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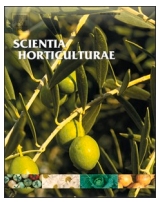

Interactive effects between salinity and nutrient deficiency on biomass production and bio-active compounds accumulation in the halophyte *Crithmum maritimum*

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ABSTRACT

Plant bioactive metabolites play a pivotal role in protection against environmental stresses and offer great potential for nutraceutical exploitation. In this sense, halophytes present an exceptional opportunity for sustainable saline agriculture since they can massively accumulate bioactive metabolites under stressful conditions. We investigated the responses of the halophyte *Crithmum maritimum* to increasing salinity, nutrient deficient and their interaction. We hypothesized that the concentration of bioactive compounds would increase together with salinity and nutrient deficient, and that the interaction between these two stresses would produce synergic effects on bioactive compounds accumulation. We studied the tolerance and foliar accumulation of nutrients and bioactive compounds (amino acids, phenols, terpenes and fatty acids) for *C. maritimum* in response to salinity and nutrient deprivation in greenhouse controlled conditions. The high salinity tolerance of *C. maritimum* was not diminished by the interaction of salinity with nutrient limitation. Mineral nutrition deficit and saturated fatty acid accumulation were induced at 200–500 mM NaCl, and accumulation of polyphenol 5-caffeoylquinic acid and some amino acids were identified as nutrient deficiency markers. *C. maritimum* responded mainly to increasing salinity by reducing foliar accumulation of five terpenes and total lipids, and to nutrient limitation by increasing the concentration of vaccenic acid and 5-caffeoylquinic acid. The interaction between salinity and nutrient availability provoked antagonistic and synergistic effects on the accumulation of terpenes and fatty acids. *C. maritimum* showed high physiological phenotypic plasticity in the response to salt stress, nutrient deficiency and their combination.

1. Introduction

Plant secondary metabolites (PSMs) play a pivotal role in protection against multiple biotic (e.g., herbivory) and abiotic (e.g., salinity) stresses (Anu et al., 2018; Yang et al., 2018). Moreover, PSMs and bioactive primary metabolites, such as polyunsaturated fatty acids, can display potent organoleptic, antioxidant, antimicrobial, anti-inflammatory and anti-tumoral activities, offering great potential for their nutraceutical exploitation (Ksouri et al., 2012; Christensen, 2020).

Plants under stress conditions accumulate more reduced PSMs in order to overcome oxidative stress through non-enzymatic defense mechanisms (Jaleel et al., 2009). Thus, suboptimal environmental conditions can result in reduced plant growth and yield, but such stressful conditions can also act as an elicitor of the biosynthesis of many bioactive compounds. In this regard, eustressors (positive stress) are stressful factors that trigger the signaling pathways leading to a higher production of bioactive compounds and an increased quality of plant products (Rouphael et al., 2018). More stressful environments are commonly associated with slow-growing plant species with high tissue

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value (Endara and Coley, 2011). Overall, tolerance to stressful conditions tends to result in increased production of PSMs with high nutraceutical and pharmaceutical value, rendering an enormous potential for industrial applications (Stevanovic et al., 2019).

Salinity and nutritional stresses are considered chemical eustressors which have been proved to affect plant chemical composition (Sun et al., 2015; Rouphael and Kyriacou, 2018; Buturi et al., 2021). Soil salinity may be applied to function as an eustressor, but salinization of soils and water bodies is increasing unintentionally under the present scenario of land management and climate change (Tan et al., 2005; Meena and Verma, 2018; Soares et al., 2019). Current attempts to adapt to these conditions through sustainable agriculture involve searching for and characterizing new wild plant species that have potential as saline crops (Debez et al., 2004; Zhao et al., 2011; Cheeseman, 2015; Fita et al., 2015).

Halophytes are plants that grow in highly saline environments such as coastal sand dunes, salt marshes, mangroves and sea cliffs. Halophytes offer an exceptional opportunity for the development of a sustainable saline agriculture (Ladeiro, 2012; Nikalje et al., 2018). Halophytes can massively accumulate bioactive metabolites under stressful conditions (Stevanovic et al., 2019). In fact, several of these bioactive metabolites, such as certain polyphenols and terpenes, are restricted to halophytic species, especially to aromatic halophytes (Buhmann and Papenbrock, 2013). Halophytes are usually exposed to nutrient limitations in addition to high salinity conditions. For example, halophytes colonizing coastal sand dunes are subjected to severe environmental stresses, including osmotic and nutritional stresses, leading to the production of PSMs with a wide range of bioactivities (Beeby et al., 2020). In fact, under saline conditions, nutritional disorders can develop and plant growth may be reduced (Grattan and Grieve, 1992). Nevertheless, halophytes have not received the attention glycophytes have in the area of salinity-mineral nutrient relations. To our knowledge, despite the importance of halophytes as model crops for sustainable agriculture, no previous study has analyzed the interactive effects of salinity and nutrient availability on their accumulation of bioactive compounds.

Our focal species to study the effects of salinity and nutritional stresses and their combination on growth and accumulation of bioactive compounds was the halophyte *Crithmum maritimum* L. (common name 'sea fennel', Family *Apiaceae*). *Crithmum maritimum* is an aromatic oilseed perennial species naturally found on maritime rocks, piers, breakwaters and sandy beaches along the Mediterranean and Atlantic coasts (Atia et al., 2011). These habitats are associated with limited nutrient availability and high salinity (Labidi et al., 2011). *C. maritimum* shows roots capable of ensuring a strong adhesion to rock from the beginning of their development (Strumia et al., 2020). *C. maritimum* is highly tolerant to environmental stresses such as salinity and drought (Hamed et al., 2007; Ventura et al., 2014; Ciccarelli et al., 2016; Fattoum et al., 2016), and a potent source of minerals, vitamin C, essential oils and other bioactive compounds (Cunsolo et al., 1993; Ruberto et al., 2000; Ben Hamed et al., 2005; Fenollosa et al., 2017; Nabet et al., 2017; Pereita et al., 2017; Sánchez-Faure et al., 2020; Souid et al., 2020). *C. maritimum* has a long history of human consumption for nutritional and medicinal purposes, and was recently suggested as a cash crop for saline agriculture (Atia et al., 2011). In fact, due to their sensorial properties (acidic and salty taste), *C. maritimum* could be used as new ingredient with health benefits to improve the diversity in modern diet and creative cuisine (Romojaro et al., 2013). *C. maritimum* has been also identified as source of botanical insecticides (Pavela et al., 2017). However, *C. maritimum* presents a great potential in different industries, it is currently underutilized (Renna et al., 2017).

The present work was undertaken in greenhouse controlled conditions to investigate the tolerance and foliar accumulation of nutrients and bioactive compounds (amino acids, phenols, terpenes and fatty acids) of *C. maritimum* to nutrient deprivation combined with different saline levels (from 0.5 to 500 mM NaCl). We hypothesized that the concentration of bioactive compounds would increase together with salinity and nutrient deficient, and that the interaction between these two stresses would produce synergic effects on bioactive compounds accumulation. Our bioprospecting results are useful to show how halophytes response to salinity and nutrient availability, and to identify the major bioactive components in the foliar profile of *C. maritimum* and how they change depending on environmental conditions.

2. Materials and methods

2.1. Plant material and experimental design

Rhizomes (c. 2 cm long) from 20 adult plants of *C. maritimum* were collected in a wild population located on sand dunes at Roche Beach 36.314138, -6.153952; Southwest Iberian Peninsula) in January 2019. Collected rhizomes were planted in wet perlite for one month until they developed roots and sprouts in the greenhouse facilities of the University of Seville. Experimental plants were selected and potted in plastic pots (14 cm diameter x 14 cm height) using sand as substrate. Plants were watered using 20% Hoagland's nutrient solution during two months until the beginning of the experiment, when they reached a canopy diameter between 15 and 20 cm (c. 0.15 g dry weight (DW) per plant).

Two nutrient treatments were established using 5% and 20% Hoagland's solution to create salinity treatments. Treatments with 20% Hoagland's solution corresponded to control conditions with non-limited nutrient concentration, whereas the treatment with 5% Hoagland's solution corresponded to a nutrient concentration lower than those recorded for *C. maritimum* populations in the field (van der Maarel, 1993). The salinity treatments were prepared using NaCl added to Hoagland's nutrient solution: 0.5 mM NaCl (control), 50 mM NaCl (3 g l^{-1}), 200 mM NaCl (12 g L^{-1}) and 500 mM NaCl (30 g L^{-1}). These salt concentrations were chosen to cover the entire salinity range to which wild plants of *C. maritimum* are exposed in the field. Plants were randomly assigned to experimental treatments (8 plants per treatment combination) and sub-irrigated (2 cm deep) with treatment solutions in randomized plastic trays. Treatment solutions were renewed once a week. This resulted in a random factorial design with 4 salt treatments, 2 nutritional treatments, 8 replicates per treatments combination, and a total of 64 experimental plants. Glasshouse conditions were maintained under natural sun radiation, controlled air temperature (+23–25 °C) and air relative humidity (40–60%). Maximum photon flux density at the canopy level measured by a LI-COR LI-250A light meter (LI-COR Inc., Lincoln, NE) was 1000 μmol m^{-2} s^{-1}. The experiment was carried out for 60 days prior to biomass harvest.

2.2. Leaf gas exchange

Measurements of net photosynthesis rate (A) and stomatal conductance (g_s) were carried out on fully expanded leaves using a LI-COR 6400-XT portable infrared CO_2 analyzer (LI-COR Biosciences, Lincoln, NE, USA) in differential mode and in an open circuit. All measurements were conducted 60 days after the start of the experiment during midday and under sunny conditions. The CO_2 concentration inside the chamber was fixed at 400 μmol mol^{-1}, flow rate was 350 μmol s^{-1}, photon flux density was 1000 μmol m^{-2} s^{-1} (LED light source 90% red and 10% blue, generating actinic light), air temperature was between 20 and 25 °C and air relative humidity between 45% and 55%. A and g_s were calculated following Von Caemmerer and Farquhar (Von Caemmerer and Farquhar, 1981).

2.3. Biomass and growth traits

At the beginning of the experiment, initial total biomass was recorded as DW (g) after oven-drying it at +80 °C for 48 h until constant weight was reached, and total biomass was calculated ($n = 8$ plants). At the end of the experiment, above-ground biomass (AGB) and below-ground biomass (BGB; roots) for live plants were recorded as reported

above. Relative growth rate (RGR, $g^{-1}day^{-1}$) was calculated as follow: RGR = (ln Bf – ln Bi) • D^{-1}, where ln Bf was the natural logarithm of the final dry total biomass, ln Bi was the natural logarithm of the initial dry total biomass and D was the duration of the experiment in days. AGB: BGB ratio was also calculated. Additionally, final plant height (cm) was recorded from the base of the plant to the apex of the youngest leaf. All biochemical foliar measurements were carried out for pools of randomly selected fully developed leaves of 2–3 plants ($n = 3$ pools per treatment combination).

2.4. Foliar nutrient concentrations

Total foliar concentrations (mg kg^{-1}) of sodium (Na), macronutrients [calcium (Ca), potassium (K), magnesium (Mg), phosphorous (P) and sulfur (S)] and micronutrients [boron (B), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo) and Zinc (Zn)] were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES Varian ICP 720-ES). Organic foliar nitrogen (N; macronutrient) concentrations (%) was estimated by the Kjeldahl method (Kjeldahl, 1883) ($n = 3$ plants per treatment combination).

2.5. Determination of amino acid composition

We recorded foliar total amino acids as markers for salinity and nutritional stresses. Amino acid composition (%) in foliar *C. maritimum* extracts was carried out according to Alaiz et al. (1992) with slight modifications. Samples (4–6 mg protein) were hydrolyzed for 24 h with 6 N HCl at +110 °C under an inert N atmosphere. After hydrolysis, samples were dried by rotary evaporator and redisolved in 1 M sodium borate buffer pH 9.0 (10 mL). The amino acid profiles were determined in the hydrolysate using ultra performance liquid chromatography (UPLC) using a reverse phase column (XSelect HSS T3 2.5 μm de 3.0 × 150 mm, Waters) in a system of binary gradient with 25 mM sodium acetate 0.02% (w/v) sodium azide pH 6.0 (Buffer A) and acetonitrile (Buffer B) as solvents and with D,L-α-aminobutyric acid (Sigma Chemical Co., St Louis, MO, USA.) as an internal standard (Martins-Noguerol et al., 2021). The amino acids used for obtaining the calibration lines were submitted to the same analytical conditions of the samples to avoid the mistakes made for the modification or loss of amino acids during acid hydrolysis. The determination of tryptophan was developed according to the method described by Yust et al. (2004).

2.6. Identification and quantification of phenolic compounds

Phenolic compounds were extracted from 20 mg of dry leaf tissue with 1 mL of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation. To perform the chromatographic analyses we used Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu Corporation, Kyoto, Japan) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. Flavonoids were quantified as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents (Moreira et al., 2021). The quantification of these phenolic compounds was achieved by external calibration using calibration curves at 0.25, 0.5, 1, 2 and 5 μg ml^{-1}. Phenolic compound concentrations were expressed in mg g^{-1} tissue on a dry weight basis.

2.7. Identification and quantification of terpenes

We extracted terpenes using 300 mg of ground fresh leaf material with 1 mL of 70% methanol in an ultrasonic bath for 20 min and stored samples at +4 °C for 24 h. We also added dodecane (Merck, #1.09658.0005) as the internal standard solution (100 ppm of dodecane in n-hexane). We injected the samples (1 μL) onto a gas chromatograph (GC, Thermo Finnegan Trace GC Ultra, Waltham, MA, USA) with a mass spectrometer (MS) detector that was fitted with a 30 m × 0.25 mm × 0.25 μ film thickness ZB-5MSi (Phenomenex, UK) in single ion monitoring mode (SIM: *m/z* 68, 69, 77, 79, 92, 93, 94, 105, 119, 121, 136, 148, 161, 175) used to make visible known terpene fragments. The GC was operated in split mode (50 mL min^{-1}) with helium as the carrier gas (flow rate 1 mL min^{-1}). The GC oven temperature program was: 2 min hold at +60 °C, 10 °C min^{-1} ramp to +70 °C, 15 min hold at +70 °C, 5 °C min^{-1} ramp to +130 °C, 30 °C min^{-1} ramp to +250 °C, and 1 min hold at +250 °C. We identified terpenes comparing their Kováts indices, calculated relative to the retention times of a series of n-alkanes (C_8—C_{20}, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) analyzed under the same chromatographic conditions, with those reported in the literature (Nabet et al., 2017; Tsoukatou et al., 2001). For each plant, we estimated the amount of terpenes by using normalized peak areas per dry weight. The normalized peak area per dry weight of each compound was obtained by dividing their integrated peak area by the integrated peak area of the internal standard and then dividing this value by the leaf dry weight. Terpene concentrations were expressed in mg g^{-1} leaf DW.

2.8. Lipid extraction and analysis of fatty acid composition

Total lipids (in mg g^{-1} fresh weight) were extracted from approximately 1 g of *C. maritimum* leaves using the method described previously by Hara and Radin (1978). The hexane-rich supernatant containing lipids was obtained and methylation of fatty acids was performed according to Garcés and Mancha (1993). Fatty acid methyl esters (FAMEs) were analyzed by GLC using a Hewlett Packard 6890 gas chromatograph (Palo Alto, CA, USA) and a Supelco SP-2380 capillary column (60 m length, 0.25 mm i.d., 0.20 μm film thickness; Supelco, Bellefonte, PA, USA). Hydrogen was used as the carrier gas at 20 cm/s, the temperature of the flame ionization detector and injector was +220 °C, the oven temperature was +185 °C and the split ratio was 100:1. Heptadecanoic acid (C-17:0) was used as internal standard for fatty acids quantification. The different methyl esters were identified by comparison with a combination of standards.

2.9. Data analyses

Analyses were carried out using IBM SPSS Statistics (version 25.0, IBM Corp., Armonk, NY, USA). Deviations were calculated as Standard Error (SE). A significance level (α) of 0.05 was applied for every analysis. Plant traits were classified into eight functional groups: (1) leaf gas exchange, (2) biomass, (3) growth, and foliar concentrations of (4) macro- and micro-nutrients, (5) amino acids, (6) phenolic compounds, (7) terpenes, and (8) fatty acids. Normality and homogeneity of variance and of raw data series were tested with the Kolmogorov-Smirnov test and the Levene test, respectively. Fifteen traits were transformed using the functions 1:x and $\sqrt{x}$ to meet the assumption of homogeneity of variances for parametric tests. To protect analyses from type I error, we used the protected analysis of variance (ANOVA) protocol: the means of the dependent variables of each trait group were compared using multivariate analysis of variance (MANOVA) and Pillai's Trace to evaluate the significance of the factors (salinity and nutrient concentration) avoiding type I error (Scheiner 2001). Redundant, highly correlated variables ($r > 0.95$) were identified prior to MANOVA analysis. Highly correlated variables (amino acids glycine, threonine and valine, and terpenes α-thujene and y-terpinene) were omitted from the statistical models. Once multivariate significance was confirmed via MANOVA, the main univariate differences were evaluated for each functional plant trait using General Lineal Models (GLMs) and Bonferroni-Dunn's test as a *post hoc* analysis. When homogeneity of variance was not achieved after data transformation, univariate differences were analyzed using the Gamma Generalized Linear Model (GGLM) with Chi-square (χ^2) de Wald (Ng and Cribbie, 2017).

Ordination analysis was used to explore patterns in our data without

constraining the analysis based on treatment (ter Braak and Smilauer, 2012). As is typical with trait data, our data responses to the imposed treatments were linear, not unimodal, suggesting that Principal Components Analysis (PCA) was a better approach than Correspondence Analysis (CA). Therefore, PCA was conducted to express covariation in numerous variables in a smaller number of composite factors. PCA was carried out analyzing the correlation matrix with 25 maximum iterations for convergence without rotation to extract independent PC factors with eigenvalues > 1. The PCA factors obtained for the response traits were correlated with both salinity (mM) and nutrient concentrations (% Hoagland's solution) treatments.

3. Results

Salinity had a significant effect on all recorded plant trait groups, except on phenolic compounds. Leaf gas exchange, and biomass and growth traits were the only plant trait groups not affected by nutrient availability. The interaction between salinity and nutrient concentration was significant only for terpenes and fatty acids accumulation (MANOVA, $P < 0.05$) (Table A1). Results of all the GLMs for the 65 measured plant traits are summarized in (Table A2). Herein we report the most significant results for each trait group.

3.1. Leaf gas exchange, biomass and growth

Net photosynthesis rate and stomatal conductance decreased 54% and 65%, respectively, at 500 mM NaCl compared to maximum values recorded at 0.5–50 mM NaCl. Both leaf gas exchange traits were independent of nutrient concentration (Fig. 1, A and B) (Table A2). All recorded biomass and growth traits decreased significantly at 500 mM NaCl. AGB and BGB were the traits showing the highest decrease with increasing salinity (−77% and −68%, respectively), whereas plant height decreased just 33% (Fig. 1, C—H) (Table A2).

3.2. Foliar nutrient concentrations

Foliar concentration of macronutrients Ca, Mg and P dropped significantly at salinities higher than freshwater, whereas the concentrations of N, K and S decreased at salinities higher than 50 mM NaCl. Additionally, foliar concentrations of all macronutrients, except Mg, were lower at 5% than 20% Hoagland's solution (Fig. 2, A-F). In contrast, leaf Na concentration increased more than 90% from freshwater to 500 mM salinity, being independent of nutrient concentration (Fig. 2, G). Foliar concentration of the micronutrient B was lower at 200–500 mM than at 0.5–50 mM NaCl. Moreover, leaf B concentration was lower at 5% than 20% Hoagland's solution (Fig. 3, A). In contrast, leaf Mo concentration increased c. 50% at 500 mM NaCl than at freshwater (Fig. 3, E) (Table A2).

3.3. Foliar amino acids

Amino acids Glu + Gln-and Tyr-increased with salinity (Fig. 4, A and D). On the contrary, five amino acids (Ser, Ala, Leu, Phes and Lys) showed lower concentration at 500 mM than at 0.5–50 mM NaCl. Respect to nutrient treatments, seven amino acid concentrations (Ser, Ala, Tyr, Trp, Leu, Phe, Lys) were higher at 5% than 20% Hoagland's solution, whereas Glu+Gln-concentration was lower at the lowest nutrient concentration (Fig. 4) (Tables A2–3).

3.4. Foliar phenolic compounds

The foliar accumulation of 3-caffeoylquinic acid increased c. 64% with increasing salinity. The foliar concentration of five phenolic compounds (5-Caffeoylquinic acid, Feruloyl quinic acid, 3,5-Di-Caffeoyl quinic acid, 4,5-Di-Caffeoylquinic acid and Kaempferol 3-glucoside-7-rhamnoside) was higher at 5% than 20% Hoagland's solution. In contrast, Rutin was not detected at 5% Hoagland's solution from 0.5 to 200 mM NaCl (Fig. 5) (Table A2).

3.5. Foliar terpenes

Leaf concentration of five terpenes (α-terpinene, p-cymene, limonene, β-phellenadrene and α-pinene) was between 50% and 76% lower at 500 mM NaCl than at freshwater conditions. Moreover, the concentration of these five terpenes and two more (β-myrcene and Thymol methyl ether) under 5% Hoagland's solution was between 30% and 50% lower than at 20% Hoagland's solution in freshwater, showing the opposite response to nutrient availability at higher salinities (Fig. 6) (Table A2).

3.6. Foliar fatty acids

Total lipid concentration decreased c. 30% with the increase of salinity, particularly in 5% Hoagland's solution. Leaf concentration of unsaturated fatty acid 16:3 was the lowest at 50 mM salinity. In contrast, the four recorded saturated fatty acids (C-16:0, C-18:0, C-20:0 and C-22:0) almost doubled their concentration at higher salinities (Table 1). In relation to nutrient concentration, total lipid concentration and the concentration of two fatty acids (C-16:3 and petroselinic acid C-18:1$^{\Delta 6}$) were c. 20% lower at 5% than at 20% Hoagland's solution. In contrast, fatty acid C-18:1$^{\Delta 11}$ showed 29% higher concentration at 5% than at 20% Hoagland's solution. In this sense, the concentration of three fatty acids (C-16:0, C-16:1$^{\Delta 9}$ and C-18:1$^{\Delta 9}$) was c. 30% higher at 5% than at 20% Hoagland's solution only at higher salinities (Table 1). Leaf concentration of fatty acid C-22:0 was c. 75% higher at 5% than at 20% Hoagland's solution at freshwater conditions, and showed the opposite response at higher salinities (Table 1) (Table A2).

3.7. Relationships between plant traits, salinity and nutrient availability

The PCA yielded 13 factors, revealing the strongest covariation among variables, and explaining 93% of the variance in plant trait responses relative to treatments (Table A4). PC1 explained 30% of the variance, and was positively correlated with foliar concentrations of five macronutrients (S, K, Mg, Ca and N), one micronutrient (B), five terpenes (α-terpinene, β-phellenadrene, limonene, p-cymene and α-pinene) and total lipids, and with RGR, plant height, AGB, total biomass and stomatal conductance traits. PC1 was negatively correlated with foliar concentrations of Na, one micronutrient (Mo), one phenolic compound (3-caffeoylquinic acid) and four saturated fatty acids (C-16:0, C-18:0, C-20:0 and C-22:0) (Table A4). PC1 decreased with salinity ($r = -0.931$, $p < 0.0001$, $n = 23$). The second factor (PC2; explaining 16% of variance) was positively correlated with the foliar concentration of one unsaturated fatty acid (C-18:1$^{\Delta 9}$), six amino acids (Tyr, Lys, Ser, Phe, Ala-and Leu) and one phenolic compound (5-caffeoylquinic acid), and negatively with foliar concentration of two amino acids (Glu + Gln) and one macronutrient (P). PC2 decreased with nutrient concentration ($r = -0.781$, $p < 0.0001$, $n = 23$) (Table A4).

4. Discussion

Our results show that the salinity tolerance of the halophyte *C. maritimum* was not diminished by the interaction of salinity with nutrient limitation. Besides the recorded high tolerance to both tested abiotic factors, mineral nutrition deficit and saturated fatty acid accumulation were both induced by the highest tested salinities (200–500 mM NaCl), and accumulation of polyphenol 5-caffeoylquinic acid and some amino acids were identified as nutrient deficiency markers. The interaction between salinity and nutrient availability provoked antagonistic and synergistic effects on the accumulation of terpenes and fatty acids.

The high salinity tolerance recorded for *C. maritimum* was reflected

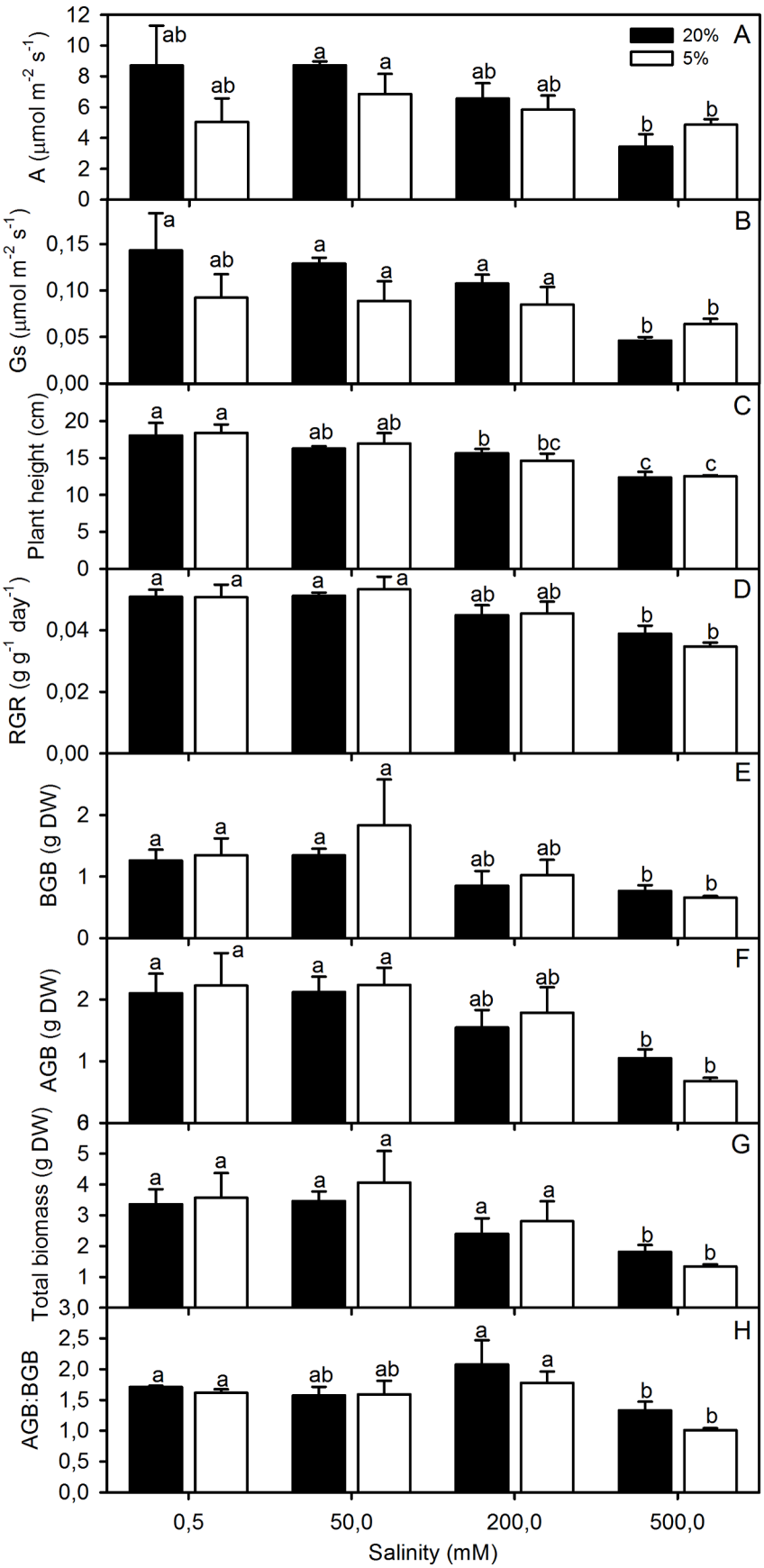

Fig. 1. Leaf gas exchange, growth and biomass responses: (A) Net photosynthesis rate, (B) stomatal conductance, (C) plant height, (D) relative growth rate, (E) below-ground biomass, (F) above-ground biomass, (G) total biomass, (H) above- to below-ground biomass ratio for *Crithmum maritimum* under different NaCl concentrations and nutritional levels (20% Hoagland's solution, black columns; 5%, Hoagland's solution, white columns). Different letters indicate significant differences between treatments. Values are mean ± SE ($n = 3$). DW, dry weight.

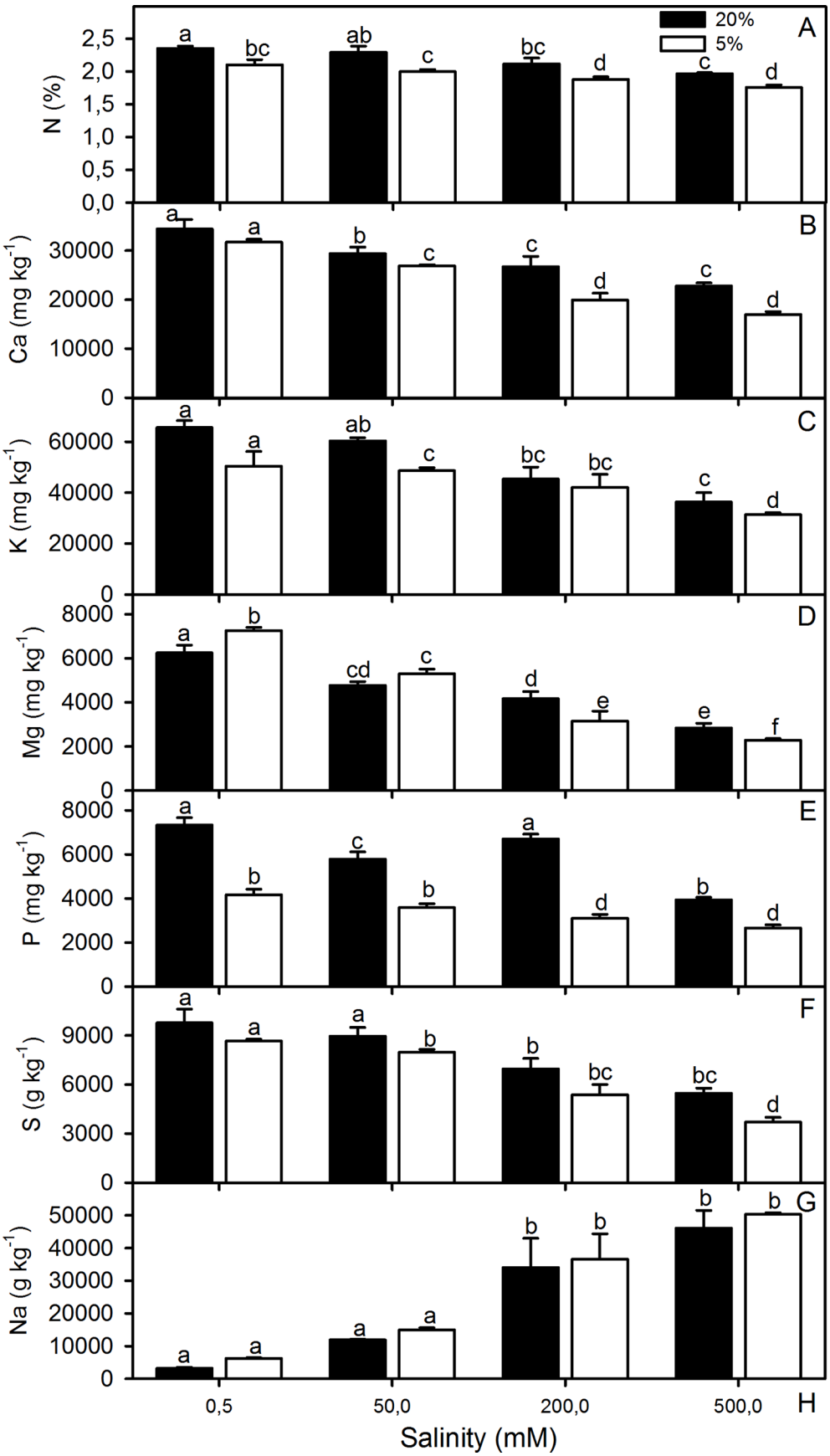

Fig. 2. Foliar macronutrient concentrations (in mg kg^{-1} DW; % for Nitrogen) for *Crithmum maritimum* under different NaCl concentrations and nutritional levels (20% Hoagland's solution, black columns; 5%, Hoagland's solution, white columns). Different letters indicate significant differences between treatments. Values are mean ± SE ($n = 3$).

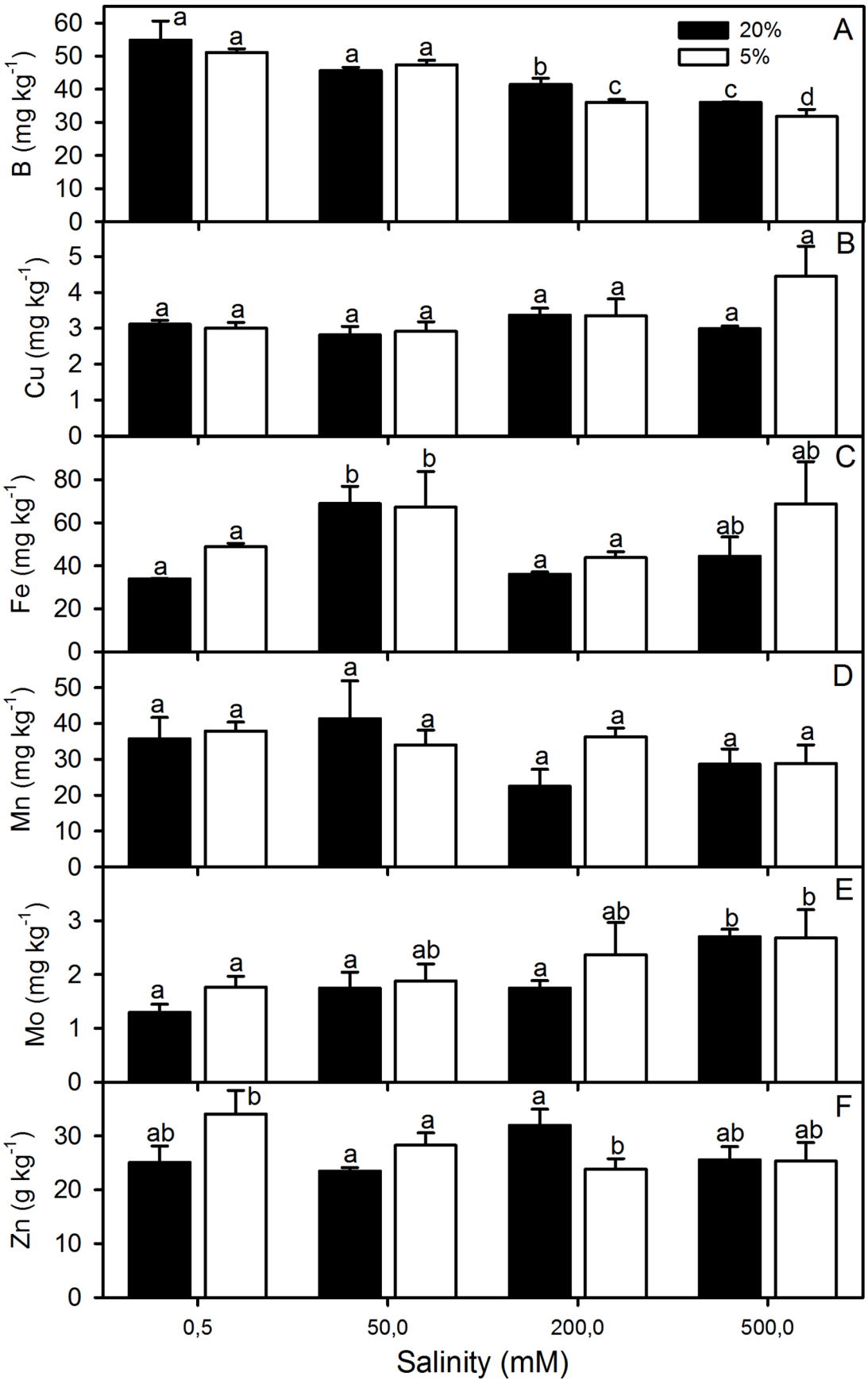

Fig. 3. Foliar micronutrient concentrations (in mg kg^{-1} DW) for *Crithmum maritimum* under different NaCl concentrations and nutritional levels (20% Hoagland's solution, black columns; 5%, Hoagland's solution, white columns). Different letters indicate significant differences between treatments. Values are mean ± SE (n = 3).

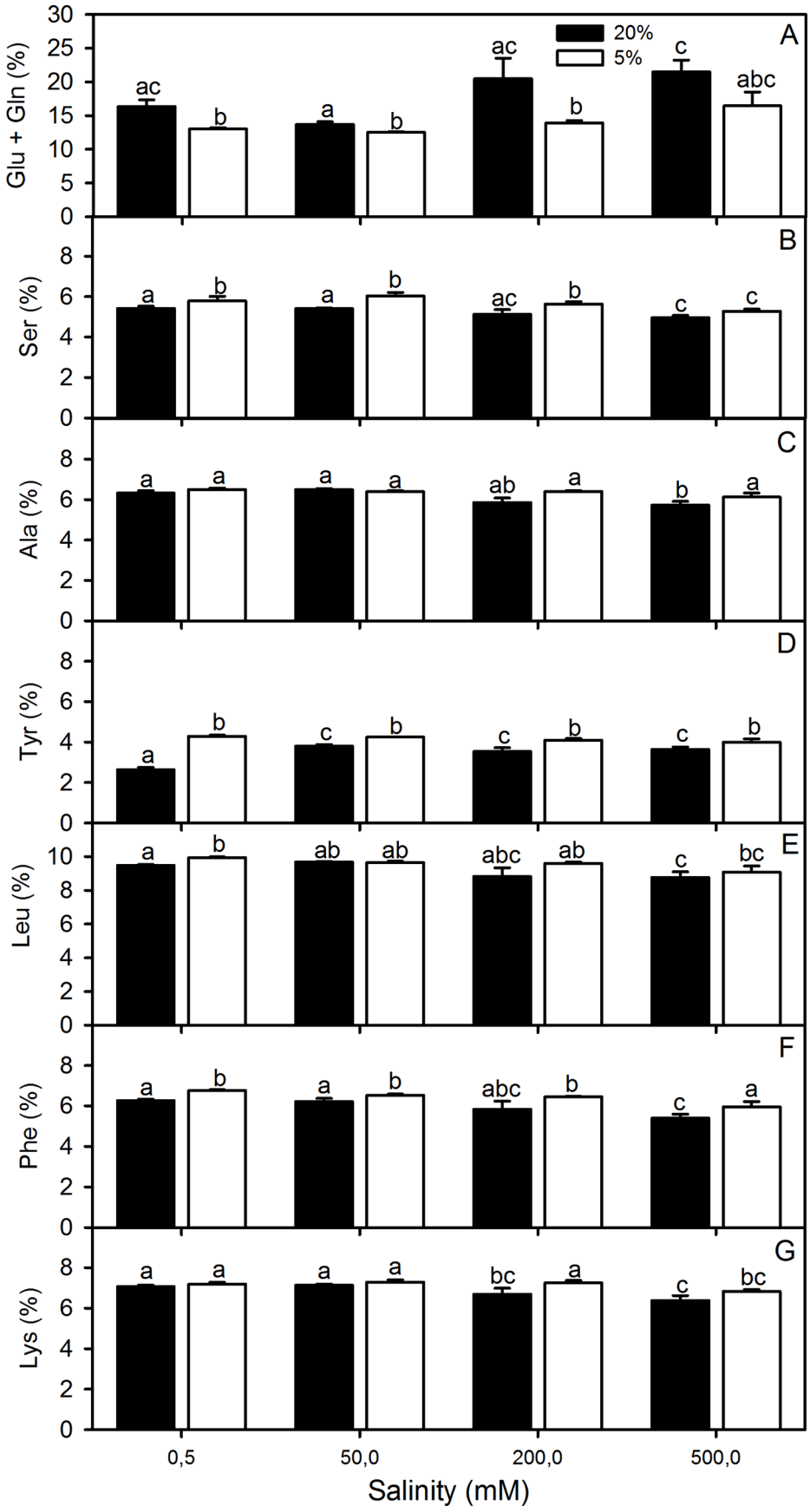

Fig. 4. Foliar amino acid concentrations (%) for *Crithmum maritimum* under different NaCl concentrations and nutritional levels (20% Hoagland's solution, black columns; 5%, Hoagland's solution, white columns). Different letters indicate significant differences between treatments. Values are mean ± SE ($n = 3$).

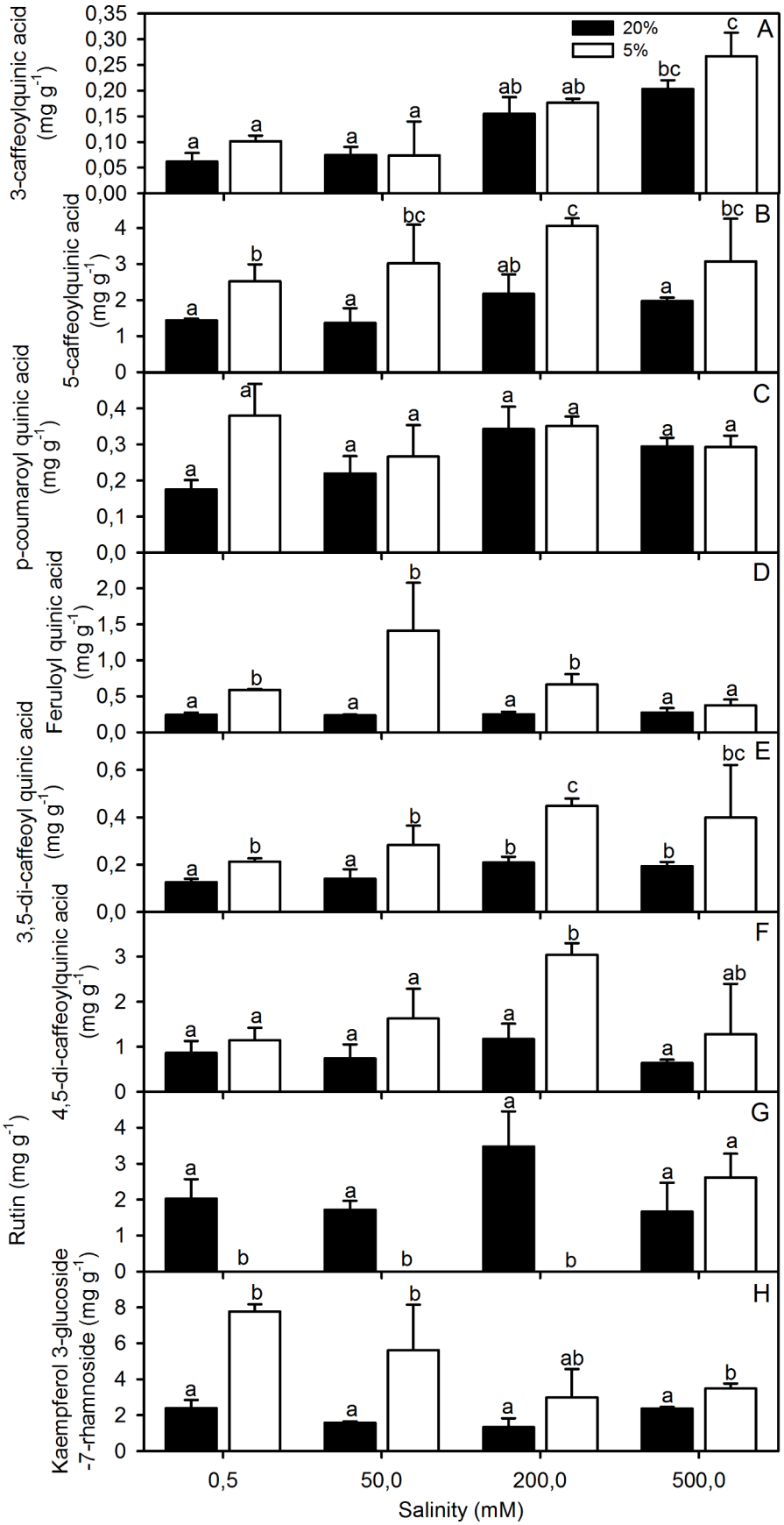

Fig. 5. Foliar phenolic compound concentrations (in mg g^{-1} DW) for *Crithmum maritimum* under different NaCl concentrations and nutritional levels (20% Hoagland's solution, black columns; 5%, Hoagland's solution, white columns). Different letters indicate significant differences between treatments. Values are mean ± SE ($n = 3$).

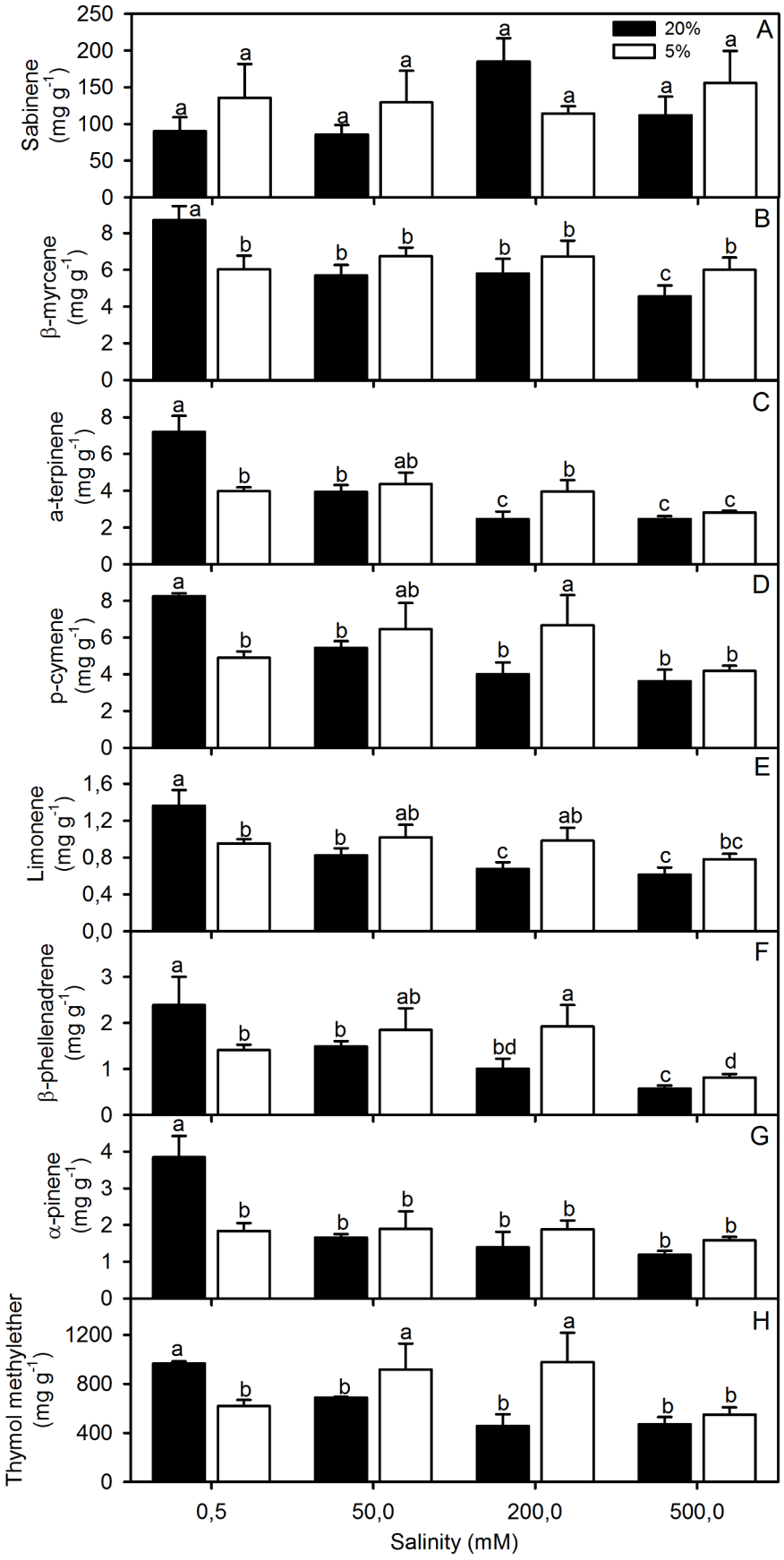

Fig. 6. Foliar terpenes concentrations (in mg g^{-1} DW) for *Crithmum maritimum* under different NaCl concentrations and nutritional levels (20% Hoagland's solution, black columns; 5%, Hoagland's solution, white columns). Different letters indicate significant differences between treatments. Values are mean ± SE ($n = 3$).

Table 1
Foliar total lipid (mg fatty acids g^{-1} fresh weight) and fatty acid (mol%) for *Crithmum maritimum* under different NaCl concentrations (0.5–500 mM NaCl) and nutritional levels (5% and 20% Hoagland's solution). Fatty acids: 16:0, palmitic acid; $16:1^{\Delta 9}$, palmitoleic acid; $16:2^{\Delta 9\Delta 12}$, palmitolinoleic acid; $16:3^{\Delta 7\Delta 10\Delta 13}$, palmitolinolenic acid; 18:0, stearic acid; $18:1^{\Delta 6}$, petroselinic acid; $18:1^{\Delta 9}$, oleic acid; $18:1^{\Delta 11}$, asclepic acid or vaccenic acid; $18:2^{\Delta 9\Delta 12}$, linoleic acid; $18:3^{\Delta 9\Delta 12\Delta 15}$, linolenic acid; 22:0, behenic acid.

	0.5 mM NaCl		50 mM NaCl		200 mM NaCl		500 mM NaCl	
	5%	20%	5%	20%	5%	20%	5%	20%
Total lipids	1.68 ± 0.26^{a}	2.00 ± 0.01^{a}	1.68 ± 0.13^{a}	1.95 ± 0.25^{a}	1.42 ± 0.11^{ab}	1.47 ± 0.21^{ab}	1.11 ± 0.03^{b}	1.60 ± 0.13^{ab}
16:0	19.05 ± 0.04^{a}	15.25 ± 0.17^{a}	17.77 ± 0.36^{a}	15.57 ± 0.40^{a}	17.65 ± 1.15^{a}	16.66 ± 1.25^{a}	26.01 ± 0.90^{b}	16.47 ± 1.04^{a}
$16:1^{\Delta 9}$	2.03 ± 0.03^{a}	1.40 ± 0.04^{b}	1.42 ± 0.03^{b}	1.78 ± 0.03^{c}	1.65 ± 0.12^{c}	1.64 ± 0.25^{c}	2.73 ± 0.15^{d}	1.65 ± 0.15^{c}
$16:2^{\Delta 9\Delta 12}$	0.90 ± 0.04^{a}	0.85 ± 0.05^{a}	0.72 ± 0.04^{b}	1.10 ± 0.09^{c}	0.73 ± 0.08^{b}	0.79 ± 0.12^{ab}	0.80 ± 0.12^{ab}	0.70 ± 0.10^{b}
$16:3^{\Delta 7\Delta 10\Delta 13}$	6.65 ± 0.64^{ab}	6.95 ± 0.15^{ab}	6.14 ± 0.31^{ab}	8.26 ± 0.38^{a}	5.18 ± 0.25^{b}	6.54 ± 1.14^{ab}	4.96 ± 0.30^{b}	5.69 ± 0.44^{ab}
18:0	4.29 ± 0.50^{ab}	3.94 ± 0.44^{ab}	3.14 ± 0.20^{a}	3.05 ± 0.30^{a}	3.67 ± 0.11^{a}	4.03 ± 0.19^{a}	5.96 ± 0.16^{b}	4.95 ± 0.63^{b}
$18:1^{\Delta 6}$	16.24 ± 8.92^{a}	18.12 ± 1.45^{a}	11.00 ± 1.71^{b}	14.93 ± 0.60^{a}	15.63 ± 3.33^{a}	16.07 ± 5.20^{a}	16.79 ± 3.28^{a}	2.74 ± 1.07^{c}
$18:1^{\Delta 9}$	2.06 ± 0.36^{a}	2.82 ± 0.19^{b}	2.96 ± 0.30^{b}	2.16 ± 0.28^{a}	2.89 ± 0.22^{b}	2.15 ± 0.39^{a}	2.33 ± 0.39^{ab}	2.61 ± 0.30^{b}
$18:1^{\Delta 11}$	0.17 ± 0.02^{a}	0.13 ± 0.01^{b}	0.22 ± 0.03^{a}	0.17 ± 0.03^{a}	0.21 ± 0.01^{a}	0.11 ± 0.04^{b}	0.19 ± 0.01^{a}	0.10 ± 0.01^{b}
$18:2^{\Delta 9\Delta 12}$	24.29 ± 0.79^{a}	22.29 ± 0.32^{b}	25.78 ± 0.76^{a}	21.74 ± 0.33^{b}	23.84 ± 1.13^{a}	22.99 ± 0.35^{ab}	22.92 ± 0.84^{ab}	2.31 ± 0.36^{c}
$18:3^{\Delta 9\Delta 12\Delta 15}$	29.67 ± 0.41^{a}	26.68 ± 0.65^{a}	28.52 ± 1.00^{a}	29.36 ± 0.97^{a}	25.97 ± 0.68^{a}	26.04 ± 3.04^{a}	28.02 ± 1.05^{a}	25.76 ± 0.95^{a}
20:0	1.35 ± 0.26^{a}	1.37 ± 0.16^{a}	1.69 ± 0.09^{a}	1.38 ± 0.07^{a}	1.93 ± 0.15^{ab}	1.60 ± 0.07^{a}	2.50 ± 0.11^{ab}	1.89 ± 0.13^{b}
22:0	0.84 ± 0.12^{a}	0.21 ± 0.02^{b}	0.63 ± 0.10^{a}	0.50 ± 0.16^{a}	0.65 ± 0.23^{a}	1.35 ± 0.02^{c}	0.84 ± 0.10^{a}	1.08 ± 0.16^{a}

Different letters indicate significant differences between treatments. Values are mean ± SE ($n = 3$).

in constant values of *A* and growth rates up to 200 mM NaCl alongside increasing foliar Na accumulation. This result is in agreement with previous studies (Fattoum et al., 2016; Boestfleisch and Papenbrock, 2017; Gil et al., 2019; Strumia et al., 2020). Hamdani et al. (2017) found that protective mechanisms against salinity were exceeded in *C. maritimum* leaves at 512 mM NaCl, which is in accordance with our results. In contrast, other works recorded significantly reduced growth for *C. maritimum* at salinities c. 200 mM NaCl (Hamed et al., 2004; Amor et al., 2005), pointing to the existence of different ecotypes regarding their response to salinity (Ventura et al., 2014; Pavela et al., 2017). *Crithmum maritimum* responded to increasing salinity mainly by reducing foliar accumulation of five terpenes and total lipids. Previous studies on aromatic plants have recorded down accumulation of terpenes and essential oil components under high salinity conditions (Shoshtari et al., 2017; Hancioglu et al., 2019). Regarding lipids, Ben Hamed et al. (2005) reported an increase in total lipid content in a Tunisian *C. maritimum* in response to irrigation with 100 mM NaCl. In addition, *C. maritimum* accumulated more Mo, a hydroxycinnamic acid (3-caffeoylquinic acid) and four saturated fatty acids in response to increasing salinity. Increasing Mo concentrations can alleviate the negative effects of NaCl (Zhang et al., 2012, 2014) since this micronutrient is involved in the biosynthesis of several enzymes that catalyze basic reactions in the N, S and C metabolism (Zdunek-Zastocka and Lips, 2003). Foliar accumulation of caffeoylquinic acids has been recorded previously in response to oxidative stress derived from increasing salinity (Yan et al., 2017). In contrast, the accumulation of four out of eight recorded phenolic compounds did not significantly change after applying salinity treatments. Similarly, a recent study found that soil salinization did not affect leaf phenolic compounds in wild cotton (*Gossypium hirsutum*) plants (Quijano-Medina et al., 2021). Although phenolic compounds are sometimes involved in resistance against salt stress, they usually play a key role in resistance against other abiotic stresses such as thermal tolerance (Close and McArthur, 2002) and biotic stresses (e.g., herbivory; Mithöfer and Boland, 2012). The accumulation of saturated fatty acids denoted *C. maritimum* limitation to acclimate to the highest tested salinities (200–500 mM NaCl) since this response seemed to be related to the inhibition of desaturases under salt stress (Okazaki and Saito, 2014). An increase in the ratio of unsaturated: saturated fatty acid has been characterized as an important plant response to salt stress because unsaturated fatty acids partly regulate membrane fluidity and permeability (Gogna et al., 2020). On the other hand, previous studies reported that salinities close to 200 mM NaCl induced mineral nutrition disturbance in *C. maritimum* (Fattoum et al., 2016; Amor et al., 2005; Atia et al., 2011). We recorded decreases close to 50% in every foliar macronutrient concentration and the micronutrient B at higher salinities. In this sense, K and N limitation diminishes the response of plants to salt stress (Ashraf et al., 2018; Nieves-Cordones et al., 2019).

Crithmum maritimum responded to nutrient limitation by increasing the concentration of vaccenic acid (C-$18:1^{\Delta 11}$) and 5-caffeoylquinic acid, and by changing its aminoacid profile. The up-accumulation of vaccenic acid in nutrient-deficient plants points to accumulation of oxylipins that create reversible storage for excess S and N under nutrient deficiency conditions (Troufflard et al., 2010). Oxylipins, known as phytoprostanes, are formed spontaneously from unsaturated fatty acids in plants belonging to the family Apiaceae (Christensen, 2020), such as *C. maritimum*, in response to different abiotic stressors (Savchenko et al., 2014). Moreover, foliar accumulation of polyphenols and free amino acids has been recorded in response to nutrient deficiency (Alves et al., 2011; Galieni et al., 2015). Additionally, *C. maritimum* reduced foliar accumulation of glutamic acid and glutamine (Glu + Gln) and the macronutrient P in response to nutrient deficit. Greater accumulation of glutamine in wheat leaves has been associated with enhanced phosphorus use efficiency (Nguyen et al., 2019).

The interaction between salinity and nutrient availability altered foliar accumulation of terpenes and fatty acids in *C. maritimum*. Tounekti et al. (2011) showed that adding nutrients to salt stressed *Rosmarinus officinalis* L. plants altered their terpenes composition due to ionic interactions between Na^{+} and other cations, such as Mg^{+}. In these sense, we recorded that the concentration of seven terpenes was lower under nutrient deficiency than in non-nutrient limited conditions in freshwater, showing the opposite response to nutrient availability at higher salinities. This response may be explained by down-regulation of antioxidant defense system, including terpenes, under the combination of nutrient deficiency and freshwater conditions, and up-regulation due to sodicity stress especially in nutrient-limited conditions (Borghesi et al., 2013; Ahanger et al., 2019). In addition, synergic effects were recorded between salinity and nutrient limitation stressors which was reflected in the concentration of one saturated (C-16:0) and two monounsaturated fatty acids (C-$16:1^{\Delta 9}$ and C-$18:1^{\Delta 9}$) increasing only for nutrient-limited plants under high salinity, but not in freshwater conditions. Increasing accumulation of unsaturated fatty acids has been recorded for other halophytes to cope with salt stress through improved plasma membrane fluidity (Yepeset al., L. 2018).

Caffeoylquinic acids were the dominant polyphenols and thymol methyl ether and sabinene were the most accumulated terpenes in analyzed *C. maritimum* leaves. These active biomolecules exhibit wide-ranging biological properties and play several therapeutic roles (Naveed et al., 2018; Reddy, 2019). Following our results, the best *C. maritimum* wild populations to maximize foliar accumulation of

phenolic compounds and terpenes would be those growing in nutrient-poor and not very salty soils, such as those colonizing sandy soils in coastal dunes and beaches and rock cracks on sea cliffs. Gil et al. (2019) showed that *C. maritimum* accumulated more polyphenols when colonizing habitats close to the coastline than in more inland habitats. The high acclimation capacity of *C. maritimum* to saline and non-saline conditions enhances the potential of the species as a promising crop of high economic interest. *Crithmum maritimum* should be cultivated in poor nutrient soils to maximize bio-active PSMs without reducing biomass production and micronutrient accumulation.

5. Conclusions

The composition of *C. maritimum* leaves includes high concentration of some phenolic compounds, terpenes, glutathione and micronutrients. Altogether, our findings suggest that *C. maritimum* is an adequate source of minerals and bio-active compounds with antioxidant properties for the food and pharmaceutical industries. Moreover, our results highlight the high physiological phenotypic plasticity of *C. maritimum* in the response to salt stress, nutrient deficiency and their combination. This study helps to optimize for most favorable exploitation of *C. maritimum* wild populations and for cultivation techniques to maximize harvesting of high quality and functional vegetable products rich in target bioactive compounds.

CRediT authorship contribution statement

Jesús M. Castillo: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Juan Manuel Mancilla-Leytón:** Conceptualization, Methodology, Writing – review & editing. **Raquel Martins-Noguerol:** Investigation, Writing – review & editing. **Xoaquín Moreira:** Investigation, Writing – review & editing. **Antonio Javier Moreno-Pérez:** Investigation, Writing – review & editing. **Sara Muñoz-Vallés:** Investigation, Writing – review & editing. **Justo J. Pedroche:** Investigation, Writing – review & editing. **Manuel Enrique Figueroa:** Conceptualization, Writing – review & editing. **Alberto García-González:** Investigation. **Joaquín J. Salas:** Investigation, Writing – review & editing. **María C. Millán-Linares:** Investigation. **Marta Francisco:** Investigation. **Jesús Cambrollé:** Conceptualization, Methodology, Data curation, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.111136.

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