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

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

3 **Running Title: Salinity improves lentil quality**

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23 **Abstract**

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

37 **Keywords:** cyanidins; lentil; *Lens culinaris*

38

39 Introduction

40 There is the need to increase the production of vegetable foods because vegan and vegetarian popu-
41 lations are increasing (Food Revolution Network, 2018), shifting the focus from starch-based crops
42 such as corn and wheat to more plant-based protein crops such as soybeans and other legumes
43 (Manners & van Etten, 2018). The European Union has also committed to reducing greenhouse
44 gases by decreasing the amount of animal products and increasing the production of protein crops
45 (Gerber et al., 2013; Van der Spiegel et al., 2013). Lentil represents a low cost high-quality protein
46 source for many people and they are cultivated around the world. However, the adaptability and
47 productivity of food legumes such as lentil (*Lens culinaris*, Med.) is becoming more limited due to
48 adverse environmental conditions. Among the environmental stresses, salinity is one of the main
49 factors limiting lentil productivity in Mediterranean and Eastern Asia countries (Katerji et al.,
50 2001). Studies have shown the effects of salt stress on seed emergence and early seedling growth of
51 different lentil genotypes (Asgharipour & Rafiei, 2011; Muscolo et al., 2007; Muscolo et al., 2015;
52 Ouji, et al., 2015; Panuccio et al., 2011; Sidari et al., 2008). Few studies evaluated the growth and
53 the productivity of lentils in fields with high salinity (e.g., 5 dS/m), as well as the changes in lentil
54 grain quality with salinity (Rameshwaran et al., 2016). Muscolo et al. (2007), Muscolo et al. (2015)
55 and Sidari et al. (2008) identified three cultivars, naturally resistant to salinity, named Ustica (UST),
56 Pantelleria (PAN), and Castelluccio di Norcia (CAST) native to southern and central Italy and eval-
57 uated the metabolic and phenotypic traits related to drought and/or salinity stress tolerance in com-
58 parison to a commercial variety native to Canada named Eston. Their results showed a relationship
59 between imposed stress and performance of the cultivars, showing variations in salinity tolerance
60 along the life cycle of each variety. According to the germination frequencies, the cultivar ranking
61 was as follows for salinity tolerance: PAN > UST > CAST > EST, while for salinity tolerance the
62 ranking was CAST $\approx$ UST > PAN $\approx$ EST. Whereas Eston, the most widespread commercial variety,
63 is sensitive to salinity, there is a need to select genotypes naturally resistant to stress, to avoid genet-
64 ic 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⁺⁺), 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-pyridyl)-s-triazine (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 dryness 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 dH₂O (Intercontinental Mod still 3/ES, Bioltechnical 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-

116 tered with Whatman 1 filter paper and used for the determination of protein, carbohydrates and fer-
117 rous chelating activity.

118

119 *2.5. Determination of proteins*

120 Soluble protein was determined using the Bradford method (1976). Coomassie Brilliant Blue G-250
121 was used (1/100 v/v) for color development (20 min). The absorbance of each sample was measured
122 at 595 nm using a 1800 UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan). Bovine serum al-
123 bumin >99% purity (Sigma) was used and soluble proteins were estimated as BSA equivalents/g fw.
124 Crude protein was determined using the Kjeldahl method (AOAC, 1990). One g of powder of len-
125 tils was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing one copper catalyst
126 tablet (1.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 2% Se) (Cu-Se, Sigma Aldrich) in a heat block (DK Series Kjeldahl
127 Digestion Units, DK6, Velp Scientifica, Usmate, MB, Italy) at 420°C for 2 hr. After cooling, dH_2O
128 was added to the hydrolysate samples before neutralization and titration. The amount of crude pro-
129 tein in the samples was calculated as nitrogen x 5.4 (Mariotti et al., 2008). All reagents were pur-
130 chased from Sigma Chemical Co.

131

132 *2.6. Detection of reducing sugars and total available carbohydrates*

133 The dinitro-salicylic reagent was used, for reducing sugar analysis, using the method of Miller
134 (1959). Briefly, 100 μL of water extract were added to 1000 μL 3,5-dinitrosalicylic acid reagent and
135 brought to a final volume of 1500 μL with dH_2O . The mixtures were heated at 100°C in a water bath
136 for 15 min and were cooled at room temperature in an ice bath for 10 min. The absorbance of the
137 samples was measured at 540 nm and quantified using a calibration curve of glucose (0-0.67
138 mg/mL) to obtain the glucose equivalents/100 g fw.

139 The total available carbohydrates were measured using the anthrone method with minor modifica-
140 tions (Hedge & Hofreiter, 1962). The samples (0.1 g) were pre-treated with 5.0 mL 52% HClO_4 and
141 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 μ L) was added to 0.1% anthrone solution in 73% H₂SO₄ 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% Na₂CO₃ 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 ($4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$)

168 Flavonoids were estimated using the aluminium chloride colorimetric method of Djerridane et al.
169 (2006). Diluted extract (1 mL) was mixed with 2% AlCl₃ methanolic solution (1 mL). After incuba-
170 tion at room temperature for 15 min, the absorbance was measured at 430 nm. Flavonoids were ex-
171 pressed as rutin (R) E/g dw on the basis of a calibration curve obtained with R.
172 For total carotenoids, lentil powders (0.10 g) were extracted with 10 mL of hexane:isopropanol so-
173 lution (3:2 v/v), with agitation, for 5 hr, centrifuged at 148 x g (1000 rpm) for 10 min. The superna-
174 tant was collected and kept in the dark, and the pellet extracted for an additional two times with
175 hexane-isopropanol solution. The supernatants were dried using nitrogen and diluted with 500 µL
176 tetrahydrofuran. The absorbance of the solution was measured at 450 nm and total carotenoid con-
177 tent was in µg/g dw (Zhang et al., 2014).

178

179 2.8. Mineral assay

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

190

191 2.9. Antioxidant activity detection

192 The method of Blois (1958) was used to determine the DPPH[•] scavenging assay. DPPH[•] concentra-
193 tion in the cuvette has been chosen to give absorbance values of ~1.0. The reaction mixtures were

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

$$198 \quad \text{Inhibition}(\%) = \frac{A_o - A_s}{A_o} \times 100 \quad (1)$$

199 where A_o is the absorbance of the control and A_s is the absorbance of the sample after 30 min of
200 incubation. DPPH activity was expressed as μ M of Trolox (T) E using a calibration curve (1.0 to 50
201 μ M T).

202 FRAP was carried out using the method of Benzie and Strain (1996) with minor modification. Daily
203 fresh prepared working FRAP reagent: 300 mM acetate buffer (20 mL) pH 3.6, 10 mM TPTZ solu-
204 tion in 40 mM HCl (2 mL) and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2.0 mL) was warmed at 37°C. The initial ab-
205 sorbance of this reagent was measured. Sample (50 μ L) was added to 1500 μ L solution and the ab-
206 sorbance was measured at 593 nm after 4 min. T (1–100 μ M) was chosen as a reference compound
207 and activity expressed as μ mol TE/ μ L of extract.

208 The ABTS assay (TE antioxidant capacity assay TEAC) was done according to Pellegrini et al.
209 (1999) with a few modifications. Solutions of 7 mM of ABTS (final concentration) and 2.45 mM
210 ammonium persulfate (final concentration) in phosphate buffered saline (PBS) were mixed and left
211 in the dark at room temperature for 12–16 hr. Before use, the absorbance of ABTS^{++} solution was
212 fixed at 0.70 ± 0.02 at 734 nm, and diluted if necessary with PBS. Aliquots of ethanol extract (25,
213 50 and 100 μ L) were added to 0.5 mL of ABTS^{++} solution and bring to the final volume of 600 μ L
214 with PBS. After 6 min of incubation in the dark at room temperature the absorbance of the samples
215 was measured at 734 nm. The inhibition (%), of radical scavenging activity was calculated as:

$$216 \quad I(\%) = 100 \times (A_o - A_s)/A_o$$

217 where A_0 is absorbance of the control and A_s is absorbance of the sample after 4 min of incubation.
218 TEAC activity was expressed as $\mu\text{M TE}$ using a reference curve (in the range from 1.0 to 50 μM) of
219 T .

220 The ferrozine-based colorimetric assay which measures chelation ability of the ethanol extract was
221 done using the method of Dorman et al. (2003) with a few modifications. Water extract (50 μL) was
222 added to a solution of 200 μL 0.2 mM FeSO_4 and 200 μL 0.5 mM ferrozine. The samples were
223 shaken and left for 10 min at room temperature. Finally, the absorbance at 562 nm was measured.
224 The inhibition (%) of the ferrozine- Fe^{2+} complex formation was obtained using the following equation:
225

$$226 \quad \% \text{ Inhibition} = [(A_c - A_s)/A_c] \times 100 \quad (2)$$

227 where A_c is the absorbance of the control and A_s is the absorbance of the samples in the presence
228 of the extracts. All reagents were purchased from Sigma Aldrich Chemical Co.

229

230 2.10. RP-DAD-HPLC identification of flavonoid components.

231 Reverse phase-diode array detector-high performance liquid chromatography (RP-DAD-HPLC)
232 analyses of samples were carried out with a Shimadzu system (Kyoto, Japan), consisting of a LC-
233 10AD pump system, a vacuum degasser, a quaternary solvent mixing, a SPD-M10A diode array detector
234 and a Rheodyne 7725i injector. Separation of each compound was done on a 250 x 4.6 mm
235 i.d., 5 μm Discovery C18 column, supplied by Supelco Park (Bellefonte, PA, USA) equipped with a
236 4.0 x 20 mm guard column. The column was placed in a column oven set at 25°C. The injection
237 loop was 20 μL , and the flow-rate was 1.0 mL/min. The mobile phase consisted of a linear gradient
238 of solvent A (acetonitrile) in 2% acidified water (acetic acid: H_2O , 2:98) as follows: 0–80% (0–55
239 min), 90% (55–70 min), 95% (70–80 min), 100% (80–90 min) and 0% (90–110 min). UV-Vis spectra
240 were measured between 200 and 600 nm, and simultaneous detection using a diode array at 278
241 and 325 nm. Compounds were measured using their retention time and UV spectra (Dueñas and Estrella,
242 2002), through comparison with purified standards (Sigma Chemical Co).

243

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 $p \leq 0.05$.

251

252 3. Results

253 3.1. Effects of salinity on minerals and nutrients in lentil seeds

254 Sodium was significantly more abundant in the seeds of salt-grown lentils (Table 1). Calcium, mag-
255 nesium, iron and phosphorous were found in the greatest amount in the grains of salt-grown-lentils
256 (Table 1). In salt-treated UST a greater amount of calcium in comparison with their own corre-
257 sponding commercial and control variety was detected, while in salt treated CAST magnesium was
258 the most abundant cation ($p \leq 0.05$) (Table 1). Results showed a significant increase in crude proteins
259 in the grains of all the salt-grown lentils compared to the lentil grown without salt and to the corre-
260 sponding commercial one (Table 2). The highest values ($p \leq 0.05$) were found in the grains of NaCl-
261 treated CAST and UST. On the other hand, the soluble proteins, decreased in the salt-grown lentils
262 compared to the commercial ones (Table 2). This data suggested a better conservation of proteins
263 during the soaking procedure that precedes cooking. The amount of total carbohydrates was similar
264 in all samples, while free glucose increased in UST and CAST salt treated seeds with respect to
265 CTR (Table 2). Ascorbic acid increased significantly in the NaCl-grown lentils and the greatest
266 amount was observed in UST in which the ascorbic acid doubled with respect to its own control and
267 its corresponding commercial sample (Table 3). In salt-grown CAST and PAN the ascorbic acid
268 was higher than their corresponding commercial sample. Vitamin E was significantly high in the
269 edible seeds of lentils grown with NaCl and the greatest increase, compared to the corresponding
270 commercial and control variety, was observed in CAST (Table 3). Carotenoids, total phenols and
271 total flavonoids were significantly more abundant in the grains of all the salt-grown lentils with re-
272 spect to the commercial sample (Table 3). In comparison to the commercial lentils, carotenoids tri-
273 pled in the grains of CAST and UST, total phenols and flavonoids doubled in all the varieties (Table
274 3).

275

276 3.2. Effect of salinity on antioxidant activities in lentil seeds

277 The results of DPPH, ABTS, FRAP and Ferrozine assays, showed that the edible seeds of lentils
278 grown in saline stressed soil had the highest antioxidant activities. ABTS and DPPH activities were
279 the highest in NaCl treated UST followed by PAN and CAST (Fig. 1A-B). The percentages of inhi-
280 bition for ABTS were 59% in UST and 54% in CAST and PAN grains (Fig. 1A). According to the
281 ability to inhibit ABTS radical formation, also the ability to scavenge DPPH was present with per-
282 centages of 12.6% (corresponding to 2.8 $\mu\text{M TE}$) in UST, 10.2% (corresponding to 2.3 $\mu\text{M TE}$) in
283 PAN and 6.5% (corresponding to 1.5 $\mu\text{M TE}$) in CAST (Fig. 1B). Ferrozine activity was the highest
284 in UST and CAST with 40% I, followed by PAN with 28% I (Fig. 1C). FRAP increased much more
285 in the edible seeds of salt-grown PAN than UST and CAST with respect to their own commercial
286 variety (Fig. 1D). FRAP values ranged from 375 $\mu\text{M TE g}^{-1}$ in the seeds of commercial UST to 620
287 $\mu\text{M TE g}^{-1}$ in the seeds of NaCl grown UST, from 214 $\mu\text{M TE g}^{-1}$ in the seeds of commercial CAST
288 to 502 $\mu\text{M TE g}^{-1}$ in the seeds of NaCl-grown CAST, and from 72 $\mu\text{M TE g}^{-1}$ in the seeds of com-
289 mercial PAN to 484 $\mu\text{M TE g}^{-1}$ in the seeds of NaCl grown PAN (Fig. 1D).

290 Correlation analysis showed relationships between each individual antioxidant compound and the
291 antioxidant capacities measured in the seeds of salt-grown, control (grown without salt) and com-
292 mercial lentils. Significant specific and positive correlations were observed between the antioxidant
293 compounds and antioxidant capacities with significant differences among the three cultivars. In
294 seeds of NaCl grown Ustica, a significant positive correlation was observed between flavonoids and
295 DPPH activity and also between ascorbic acid and vitamin E with FRAP, ABTS and Ferrozine ac-
296 tivities. Moreover in seeds of NaCl grown CAST, a positive correlation between ascorbic acid and
297 vitamin E with FRAP, DPPH and Ferrozine was seen. In seeds from PANT NaCl treated-plants, ca-
298 rotenoids and flavonoids were positively correlated to FRAP and DPPH antioxidant activities, while
299 phenols were correlated with DPPH and Ferrozine (Table 5). In the edible seeds of salt grown len-
300 tils the majority of antioxidant activities were significantly and positively correlated with the anti-
301 oxidant compounds in comparison with commercial and control lentils (Table 5).

302

303 3.3 RP-HPLC-DAD separation and identification of compounds.

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

312

313 4. Discussions

314 Lentils have functional characteristics suggesting their involvement in nutritional, health-promoting
315 and disease-preventing effects. They are a biological source of high quality proteins, complex car-
316 bohydrates (resistant starches, oligosaccharides and dietary fibers), carotenoids and vitamins C and
317 E (Ganesan & Xu, 2017). The amounts of these substances observed in the commercial seeds of the
318 three landraces conformed to the data reported in literature for other lentil cultivars (Urbano et al.,
319 2007). Salt-grown lentils led to more proteins to contrast osmotic stress unlike other crops which
320 preferentially led to sugars or other molecules as osmolytes (Nadeem et al., 2019). These results
321 are consistent with results of Amini et al. (2007) that showed in tomato seedlings a greater produc-
322 tion of proteins in response to salt stress. Additionally, Klessig and Malamy (1994) showed that
323 plants generated activated proteins as signal transducers or messengers after environmental stresses.
324 This represents a nutritional advantage because vegetable higher in proteins are now recommended.
325 The increase in calcium, magnesium, phosphorous and iron is related to the tendency of plants to
326 accumulate ions as chemical osmo-regulators and to avoid the toxic effects that result from chloride
327 accumulation (Chen & Jang, 2010). Additionally, Kudo et al. (2010) suggested that cations in-
328 creased 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

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

384

385 5. Conclusions

386 Even if lentil is considered a legume sensitive to salinity, three salt resistant varieties have been
387 identified. Although, NaCl decreased lentil productivity in terms of growth and seed production (-
388 20%) the results showed that salinity was able to increase their nutritional values such as proteins,
389 vitamin C and E and antioxidant compounds increasing the reaction with free radical and the ability
390 to inhibit the oxidation processes. NaCl favored the synthesis of different secondary metabolites
391 with multiple effects on different target organs, with the advantage of protecting, at the same time,
392 the human body against various diseases ranging from specific types of cancer to cardiovascular
393 diseases. These three lentil varieties can be cultivated, without compromising the quality of their
394 final product, in marginal lands of the Mediterranean and semi-arid areas where, for water scarcity
395 and/or low quality water, soil can reach a salinity of 8 dS/m.

396

397 Declaration of competing interest

398 The authors confirm that they have no conflicts of interest with respect to the work described in this
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400

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404

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562

Table 1 Sodium, potassium, calcium, magnesium, iron and phosphorous in the grains of Pantelleria (PAN) Ustica (UST) and Castelluccio di Norcia (CAST) landraces. CTR (control without salinity), Com (commercial local variety), NaCl (lentils grown with 80 mM NaCl). The data are expressed 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

567 Table 2. Proteins and carbohydrates in the grains of three lentil varieties. Different lower-case let-
568 ters indicate significant differences among the different treatments within each variety. Different
569 upper-case letters refer to differences among the varieties for the same treatment (Tukey's test,
570 $p \leq 0.05$).

572

Samples	Crude Protein mg/100 g	Soluble Protein mg BSE equivalents/100 g	Total carbohydrates g/100 g	Free glucose mg/100 g
UST				
CTRL	21±0.2 ^{bA}	(5.9±0.6)*10 ^{2bB}	41±2 ^{aA}	0.49±0.01 ^{cC}
NaCl	23±0.1 ^{aA}	(6.4±0.6)*10 ^{2bB}	42±1 ^{aA}	0.73±0.02 ^{aC}
Com	22±0.1 ^{bA}	(112±1)*10 ^{2aA}	42±1 ^{aA}	0.66±0.02 ^{bC}
CAST				
CTRL	21±0.2 ^{cA}	(6.7±0.3)*10 ^{2bB}	40±1 ^{aA}	1.39±0.02 ^{bB}
NaCl	23±0.4 ^{aA}	(6.5±0.2)*10 ^{2bB}	39±1 ^{aA}	1.83±0.02 ^{aA}
Com	22±0.3 ^{bA}	(9.4±0.8)*10 ^{2aA}	42±1 ^{aA}	1.75±0.01 ^{aA}
PAN				
CTRL	17±0.4 ^{bB}	(10±1)*10 ^{2aA}	39±2 ^{aA}	1.65±0.01 ^{aA}
NaCl	18±0.3 ^{aB}	(7.3±0.2)*10 ^{2bA}	41±1 ^{aA}	1.44±0.01 ^{bB}
Com	17±0.4 ^{bB}	(10±1)*10 ^{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

581
582

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

USTICA				
CAROTENOIDS	FRAP	ABTS	DPPH	FERROZINE
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

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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

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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