Soil salinity improves nutritional and health promoting compounds in three varieties of lentil (*Lens culinaris* Med.).

Running Title: Salinity improves lentil quality

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Abstract

Lentils are salt sensitive low cost, high-quality protein crop, cultivated in many part of the world. In this work lentils were cultivated using increased soil salinity conditions to evaluate the nutritive and bioactive compounds of the edible part of different lentil varieties. Growth and nutritive properties of each local variety were compared to its own control (lentils cultivated in no saline soil, <4 dS/m) and to the same local variety sold in the market. Salinity improved nutritive properties (proteins, minerals, ascorbic acid, tocopherol, carotenoids, flavonoids and total phenols) and led to the synthesis of dimeric and trimeric cyanidins. Additionally, the antioxidant capacity assays (ABTS, DPPH, FRAP and ferrozine) of the edible seeds, all showed increases for all three varieties. . In conclusion, these three lentil varieties can be cultivated, on marginal lands including semi-arid areas where soil salinity can reach 8 dS/m which is beneficial where water is scarce or has a high salinity. At the same time the functional properties of the final product may be improved. The resistant varieties might be used in breeding programs to develop salinity resistant lentil cultivars with high nutritive values.

Keywords: cyanidins; lentil; *Lens culinaris*

Introduction

There is the need to increase the production of vegetable foods because vegan and vegetarian populations are increasing (Food Revolution Network, 2018), shifting the focus from starch-based crops such as corn and wheat to more plant-based protein crops such as soybeans and other legumes (Manners & van Etten, 2018). The European Union has also committed to reducing greenhouse gases by decreasing the amount of animal products and increasing the production of protein crops (Gerber et al., 2013; Van der Spiegel et al., 2013). Lentil represents a low cost high-quality protein source for many people and they are cultivated around the world. However, the adaptability and productivity of food legumes such as lentil (*Lens culinaris,* Med.) is becoming more limited due to adverse environmental conditions. Among the environmental stresses, salinity is one of the main factors limiting lentil productivity in Mediterranean and Eastern Asia countries (Katerji et al., 2001). Studies have shown the effects of salt stress on seed emergence and early seedling growth of different lentil genotypes (Asgharipour & Rafiei, 2011; Muscolo et al., 2007; Muscolo et al., 2015; Ouji, et al., 2015; Panuccio et al., 2011; Sidari et al., 2008). Few studies evaluated the growth and the productivity of lentils in fields with high salinity (e.g., 5 dS/m), as well as the changes in lentil grain quality with salinity (Rameshwaran et al., 2016). Muscolo et al. (2007), Muscolo et al. (2015) and Sidari et al. (2008) identified three cultivars, naturally resistant to salinity, named Ustica (UST), Pantelleria (PAN), and Castelluccio di Norcia (CAST) native to southern and central Italy and evaluated the metabolic and phenotypic traits related to drought and/or salinity stress tolerance in comparison to a commercial variety native to Canada named Eston. Their results showed a relationship between imposed stress and performance of the cultivars, showing variations in salinity tolerance along the life cycle of each variety. According to the germination frequencies, the cultivar ranking was as follows for salinity tolerance: PAN > UST > CAST > EST, while for salinity tolerance the ranking was CAST ≈ UST > PAN ≈ EST. Whereas Eston, the most widespread commercial variety, is sensitive to salinity, there is a need to select genotypes naturally resistant to stress, to avoid genetic manipulation that could negatively affect lentil nutritional values or soil microbiota. Based on the

above considerations, lentils were cultivated in salt conditioned soils and compared to those grown in un-salinized soils for the whole vegetative cycle to verify their ability to produce seeds in the salinized environment, and to determine the quality of these edible seeds. The aim of this work was to evaluate if salinity affected the nutritive values and phytochemicals of the edible part of the different lentil accessions. Growth and nutritive properties of each local variety were compared to its own control (lentil cultivated in un-salinized soil) and to the same local variety sold in the market.

1. Materials and methods

2.1. Chemicals

Metaphosphoric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), NaOH, nitroblue tetrazolium, dichlorophenol-indophenol (DCPID), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxyl acid (Trolox), phenazine methosulphate, ethanol, gallic acid, ethylenediaminetetraacetic acid (EDTA), ferrozine, 2,4,6-tris(2-piridil)-s-triazina (TPTZ) and iron sulphate heptahydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and acetic acid were HPLC-grade and were purchased from Merck (Darmstadt, Germany). All the phenolic standards were obtained from Extra Syntheses (Genay, France). Solvents and reagents for carotenoid detection were purchased from Panreac (Barcelona, Spain). Other chemicals were of analytical grade purchased from Carlo Erba Reagents s.r.l. (Cornaredo, MI, Italy).

2.2. Plant material and stress treatment

Three varieties of lentils, the accessions PAN and UST, native and cultivated in a protected area (National Park of Pantelleria island and volcanic soils of Ustica island) of small islands close to Sicily (southern Italy) and CAST a local population cultivated in the Umbria region (central Italy),

were sown in October 2016 in pots (30 cm diameter) filled with sandy-loam soil (11.85% clay, 23.21% silt, and 64.94% sand). Soil was taken from Motta San Giovanni, Loc. Liso, Italy (LAT: 38°0'15"12 N; LONG: 15°41'45"24 E). The irrigation was carried out with distilled water (control) or with 80 mM NaCl to result in salt stress conditions. During the whole experiment (9 months) the moisture was kept at ~ 70% of field capacity by supplying fresh water (Kirkham, 2014). At the end of the experiment (June 2017), lentil seeds were collected and stored in a refrigerator at 4°C until analysis (28 days). Salt stressed seeds were compared to control seeds and authenticated seeds of the same varieties sold in the supermarket (Errera s.r.l, Pantelleria, Italy and Pagliuzzo s.r.l, Ustica, Italy).

2.3. Sample preparation

A fixed amount (1.5 g) of all seeds were ground using a mortar and pestle to a fine powder and sifted with a 0.5 mm sieve. The powder was stored at 4°C, until the preparation of the extracts (28 days).

2.4. Preparation of ethanol and water extracts

The extracts were obtained using the method described by Kang (2015) with some modifications. Briefly, 250 mg were weighed and three different samples extracted at room temperature (22 to 25°C) with continuous stirring for 90 min with 15 mL 95% ethanol . The samples were immediately centrifuged (Unicen 21 RT167, Ortoalresa Inc., Madrid, Spain) at 2,370 x g (4000 rpm) for 15 min and the supernatants were filtered with 1 mm Whatman 185 filter paper (Merck), evaporated to dry-ness in a rota-vapour (Diagonal condenser RE 400, Stuart Equipment, ST 15, OSA) and re-suspended in a final volume of 3.0 mL 95% ethanol.

One hundred mg were weighed and extracted at room temperature with continuous stirring for 60 min with 2.0 mL dH_20 (Intercontinental Mod still 3/ES, Bioltecnical Service, s.n.c., Rome, Italy). The samples were then centrifuged at 590 x g (2000 rpm) for 10 min and the supernatants were fil-

tered with Whatman 1 filter paper and used for the determination of protein, carbohydrates and ferrous chelating activity.

2.5. Determination of proteins

Soluble protein was determined using the Bradford method (1976). Coomassie Brilliant Blue G-250 was used (1/100 v/v) for color development (20 min). The absorbance of each sample was measured at 595 nm using a 1800 UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan). Bovine serum albumin >99% purity (Sigma) was used and soluble proteins were estimated as BSA equivalents/g fw. Crude protein was determined using the Kjeldahl method (AOAC, 1990). One g of powder of lentils was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing one copper catalyst tablet (1.5% $CuSO_4.5H_2O$ + 2% Se) (Cu-Se, Sigma Aldrich) in a heat block (DK Series Kjeldahl Digestion Units, DK6, Velp Scientifica, Usmate, MB, Italy) at 420°C for 2 hr. After cooling, dH_2O was added to the hydrolysate samples before neutralization and titration. The amount of crude protein in the samples was calculated as nitrogen x 5.4 (Mariotti et al., 2008). All reagents were purchased from Sigma Chemical Co.

2.6. Detection of reducing sugars and total available carbohydrates

The dinitro-salicylic reagent was used, for reducing sugar analysis, using the method of Miller (1959). Briefly, 100 µL of water extract were added to 1000 µL 3,5-dinitrosalicylic acid reagent and brought to a final volume of 1500 µL with dH_20. The mixtures were heated at 100°C in a water bath for 15 min and were cooled at room temperature in an ice bath for 10 min. The absorbance of the samples was measured at 540 nm and quantified using a calibration curve of glucose (0-0.67 mg/mL) to obtain the glucose equivalents/100 g fw.

The total available carbohydrates were measured using the anthrone method with minor modifications (Hedge & Hofreiter, 1962). The samples (0.1 g) were pre-treated with 5.0 mL 52% $HClO_4$ and stored for 18 hr in the dark. Distilled water was added and the samples were filtered using Whatman

1. Finally the volume of the filtrate was adjusted to 10 mL. Filtrate (100 µL) was added to 0.1% anthrone solution in 73% H_2SO_4 and brought to a final volume of 5.0 mL. Samples were boiled for 10 min, cooled at room temperature and the absorbance was measured at 630 nm. The amount of available carbohydrates was calculated using a glucose calibration curve (range of 10–100 mg/mL). The results were expressed as glucose equivalents/100 g fw.

2.7. Determination of total phenolic compounds, ascorbic acid, total carotenoids, total flavonoids and vitamin E.

Total phenols were measured using the Folin-Ciocalteu assay (Velioglu et al., 1998) with a few changes. Ethanol extracts (0.04 mL) were added to 0.1 mL of Folin-Ciocalteu reagent. The flasks were vortexed (MIX ARGOlab, Vortex Mixer, GIORGIO BORMAC s.r.l, Modena, Italy) and incubated at room temperature for 10 min. Finally, 20% Na_2CO_3 solution was added and left at 40°C for 20 min in a water bath, with intermittent shaking. The absorbance of the samples was measured at 760 nm. Phenol was expressed as mg/100 g fw on the basis of a standard curve obtained with gallic acid (0-200 mg/L).

For ascorbic acid determinations, the method of Davies and Masten (1991) was used. Lentil powders (0.10 g) were extracted with 10 mL of 3% meta-phosphoric acid - 7.98% acetic acid, centrifuged at 2,370 x g (4000 rpm) for 10 min and the supernatant was used for the determination of ascorbic acid.

For vitamin E analysis, lentil powders (0.10 g) were extracted with 10 mL of hexane:isopropanol solution (3:2 v/v), with agitation for 5 hr, and centrifuged at 1,330 x g (3000 rpm) for 10 min. The supernatant was used for the determination of vitamin E (Prieto et al., 1999). Hexane-isopropanol extract (0.05 mL) was mixed with 0.5 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 37 °C for 90 min with shaking and then the absorbance measured at 695 nm. Quantification of vitamin E and of other reducing species was based on the molar absorption coefficient of vitamin E ($4 \times 10^3\ M^{-1}\ cm^{-1}$)

Flavonoids were estimated using the aluminium chloride colorimetric method of Djeridane et al. (2006). Diluted extract (1 mL) was mixed with 2% $AlCl_3$ methanolic solution (1 mL). After incubation at room temperature for 15 min, the absorbance was measured at 430 nm. Flavonoids were expressed as rutin (R) E/g dw on the basis of a calibration curve obtained with R.

For total carotenoids, lentil powders (0.10 g) were extracted with 10 mL of hexane:isopropanol solution (3:2 v/v), with agitation, for 5 hr, centrifuged at 148 x g (1000 rpm) for 10 min. The supernatant was collected and kept in the dark, and the pellet extracted for an additional two times with hexane-isopropanol solution. The supernatants were dried using nitrogen and diluted with 500 µL tetrahydrofuran. The absorbance of the solution was measured at 450 nm and total carotenoid content was in µg/g dw (Zhang et al., 2014).

2.8. Mineral assay

Cations (Na, K, Ca, Mg) were extracted from seeds and analysed using ion chromatography (DIONEX ICS-1100, Thermo Fisher Scientific Waltham, MA, USA). One g of dry material was ashed at 550°C for 6 hr in a porcelain capsule. The ash was then acidified for 30 min at 100°C using 1M HCl solution (10 mL). Finally, it was filtered using Whatman 1 and measured using the ion chromatograph with 20 mM methane-sulfonic acid as eluent. Fe concentration was determined using atomic absorption spectrophotometry (model 2380, Perkin Elmer Co., Waltham, MA, USA). The amount of each cation was calculated using its own standard curve. P was measured using ion chromatography (DIONEX) and comparing the results with a multi ion cation standard curve (Multi Ion cation IC standard solution, Specpure® , Dionex) (Anon, 1990). All solvents and reagents were purchased from Panreac (Barcelona, Spain).

2.9. Antioxidant activity detection

The method of Blois (1958) was used to determine the DPPH$^{\bullet}$ scavenging assay. DPPH$^{\bullet}$ concentration in the cuvette has been chosen to give absorbance values of ~1.0. The reaction mixtures were

composed of: 10 μL of each extract, 700 μL DPPH• and 95% ethanol brought to 1.0 mL. A blank of each sample, without ethanol extract, was used. The change in absorbance of the violet solution was measured at 517 nm after 30 min of incubation at 37°C. The inhibition (%) of radical scavenging activity was calculated using the following equation:

$$Inhibition(\%) = \frac{A_O - A_S}{A_O} \times 100 \quad (1)$$

where A_O is the absorbance of the control and A_S is the absorbance of the sample after 30 min of incubation. DPPH activity was expressed as μM of Trolox (T) E using a calibration curve (1.0 to 50 μM T).

FRAP was carried out using the method of Benzie and Strain (1996) with minor modification. Daily fresh prepared working FRAP reagent: 300 mM acetate buffer (20 mL) pH 3.6, 10 mM TPTZ solution in 40 mM HCl (2 mL) and 20 mM $FeCl_3 \cdot 6H_2O$ (2.0 mL) was warmed at 37°C. The initial absorbance of this reagent was measured. Sample (50 μL) was added to 1500 μL solution and the absorbance was measured at 593 nm after 4 min. T (1−100 μM) was chosen as a reference compound and activity expressed as μmol TE/μL of extract.

The ABTS assay (TE antioxidant capacity assay TEAC) was done according to Pellegrini et al. (1999) with a few modifications. Solutions of 7 mM of ABTS (final concentration) and 2.45 mM ammonium persulfate (final concentration) in phosphate buffered saline (PBS) were mixed and left in the dark at room temperature for 12–16 hr. Before use, the absorbance of $ABTS^{+\bullet}$ solution was fixed at 0.70 ± 0.02 at 734 nm, and diluted if necessary with PBS. Aliquots of ethanol extract (25, 50 and 100 μL) were added to 0.5 mL of $ABTS^{+\bullet}$ solution and bring to the final volume of 600 μL with PBS. After 6 min of incubation in the dark at room temperature the absorbance of the samples was measured at 734 nm. The inhibition (%), of radical scavenging activity was calculated as:

$I (\%) = 100 \times (A_0 - A_S)/A_0$

where A_0 is absorbance of the control and A_S is absorbance of the sample after 4 min of incubation. TEAC activity was expressed as µM TE using a reference curve (in the range from 1.0 to 50 µM) of T.

The ferrozine-based colorimetric assay which measures chelation ability of the ethanol extract was done using the method of Dorman et al. (2003) with a few modifications. Water extract (50 µL) was added to a solution of 200 µL 0.2 mM $FeSO_4$ and 200 µL 0.5 mM ferrozine. The samples were shaken and left for 10 min at room temperature. Finally, the absorbance at 562 nm was measured. The inhibition (%) of the ferrozine-Fe^{2+} complex formation was obtained using the following equation:

$$\% \text{ Inhibition} = [(Ac - As)/Ac] \times 100 \quad (2)$$

where Ac is the absorbance of the control and As is the absorbance of the samples in the presence of the extracts. All reagents were purchased from Sigma Aldrich Chemical Co.

2.10. RP-DAD-HPLC identification of flavonoid components.

Reverse phase-diode array detector-high performance liquid chromatography (RP-DAD-HPLC) analyses of samples were carried out with a Shimadzu system (Kyoto, Japan), consisting of a LC-10AD pump system, a vacuum degasser, a quaternary solvent mixing, a SPD-M10A diode array detector and a Rheodyne 7725i injector. Separation of each compound was done on a 250 x 4.6 mm i.d., 5 µm Discovery C18 column, supplied by Supelco Park (Bellefonte, PA, USA) equipped with a 4.0 x 20 mm guard column. The column was placed in a column oven set at 25°C. The injection loop was 20 µL, and the flow-rate was 1.0 mL/min. The mobile phase consisted of a linear gradient of solvent A (acetonitrile) in 2% acidified water (acetic acid:H_2O, 2:98) as follows: 0–80% (0–55 min), 90% (55–70 min), 95% (70–80 min), 100% (80–90 min) and 0% (90–110 min). UV-Vis spectra were measured between 200 and 600 nm, and simultaneous detection using a diode array at 278 and 325 nm. Compounds were measured using their retention time and UV spectra (Dueñas and Estrella, 2002), through comparison with purified standards (Sigma Chemical Co).

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244 *2.11. Statistical analysis*

245 Analysis of variance was carried out for all the data sets. One-way ANOVA with Tukey's Honestly
246 Significant Difference test were carried out to analyze the effects of salinity on each of the various
247 parameters measured. Correlation among antioxidant compounds and antioxidant activities of the
248 tree lentil varieties was analyzed. SYSTAT 13.2, Inc. Richmond, CA USA – Powerful Statistical
249 Analysis and Graphics Software for Windows 7, was used for all the statistical analyses. Effects
250 were significant at $p \leq 0.05$.

3. Results

3.1. Effects of salinity on minerals and nutrients in lentil seeds

Sodium was significantly more abundant in the seeds of salt-grown lentils (Table 1). Calcium, magnesium, iron and phosphorous were found in the greatest amount in the grains of salt-grown-lentils (Table 1). In salt-treated UST a greater amount of calcium in comparison with their own corresponding commercial and control variety was detected, while in salt treated CAST magnesium was the most abundant cation ($p \leq 0.05$) (Table 1). Results showed a significant increase in crude proteins in the grains of all the salt-grown lentils compared to the lentil grown without salt and to the corresponding commercial one (Table 2).The highest values ($p \leq 0.05$) were found in the grains of NaCl-treated CAST and UST. On the other hand, the soluble proteins, decreased in the salt-grown lentils compared to the commercial ones (Table 2). This data suggested a better conservation of proteins during the soaking procedure that precedes cooking. The amount of total carbohydrates was similar in all samples, while free glucose increased in UST and CAST salt treated seeds with respect to CTR (Table 2). Ascorbic acid increased significantly in the NaCl-grown lentils and the greatest amount was observed in UST in which the ascorbic acid doubled with respect to its own control and its corresponding commercial sample (Table 3). In salt-grown CAST and PAN the ascorbic acid was higher than their corresponding commercial sample. Vitamin E was significantly high in the edible seeds of lentils grown with NaCl and the greatest increase, compared to the corresponding commercial and control variety, was observed in CAST (Table 3). Carotenoids, total phenols and total flavonoids were significantly more abundant in the grains of all the salt-grown lentils with respect to the commercial sample (Table 3). In comparison to the commercial lentils, carotenoids tripled in the grains of CAST and UST, total phenols and flavonoids doubled in all the varieties (Table 3).

3.2. Effect of salinity on antioxidant activities in lentil seeds

The results of DPPH, ABTS, FRAP and Ferrozine assays, showed that the edible seeds of lentils grown in saline stressed soil had the highest antioxidant activities. ABTS and DPPH activities were the highest in NaCl treated UST followed by PAN and CAST (Fig. 1A-B). The percentages of inhibition for ABTS were 59% in UST and 54% in CAST and PAN grains (Fig. 1A). According to the ability to inhibit ABTS radical formation, also the ability to scavenge DPPH was present with percentages of 12.6% (corresponding to 2.8 μM TE) in UST, 10.2% (corresponding to 2.3 μM TE) in PAN and 6.5% (corresponding to 1.5 μM TE) in CAST (Fig. 1B). Ferrozine activity was the highest in UST and CAST with 40% I, followed by PAN with 28% I (Fig. 1C). FRAP increased much more in the edible seeds of salt-grown PAN than UST and CAST with respect to their own commercial variety (Fig. 1D). FRAP values ranged from 375 μm TE g^{-1} in the seeds of commercial UST to 620 μm TE g^{-1} in the seeds of NaCl grown UST, from 214 μm TE g^{-1} in the seeds of commercial CAST to 502 μm TE g^{-1} in the seeds of NaCl-grown CAST, and from 72 μm TE g^{-1} in the seeds of commercial PAN to 484 μm TE g^{-1} in the seeds of NaCl grown PAN (Fig. 1D).

Correlation analysis showed relationships between each individual antioxidant compound and the antioxidant capacities measured in the seeds of salt-grown, control (grown without salt) and commercial lentils. Significant specific and positive correlations were observed between the antioxidant compounds and antioxidant capacities with significant differences among the three cultivars. In seeds of NaCl grown Ustica, a significant positive correlation was observed between flavonoids and DPPH activity and also between ascorbic acid and vitamin E with FRAP, ABTS and Ferrozine activities. Moreover in seeds of NaCl grown CAST, a positive correlation between ascorbic acid and vitamin E with FRAP, DPPH and Ferrozine was seen. In seeds from PANT NaCl treated-plants, carotenoids and flavonoids were positively correlated to FRAP and DPPH antioxidant activities, while phenols were correlated with DPPH and Ferrozine (Table 5). In the edible seeds of salt grown lentils the majority of antioxidant activities were significantly and positively correlated with the antioxidant compounds in comparison with commercial and control lentils (Table 5).

3.3 RP-HPLC-DAD separation and identification of compounds.

A higher number of compounds were found in the ethanol extracts of seeds of all the NaCl-grown lentils compared to those grown in the same soil without salts or to the corresponding commercial varieties (Table 4). Regardless of the treatments, in the seeds of all lentils, 3,4,5-trihydroxybenzaldehyde, cyanidin dimers, catechin, trans p-coumaric acid and trans-resveratrol-5-glucoside were found. In particular, dimeric cyanidins, at different retention times, and epicatechin, was found only in the salt-grown Castelluccio di Norcia, while trimeric cyanidins at different retention times were present only in the seeds of the commercial, control and salt grown PAN and UST (Table 4).

4. Discussions

Lentils have functional characteristics suggesting their involvement in nutritional, health-promoting and disease-preventing effects. They are a biological source of high quality proteins, complex carbohydrates (resistant starches, oligosaccharides and dietary fibers), carotenoids and vitamins C and E (Ganesan & Xu, 2017). The amounts of these substances observed in the commercial seeds of the three landraces conformed to the data reported in literature for other lentil cultivars (Urbano et al., 2007). Salt-grown lentils led to more proteins to contrast osmotic stress unlike other crops which preferentially led to sugars or other molecules as osmolytes (Nadeem et al., 2019). These results are consistent with results of Amini et al. (2007) that showed in tomato seedlings a greater production of proteins in response to salt stress. Additionally, Klessig and Malamy (1994) showed that plants generated activated proteins as signal transducers or messengers after environmental stresses. This represents a nutritional advantage because vegetable higher in proteins are now recommended. The increase in calcium, magnesium, phosphorous and iron is related to the tendency of plants to accumulate ions as chemical osmo-regulators and to avoid the toxic effects that result from chloride accumulation (Chen & Jang, 2010). Additionally, Kudo et al. (2010) suggested that cations increased with salinity to balance the excess of external sodium, thus their increase can be considered

329 an index of Na tolerance in glycophytes. The enhancement of the above mentioned nutrients confers
330 to treated lentils an additional nutritional value because minerals have important roles in human
331 health such as maintaining blood pressure, fluid and electrolyte balance, and bone health (Abram &
332 Atkinson, 2003). These minerals are also involved in making new cells and in delivering oxygen to
333 cells contributing to the normal muscle and nerve functioning. Additionally, Ca^{2+} increased in salt
334 grown lentils because it is one of the important ubiquitous second messengers in signal transduction
335 pathways and usually its concentration increases in response to external stimuli, including stress
336 signals (Tuteja, 2007). Kurusu et al. (2013) and Choi et al. (2014) showed that plant hyperosmotic
337 sensors were coupled with Ca^{2+} channels. Within sec after exposure to NaCl, a rapid increase in
338 Ca^{2+} was the result of searching for a new ion balance. Physiological and epidemiological evidence
339 showed a possible direct correlation in humans between regular lentil ingestion and colon cancer
340 prevention, post-prandial glycemic response, blood pressure, cholesterolemic and lipid lowering ef-
341 fects along with reducing the incidence of type-2 diabetes (Aslani et al., 2015; Shahwar et al.,
342 2017). These properties have been linked to the specific composition of this legume, high in phyto-
343 chemical compounds. Phenolic compounds, flavonoids and carotenoids increased significantly in
344 the salt-grown lentils in comparison with the corresponding commercial and control ones. The in-
345 crease in antioxidant compounds in salt-treated lentils is due to salinity that changes the biosynthe-
346 sis of primary and secondary metabolites in plants as already shown in fennel (Bettaieb Rebey et al.,
347 2017), in coriander (Neffati et al., 2010), in black fennel (Bourgou et al., 2010), in sweet majorana
348 (Baatour et al., 2012) and in maize (Panuccio et al., 2018). The enhancement of ascorbic acid, vita-
349 min E, carotenoids, total phenols and total flavonoids, with stress conditions, contributed to natural-
350 ly improving the nutritional value of lentils. Recent research (Aqil et al., 2013; Huyut et al., 2017;
351 Panuccio et al., 2016) indicated that phenolic compounds have effective antioxidant properties, and
352 their beneficial effects were attributed to their reducing power and free radicals scavenging. Kiokias
353 et al. (2008) and Anbudhasan et al. (2014) showed that carotenoids and flavonoids have the capaci-
354 ty to improve food quality and stability, terminating free radical chain reactions in biological sys-

355 tems, providing additional health benefits to consumers, with the reduction of chronic diseases (e.g.,
356 cardiovascular disease, hypertension, diabetes, and cancer) (Zhao et al., 2014). Epidemiological
357 studies were consistent with the previous statement, showing that the oral supplementation of toma-
358 to extract (rich in carotenoids and lycopene) significantly controlled the risk of hyperlipidemia,
359 CVD, metabolic syndrome by regulating several physiological phenomenon such as the reduction
360 of blood pressure, and decreased low density lipoprotein oxidation and hypertension (Di Pietro et
361 al., 2016). The increase in phytochemicals with antioxidant properties in salt stressed lentils in-
362 creased in turn the defense systems necessary to detoxify or prevent the detrimental effect of the in-
363 creased production of ROS that occurs with stress conditions. This was expressed in the antioxidant
364 capacity of seeds produced in saline conditions, in which the ability of avoiding or scavenging the
365 different radical species was significantly higher and diversified in such a way as to eliminate all
366 types of free radicals.
367 The increases in antioxidant compounds and antioxidant activities in all the lentil varieties treated
368 with NaCl, showed that the correlation among the antioxidant compounds and antioxidant activities
369 was specific and significant. In the ethanol extracts of lentils a greater amount of dimeric and tri-
370 meric cyanidins in salt treated lentils was detected. Cyanidins, the most common type of anthocya-
371 nidins are antiaging agents and have been linked to a variety of health benefits (Khoo et al., 2017),
372 such as 1) anticancer effect, for the ability to inhibit the growth of human HT-29 colon cancer cells,
373 to increase the expression of tumor suppression genes (p21WAF1 and p27KIP1) and to decrease of
374 cyclooxygenase-2 gene expression (Malik et al., 2003), 2) cardio-protective effects, inhibiting plate-
375 let aggregation (*in vitro* antithrombotic properties), and decreasing the susceptibility to ischemia-
376 reperfusion injury and infarct size with increased myocardial antioxidant enzymes (Toufektsian et
377 al., 2008; Zheng & Zhang, 2017). The increase of antioxidative compounds and the appearance of
378 new cyanidins in the edible part of lentils grown with salinity suggested that salinity represented a
379 source of stress that activates the secondary metabolism of plants, as generally occurs in organisms
380 subjected to biotic and abiotic stresses, causing an overproduction of phytochemicals with positive

effects on human health. The greatest number of bioactive compounds appeared in Castelluccio di Norcia, the landrace that always showed a greater salinity tolerance with better adaptive biological and phenotypic traits in comparison with the other two varieties (Muscolo et al., 2015).

5. Conclusions

Even if lentil is considered a legume sensitive to salinity, three salt resistant varieties have been identified. Although, NaCl decreased lentil productivity in terms of growth and seed production (-20%) the results showed that salinity was able to increase their nutritional values such as proteins, vitamin C and E and antioxidant compounds increasing the reaction with free radical and the ability to inhibit the oxidation processes. NaCl favored the synthesis of different secondary metabolites with multiple effects on different target organs, with the advantage of protecting, at the same time, the human body against various diseases ranging from specific types of cancer to cardiovascular diseases. These three lentil varieties can be cultivated, without compromising the quality of their final product, in marginal lands of the Mediterranean and semi-arid areas where, for water scarcity and/or low quality water, soil can reach a salinity of 8 dS/m.

Declaration of competing interest

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

Acknowledgments

This research was supported by the Mediterranea University of Reggio Calabria Programmi di Ricerca Scientifica. No outside funding was received.

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406 **References**

407 Abram, S. A. & Atkinson, S. A. (2003). Calcium, magnesium, phosphorus and vitamin D fortifica-
408 tion of complementary foods. *The Journal of Nutrition*, 133, (9), 2994S–2999S.

409 Amini, A., Ehsanpour, A., Hoang, Q. T. & Shin, J. S. (2007). Protein pattern changes in tomato un-
410 der *in vitro* salt stress. *Russian Journal of Plant Physiology*, 54, 464–471.

411 Anbudhasan, P., Surendraraj, A., Karkuzhali, S. & Sathishkumaran, P. (2014). Natural antioxidants
412 and its benefits. *International Journal of Food and Nutritional Sciences*, 6, 225–232.

413 Anonymous. (1990). Recommended practice for chemical analysis by ion chromatography. Austral-
414 ian Standard AS 3741, Sidney, Australia.

415 AOAC (1990). Official Methods of Analysis, Vol. 1, 15th ed. pp. 342. Arlington, VA, USA: Asso-
416 ciation of Official Analytical Chemists.

417 Aqil, F., Munagala, R., Jeyabalan, J. & Vadhanam, M. V. (2013). Bioavailability of phytochemicals
418 and its enhancement by drug delivery systems. *Cancer Letters*, 334(1), 133–141.

419 Asgharipour, M. R., & Rafiei, M. (2011). Effect of salinity on germination and seedling growth of
420 lentils. *Australian Journal of Basic and Applied Sciences*, 5(11), 2002-2004.

421 Aslani, Z., Mirmiran, P., Alipur, B., Bahadoran, Z. & Farhangi M. A. (2015). Lentil sprouts effect
422 on serum lipids of overweight and obese patients with type 2 diabetes. *Health Promotion Per-*
423 *spectives*, 5, 215–224. doi: 10.15171/hpp.2015.026.

424 Bâatour, O., Mahmoudi, H., Tarchoun, I., Nasri, N., Kaddour, R. M., Wissal, A., Hamdaoui, G.,
425 Lachaâl, M. & Marzouk, B (2012). Salt effect on phenolics and antioxidant activities of Tunisian
426 and Canadian sweet marjoram (*Origanum majorana* L.) shoots. *Journal of Science Food and*
427 *Agriculture*, 93, 134–141.

Bettaieb Rebey, I., Bourgou, S., Marzouk, B., Fauconnier, M.l. & Ksouri, R. (2017). Salinity impact on seed yield, polyphenols composition and antioxidant activity of fennel (*Foeniculum vulgarae* Mill) extracts. *Journal of New Sciences, Agriculture and Biotechnology*, CSIEA, 3, 2610–2619.

Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical, *Nature*, 181, 1199-1200.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72, 248–254.

Bourgou, S., Bettaieb, I., Saidani, M. & Marzouk, B. (2010). Fatty acids, essential oil and phenolics modifications of black cumin fruit under NaCl stress conditions. *Journal of Agriculture and Food Chemistry,* 58, 12399–12406.

Chen , H. & Jiang, J-G. (2010). Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environmental Reviews,* 18, 309-319.

Choi, W. G., Toyota, M., Kim, S. H., Hilleary, R. &, Gilroy S. (2014). Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. *Proceeding of the National Academy of. Science U.S.A.* 111, 6497–6502. 10.1073/pnas.1319955111.

Davies, S. H. R. & Masten, S. J. (1991). Spectrophotometric method for ascorbic acid using dichlo-rophenolindophenol: Elimination of the interference due to iron. *Analytica Chimica Acta,* 248, 225-227.

Di Pietro N., Di Tomo P. & Pandolfi A. (2016). Carotenoids in cardiovascular disease prevention. *SM Atherosclerosis,* 1(1):1–13.

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P. & Vidal, N. (2006). Antioxi-dant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry*, 97, 654–660.

Dorman, H. J. D. Kosar, M., Kahlos, K., Holm, Y. & Hiltunen, R. (2003). Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *Journal of Agricultural & Food Chemistry,* 51, 4563–4569.

454 Dueñas, M. & Estrella, T. H. I. (2002). Phenolic composition of the cotyledon and the seed coat of
455 lentils (*Lens culinaris* L.). *European Food Research Technology,* 215, 478–483.
456 Food Revolution Network (2018), Food Revolution Network, accessed 25 May 2018
457 <https://foodrevolution.org/blog/vegan-statistics-global/>
458 Gerber, P. J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. & Tem-
459 pio, G. (2013). Tackling climate change through livestock – A global assessment of emissions
460 and mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO),
461 Rome
462 Ganesan, K. & Xu, B. (2017). Polyphenol-rich lentils and their health promoting effects. *Interna-*
463 *tional Journal of Molecular Science,* 18, 2390. DOI: 10.3390/ijms18112390.
464 Hedge, J. E. & Hofreiter, B. T. (1962).) Estimation of Carbohydrate In: Methods in Carbohydrate
465 Chemistry (Eds Whistler R L and Be Miller, J N) Academic Press, New York, 17–22.
466 Huyut, Z., Beydemir, S. & Gülçin, I. (2017). Antioxidant and antiradical properties of selected fla-
467 vonoids and phenolic compounds. *Biochemical Research International*, 7616791. Doi:
468 10.1155/2017/7616791.
469 Kang, H. W. (2015). Antioxidant activity of ethanol and water extracts from lentil (*Lens culinaris*
470 Medik). *Journal of Food and Nutrition Research*, 3(10), 667–669.
471 Katerjia, N., van Hoornb, J. W., Hamdy A., Mastrorilli, M., Oweise T., Erskine, W. (2001). Re-
472 sponse of two varieties of lentil to soil salanity. *Agricultural Water Management,* 47, 179–190.
473 Klessig D. F. & Malamy J. (1994). The salicylic acid signal in plants. *Plant Molecular Biology,* 26,
474 1439–1458.
475 Khoo, H. E., Azlan, A., Tang, S. T., Lim, S.M. (2017). Anthocyanidins and anthocyanins: Colored
476 pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food Nutrition*
477 *Research,* 61(1), 1361779 https://doi.org/10.1080/16546628.2017.1361779.

478 Kiokias, O., Varzakas, T. & Oreopoulou, V. (2008). *In vitro* activity of vitamins, flavonoids, and
479 natural phenolic antioxidants against the oxidative deterioration of oil-based systems. *Critical*
480 *Reviews in Food Science and Nutrition*, 48, 78–93.
481 Kirkham, M. B. (2014). Field capacity, wilting point, available water, and the nonlimiting water
482 range. In: Principles of Soil and Plant Water Relations (Second Edition), pp 598, Elsevier Inc.
483 Manhattan, USA, ISBN 978-0-12-420022-7.
484 Kudo, N., Sugino, T., Oka, M. & Fujiyama, H. (2010). Sodium tolerance of plants in relation to ion-
485 ic balance and the absorption ability of microelements. *Soil Science and Plant Nutrition,* 56,
486 225–233.
487 Kurusu, T., Kuchitsu, K., Nakano, M., Nakayama, Y. &, Lida, H. (2013). Plant mechanosensing
488 and Ca^{2+} transport. *Trends in Plant Science,* 18, 227–233. 10.1016/j.tplants.2012.12.002
489 Malik, M., Zhao, C., Schoene, N., Guisti, M. M., Moyer, M. P. & Magnuson, B. A. (2003). Antho-
490 cyanin-rich extract from *Aronia meloncarpa* E induces a cell cycle block in colon cancer but not
491 normal colonic cells. *Nutrition and Cancer,* 46(2), 186–96.
492 Manners, R. & van Etten, J. (2018). Are agricultural researchers working on the right crops to ena-
493 ble food and nutrition security under future climates? *Global Environmental Change*, 53, 183–
494 194.
495 Mariotti, F., Tomè, D. & Mirand, P. P. (2008). Converting nitrogen into protein beyond 6.25 and
496 Jones' factors. *Critical Review in Food Science and Nutrition*, 48(2),177–184..
497 Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Ana-*
498 *lytical Chemistry*, 31, 426–428.
499 Muscolo, A., Junker, A., Klukas, C., Weigelt-Fischer, K., Riewe, D. & Altman T. (2015). Pheno-
500 typic and metabolic responses to drought and salinity of four contrasting lentil accessions *Jour-*
501 *nal of Experimental Botany*, 66, (18), 5467–5480. doi: 10.1093/jxb/erv208.
502 Muscolo, A., Sidari, M., Santonoceto, C., Anastasi, U. & Preiti G. (2007). Response of four geno-
503 types of lentil to salt stress conditions. *Seed Science & Technology*, 35, 497–503.

Nadeem, M., Li, J., Yahya M., Wang M., Ali A., Cheng A., Wang X. & Ma, C. (2019). Grain legumes and fear of salt stress: Focus on mechanisms and management strategies. *International Journal of Molecular Science,* 20(4), 799. doi:10.3390/ijms20040799.

Neffati, M., Sriti, J., Hamdaoui, G., Kchouk, M. E. & Marzouk, B. (2010). Salinity impact on fruit yield, essential oil composition and antioxidant activities of *Coriandrum sativum* fruit extracts. *Food Chemistry*, 124, 221–225.

Ouji, A., El-Bok, S., Mouelhi, M., Ben-Younes, M. & Kharrat, M. (2015). Effect of salinity stress on germination of five Tunisian lentil (*Lens Culinaris* L.) genotypes. *European Scientific Journal,* 11, 21 ISSN: 1857 – 7881 (Print) e - ISSN 1857- 7431.

Panuccio. M. R, Logoteta, B., De Lorenzo, F., & Muscolo, A. (2011). Root plasticity improves salt tolerance in different genotypes of lentil (*Lens culinaris*), *Ecological Questions*, 14, 95-99.

Panuccio, M. R., Fazio, A., Papalia, T., & Barreca, D. (2016). Antioxidant properties and flavonoid profile in leaves of Calabrian *Lavandula multifida* L., an autochthon plant of Mediterranean southern regions *Chemistry and Biodiversity*, 13, 416–421.

Panuccio, M. R., Chaabani, S., Roula, R. & Muscolo, A. (2018). Bio-priming mitigates detrimental effects of salinity on maize improving antioxidant defense and preserving photosynthetic efficiency. *Plant Physiology and Biochemistry*, 132, 465–474.

Pellegrini, N., Re, R., Yang, M., Rice-Evans, C. A., & Lester, P. (1999). Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying the 2,2'-azobis(3-ethylenebenzothiazoline-6-sulfonic acid) radical cation decolorization assay. *Methods in Enzymology*, 299, 379–389.

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Analytical Biochemistry,* 1, 269(2), 337–341.

528 Rameshwaran, P., Qadir, M., Ragab, R., Arslan, A., Majid, G. & Abdallah, K. (2016). Tolerance
529 of faba bean, chickpea and lentil to salinity: Accessions salinity response functions. *Irrigation*
530 *and Drainage*, 65, 49–60.
531 Shahwar, D., Bhat, T. M.,. Ansari, M. Y. K., Chaudhary, S. & Aslam, R. (2017). Health functional
532 compounds of lentil (*Lens culinaris Medik*): A review. *International Journal of Food Proper-*
533 *ties*, 20, S1–S15, DOI:10.1080/10942912.2017.1287192.
534 Sidari, M., Santonoceto, C., Anastasi, U, Preiti, G. & Muscolo, A. (2008). Variations in four geno-
535 types of lentil under NaCl-salinity stress. *American Journal of Agricultural and Biological Sci-*
536 *ence*, 3, 410–416.
537 Toufektsian, M. C., de Lorgeril, M., Nagy, N., Salen, P., Donati, M. B., Giordano, L., Mock, H. P.,
538 Peterek, S., Matros, A., Petroni, K, Pilu, R., Rotilio, D., Tonelli, C., de Leiris, J., Boucher, F., &
539 Martin, C. (2008). Chronic dietary intake of plant-derived anthocyanins protects the rat heart
540 against ischemia-reperfusion injury. *Journal of Nutrition*, 138(4), 747–752.
541 Tuteja, N. (2007). Mechanisms of high salinity tolerance in plants. *Methods in Enzymology*, 428,
542 419–38.
543 Urbano, G., Porres, J. M., Frıas, J. & Vidal-Valverde, C. (2007). Chapter 5: Nutritional value. In:
544 Lentil: An Ancient Crop For Modern Times (Eds Yadav, S. S., McNeil, D. & Stevenson, P. C.)
545 Berlin, Springer, pp 4793.
546 Van der Spiegel, M., Noordam, M. Y. & van der Fels-Klerx, H. J. (2013). Safety of novel protein
547 sources (insects, microalgae, seaweed, duckweed, and rapeseed) and legislative aspects for their
548 application in food and feed production. *Comprehensive Reviews in Food Science and Food*
549 *Safety*, 12 (6), 662–678.
550 Velioglu, Y. S., Mazza, M., Gao, L. & Oomah, B. D. 1998. Antioxidant activity and total phenolics
551 in selected fruits, vegetables, and grain products. *Journal of Agriculture of Food Chemistry*
552 1998, 46, 4113–4117.

553 Zhang, B., Deng, Z., Tang, Y., Chen, P., Liu, R, Ramdath, D. D., Liu, Q., Hernandez, M. & Tsao,
554 R. (2014). Fatty acid, carotenoid and tocopherol compositions of 20 Canadian lentil cultivars and
555 synergistic contribution to antioxidant activities. *Food Chemistry,* 161, 296–304
556 Zhao, H., Zhang, H. & Yang, S. (2014). Phenolic compounds and its antioxidant activities in etha-
557 nolic extracts from seven cultivars of Chinese jujube. *Food Science and Human Wellness,* 3, (3–
558 4), 183–190.
559 Zheng, F. M. & Zhang, H. (2017). Phytochemical constituents, health benefits, and industrial appli-
560 cations of grape seeds: A mini-review. *Antioxidants*, 6(3), 71
561 https://doi.org/10.3390/antiox6030071.
562

563 Table 1 Sodium, potassium, calcium, magnesium, iron and phosphorous in the grains of Pantelleria (PAN) Ustica (UST) and Castelluccio di Norcia
564 (CAST) landraces. CTR (control without salinity), Com (commercial local variety), NaCl (lentils grown with 80 mM NaCl). The data are expressed
565 in mg/g dw. Different letters, in the same row, indicate significant differences at $p \leq 0.05$.

566

Minerals	**PAN CTR**	**PAN Com**	**PAN NaCl**	**UST CTR**	**UST Com**	**UST NaCl**	**CAST CTR**	**CAST Com**	**CAST NaCl**
Na^{+}	0.22 ± 0.01^{c}	0.17 ± 0.01^{d}	0.39 ± 0.02^{a}	0.26 ± 0.03^{b}	0.20 ± 0.02^{dc}	0.41 ± 0.02^{a}	0.17 ± 0.07^{d}	0.24 ± 0.03^{c}	0.22 ± 0.01^{c}
K^{+}	7.3 ± 0.1^{d}	8.0 ± 0.1^{b}	7.5 ± 0.2^{d}	6.9 ± 0.02^{e}	7.1 ± 0.03^{e}	7.0 ± 0.01^{e}	7.8 ± 0.01^{c}	9.4 ± 0.1^{a}	8.3 ± 0.04^{b}
Ca^{++}	1.2 ± 0.04^{c}	1.3 ± 0.1^{c}	2.0 ± 0.2^{a}	0.7 ± 0.01^{e}	0.7 ± 0.02^{e}	1.8 ± 0.1^{a}	1.1 ± 0.05^{d}	1.2 ± 0.03^{d}	1.7 ± 0.02^{b}
Mg^{++}	1.1 ± 0.1^{c}	1.0 ± 0.03^{d}	1.8 ± 0.03^{b}	1.0 ± 0.02^{d}	1.0 ± 0.01^{d}	1.7 ± 0.1^{b}	1.1 ± 0.03^{c}	1.3 ± 0.1^{c}	2.0 ± 0.02^{a}
Fe^{++}	0.075 ± 0.001^{b}	0.072 ± 0.001^{c}	0.080 ± 0.003^{a}	0.072 ± 0.001^{c}	0.075 ± 0.001^{b}	0.088 ± 0.002^{a}	0.070 ± 0.002^{c}	0.075 ± 0.001^{b}	0.090 ± 0.007^{a}
P	4.3 ± 0.02^{b}	4.4 ± 0.1^{b}	4.5 ± 0.01^{a}	4.0 ± 0.04^{d}	4.0 ± 0.04^{d}	4.2 ± 0.01^{c}	4.2 ± 0.1^{c}	4.3 ± 0.03^{b}	4.6 ± 0.1^{a}

Table 2. Proteins and carbohydrates in the grains of three lentil varieties. Different lower-case letters indicate significant differences among the different treatments within each variety. Different upper-case letters refer to differences among the varieties for the same treatment (Tukey's test, $p \leq 0.05$).

Samples	Crude Protein mg/100 g	Soluble Protein mg BSE equivalents/100 g	Total carboydrates g/100 g	Free glucose mg/100 g
UST				
CTRL	21 ± 0.2^{bA}	$(5.9\pm0.6)*10\text{^}^{2bB}$	41 ± 2^{aA}	0.49 ± 0.01^{cC}
NaCl	23 ± 0.1^{aA}	$(6.4\pm0.6)*10\text{^}^{2bB}$	42 ± 1^{aA}	0.73 ± 0.02^{aC}
Com	22 ± 0.1^{bA}	$(112\pm1)*10\text{^}^{2aA}$	42 ± 1^{aA}	0.66 ± 0.02^{bC}
CAST				
CTRL	21 ± 0.2^{cA}	$(6.7\pm0.3)*10\text{^}^{2bB}$	40 ± 1^{aA}	1.39 ± 0.02^{bB}
NaCl	23 ± 0.4^{aA}	$(6.5\pm0.2)*10\text{^}^{2bB}$	39 ± 1^{aA}	1.83 ± 0.02^{aA}
Com	22 ± 0.3^{bA}	$(9.4\pm0.8)*10\text{^}^{2aA}$	42 ± 1^{aA}	1.75 ± 0.01^{aA}
PAN				
CTRL	17 ± 0.4^{bB}	$(10\pm1)*10\text{^}^{2aA}$	39 ± 2^{aA}	1.65 ± 0.01^{aA}
NaCl	18 ± 0.3^{aB}	$(7.3\pm0.2)*10\text{^}^{2bA}$	41 ± 1^{aA}	1.44 ± 0.01^{bB}
Com	17 ± 0.4^{bB}	$(10\pm1)*10\text{^}^{2aA}$	42 ± 1^{aA}	1.43 ± 0.02^{bB}

573 Table 3. Antioxidant compounds in ethanol extracts of the grains of the three lentil varieties. Dif-
574 ferent lower-case letters indicate significant differences among the different treatments within each
575 variety. Different upper-case letters refer to differences among the varieties for the same treatment.
576 (Tukey's test, $p \leq 0.05$).

Samples	Ascorbic Acid mg/100 g	Vitamin E µg/g	Carotenoids µg/g	Total Phenols mg/100 g	Total Flavonoids mg/100g
UST					
CTR	7.2 ± 0.5^{bB}	30 ± 6^{cB}	95 ± 0.4^{bA}	208 ± 5^{aA}	180 ± 10^{aA}
NaCl	12 ± 1^{aA}	113 ± 1^{aA}	116 ± 1^{aA}	204 ± 2^{aB}	185 ± 5^{aB}
Com	6.6 ± 1.5^{bB}	80 ± 6^{bA}	32 ± 2^{cA}	130 ± 10^{bA}	99 ± 7^{bB}
CAST					
CTR	6.1 ± 1^{bC}	50 ± 2^{bA}	34 ± 1^{bB}	163 ± 3^{bB}	150 ± 10^{bB}
NaCl	8.6 ± 1^{aB}	81 ± 3^{aB}	103 ± 2^{aB}	216 ± 5^{aA}	190 ± 10^{aB}
Com	4.5 ± 1^{cC}	28 ± 5^{cB}	16 ± 2^{cB}	124 ± 1^{cA}	103 ± 4^{cB}
PAN					
CTR	8.8 ± 1^{bA}	45 ± 6^{bA}	12 ± 1^{abC}	220 ± 30^{aA}	172 ± 3^{bA}
NaCl	14 ± 1^{aA}	65 ± 6^{aC}	15 ± 2^{aC}	230 ± 10^{aA}	201 ± 3^{aA}
Com	7.9 ± 1^{bA}	33 ± 5^{cB}	10 ± 2^{bC}	131 ± 5^{bA}	115 ± 4^{cA}

577

578 **Table 4**. Compounds in the three lentil varieties were identified on the basis of their retention time

579 (Rt) by comparison with purified standards, for every one of these compounds.

580

Rt	Compounds	CAST COM	CAST CTR	CAST NaCl	PAN COM	PAN CTR	PAN NaCl	UST COM	UST CTR	UST NaCl
7.2	Gallic acid		*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
8.4	3,4,5-trihydroxybenzaldehyde	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
9.1	Cyanidin dimer	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
12.3	Cyanidin dimer			*X*						
12.4	Cyanidin dimer			*X*						
12.9	Cyanidin dimer			*X*						
13.1	Cyanidin dimer			*X*						
14.2	Cyanidin dimer			*X*						
14.5	Cyanidin dimer			*X*		*X*	*X*	*X*	*X*	*X*
16.0	Cyanidin B3		*X*	*X*	*X*				*X*	
17.8	Cyanidin B1			*X*	*X*					*X*
19.4	Cyanidin dimer			*X*			*X*			
19.8	Phenolic acid derivative		*X*	*X*						
22.1	Catechin	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
25.2	Cyanidin B2				*X*		*X*		*X*	*X*
26.4	Cyanidin trimer						*X*		*X*	*X*
28.1	Cyanidin trimer					*X*	*X*	*X*	*X*	*X*
29.2	Cyanidin trimer				*X*	*X*	*X*	*X*	*X*	*X*
31.7	Epicatechin	*X*	*X*	*X*						
32.7	Trans para-cumaric acid	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
38.1	Cyanidin dimer			*X*		*X*	*X*			*X*
39.5	Cyanidin dimer			*X*						
40.3	Trans-resveratrol-5-glucoside	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*

Table 5. Correlation among antioxidant compounds and antioxidant activities of the tree lentil varieties.

USTICA	FRAP	ABTS	DPPH	FERROZINE
CAROTENOIDS				
COMM	r = - 0.756 R^2 = 0.571	r = - 0.866 R^2 = 0.75	N.S.	r = - 0.803 R^2 = 0.645
CONTR	r = - 0.983 R^2 = 0.879	r = - 0.866 R^2 = 0.75	r = 0.756 R^2 = 0.571	r = 0.945 R^2 = 0.893
NaCl	r = - 0.814 R^2 = 0.662	r = - 0.971 R^2 = 0.942	N.S.	r = - 0.756 R^2 = 0.571
PHENOLS				
COMM	N.S.	r = - 0.998 R^2 = 0.996	r = - 0.910 R^2 = 0.825	N.S.
CONTR	r = 0.721 R^2 = 0.520	N.S.	r = - 0.954 R^2 = 0.911	r = - 0.736 R^2 = 0.541
NaCl	N.S.	N.S.	r= - 0.809 R^2 = 0.655	N.S.
FLAVONOIDS				
COMM	r = - 0.997 R^2 = 0.994	N.S.	N.S.	r = - 1.00 R^2 = 1.00
CONTR	N.S.	N.S.	r = - 0.965 R^2 = 0.930	r = - 0.711 R^2 = 0.505
NaCl	N.S.	N.S.	r = 1.00 R^2 = 1.00	N.S.
ASCORBIC ACID				
COMM	r = 0.983 R^2 = 0.965	N.S.	N.S.	r = 0.994 R^2 = 0.987
CONTR	r = - 0.984 R^2 = 0.969	r = - 1.00 R^2 = 1.00	N.S.	r = - 0.980 R^2 = 0.961
NaCl	r = 0.995 R^2 = 0.991	r = 0.961 R^2 = 0.923	N.S.	r = 0.982 R^2 = 0.964
VITAMIN E				
COMM	r = -- 0.982 R^2 = 0.965	N.S.	N.S.	r = -- 0.993 R^2 = 0.987
CONTR	r = 0.983 R^2 = 0.972	r = 1.00 R^2 = 1.00	N.S.	r = - 0.982 R^2 = 0.964
NaCl	r = 0.995 R^2 = 0.991	r = 0.961 R^2 = 0.923	N.S.	r = 0.982 R^2 = 0.964

584

CASTELLUCCIO	FRAP	ABTS	DPPH	FERROZINE
CAROTENOIDS				
COMM	r = - 0.8543 R^2 = 0.729	r = 0.786 R^2 = 0.617	r = 0.756 R^2 = 0.571	N.S.
CONTR	N.S.	r = - 0.803 R^2 = 0.647	N.S.	N.S.
NaCl	N.S.	N.S.	r = - 0.747 R^2 = 0.558	N.S.
PHENOLS				
COMM	r = - 0.963 R^2 = 0.928	r = 0.924 R^2 = 0.855	N.S.	r = - 0.836 R^2 = 0.699
CONTR	N.S.	N.S.	N.S.	N.S.
NaCl	r = - 0.772 R^2 = 0.596	N.S.	r = - 0.868 R^2 = 0.754	r = - 0.790 R^2 = 0.624
FLAVONOIDS				
COMM	N.S.	N.S.	r = - 0.866 R^2 = 0.750	N.S.
CONTR	N.S.	N.S.	N.S.	N.S.
NaCl	r = - 0.988 R^2 = 0.976	N.S.	r = - 1.000 R^2 = 1.000	r = - 0.992 R^2 = 0.968
ASCORBIC ACID				
COMM	r = - 0.950 R^2 = 0.902	r = 0.980 R^2 = 0.961	N.S.	r = - 1.000 R^2 = 1.000
CONTR	r = - 0.990 R^2 = 0.980	r = - 0.862 R^2 = 0.743	r = - 0.991 R^2 = 0.982	r = - 0.958 R^2 = 0.919
NaCl	r = 0.990 R^2 = 0.980	N.S.	r = 0.997 R^2 = 0.995	r = 0.997 R^2 = 0.995
VITAMIN E				
COMM	r = - 0.952 R^2 = 0.907	r = 0.982 R^2 = 0.964	N.S.	r = - 1.000 R^2 = 1.000
CONTR	r = 0.991 R^2 = 0.983	r = 0.867 R^2 = 0.752	r = 0.989 R^2 = 0.978	r = 0.961 R^2 = 0.924
NaCl	r = 0.999 R^2 = 0.997	N.S.	r = 0.979 R^2 = 0.958	r = 0.998 R^2 = 0.996

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PANTELLERIA	FRAP	ABTS	DPPH	FERROZINE
CAROTENOIDS				
COMM	N.S.	r = - 0.912 R^2 = 0.832	N.S.	r = 0.756 R^2 = 0.571
CONTR	r = - 0.991 R^2 = 0.983	N.S. .	N.S.	r = - 0.866 R^2 = 0.750
NaCl	r = 0.945 R^2 = 0.893	r = - 0.995 R^2 = 0.989	r = 0.900 R^2 = 0.810	N.S.
PHENOLS				
COMM	N.S.	r = 0.942 R^2 = 0.887	r = - 0.996 R^2 = 0.992	N.S.
CONTR	r = 0.930 R^2 = 0.865	N.S.	N.S.	r = 0.720 R^2 = 0.518
NaCl	N.S.	r = - 0.783 R^2 = 0.614	r = 0.948 R^2 = 0.899	r = 0.895 R^2 = 0.801
FLAVONOIDS				
COMM	r = - 0.982 R^2 = 0.964	N.S.	N.S.	r = 0.786 R^2 = 0.617
CONTR	r = 0.991 R^2 = 0.983	N.S.	N.S.	r = 0.866 R^2 = 0.75
NaCl	r = 0.945 R^2 = 0.893	r = - 0.995 R^2 = 0.989	r = 0.900 R^2 = 0.810	N.S.
ASCORBIC AC-ID				
COMM	r = - 1.00 R^2 = 1.00	N.S.	r = - 0.756 R^2 = 0.571	N.S.
CONTR	r = 0.924 R^2 = 0.855	r = 0.965 R^2 = 0.930	N.S	r = 1.00 R^2 = 1.00
NaCl	N.S.	N.S.	r = - 0.828 R^2 = 0.685	r = - 0.982 R^2 = 0.964
VITAMIN E				
COMM	r = 1.00 R^2 = 1.00	N.S.	N.S.	N.S.
CONTR	r = 0.924 R^2 = 0.855	r = 0.965 R^2 = 0.931	N.S.	r = 1.00 R^2 = 1.00
NaCl	N.S.	N.S.	r = - 0.827 R^2 = 0.685	r = - 0.982 R^2 = 0.9

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588 **Figure Captions**

589 **Figure 1** Antioxidant capacity (ABTS, DPPH, FRAP and Ferrozine) detected in the edible part
590 (seeds) of the three different lentils Ustica (UST), Pantelleria (PAN) and Castelluccio di Norcia
591 (CAST) grown in presence of NaCl, water (CTR) and commercial (Com). Different letters indicate
592 significant differences at $p \leq 0.05$.

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599 **Figure 1**

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