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SALINITY STRESS

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Salinity tolerance of lentil is achieved by enhanced proline accumulation, lower level of sodium uptake and modulation of photosynthetic traits

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Abstract

Lentil (*Lens culinaris*, Medik.), an accessible low-cost high-quality protein form for many people, is a salt-sensitive legume, which already at an electrical conductivity (EC) of 3 dS/m (~30 mM NaCl) has a yield loss of about 90% compared to other crops. Identifying salinity-tolerant lentil germplasm is nowadays of primary importance for ensuring the production of superfood and the sustainability of the lentil industry. In this study, four cultivars Castelluccio di Norcia, Eston, Ustica and Pantelleria were grown up to a complete life cycle, in an open field, in soils conditioned with 100 mM NaCl and were compared with the same cultivars grown in unsalinized soils. Growth parameters, osmolytes and phenotypic characteristics of lentils were assessed. Our results evidenced different mechanisms specific for each tolerant cultivar. Pantelleria was the cultivar that mostly accumulated sodium in shoot and root and used it in addition to proline as osmoregulatory. Ustica accumulated less sodium and calcium than Pantelleria but more chlorine in root and enhanced also the production of the osmoregulatory. Castelluccio accumulated less sodium but more calcium and sulphates than the other two resistant cultivars, producing at the same time also osmolytes. The preference of ion uptake and compartmentalization can depend on the growth environment. PANT and UST are islander, therefore, prevalently in contact with sodium and chlorine, while CAST originated from central Italy is cultivated in soil where calcium and sulphate are the most abundant element.

KEYWORDS
chlorophyll, ions, osmoregulation, photosynthetic efficiency, salinity

1 | INTRODUCTION

Currently, more than 800 million hectares of land are saline, and is projected to increase in future for the climate changes (Shahid et al., 2018). It is forecast that 20% of total cultivated and 33% of irrigated agricultural land worldwide may be affected by high salinity. By 2050, more than 50% of arable lands are expected to become saline (Machado & Serralheiro, 2017). Salinity is a limiting factor

for the productivity of crops because it affects mostly the uptake of water and necessary nutrients, thereby reducing plant growth, development and crop yields (Hussain et al., 2016; Schleiff, 2008; Shrivastava & Kumar, 2015). Soil salinity acts through ion toxicity, creating osmotic stress and oxidative damage on crops and consequently limiting the water absorption (AbdElgawad et al., 2016; Hussain, Elnaggar, et al., 2020).

By 2050, cultivated land is expected to decrease for salinity, while the world's population will reach 9.1 billion, 34% higher than today. In order to feed this larger population, food production must increase by 70% (AbdElgawad et al., 2016). There is the need to find sustainable solution, searching for crop rich in nutrients that better adapt to saline lands to increase yield. The focus on starch-based crops like corn is shifting more to plant rich in proteins like soybeans and other legumes (Hertzler et al., 2020). Lentil is a legume with a significant role in human and animal alimentation and in maintaining soil fertility (Abraham, 2015). Lentil is generally cultivated in rotation to cereal crops because it benefits succeeding crops for its well-known potentiality of fixing free nitrogen, maintaining over time soil fertility. Lentil represents an accessible low-cost high-quality protein source for many people, and having an indeterminate adaptability to grow in different habitats, it is easily cultivated around the world (Khazaei et al., 2019). Lentil (*Lens culinaris*, Medik.) is a salt-sensitive legume (Mamo et al., 1996; Singh et al., 1993) and already at an electrical conductivity (EC) of 3 dS/m (~ 30 mM NaCl) has a yield loss of about 90% compared to other crops such as barley (10% yield loss at 66 mM NaCl), wheat (10% yield loss at 49 mM NaCl) and canola (10% yield loss at 73 mM NaCl) (Nadeem et al., 2019). Hence, to identify salinity-tolerant lentil germplasm is nowadays important to ensure the production of superfood and the sustainability of the pulses food industry. The majority of studies on lentil and salinity have been conducted in vitro on germination and in controlled conditions on seedlings (Foti et al., 2019; Kökten et al., 2010; Singh et al., 2017). No so much studies evaluated performance and growth of lentils in open fields with a salinity higher than 5 dS/m. Previous works (Muscolo et al., 2015; Sidari et al., 2007, 2008) identified three cultivars, in southern and central Italy that were tolerant to different salinity concentrations: two cultivars known as Ustica (UST) and Pantelleria (PANT) in the homonym islands, and Castelluccio di Norcia (CAST) cultivated in central Italy. The studies conducted with respect to the commercial variety cultivated in Canada and named Eston evidenced that the responses to the imposed salinity varied with the growth stage of the plants and depended mainly on each variety. The responses to salt stress have been evaluated at 0, 50, 100, 150 and 200 mM NaCl to evaluate the variation in salt tolerance. At 200 mM, the cultivars decreased their germination: Pantelleria and Ustica germinated more than the other ones (74% and 82%, respectively), exhibiting a fair degree of salt tolerance. According to the germination frequencies, salinity cultivar tolerance ranking was as follows: PANT >UST > CAST >EST, while for salinity seedling tolerance, the ranking was CAST ≈ UST >PANT ≈ EST. Eston resulted in salinity sensitive, and from this, arose the need to identify, through open field studies, salt-tolerant cultivars to avoid genetic manipulations that, in the long term, could affect soil biodiversity and in turn soil fertility (Dunfield & Germida, 2004; Tsatsakis et al., 2017).

Considering that salinity differently affected the stage of plants with respect to the type of cultivars, our research hypothesis was that the different cultivars lentils in fields might respond differently to salinity, also changing the cultivar tolerance ranking observed at the different growth stages. Hence, to fill the research gap, Castelluccio di Norcia, Eston, Ustica and Pantelleria were grown, up to a complete life cycle, in open field, in soils conditioned with 100 mM NaCl and were compared with the same varieties grown in non-saline soils. The soils have been analysed and the whole vegetative cycle was monitored to verify if and how salinity (NaCl) affected growth parameters, and phenotypic characteristics of lentils.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Experimental sites were located in Motta San Giovanni, in the Agricultural Farm Orfei Loc. Liso, Italy (x: 561023,1; y: 4204908,9; WGS 84 UTM Zone 33 N). The soil was a sandy-loam (11.85% clay, 23.21% silt and 64.94% sand) textural class according to the Food and Agriculture Organization of the United Nations (FAO) soil classification system (2014). Before the sowing, artificially salinized soils were prepared adding NaCl solution up to EC 10 $dS{\cdot}m^{-1}$ in saturated extracts. Unsalinized soil was used as control. The lentils were sown in October 2017–2018–2019. Each independent experiment was performed in triplicates in field, separated in parcel of 1 m square each. In each parcel, 10 plants/m^2 were cultivated. Each lentil variety completed its life cycle in soil conditioned with NaCl and was compared with the same variety grown in unsalinized soils. The experiment was arranged in a randomized complete block design. The reported data are the mean of the 3 consecutive years as no significant differences were observed among the results coming from the different years. The climate is typically Mediterranean, with summer medium temperature of 24°C and winter medium temperature of 15 °C. The month warmest is August with 28°C in media, and the coldest month is February with temperature ranging from 6 to 12 degree. The rainy period in the year lasts 9.7 months, from 10 August to 31 May, with a rolling period of 31 days of at least 13 millimetres. Most rain falls during the 31 days centred around December 1, with an average total accumulation of 1.0 inches. The rainless period of the year lasts for 2.3 months, from May 31 to August 10. The least rain is around July 9, with an average total accumulation of 0.3 inches (Figure 1). The parameters of rainfall and temperature during the cultivation period do not interfere with the progress of the experiment.

During the whole experiment (9 months), salinity was weekly monitored collecting soil up to the depth explored by the root (50 cm) to maintain 100 mM NaCl constant for the whole experiments, and the moisture was kept at ~70% of field capacity by supplying fresh water (Kirkham, 2014).

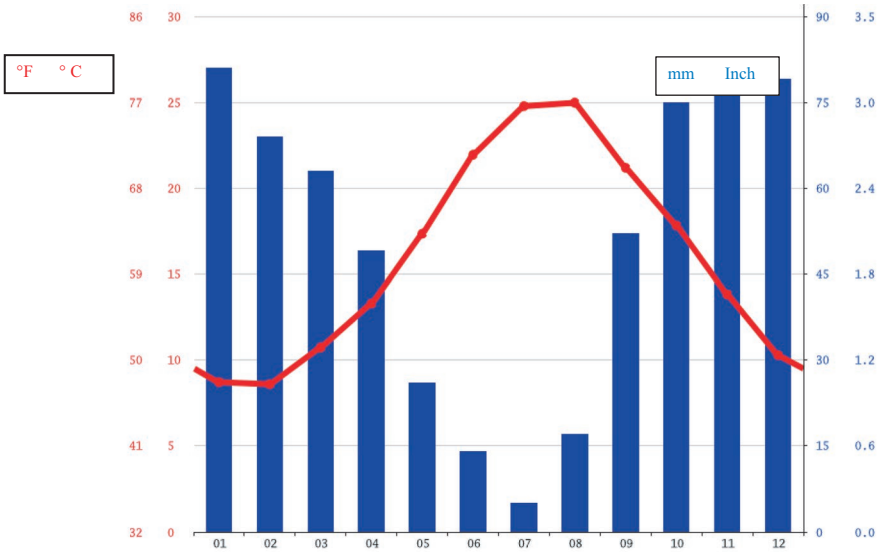

FIGURE 1 The thermo-pluviometric diagram during the experimental time

2.2 | Plant material

One commercial variety against three Italian lentil accessions has been studied. Eston (EST) the commercial variety is native to Canada, Pantelleria (PANT) comes from a protected area (National Park of Pantelleria Island), Ustica (UST) is native to a volcanic soil in Ustica Island and Castelluccio di Norcia (CAST) is a local population cultivated in Umbria (central Italy).

2.3 | Physical and chemical soil analysis

Analysis were done in three replicates. Particle size analysis was carried out with hydrometer method, using sodium hexametaphosphate as dispersant (Bouyoucos, 1962); dry matter of saline and non-saline soils was determined at 105°C until the mass loss of the sample during 24 h was lower than 0.5% of its weight; pH was measured in distilled water and 1 M KCl using a 1:2.5 (soil/water) suspension; electrical conductivity (EC) was determined in distilled water by using 1:5 soil: water suspension, after shaking at 15 rpm for 1 h to dissolve soluble salts and then detected by Eutech instrument conductivity meter; organic carbon (OC) was determined with Walkley–Black method based on the determination of the Cr^{3+} deriving from organic C oxidation (Walkley & Black, 1934), and OC percentage was multiplied for 1.72 to be converted into organic matter; and total nitrogen was detected with Kjeldahl method (Kjeldahl, 1883). The C/N ratio was calculated as TOC/Ntot. Microbial biomass carbon (MBC) was determined by the chloroform fumigation-extraction procedure (Vance et al., 1987) with soil moist samples (equivalent to 20 g d.s.). Soil samples were fumigated with alcohol-free $CHCl_3$ for 24 h at 24°C. Both fumigated and non-fumigated samples were extracted with 0.5 M K_2SO_4 (1:4 w/v) and filtered with Whatman's no. 42 paper and then were analysed for soluble organic C, using the method of Walkley and Black (1934). MBC was estimated based on the difference between the organic C extracted from the fumigated soil and unfumigated soil, using 0.38 as extraction efficiency coefficient to convert soluble C into biomass C (Vance et al., 1987).

2.4 | Plant analysis

At the end of each experiment, growth parameters in terms of plant height (cm), leaf number, leaf area (cm^2), flower and fruit numbers, pod and seed numbers were evaluated. Lentils were collected separated in leaves and roots and stored in a refrigerator at 4°C until analysis (28 days). Cations and anions were detected in leaves and roots. Photosynthetic efficiency, total chlorophyll, proline content, total soluble sugar and relative water content (RWC) were detected in leaves.

2.5 | Cation and anion analysis

Cations and anions extracted from leaves, root and soil were detected by ion chromatography (DIONEX ICS-1100). For anions, 0.5 g of dried material was extracted using 50 ml of anion solution (Na_2CO_3/$NaHCO_3$ 3.5 mM) stirring for 20 min. The extracts were filtered and the chromatographic analysis was carried out. For cations, 1 g of dry material was ashed at 550°C for 5–6 h in a porcelain capsule. The ash was then mineralized for 30 min at 100°C using 1 M HCl solution. The solution was subsequently filtered and analysed by ion chromatograph (eluent meta-sulfonic acid 20 mM). Bioconcentration factor (cation or anion in root/cation or anion in soil), bioaccumulation coefficient (cation or anion in leaves/cation or anion in soil) and translocation factor (cation or anion in leaves/cation or anion in roots) were detected.

2.6 | Chlorophyll fluorescence imaging

Photosynthetic efficiency of primed and unprimed seedlings in absence and in presence of salinity was evaluated by using an Imaging PAM Fluorometer (Walz). The chlorophyll fluorescence parameters detected were as follows: Maximum quantum yield of PSII photochemistry (F_v/F_m); Effective quantum yield of PSII photochemistry (Y(II)); Quantum yield of regulated energy dissipation at PSII

(Y(NPQ)); Quantum yield of non-regulated energy dissipation at PSII (Y(NO)); Non-photochemical quenching coefficient (NPQ) and Electron transport rate (ETR).

2.7 | Preparation of leaf extracts

One gram of fresh leaves was extracted at room temperature using 3.0 ml dH_20 (Intercontinental Mod still 3/ES, Bioltecnical Service, s.n.c.). The samples were centrifuged at 590 × g (2000 rpm) for 10 min and the supernatants were filtered with Whatman no. 1 filter paper and used for the determination of proline and total carbohydrates.

2.8 | Determination of total chlorophyll content, proline content, total soluble sugar and relative water content (RWC)

For the estimation of total chlorophyll content, 100 mg leaf tissue was finely ground in liquid nitrogen and suspended in DMSO. The suspension was maintained at 65°C for 30 min. The final volume was adjusted to 10 ml with DMSO and absorbance was recorded at 645 and 663 nm. Total chlorophyll content was calculated as reported in Hiscox and Israelstam (1979).

Proline was analysed following the method of Bates et al. (1973). Leaf was homogenized in sulphosalicylic acid (3% in distilled water) and centrifuged. The 0.5 ml of extract was added to an equal volume of acid ninhydrin reagent and glacial acetic acid and incubated for 1 h. The reaction was stopped by adding 1 ml toluene. The absorbance was measured at 520 nm. The concentration of proline was calculated using standard curve of proline.

The total soluble sugars were detected with the anthrone method (Hedge & Hofreiter, 1962). The samples (0.1 g) were processed with 5.0 ml 52% $HClO_4$ and conserved at the dark for 18 h. Samples were successively filtered using Whatman no. 1 and then volumized to 10 mL with distilled water. Filtrate (100 µl) was added to 0.1% anthrone solution up to a final volume of 5.0 ml. After a boiling of 10 min, were chilled and the absorbance was read at 630 nm. The amount of total soluble sugars was calculated on a glucose calibration curve (range 10–100 mg/ml). The results were expressed as µg glucose/g dw.

RWC was determined in each sample by weight and its fresh weight was recorded (FW), then dried at 104°C for 2 and 72 h at 80°C. The dry matter was weighed and recorded as DW. Water content was calculated as the following:

$$\text{Water content (\%)} = (\text{FW} - \text{DW}) / \text{FW} * 100.$$

2.9 | Statistical analysis

Analysis of variance was carried out for all the data sets. One-way ANOVA with Tukey's honestly significant difference test were carried out to analyze the effect of salinity on plant properties. Significant difference test was carried out to analyse the effects of salinity on each of the various parameters measured. Two-way ANOVA was used to test the effects of the factors (salinity and cultivars) on the growth parameters and cations and anions. Correlation among growth parameters, salinity and lentil varieties was analysed. SYSTAT 13.2 (SYSTAT Inc.), Powerful Statistical Analysis and Graphics Software for Windows 7, was used for all the statistical analyses.

To explore relationships among photosynthetic parameters, total ions (shoot/root) and growth parameters of CAST, PANT, UST and EST cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR), data sets were analysed using principal component analysis (PCA) and the results are summarized in an ordination diagram (Hammer et al., 2001). Because the data are expressed in different units, the results are standardized with the following formula:

$$z = ((x_i - \bar{x})) / \text{SD}.$$

where x_i is the individual value of each parameter, $\bar{x}$ is the mean and SD is the standard deviation.

TABLE 1 Soil chemical analysis before lentil sowing. Electric CONDUCTIVITY (EC, µS cm^{-1}), water content (WC, %), water soluble phenols (WSP, µg TAE g^{-1} dry soil), total organic carbon (TOC %), total nitrogen (TN%), carbon/nitrogen ratio (C/N), organic matter (OM%), fluorescein diacetate (FDA µg g^{-1} dry soil) hydrolysis, dehydrogenase (DH, µg INTF g^{-1} dry soil h^{-1}), microbial biomass (MBC, µg C g^{-1} f.s.) and cation exchange capacity (CEC, cmol(+) Kg^{-1})

Texture	Sandy-Loam
pH	8.5 ± 0.60
EC	350 ± 14
WC	21.5 ± 2.81
WSP	14 ± 2.80
TOC	3.1 ± 0.16
TN	0.15 ± 0.01
C/N	20.6 ± 0.31
SOM	5.33 ± 0.27
FDA	42 ± 1.39
DH	57 ± 2.81
MBC	835 ± 18
CSC	18.7 ± 1.42
Na^+	0.07 ± 0.01
K^+	0.22 ± 0.05
Mg^{++}	0.10 ± 0.01
Ca^{++}	0.25 ± 0.05
Cl^-	0.20 ± 0.03
NO_3^-	0.05 ± 0.01
PO_4^-	0.05 ± 0.02
SO_4^-	0.15 ± 0.04

Note: The data are the mean of three replicates ±standard deviation.

For PCA diagram of plants equilibrated with NaCl (100 mM) and without NaCl (control, CTR), the first two components (Eigenvalues >1) have been extracted. Component 1 (PC1) explains about 37% of all the variability in the parameters, while component 2 explains about 32%.

3 | RESULTS AND DISCUSSION

3.1 | Soil chemical analysis

The soil used for the cultivation of lentils was alkaline, non-saline, with a low phenol content, a C/N value corresponding to an equilibrium state between mineralization and immobilization processes, an elevated amount of organic matter and a medium CEC value (Table 1). It is well known that lentil adapts perfectly to soils with a pH ranging from 6 to 8 as reported by Singh et al. (2018) and on soils belonging to sandy or sandy-loam textural classes (Mitiku, 2016). Our data evidenced that the characteristics of the soil used in this experiment were suitable for lentil growth.

3.2 | Soil biochemical properties

The high amount of microbial biomass was explained by the high content of organic matter in the soil. The values of dehydrogenase activity (DH) and FDA hydrolysis (FDA) suggested a balance between hydrolytic and oxidative activities in soil (Table 1). FDA and DH are enzymes involved in biogeochemical cycles (C, N and S), which in turn mirror the quantitative changes in the SOM and metabolic processes of the soil (Martínez-Espinosa, 2020). These enzymes, present in all intact and viable microbial cells, with soil microbial biomass can be considered sensitive indicators of damage to the biological properties of the soil (Bhowmik et al., 2019; Cardoso et al., 2013). In our study, the value of these enzymatic activities indicated high metabolic intensity of MBC in soil. The most abundant cations in soil were calcium, followed by magnesium (Table 1). Chloride and sulphate were the most abundant anions. In non-saline soil under lentils, cations and anions maintained the same trend of control soil over time (Table 2). In contrast, in presence of NaCl, in soil without and with lentils, sodium increased. Potassium maintained its value in soil without lentils, while in soil with lentils, decreased only under CAST and EST (Table 2). Calcium and magnesium were similar to control in soil without salinity beneath the different cultivars. In saline soils under all the cultivars magnesium unchanged, while calcium increased only in soil under Ustica and Eston (Table 2). Chloride decreased in presence of salinity under all the cultivars with respect to control. Sulphate was the lowest in CTR under salinity suggesting a slow release of this anion from soil due to its uptake by roots of the different cultivars (Table 2). Two-way ANOVA showed that salinity (NaCl) was the factor that mostly induced changes in sodium and calcium content and also in anions with the exception of nitrate, whose variation was not significant for any of the factors. Soil and soil–salt interaction caused significant variations in K and Mg contents.

TABLE 2 Cations and anions detected at the end of the experiment (9 months after lentil sowing) in soils without or with 100 mM NaCl

ID	Na^+	K^+	Mg^{++}	Ca^{++}	Cl^-	NO_3^-	SO_4^-	PO_4^-
Soil without NaCl								
CTR	0.070[a]	0.22[a]	0.10[a]	0.26[b]	0.20[a]	0.06[a]	0.006[a]	0.14[a]
CAST	0.075[a]	0.18[a]	0.11[a]	0.29[a]	0.20[a]	0.08[a]	0.007[a]	0.17[a]
PANT	0.034[c]	0.20[a]	0.08[a]	0.25[b]	0.19[a]	0.09[a]	0.011[a]	0.15[a]
UST	0.051[b]	0.19[a]	0.10[a]	0.23[b]	0.18[a]	0.08[a]	0.009[a]	0.14[a]
EST	0.073[a]	0.20[a]	0.11[a]	0.22[b]	0.15[b]	0.07[a]	0.007[a]	0.15[a]
Soil with NaCl								
CTR	2.4a	0.22a	0.10a	0.26b	3.7a	0.06a	0.06a	0.14c
CAST	0.26d	0.17b	0.09a	0.30b	0.22bc	0.08a	0.01b	0.45b
PANT	0.33c	0.21a	0.09a	0.27b	0.16c	0.09a	0.01b	0.52b
UST	0.43b	0.25a	0.14a	0.41a	0.27b	0.08a	0.01b	0.61a
EST	0.19e	0.11c	0.12a	0.44a	0.17c	0.09a	0.01b	0.58a
F-ratios								
NaCl	8092***	7*	n.s.	321***	3078***	n.s.	194***	3248***
Soil	3479***	32***	4*	37***	2995***	n.s.	121***	247***
NaCl × Soil	3413***	30***	3*	99***	2927***	n.s.	137***	240***
R^2	0.999	0.927	0.607	0.977	0.999	0.370	0.984	0.996

Note: CTR represents the soils free of lentil cultivation for both situations. The data are the mean of three replicates. Different letters in the same column and for the same treatment indicate significant differences at $p < .05$. Two-way ANOVA showed the effects of salinity, cultivars and their interaction (NaCl × lentils) on growth parameters.

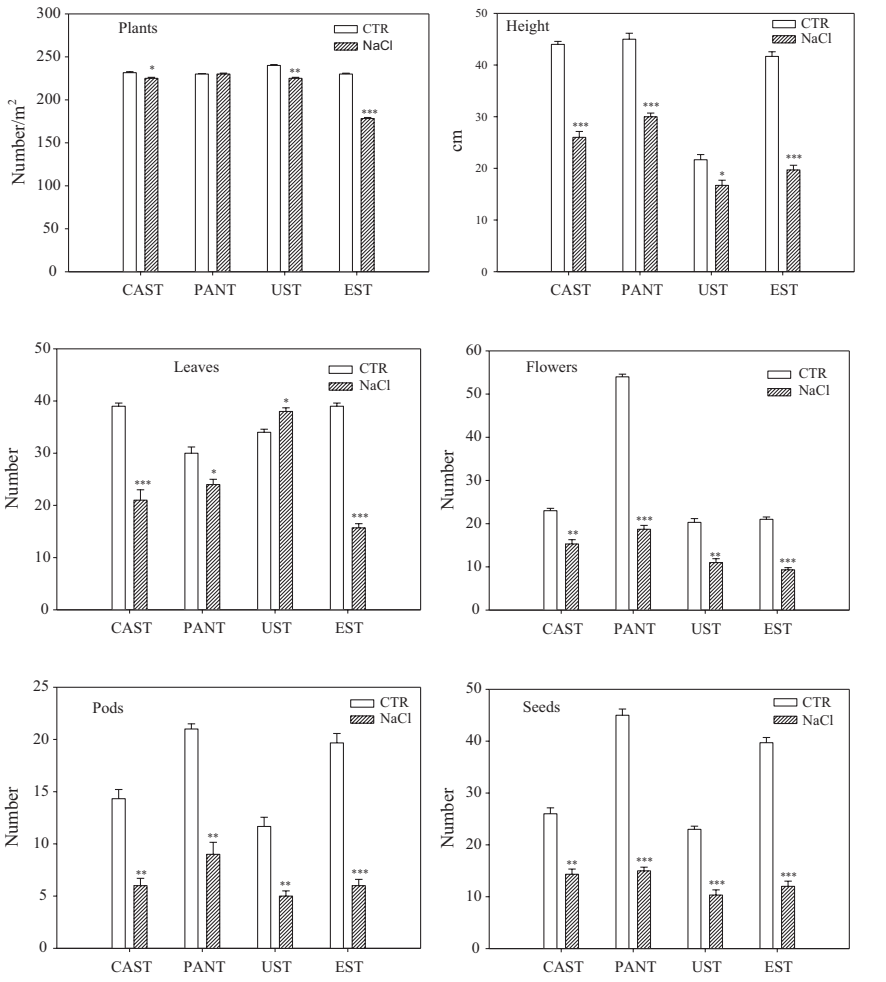

FIGURE 2 (A-F) Plant height and number, leaf, flower, pod and seeds number in Castelluccio (CAST), Pantelleria (PANT), Ustica (UST) and Eston (EST) cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR). Data represent the mean of 3 replicates ±SE. According to one-way ANOVA, asterisks (*) indicate significant difference between CTR and NaCl treatment (*$p \leq .05$; **$p < .01$; *** $p < .001$). Two-way ANOVA showed the effects of salinity, cultivars and their interaction (NaCl × Lentils) on growth parameters

	Plant number	Height	Leaf number	Flower number	Pod number	Seed number
R^2	0.993	0.987	0.982	0.994	0.965	0.990
F-ratios						
NaCl	628***	589***	348***	922***	331***	1121***
Lentils	324***	172***	57***	359***	27***	93***
NaCl× Lentils	248***	34***	120***	152***	8**	62***

3.3 | Plant growth parameters

Salinity reduced differently the growth parameters. The reduction was different in percentage and dependent on the cultivars. Plant numbers per square meter were mainly reduced for Eston (22%) and Ustica (−6.6%), while no change was observed for Pantelleria (Figure 2). Plant height decreased in presence of salinity, showing a 43% reduction in Castelluccio, 31% in Pantelleria, 22% in Ustica and 52% in Eston. For Ustica, unlike the other cultivars, a reduction in growth was opposed to an increase in the number of leaves in plants grown with NaCl. Under salinity, flower, pod and seed numbers per plant were the most inhibited growth parameters.

Statistical analysis confirmed that the growth parameters significantly differentiated the various cultivars regardless of the NaCl treatment (Figure 2). In any case, salinity induced the greatest inhibitory effect on plants (*F* ratios) and the interactions between NaCl and cultivars were less significant if compared to the effects individually considered. These results evidenced different physiological responses by the different cultivars. Numerous studies highlighted that the deleterious effects of salinity affected different physiological and metabolic processes of plants and the responses to these changes were different, dependent on species and cultivars and were expressed with different symptoms (Hussain et al., 2018; Hussain, Al-Shamsi, et al., 2020; Hussain, Elnaggar, et al., 2020; Parida & Das, 2005).

3.4 | Stress damage to plant metabolism

Numerous authors (Al-Dakheel & Hussain, 2016; Hussain et al., 2016; Panuccio et al., 2014; Pardo, 2010; Roy et al., 2014) related the damage to taking up high amount of salts by plants and the detrimental effects were ascribed to osmotic factor caused by an inhibition of water uptake by roots, and/or ionic specific, due to

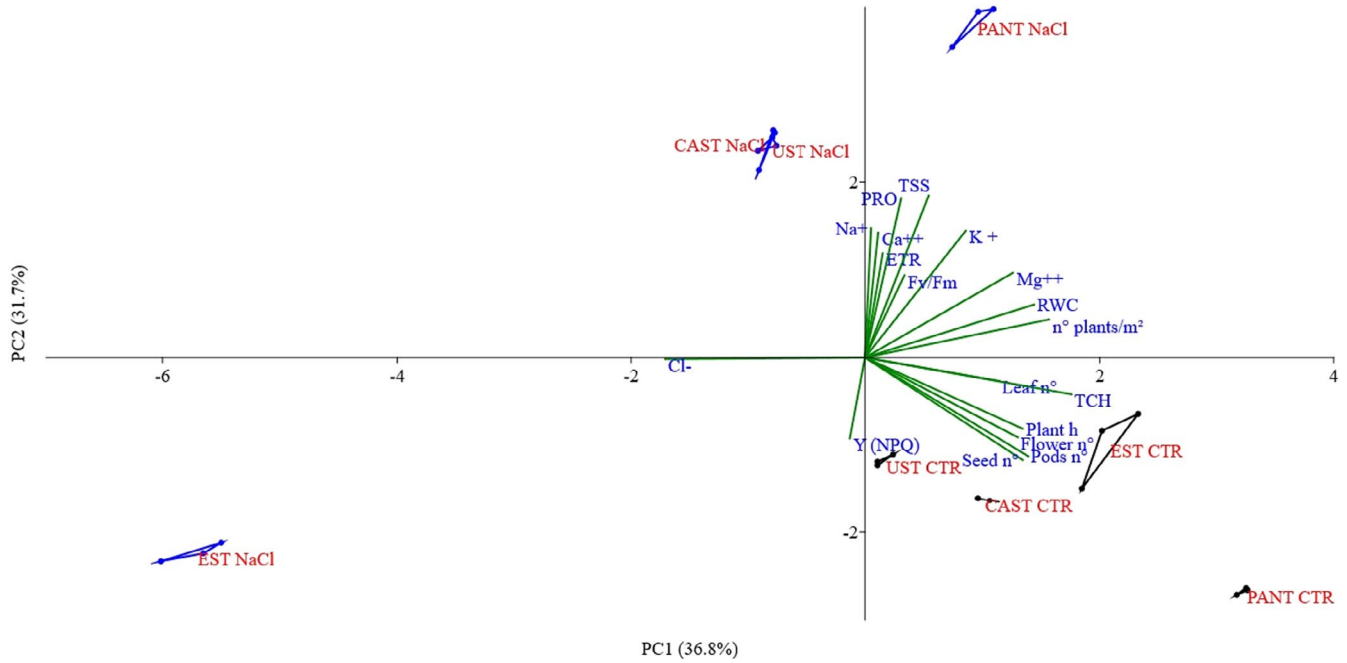

FIGURE 3 PCA (principal component analysis) diagram for Castelluccio (Cast), Pantelleria (Pant), Ustica (Ust) and Eston (Est) cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR)

the accumulation of toxic concentrations of sodium and/or chloride ions that induce ion imbalance (Panda & Khan, 2009). For their similar physicochemical characteristics (ionic radius and ion hydration energy), sodium can compete directly with potassium for K^+-binding sites causing severe metabolism damage. The uptake of other cations such as Ca^{2+} and Mg^{2+}, with well-known beneficial effects on plants (Huang et al., 2020; Tobe et al., 2002), can be also reduced by the passive Na^+ uptake into root cells at high soil salinity mediated by a family of non-selective cation channels (NSCCs family) (Blumwald et al., 2000; Kronzucker & Britto, 2011). In this study, the results on ion content showed that K^+, essential to plant growth and metabolism, was not significantly affected by salinity both in lentil root and shoot, except for Eston where a significant decrease (−50%) was observed only in shoot (Table 4). No significant changes in Mg^{2+}content were observed between salinity-grown and non-salinity–grown lentil cultivars, both in root and shoot (Table 4). Conversely, salinity increased calcium amount in shoot and root of Pantelleria and Castelluccio and in shoot of Ustica. Calcium concentration decreased significantly in Eston shoots (−43%) in salinity condition (Table 4). Salinity can induce a quick and transient enhancement of free cytosolic Ca^{2+} (Pottosin & Shabala, 2014), which acts as a second messenger linking numerous extracellular stimuli with different intracellular responses, all leading to re-establishment of ion homeostasis (Conde et al., 2011). Our results evidenced that EST was the cultivar most affected by salinity that inhibited the absorption and use of calcium and potassium, causing nutritional imbalances in the plants. This is also widely displayed in Figure 3, where NaCl-grown EST is located in the lower left quadrant (negative weight), opposite to all selected total ions. Considering that Ca^{++} and K^+ are macronutrients with specific and important functions on plant physiology, their reduced availability under saline conditions can induce deficiency and potentiate the negative effect of such stress on EST growth and productivity. Data related to the total cations and anions contents (Figure 4), a part the significant increase in total cation and anion in each cultivar in the presence of NaCl, evidenced a different distribution between shoot and root. Unlike all other cultivars, salinity did not change cation content of UST root. The significant increases in cations in CAST and PANT shoot were mainly due to Na^+ and Ca^{++} accumulation (Table 3). Under salinity, total anion content doubled in UST, PANT and EST (Figure 4). The most significant increases in anion amount were observed in EST and UST shoot due to sodium accumulation and in PANT root mainly for increase in sulphate concentration (Table 3). Important are the mechanisms that regulate ion homeostasis while mediating osmotic adjustment through the accumulation and intracellular compartmentation of ions that have very high concentrations in the external environment (Niu et al., 1995).

Bioconcentration factor, bioaccumulation coefficient and translocation factor gave a better view on how the ions were uptaken, transported and accumulated in the different lentil cultivars. The bioconcentration factor, defined as the root uptake of ions from the circulant soil solution (ion root/ion soil), showed an increase in potassium, calcium and magnesium concentration in roots of CAST and PANT and a reduction in root of Eston (Table 4). As regards the anions, Cl^- concentration increased in root of lentils grown with salts, except for CAST, while sulphate significantly decreased in all cultivar roots (Table 4). Bioconcentration factors showed a strong increase in phosphate concentrations in roots and this could be due to the fact that in saline soil or in soil irrigated with saline water, in the presence of sodium salts, the phosphate

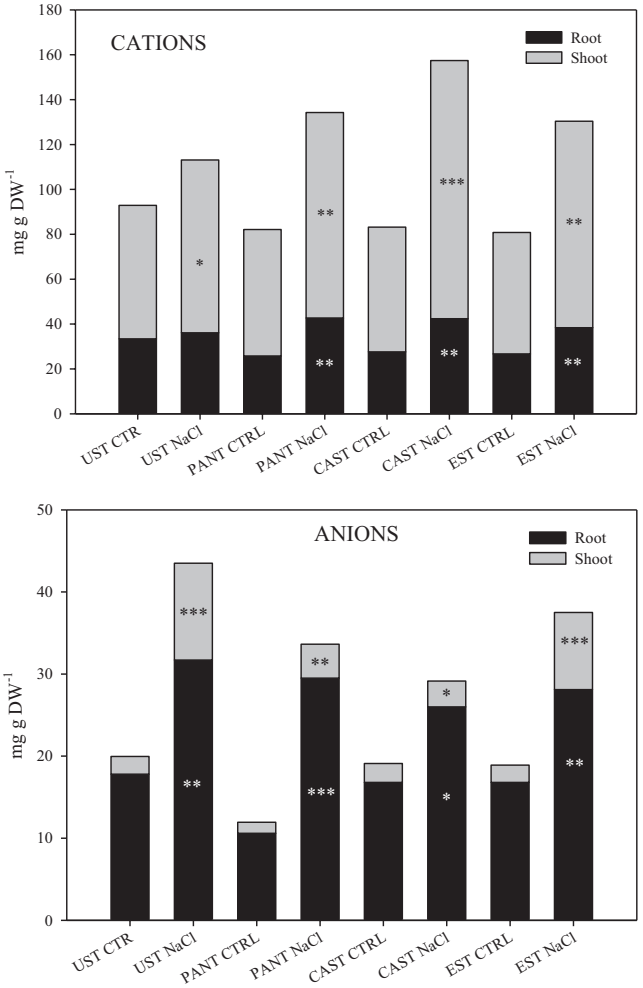

FIGURE 4 Total cations and anions in root and shoot of Castelluccio (CAST), Pantelleria (PANT), Ustica (UST) and Eston (EST) cultivated for 9 months in soil equilibrated with NaCl (100 mM) in comparison with untreated plants (CTR). Significance: $^{*}p \leq .05$; $^{**}p < .01$; $^{***}p < .001$ according to one-way ANOVA

may exist as sodium phosphate salt, which is highly soluble and can be easily absorbed (Beji et al., 2017).

The bioaccumulation factor, which is the ratio between ion in shoot and ion in soil, evidenced in CAST and PANT an increase in sodium, calcium and magnesium. In EST and UST leaves, a significant decrease in calcium concentration was detected. The greatest bioaccumulation of anion was in EST for Cl^- (Table 5). Its less growth may be potentially ascribed to the toxic effect of Cl^- and to the contemporary decrease in other cations with well-known beneficial effects on plants. However, as reported by Maathuis (2014) and Geilfus (2018), ion toxicity due to Cl^- and Na^+ accumulation not occurs instantly, but only when the perception of actual changes in ionic concentration takes places at later stages of plant growth.

Translocation factor, which expresses the ability of the plant to split up the absorbed ions between leaves and roots, showed that, in the presence of NaCl, all the cultivars translocated sodium to leaves (Table 6). Eston was the cultivar that translocated less all the cations. These data suggested that in PANT, UST and CAST, the increased accumulation of inorganic ions (Na^+, Cl^- and K^+) is an useful mechanism to maintain leave osmotic and turgor pressure under saline conditions as halophytes do (Flowers et al., 1977; Glenn et al., 1999; Storey, 1995).

3.5 | Photosynthetic efficiency

The decrease in growth observed in many plants subjected to salinity stress is often associated with a decline in their photosynthetic capacity. Chlorophyll fluorometers method gives an insight into the health of the photosynthetic plant systems measuring the variable fluorescence of photosystem II (Ivanov & Bernards, 2016). Regarding the photosynthetic parameters, F_v/F_m is the ratio of variable fluorescence (F_v) over maximal fluorescence (F_m) and it is the most used indicator of the conversion efficiency of primary light energy and the maximal efficiency of PSII photochemistry (Adhikari et al., 2019; Hussain & Reigosa, 2011, 2017). Greater the plant stress, fewer open reaction centres are available, and lower is the F_v/F_m ratio (Moustakas et al., 2019; Oxborough, 2004). Our results evidenced the lowest value of F_v/F_m in salt-grown Eston. Conversely, an increase in F_v/F_m ratios was observed in the other cultivars grown with NaCl compared to their own controls (Table 7). Y(II) represents a measurement ratio of plant efficiency, indicates the quantity of energy used by the photosystem II under a steady-state photosynthetic lighting condition, and it is directly related to electron transport rate (ETR) and to plant carbon assimilation (Del Pozo et al., 2020; Moustakas et al., 2019). In the PCA diagram, the bottom left position of EST with NaCl, with respect to own control and to the other NaCl-treated cultivars (located in opposite quadrants), evidenced a sufferance state of this cultivar in the presence of salinity (Figure 3). Among the quenching measurements, qP gives an indication of the proportion of PSII reaction centres that are open and represents an intrinsic/maximum efficiency of PSII, while qN estimates the heat dissipation (Ruban, 2016). The lower values of these two coefficients in Eston grown with NaCl with respect to own control and to the other NaCl-treated cultivars suggested that, in this cultivar, an inhibition of potential activity of PSII occurred (Hussain & Reigosa, 2011). NPQ reflects heat dissipation of light energy in the antenna system to avoid the photodamage and it is considered the most important short-term photoprotective process in higher plants. NPQ values increased in all cultivars grown under salinity, except for Eston, pointing out the occurrence of an oxidative damage of the photosynthetic apparatus for this plant. These data agreed with the total chlorophyll content (TChl) that was lower in salt-grown Eston than in the other salt-grown cultivars. Chlorophyll reflects plant photosynthetic capacity and its amount is one of the most important indicators of salt tolerance in plant (Li et al., 2018). The loss of TChl is usually accompanied by the inactivation of photochemical reactions, especially those mediated by PSII in plants exposed to salt stress (Ghassemi-Golezani et al., 2020). In this study, a strong decrease in TChl was observed in Eston, no decrease in PANT, while a slight

TABLE 3 Cation and anion in root and shoot of Castelluccio (CAST), Pantelleria (PANT), Ustica (UST) and Eston (EST) cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR)

Shoot						Root							
ID	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	NO_3^-	SO_4^-	PO_4^-
CAST CTR	0.45b^{e}	2.16a^{d}	52b^{f}	1.08a^{d}	2.1b^{d}	3.0a^{c}	2.2b^{b}	19b^{c}	3.6a^{a}	2.3b^{d}	Nd	13.2b^{d}	0.2a^{a}
CAST NaCl	3.38a^{b}	2.55a^{b}	108a^{a}	1.06a^{d}	3.3a^{c}	3.3a	3.3a^{a}	33a^{a}	3.6a^{a}	3.5a^{c}	Nd	21.1a^{a}	0.23a^{a}
PANT CTR	0.34b^{f}	2.62b^{b}	52b^{f}	1.31b^{c}	1.3a^{e}	3.6b^{b}	2.6b^{b}	15b^{d}	3.5a^{a}	1.3b^{e}	nd	8.4b^{e}	0.12b^{b}
PANT NaCl	8.25a^{a}	3.61a^{a}	78a^{c}	1.77a^{a}	1.4a^{e}	8.2a^{a}	3.5a^{a}	25a^{b}	3.2a^{b}	4.7a^{b}	nd	22.6a^{a}	0.19a^{a}
UST CTR	0.40b^{e}	2.00a^{d}	56b^{e}	1.11b^{d}	2.2b^{b}	2.7a^{d}	2.0a^{c}	25a^{b}	3.7a^{a}	2.2b^{d}	nd	15.7a^{c}	0.11a^{b}
UST NaCl	3.18a^{b}	2.32a^{c}	70a^{d}	1.48a^{b}	4.6a^{d}	3.1a^{c}	2.3a^{b}	25a^{b}	3.7a^{a}	12a^{a}	nd	18.8a^{b}	0.15a^{b}
EST CTR	0.55b^{d}	2.21a^{cd}	88a^{b}	1.30a^{c}	1.5b^{e}	2.1b^{e}	2.0a^{c}	19a^{c}	3.5a^{a}	2.1b^{d}	nd	14.1a^{d}	0.13a^{b}
EST NaCl	1.41a^{c}	1.13b^{e}	50b^{f}	1.25a^{c}	10a^{a}	3.0a^{c}	1.9a^{c}	19a^{c}	2.8b^{b}	12a^{a}	nd	15.1a^{c}	0.15a^{b}

Note: Data are the mean of three independent experiments. Values in the same column with different superscript letters are significantly different. Different upper-case letters in the same column refer to differences within each cultivar (Tukey's test at $p < .05$).

ID	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	SO_4^-	PO_4^-
CAST CTR	4b^{cd}	12a^{b}	68b^{c}	34a^{c}	12a^{e}	76a^{c}	4b^{d}
CAST NaCl	13a^{b}	15a^{a}	110a^{a}	40a^{b}	15a^{d}	28b^{f}	23a^{a}
PANT CTR	10b^{b}	13b^{b}	64b^{c}	43b^{b}	15b^{d}	56a^{d}	11b^{c}
PANT NaCl	25a^{a}	17a^{a}	96a^{b}	58a^{a}	29a^{c}	44b^{e}	19a^{a}
UST CTR	5a^{c}	9a^{c}	108a^{a}	37a^{bc}	12b^{e}	107a^{a}	1b^{e}
UST NaCl	7a^{c}	9a^{c}	60b^{d}	27b^{d}	44a^{b}	29b^{f}	15a^{b}
EST CTR	2a^{d}	13a^{b}	95a^{b}	31a^{d}	10b^{e}	93a^{b}	2b^{e}
EST NaCl	11a^{b}	10a^{c}	56b^{d}	29a^{d}	59a^{a}	26b^{f}	15a^{b}

TABLE 4 Bioconcentration factors (cation root/cation soil and anion root/anion soil) of Castelluccio (CAST), Pantelleria (PANT), Ustica (UST) and Eston (EST) cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR)

Note: Data are the mean of three independent experiments. Different letters in the same column indicate significant differences at $p < .05$.

TABLE 5 Bioaccumulation coefficient (cation shoot/cation soil and anion shoot/anion soil) of Castelluccio (CAST), Pantelleria (PANT), Ustica (UST) and Eston (EST) cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR)

ID	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-
CAST CTR	6b^{c}	12a^{c}	179b^{f}	9a^{c}	10b^{d}
CAST NaCl	13a^{b}	15a^{a}	360a^{a}	12a^{c}	15a^{d}
PANT CTR	10b^{b}	13b^{b}	208b^{e}	16a^{b}	15a^{d}
PANT NaCl	25a^{a}	17a^{a}	286a^{b}	19a^{a}	29b^{c}
UST CTR	8a^{bc}	11a^{cd}	245a^{c}	11a^{c}	12b^{d}
UST NaCl	7a^{c}	9a^{d}	170b^{f}	9a^{c}	44a^{b}
EST CTR	8a^{bc}	1b^{cd}	229a^{d}	12a^{c}	10b^{d}
EST NaCl	7a^{c}	11a^{cd}	201b^{e}	10a^{c}	59a^{a}

Note: Data are the mean of three independent experiments. Values in the same column with different superscript letters are significantly different. Different upper-case letters in the same column refer to differences within each cultivar (Tukey's test at $p < .05$).

TABLE 6 Translocation factors (cation shoot/cation root) of Castelluccio (CAST), Pantelleria (PANT), Ustica (UST) and Eston (EST) cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR)

ID	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-
CAST CTR	0.15b^{d}	1a^{a}	2.7b^{b}	0.27a^{c}	0.87a^{b}
CAST NaCl	1.0a^{b}	1a^{a}	3.3a^{a}	0.27a^{c}	0.94a^{a}
PANT CTR	0.09b^{e}	1a^{a}	3.4a^{a}	0.37a^{b}	1a^{a}
PANT NaCl	1.0a^{b}	1a^{a}	3.2a^{a}	0.37a^{b}	0.97a^{a}
UST CTR	0.13b^{d}	1a^{a}	2.2b^{d}	0.30a^{c}	1a^{a}
UST NaCl	2.3a^{a}	1a^{a}	2.8a^{b}	0.28a^{c}	1a^{a}
EST CTR	0.3a^{c}	1a^{a}	2.5a^{c}	0.37a^{b}	0.75b^{c}
EST NaCl	0.5a^{c}	0.5b^{b}	2.6a^{c}	0.43b^{a}	0.83a^{bc}

Note: Data are the mean of three independent experiments. Values in the same column with different superscript letters are significantly different. Different upper-case letters in the same column refer to differences within each cultivar (Tukey's test at $p < .05$).

decrease was detected in Cast and UST grown with salt. It is known that salt-tolerant plants increase or unchanged their chlorophyll content, whereas salt-sensitive plants lower their chlorophyll levels (Ashraf & Harris, 2013; Stepien & Johonson, 2009). Chlorophyll decrease is the result of slow synthesis or fast breakdown of the pigments in cells (Ashraf, 2003) due to a shift of metabolic pathway for producing osmolytes to contrast the osmotic stress created by salts. In CAST, UST and PANT, an increase in proline (Figure 5) was observed under saline stress (Hasegawa et al., 2000). In addition to its role as compatible osmolyte, proline can act as an enzyme

TABLE 7 Photosynthetic parameters of Castelluccio (CAST), Pantelleria (PANT), Ustica (UST) and Eston (EST) cultivated for 9 months in soil equilibrated with NaCl (100 mM) and without NaCl (control, CTR)

ID	UST CTR	UST NaCl	CAST CTR	CAST NaCl	PANTT CTR	PANT NaCl	EST CTR	EST NaCl
F_v/F_m	0.61^{c}	0.63^{b}	0.63^{b}	0.65^{a}	0.60^{c}	0.65^{a}	0.65^{a}	0.60^{c}
Y (II)	0.27^{c}	0.33^{a}	0.24^{d}	0.32^{a}	0.24^{c}	0.27^{c}	0.30^{b}	0.25^{d}
Y (NPQ)	0.36^{a}	0.23^{b}	0.24^{b}	0.33^{a}	0.26^{b}	0.23^{b}	0.33^{a}	0.34^{a}
Y (NO)	0.33^{a}	0.22^{b}	0.25^{b}	0.35^{a}	0.24^{b}	0.23^{b}	0.31^{a}	0.25^{b}
NPQ	0.24^{c}	0.28^{b}	0.22^{c}	0.31^{a}	0.23^{c}	0.29^{b}	0.32^{a}	0.20^{c}
qN	0.47^{c}	0.69^{a}	0.45^{c}	0.61^{a}	0.44^{c}	0.48^{c}	0.55^{b}	0.50^{b}
qP	0.55^{a}	0.59^{a}	0.38^{c}	0.52^{a}	0.39^{c}	0.41^{c}	0.55^{a}	0.50^{b}
qL	0.39^{a}	0.38^{a}	0.25^{d}	0.32^{b}	0.29^{d}	0.28^{d}	0.33^{b}	0.35^{b}
ETR	26.1^{ab}	28.4^{a}	25.2^{b}	25.2^{b}	24.3^{c}	27.1^{ab}	27.3^{ab}	24.9^{b}

Note: Data are the mean of three independent experiments. Different letters, in the same row, indicate significant differences at $p < .05$.

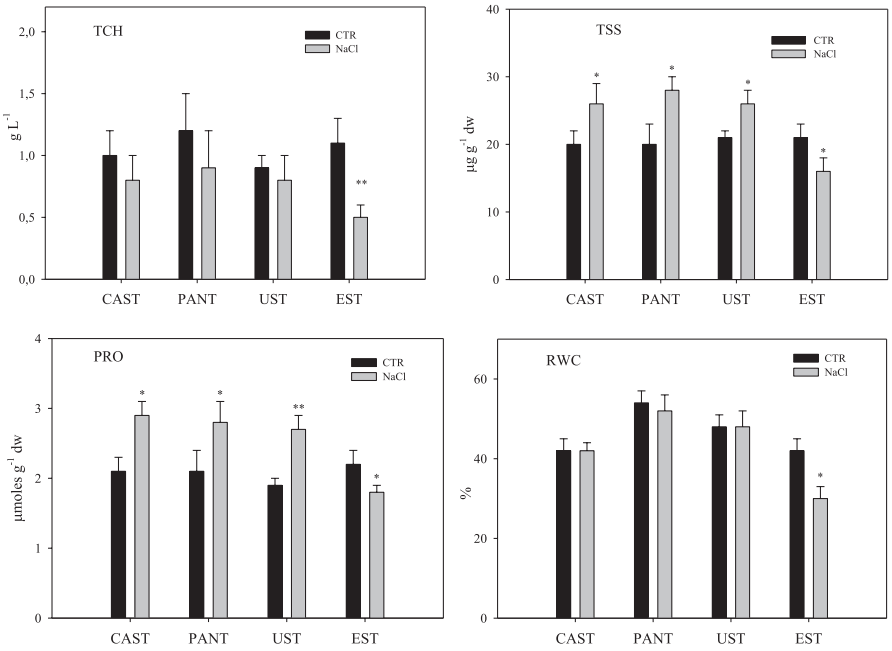

FIGURE 5 Changes in total chlorophyll content (TCH, g L^{-1}), total soluble sugar (TSS, µg g^{-1} dw), proline content (PRO, µmoles g^{-1} dw) and relative water content (RWC %) in lentils cultivated for 9 months in soil with NaCl (100 mM) or without NaCl (control, CTR). Data are the mean of 3 independent experiments ±SE. According to one-way ANOVA, asterisks (*) indicate significant difference between CTR and NaCl treatment within each cultivar (*$p \leq .05$; **$p < .01$; *** $p < .001$)

protectant, free radical scavenger, cytosolic pH and cell redox balancer (Verbruggen & Hermans, 2008). Therefore, a great accumulation of proline could have contributed to a better growth of CAST, UST and PANT under salinity in comparison with EST. The accumulation of soluble sugars, in CAST, UST and PANT (Figure 5), not only represented an energetic reserve in stress conditions but, due to the high water-binding capacity of their hydroxyl groups, also allows the plants with the possibility to resist the physiological drought caused by salts (Zhao et al., 2020). RWC data confirmed this assumption, being lower in salt-grown EST compared to the other cultivars (Figure 5). No differences were observed in RWC content between control and saline-grown CAST, UST and PANT, indicating no loss of leaf turgor. CAST, PANT and UST were the cultivars with high water content, suggesting that, beyond the well-known osmolytes, also calcium and potassium could serve as osmoregulatory helping plants to keep water against the high osmotic soil potential, as already demonstrated by Cochrane and Cochrane (2009).

4 | CONCLUSIONS

In short, our results confirm that variations in salt tolerance exist, and the degree of salt tolerance can vary with plant species and also within a species. PCA of lentil genotypes revealed diverse grouping pattern. The separation on the basis of PC1 and PC2 revealed that the genotypes were scattered in all the quarters, which show the high level of genotypic variation among the cultivar. Lentils to adapt and grow in different saline environment have brought into play different physiological and biochemical mechanisms in order to survive in soils with high salt concentration. The mechanisms were different and specific for each cultivar. The principle salt tolerance mechanisms in PANT included, but not limited to, ion uptake, transport and accumulation. PANT was the cultivar that mostly accumulated sodium in shoot and root and used it in addition to proline as osmoregulatory. UST accumulated less sodium

and calcium than PANT both in shoot and root but more chlorine in root, and enhanced also the production of the osmoregulators. CAST accumulated less sodium but more calcium and sulphates than the other two resistant cultivar, producing at the same time also osmolytes. Thus, salt tolerance of these three cultivars can be ascribed to an adaptative response mechanism caused by the environmental conditions they have been in contact with for a long time. The preference for sodium and/or chlorine of PANT and UST can depend on the fact that they are islander and therefore prevalently in contact with sodium and chlorine, while CAST originates from central Italy in soil where calcium and sulphate are the most abundant elements. In short, these results allow to put in light that Ustica, Pantelleria and Castelluccio native to a central/southern Italian semiarid environment can be cultivated in soils where salinity is one of the most significant constraint and temperatures are high and typical of the Mediterranean environment. Salinity tolerance is too complex to be easily amenable to improvement through selection as a trait in itself, but traits that are hypothesized to contribute to salinity tolerance can be highlighted and identified using molecular genetics tools and genomics. All these three cultivars can be also used in breeding programmes and for genetic manipulations to ameliorate the tolerance to salinity of other sensitive legumes or vegetal species.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

DATA AVAILABILITY STATEMENT

The data are available from the corresponding author upon a reasonable request.

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