn. Even if the experiment was carried out in a greenhouse with a proper photoperiod and sheltered walls, the lower outside temperatures might have affected the growth and development of plants, acting as an additional stress factor.

Unlike soybean, growth parameters confirmed *C. album* to be a halophytic plant, displaying various mechanisms of salt tolerance *(Tanveer and Shah, 2017)*. Our results confirm the findings of *Yao et al. (2010)*, who observed that plant growth was not inhibited by salt stress up to 300 mM. Conversely, *A. retroflexus* appeared to be affected by salinity, in accordance with *Hao et al. (2017)* and *Khan et al. (2022)*, who found that seed germination was significantly affected by salt stress above and equal to NaCl 100 mM, and *Sharma et al. (2021)*, who found a reduction in plant height and leaf number at NaCl 150 mM. However, no synergistic effect of competition was found for *A. retroflexus*, demonstrating its ability to adapt to a variety of environments and climatic conditions and justifying the concerns around it becoming an increasingly aggressive weed in semiarid regions, including the Mediterranean area *(Sharma et al., 2021)*.

Both *C. album* and *A. retroflexus* showed lower SPAD values in salt-treated plants. This is in agreement with the observations of *Omami and Hammes (2010)* and *Zhang, Mutailifu, and Lan (2022)*, who recorded the inhibition of photosynthesis in different salt-treated *Amaranthus* species and *C. album*, respectively. However, *Yao et al. (2010)* observed that chlorophyll content was not reduced in *C. album* exposed for two months to salt stress up to 300 mM. This discrepancy might be due to differences among ecotypes.

In terms of K^+ and Na^+ leaf content, all species had an increase in Na^+ content in salt-treated plants, in agreement with literature data, that show how high Na^+ in the soil can induce an increase in Na^+ content in the plant tissues *(Almeida et al., 2017)*. Due to the similar

physicochemical properties of K^+ and Na^+, Na^+ can inhibit K^+ uptake and substitute K^+ in important metabolic binding sites, causing ion imbalance in plant cells. The tolerance of plant species and varieties is often correlated with their ability to maintain a high K^+ / Na^+ ratio in the cytosol *(Almeida et al., 2017)*. In this case, soybean increased or at least maintained K^+ content in an attempt to balance the K^+ / Na^+ ratio. This is in agreement with our previous results observed in hydroponic setups, where the K^+ leaf content of soybean plants was not reduced by salt treatment *(Ghirardelli et al., 2021)*. In the same experiment, salinity reduced K^+ content in leaves of *C. album*, which was explained by speculating that elevated initial levels of K+ had counteracted the early negative effects of Na^+, also considering the very high K^+ content in the control plants. In the present study, however, *C. album* always maintained or even increased K^+ leaf content, more in agreement with the literature, that sees this species as an extremely salt-tolerant plant, able to increase K^+ / Na^+ ratio by accumulating K^+ *(Ivanova et al., 2016; Tanveer and Shah, 2017; Yao et al., 2010)*. In the case of *A. retroflexus*, no data is available regarding the effects of salt stress on the K^+ / Na^+ ratio, but other *Amaranthus* species showed no reduction in K^+ content in the presence of NaCl treatment *(Makus, 2003)*.

A. retroflexus was also the only species that consistently showed an increase in CP leaf content in salt-treated plants, except for the second trial. This is consistent with *Vecchio and Casini (1983)*, who observed an increase in N content (and therefore CP) in *A. retroflexus* exposed to increased salinity levels and grown in competition with chickpea (*Cicer arietinum* L.). The fact that the second trial showed no difference in terms of CP content might be linked to the fact that the trial was carried out in autumn, and the lower temperatures might have affected the plant metabolism. On the other hand, soybean was the only species to show a significant increase in MDA content in response to salt stress, corresponding to an increase in lipid peroxidation processes. This trend has been frequently observed in literature *(Liu et al., 2017; Phang et al., 2008; Weisany et al., 2012)*, and is consistent with our previous experiment, where salt stress increased lipid peroxidation in soybean *(Ghirardelli et al., 2021)*. The fact that neither weed species showed a similar increase in MDA content confirmed that soybean was more affected than *C. album* and *A. retroflexus* also at the metabolic level.

Only *A. retroflexus* and soybean grown in mixed-species tanks showed some differences in proline content. This compound, known as an osmoregulator or osmoprotectant *(Aghaleh and Niknam, 2009),* is often increased in plants exposed to salt stress, including soybean

(Phang et al., 2008). The fact that the increase was significant only in the mixed-species tanks might be linked to the increased salt stress caused by the combination with inter-specific competition, but it also shows that the plants were reacting to the increased stress, which would not occur in the case of highly salt-sensitive species or cultivars *(Heuer, 2010)*.

5. Conclusions

This study had the objective to evaluate the impact of salinity on crop and weed competition on a soil medium. The experiment was designed as a follow-up of the hydroponic trials we conducted on soybean and *C. album*, to represent more accurately the plant-soil interactions that might occur in field conditions. The experiment confirmed that salinity affected soybean more than the studied weed species. This resulted in significant differences in plant height, SPAD values, biomass and lipid peroxidation between treated and untreated plants, although this cultivar (PD1T45) showed some abilities to cope with salt stress, as demonstrated by the increase in K^+ and proline content. *A. retroflexus* was moderately sensitive to salinity, as demonstrated by the reduction in plant height and biomass, while *C. album* confirmed to be highly tolerant to salt stress, with no consistent changes in plant height, biomass, CP, proline and lipid peroxidation. However, in both species, no consistent effect of competition was observed, both in the case of salt-treated and control plants. On the other hand, the presence of *A. retroflexus*, and even more of *C. album* combined with salt stress, consistently affected SPAD values, biomass, root nodulation and proline content throughout the different trials. The significant effects of competition in salt-treated pots corroborate the hypothesis of increased weed competitiveness in salty soils. In this context, monitoring weed species, especially those that are salt-tolerant like *C. album*, is of crucial importance.

Given the lack of information on weed physiological responses to salt stress, future experiments should focus more on metabolic pathways and plant osmoregulation. Also the role of allelochemicals, commonly produced by some of the weed species infesting major crops, including *A. retroflexus* and *C. album*, should be further investigated in the presence of salt stress.

Our findings lay the foundation to develop better weed management strategies required in the context of expanding soil salinization and climate change. The results suggest the need for crop amelioration towards a higher tolerance to salinity, combined with careful weed control.

Appendix

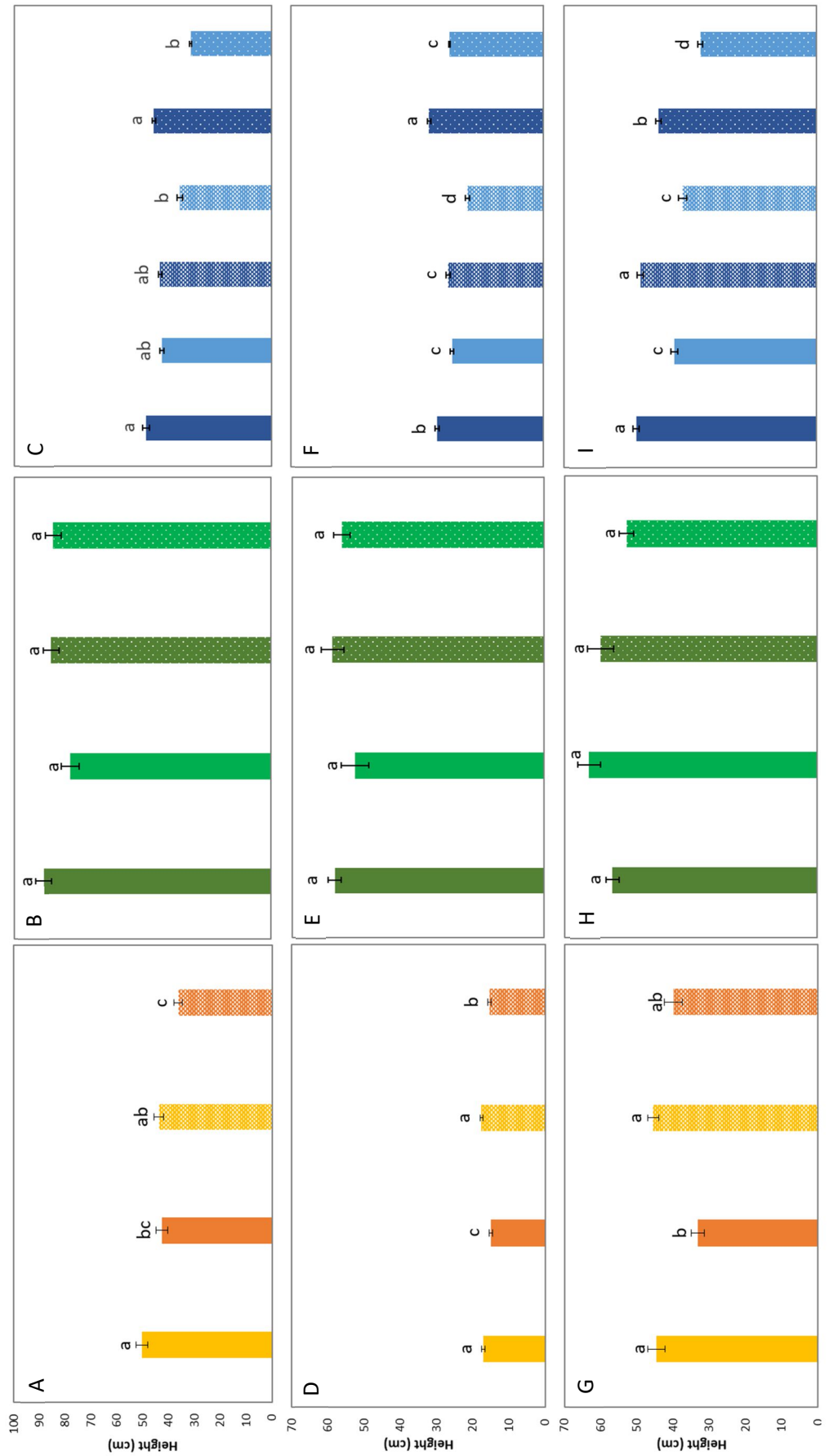

Figure A 1. *Final plant height of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, $n=18$. Tukey test on C. album and A. retroflexus (3^{rd} trial) was performed on transformed data (squared root transformation). A=A. retroflexus; C= C. album; S=soybean.*

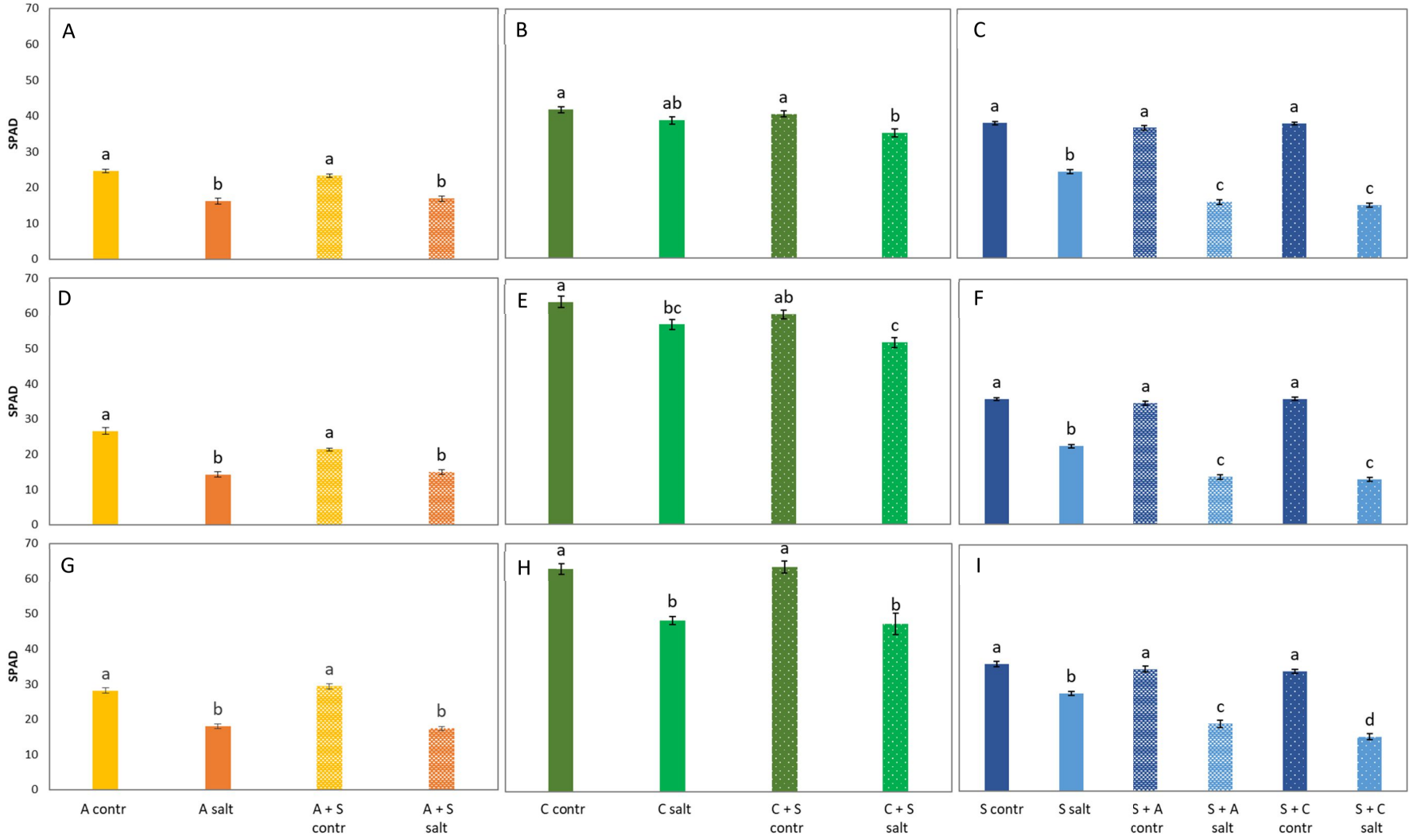

Figure A 2. *Final SPAD values of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). A=A. retroflexus; C= C. album; S=soybean. Different letters within each group of bars indicate significant differences at p<0.05, n=18. Tukey test on A. retroflexus (1st and 2nd trial) was performed on transformed data (squared root transformation). A=A. retroflexus; C= C. album; S=soybean*

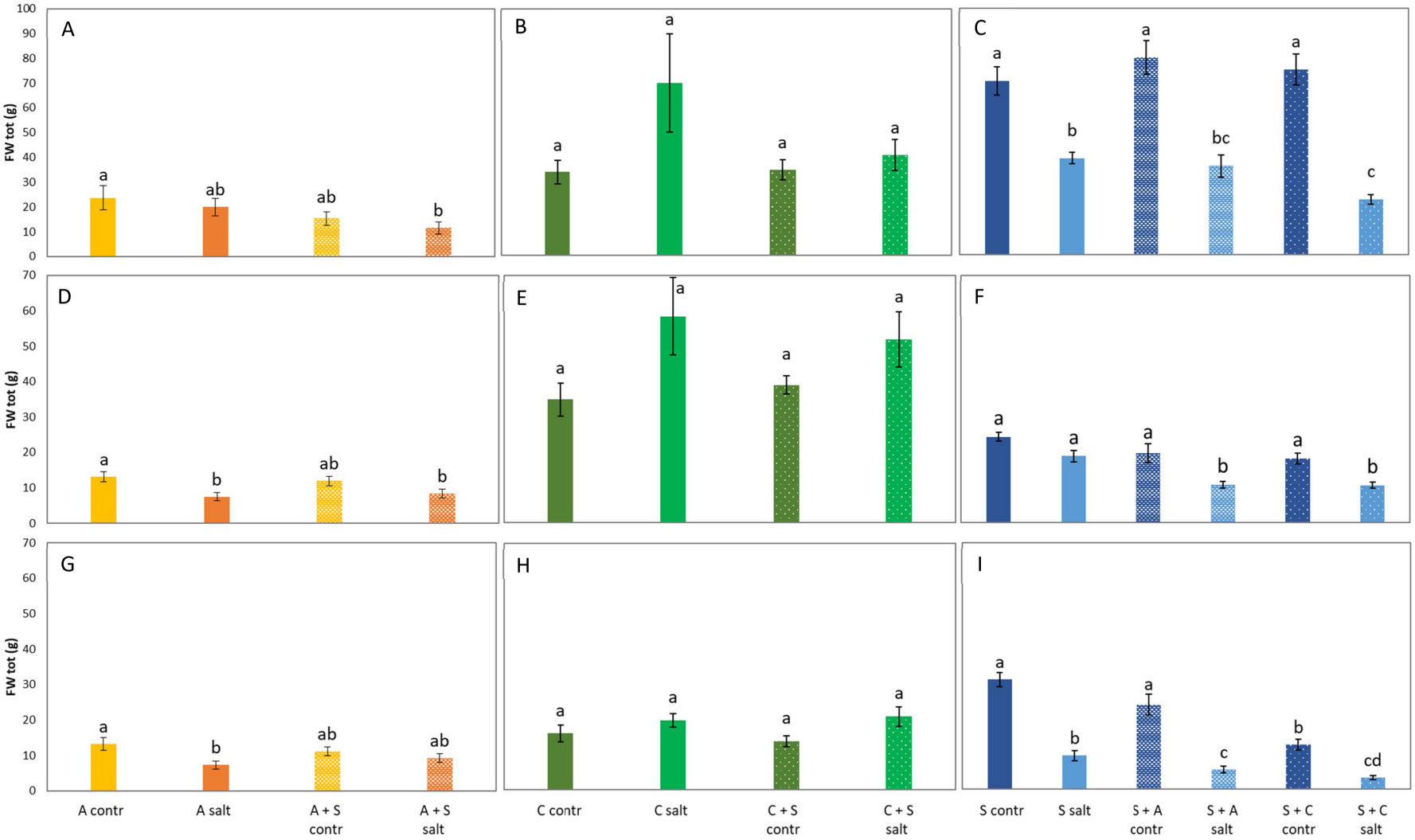

Figure A 3. *Total fresh weight (FW) of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, n=12. Tukey test on A. retroflexus (1st trial), C. album (1st and 2nd trial) and soybean (3rd trial) was performed on transformed data (logarithmic transformation in the weeds and squared root transformation in soybean). A=A. retroflexus; C= C. album; S=soybean.*

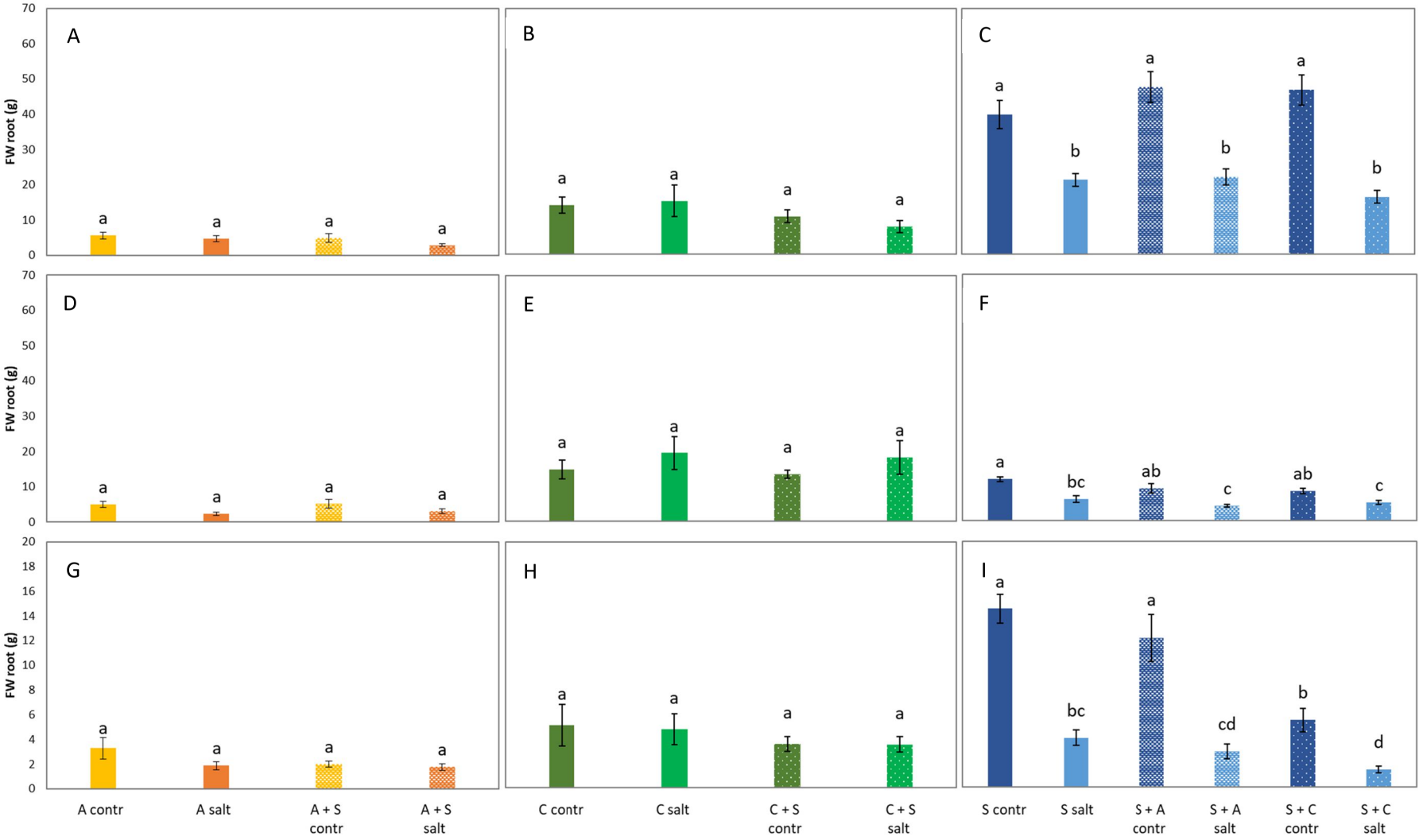

Figure A 4. *Root fresh weight (FW) of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at p<0.05, n=12. Tukey test on A. retroflexus (2nd trial), C. album 1st and 2nd trial) and soybean (1st, 2nd and 3rd trials) was performed on transformed data (logarithmic transformation in the weeds and squared root transformation in soybean). A=A. retroflexus; C= C. album; S=soybean.*

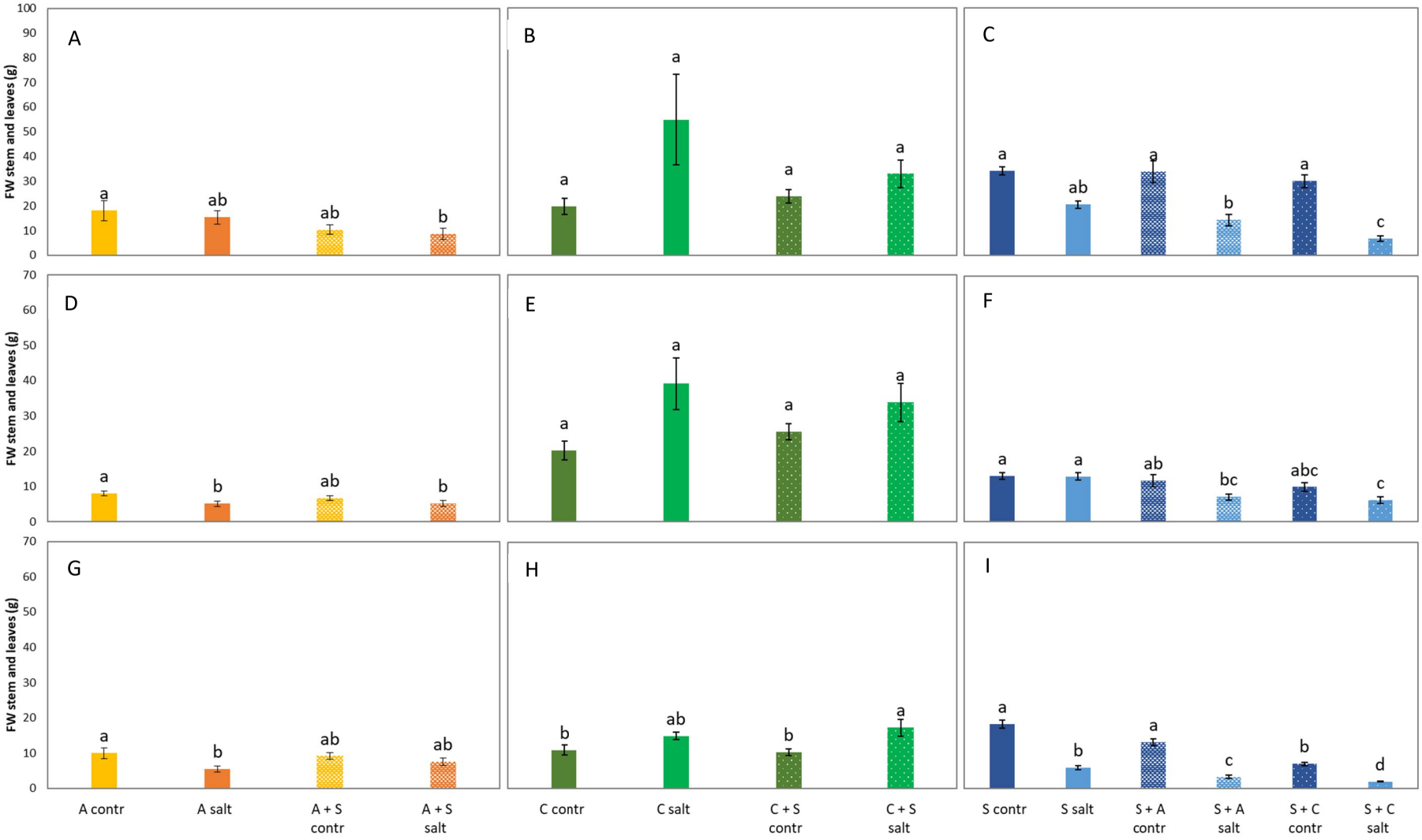

Figure A 5. *Stem and leaves fresh weight (FW) of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, $n=12$. Tukey test on soybean (1^{st} and 3^{rd} trial) was performed on transformed data (logarithmic transformation). A=A. retroflexus; C= C. album; S=soybean.*

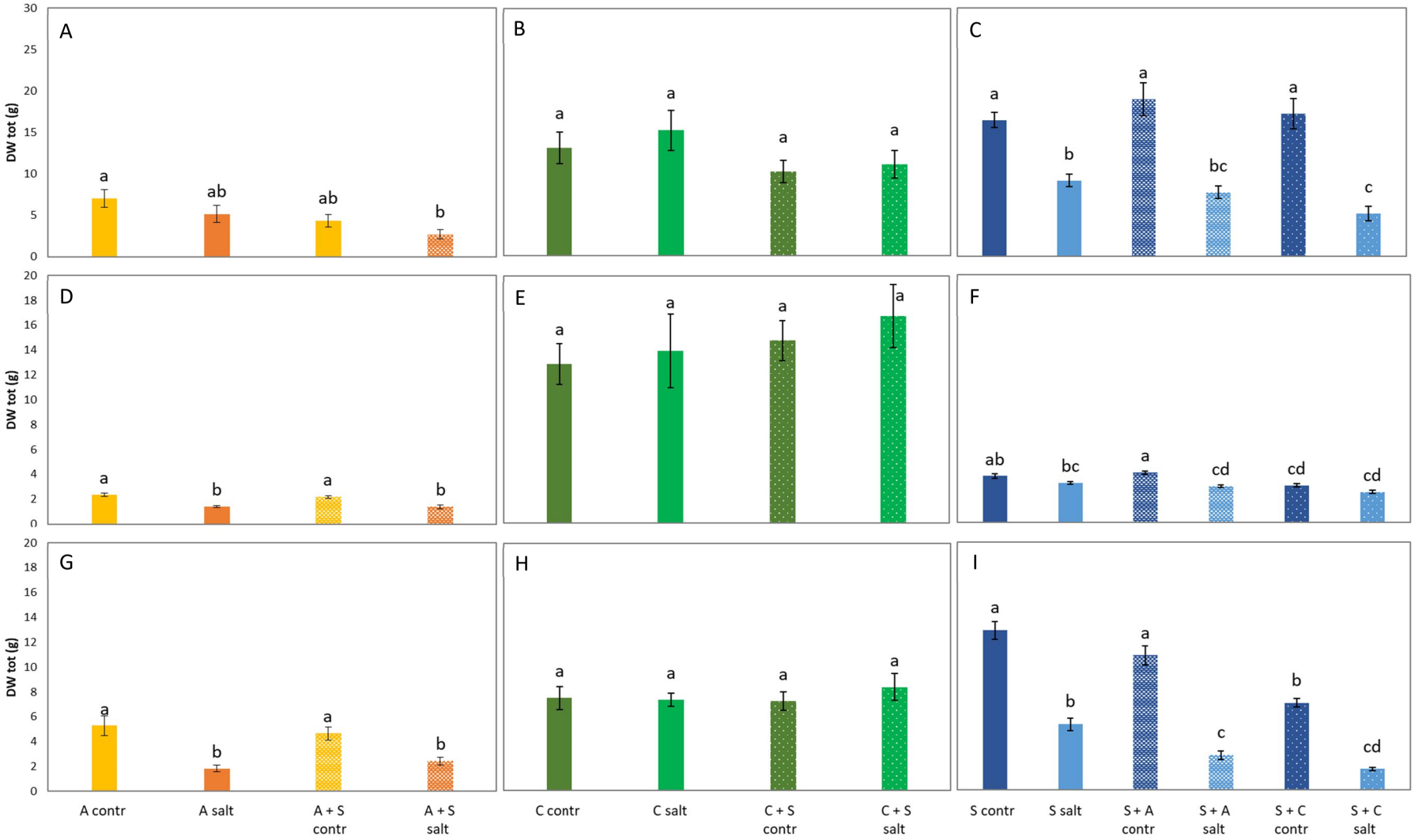

Figure A 6. *Total dry weight (DW) of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, n=12. Tukey test on soybean (1st, 2nd and 3rd trial) was performed on transformed data (squared root transformation). A=A. retroflexus; C= C. album; S=soybean.*

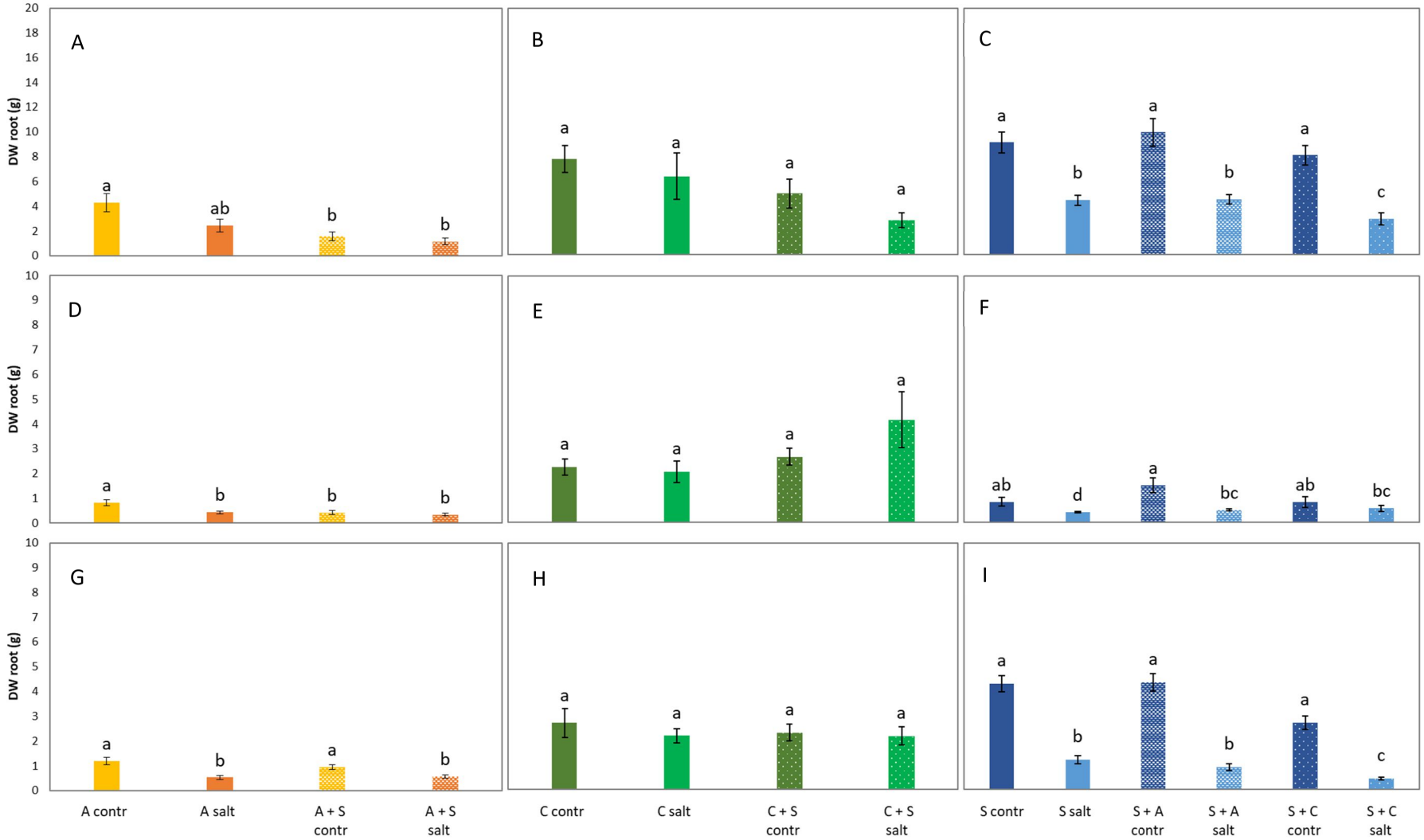

Figure A 7. *Root dry weight (DW) of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, n=12. Tukey test on A. retroflexus (1st and 3rd trial), and soybean (1st, 2nd and 3rd trial) was performed on transformed data (logarithmic transformation). A=A. retroflexus; C= C. album; S=soybean.*

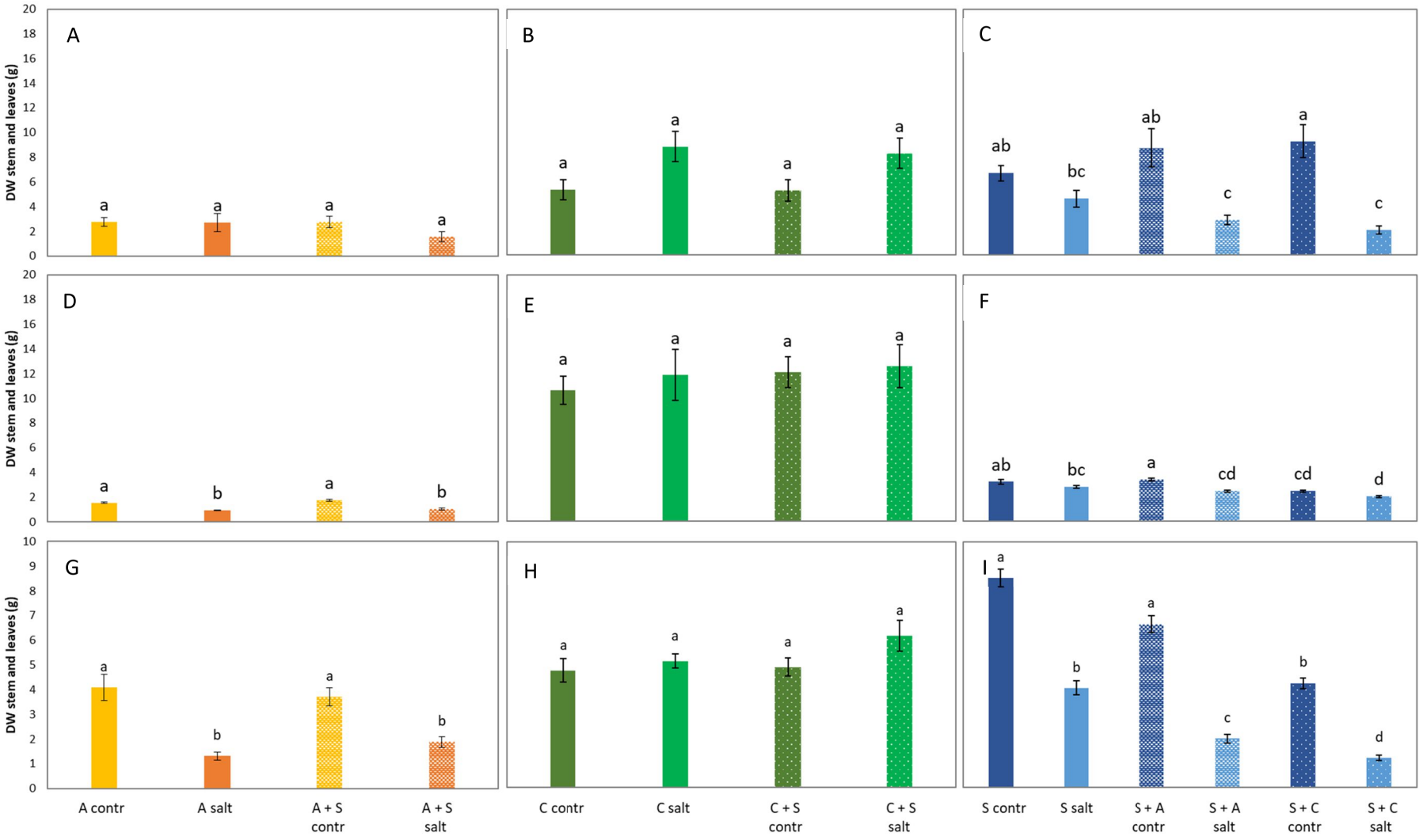

Figure A 8. *Stem and leaves dry weight (DW) of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, n=12. Tukey test on A. retroflexus (2nd and 3rd trial) and soybean (1st, 2nd and 3rd trial) were performed on transformed data (logarithmic transformation in A. retroflexus and squared root transformation in soybean). A=A. retroflexus; C= C. album; S=soybean.*

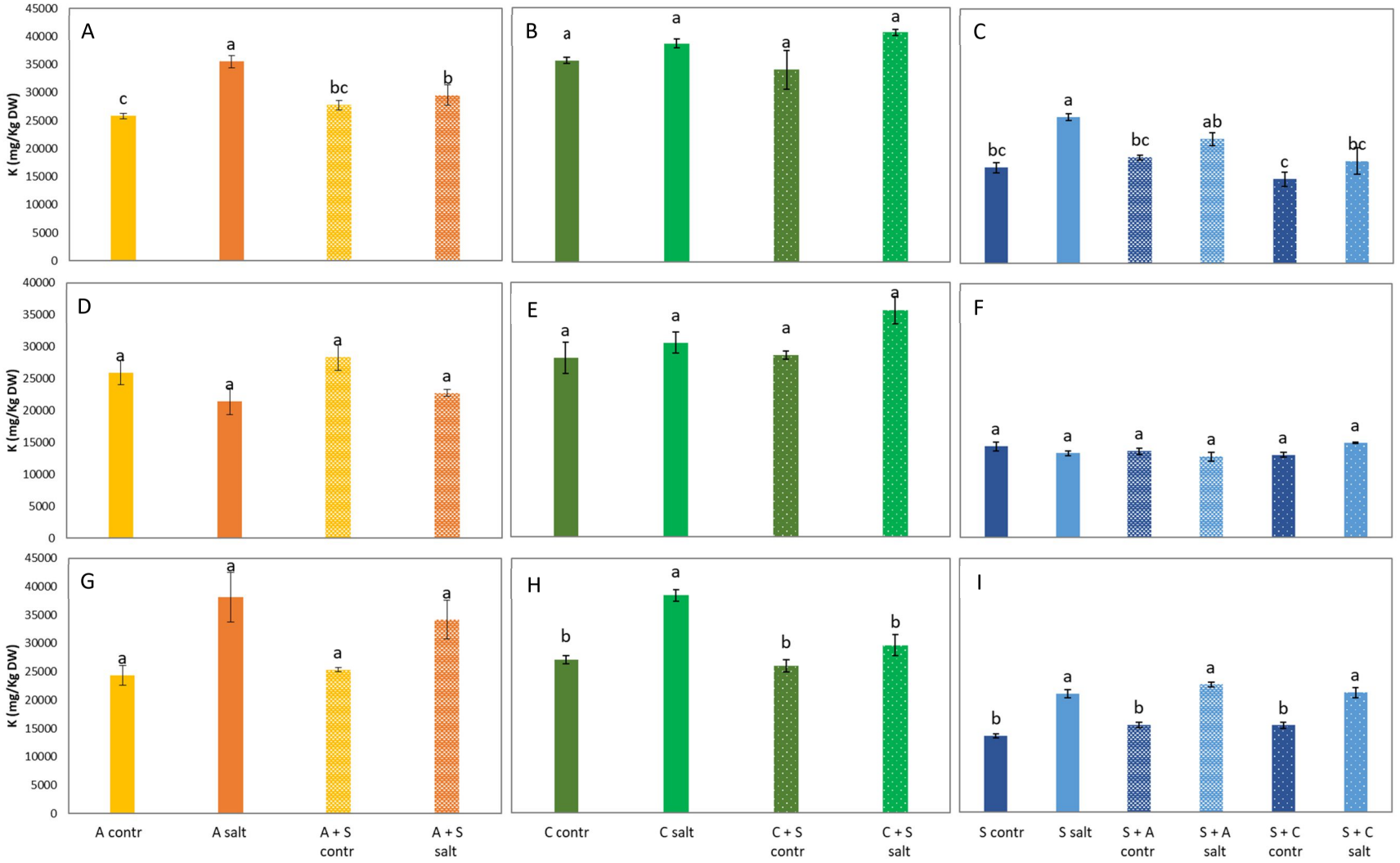

Figure A 9. *Potassium leaf content of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, n=12. A=A. retroflexus; C= C. album; S=soybean; DW=dry weight.*

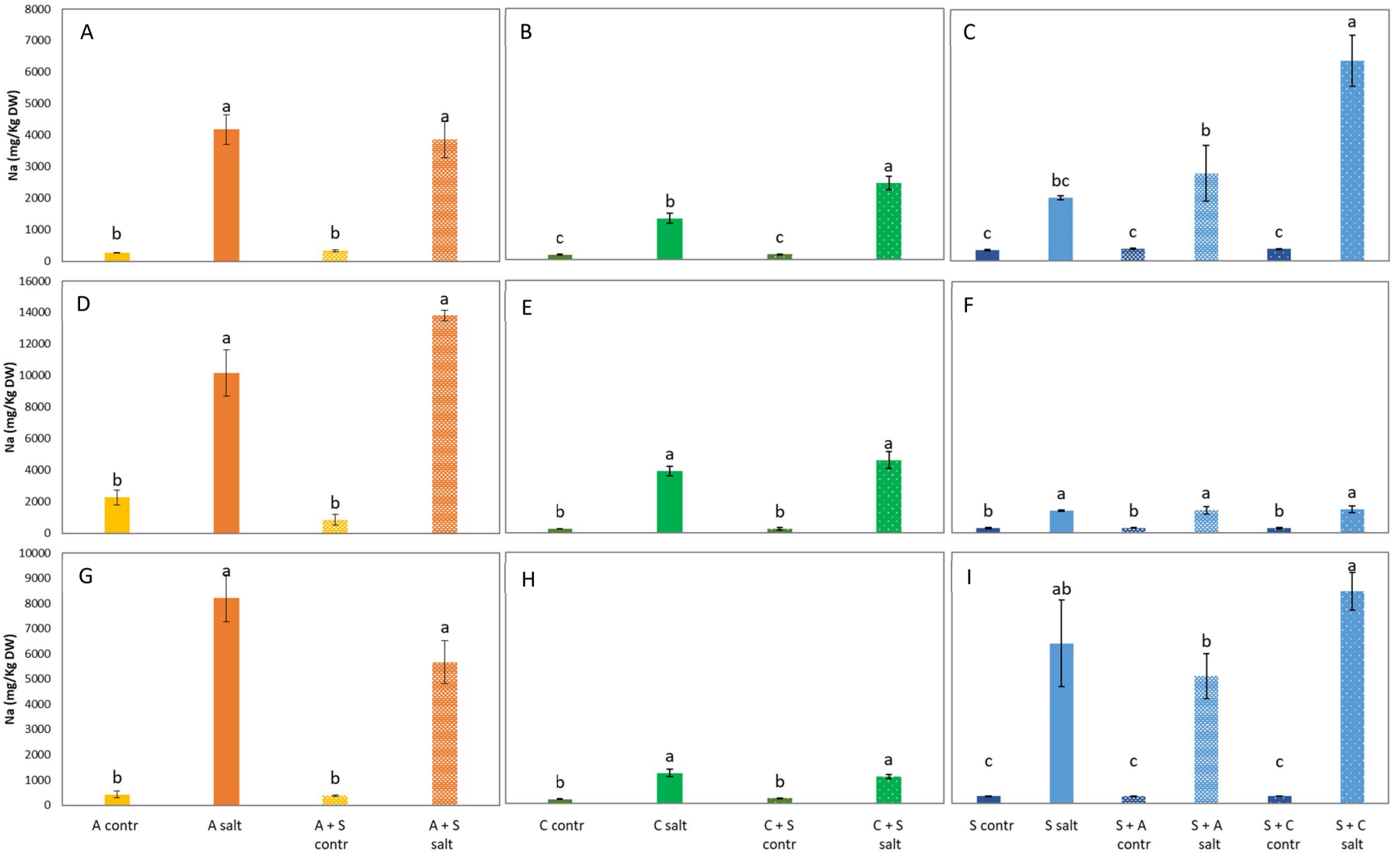

Figure A 10*. Sodium leaf content of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at p<0.05, n=12. A=A. retroflexus; C= C. album; S=soybean; DW=dry weight.*

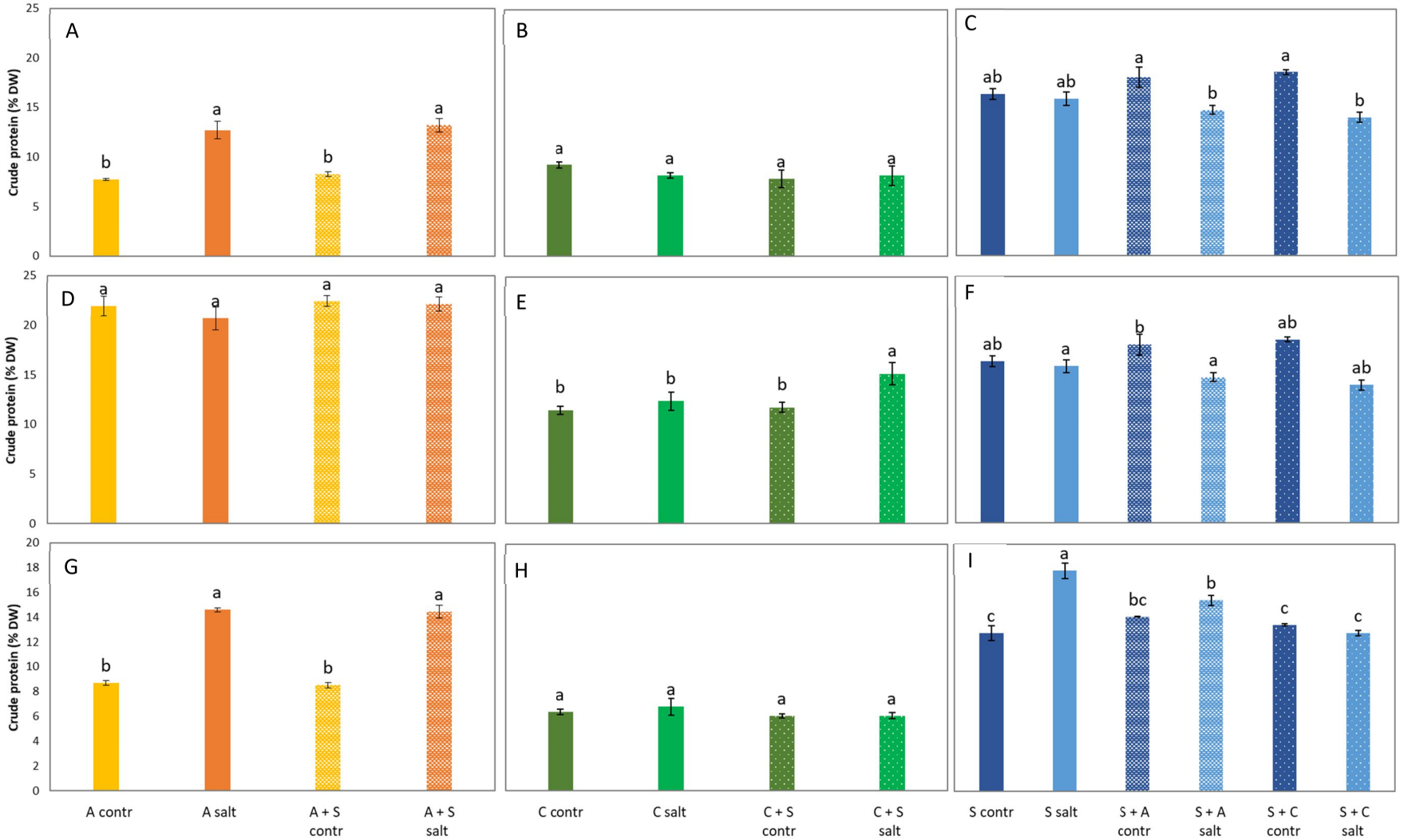

Figure A 11. *Crude protein leaf content of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at p<0.05, n=12. A=A. retroflexus; C= C. album; S=soybean; DW=dry weight.*

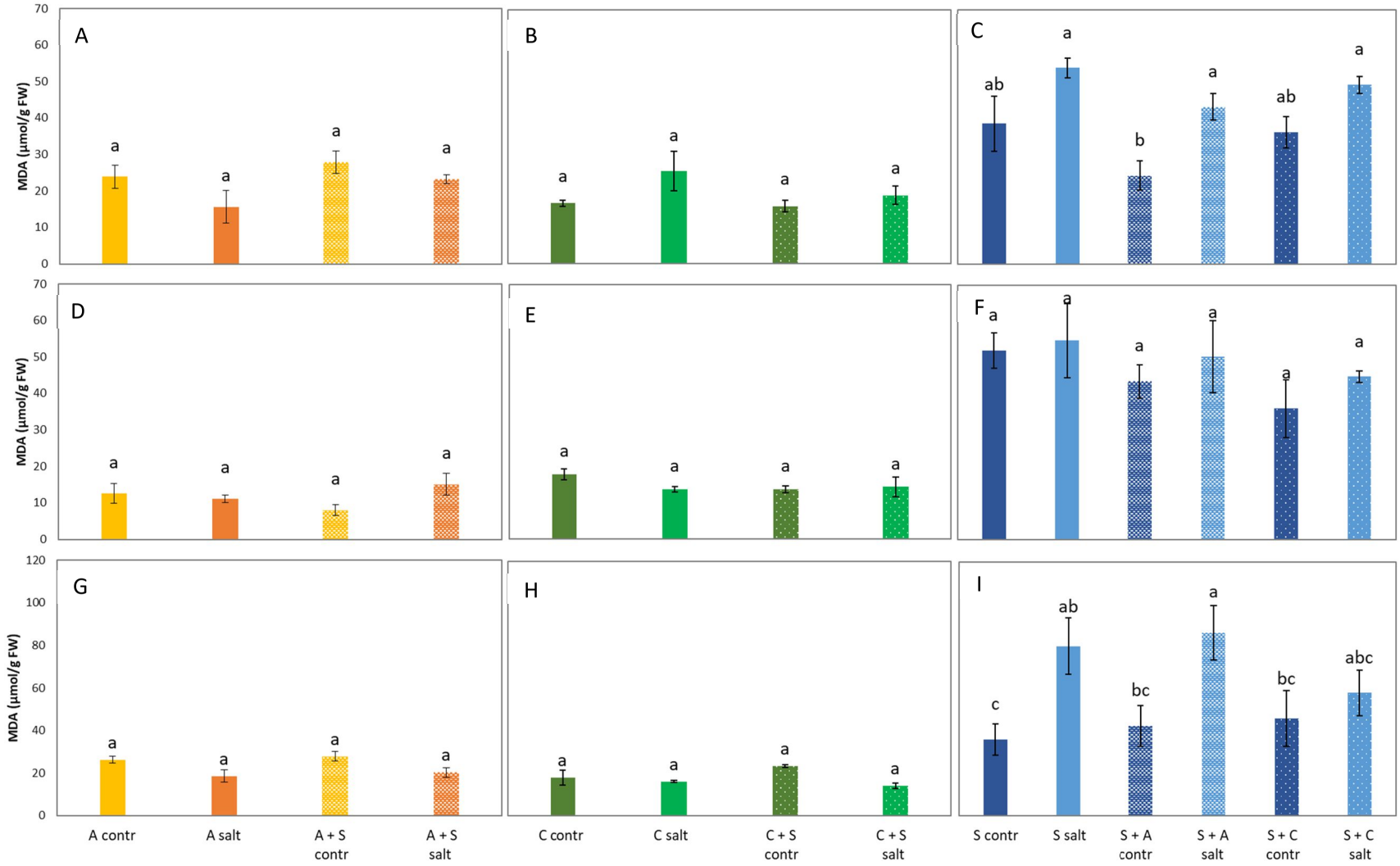

Figure A 12. *Malondialdehyde (MDA) leaf content of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at p<0.05, n=6. A=A. retroflexus; C= C. album; S=soybean; FW=fresh weight.*

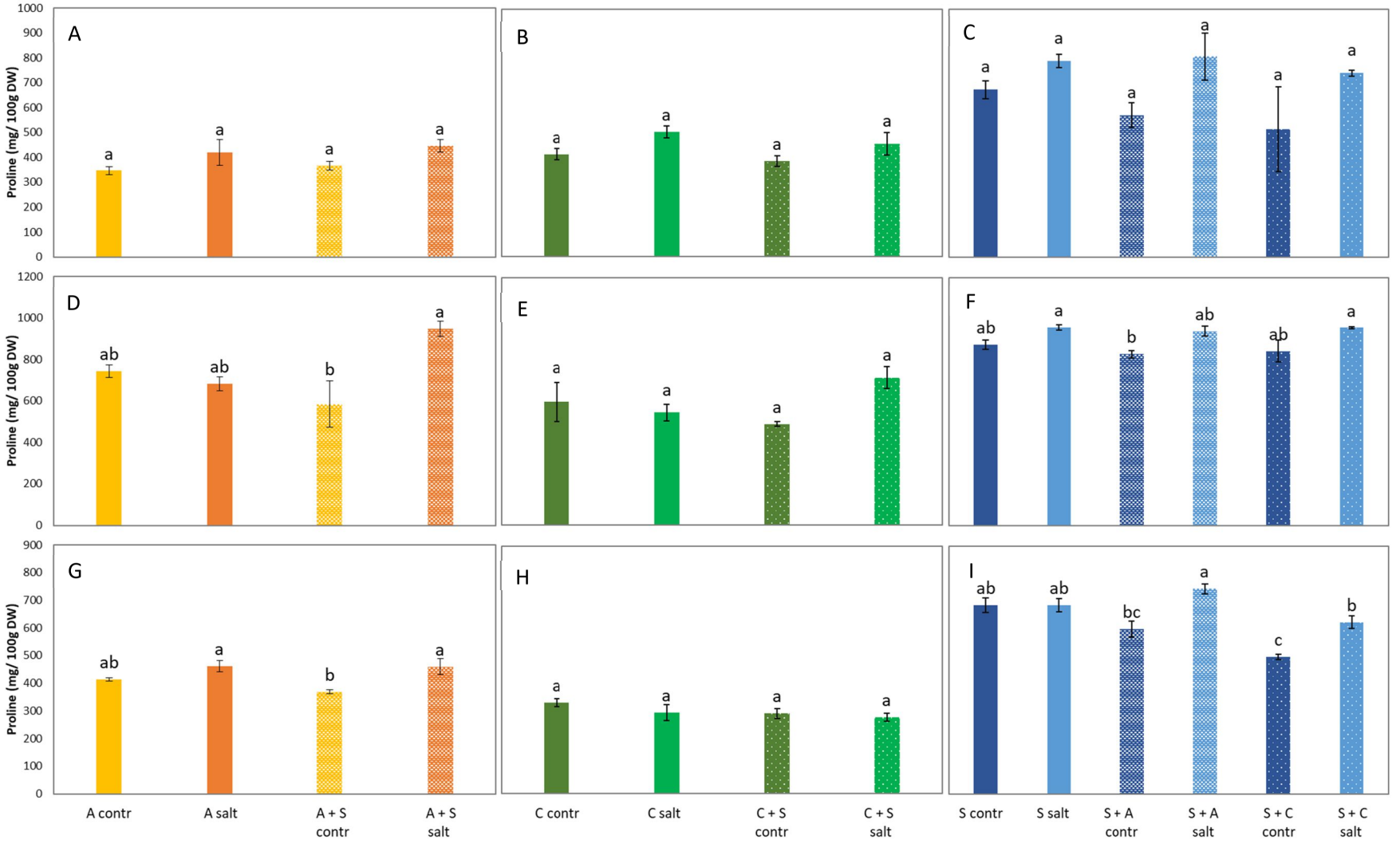

Figure A 13. *Proline leaf content of Amaranthus retroflexus, Chenopodium album and soybean (Glycine max), at the end of the first (A-C), second (D-F) and third experiment (G-I). Different letters within each group of bars indicate significant differences at $p<0.05$, n=12. A=A. retroflexus; C= C. album; S=soybean; DW=dry weight.*

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VII General conclusions and future developments

Results indicate that many weed species associated with spring-summer crops in the Mediterranean basin and other temperate and semi-arid regions show high adaptability and resilience to salt stress. Although germination and early growth stages are considered to be the most sensitive to salt for many plant species, we demonstrated that even weed ecotypes never exposed to salinity are capable of germinating and developing roots and shoots when exposed to moderate to strong salt stress. However, it was clear that a high tolerance at the germination stage does not always correspond to the same tolerance in early-stage development. *Abutilon theophrasti*, *Chenopodium album* and *Setaria pumila* appeared to be the most tolerant weed species at these stages, potentially representing an increased threat in semi-arid and temperate regions affected by salinization. During germination and seedling development, the temperature played a key role: when far from the optimum, the effects of salt stress were emphasized in both winter and summer species. This was particularly evident in most of the weeds incubated at lower temperatures, suggesting that the increase in temperature due to global warming might enhance their salt-tolerance at the germination and early growth stages. Considering also the weed-crop competition factor, *C. album* was confirmed to be the most tolerant among the analyzed species, causing a decrease in height, SPAD values and biomass production and increased lipid peroxidation in soybean grown with the weed. However, even weed species that appeared to be more sensitive, such as *Amaranthus retroflexus*, maintained their competition potential when grown with a crop, meaning that the presence of the crop did not exacerbate the effects of salinity in weed species. These results corroborate the hypothesis of increased weed competitiveness in salty environments. In this context, proper monitoring and management of weeds, especially the salt-tolerant ones, is crucial. The comparison between winter and summer species (barley, *Avena sterilis* and *Lolium rigidum* VS rice, *Echinochloa crus-galli* and weedy rice) confirmed that barley is a salt-tolerant crop and showed that the competition with *L. rigidum* did not affect its height, biomass, hydrogen peroxide (H_2O_2) content and lipid peroxidation.

These results are particularly relevant to the Mediterranean basin and other regions characterized by a semi-arid climate, that are increasingly exposed to the threat of salinization, especially near the coastline and river estuaries. Our findings also lay the foundation to develop better weed management strategies in the context of soil salinization. Possible sustainable strategies to mitigate crop losses due to weed competition should follow multiple directions:

- At the genetic level, the breeding of crop cultivars more tolerant to salt stress and/or allelochemicals released by weeds should be prioritized. This is particularly important for staple crops that are now highly susceptible to salt stress, such as soybean.
- At the agronomic level, summer crops should be sown in correspondence with the highest probability of precipitation so that salt can be partly leached from the soil by rainfall.
- In terms of weed management strategies, the false seedbed technique might help remove weed seedlings from the topsoil before sowing the crop.
- Whenever yield loss on summer crops becomes too extreme due to secondary salinization, winter crops, especially salt-tolerant ones such as barley, might be a cost-effective option.

In a climate-change scenario characterized by increasing salinization and high salinity levels in irrigation water, future studies should assess the response to salt stress of more weed species at different growth stages. Given the high intra-specific variability of weed species, also comparisons between weed ecotypes grown in saline and non-saline environments should be made. The competition between crops and weeds and the interactions between different weed species should also be studied in more depth, both in controlled conditions and in the field, for instance by monitoring the distribution and abundance of selected weed species in salt-affected cropland. At the molecular and plant level, given the lack of information on weed physiology in response to salt stress, future experiments should focus more on metabolic pathways and plant osmoregulation. Also the role of allelopathic compounds, commonly produced by some of the major weed species, including *A. retroflexus* and *C. album*, should be further investigated.

Salinity is only one of the threats to crop production, as many intertwined problems are challenging modern agriculture, from population growth to climate change and land degradation. To face the issue, it is extremely important to consider all the elements constituting the agro-ecosystem from an all-round perspective, from the macroscopic to the microscopic level. Overall, this PhD project proved the importance of including weed species in the picture, as they are capable of altering the crop response to salt stress.

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