Comparative proteomic analysis and plant salinity response of two quinoa genotypes

Running head: Variation of salt resistance quinoa

Walid Derbali[a,b,c], Arafet Manaa[a], Bernhard Spengler[d], Rahma Goussi[a,b], Zainul,Abideen[f], Parviz Ghezellou[d], Chedly Abdelly[a], Christoph Forreiter[e], Hans-Werner Koyro[c*]

[a]Laboratory of Extremophile Plants, Centre of Biotechnology of Borj Cedria, B.P. 901, Hammam-Lif 2050, Tunisia.

[b]Faculté des Sciences de Tunis, Université Tunis El Manar, 2092. Tunisia

[c]Institute for Plant Ecology, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany.

[d]Institute of Inorganic and Analytical Chemistry, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 17, 35392 Giessen, Germany.

[e]Institut für Biologie, University of Siegen, Naturwissenschaftlich-Technische Fakultät, Adolf-Reichwein-Str. 2, 57068 Siegen

[f]Dr. Muhammad Ajmal Khan Institute of Sustainable Halophyte, University of Karachi, Karachi, Pakistan

Author to whom correspondence should be addressed:

Hans-Werner Koyro

Institute for Plant Ecology, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

E-mail: Hans-Werner.Koyro@bot2.bio.uni-giessen.de

Phone (++49) 641 9935313/ Fax (++49) 9919965

ABSTRACT

The aim of this study was to investigate the effect of NaCl salinity (0, 100 and 300 mM) on the individual response of the quinoa varieties Kcoito (Altiplano Ecotype) and UDEC-5 (Sea-level Ecotype) with physiological and proteomic approaches. UDEC-5 showed an enhanced capacity to withstand salinity stress compared to Kcoito. In response to salinity, we detected overall the following differences between both genotypes: Toxicity symptoms, plant growth performance, photosynthesis performance and intensity of ROS-defense.

We found a mirroring of these differences in the proteome of each genotype. Among the 700 protein spots reproducibly detected, 24 exhibited significant abundance variations between samples. These 24 proteins were involved in energy and carbon metabolism, photosynthesis, ROS scavenging and detoxification, stress defense and chaperone functions, enzyme activation and ATPases. A specific set of proteins predominantly involved in photosynthesis and ROS scavenging showed significantly higher abundance under high salinity (300 mM NaCl). The adjustment was accompanied by a stimulation of various metabolic pathways to balance the supplementary demand for energy or intermediates. However, the more salt-resistant genotype

UDEC-5 presented a beneficial and significantly higher expression of nearly all stress-related altered enzymes than Kcoito.

Keywords: Salinity, halophyte, quinoa, proteomic, photosynthesis, antioxidant, salt resistance, oxidative stress.

1. Introduction

There are increasing concerns about threats to the global food supply from growing competition between feed production for livestock, biofuels and climate change (van Beek et al., 2010). It fits to a worst-case scenario that (i) climate change models predict increasing drought and soil salinization, affecting more than 50% of all arable lands by the year 2050 (Kopittke et al., 2019) and (ii) increasing pressure on the world's food production due to an expanding human population which will reach nine billion within the next decades (about 9.2 billion by 2050). This represents an urgent concern since already 870 million people suffer through hunger in underdeveloped countries (highly susceptible to the effects of climate change) (Tewari et al., 2017). Facing this situation, it is necessary to provide adequate food by an increase of the current production within the agricultural sector. Both farmers and scientists need new tools to adapt to these changes by adaptation of agriculture to changing climatic conditions and dietary needs through the optimization of growth conditions and the use of suitable crops. Latter one could be for example species or genotypes within species resistant to abiotic stresses such as drought, high temperature and saline soils. As one of many strategies, we can deal with this situation by introduction of new halophytic (salt-loving) crops such as the facultative halophyte Quinoa, *Chenopodium quinoa* Willd (Santos et al., 2016). Originating from the Andean region, quinoa

grain is an extraordinarily good source of high-quality protein and is classified as the most gluten-free grain (Bazile et al., 2016; Délano-Frier et al., 2011; Waqas, Yaning, et al., 2019). This pseudocereal contains in addition high amounts of essential amino acids, dietary fiber, vitamins, polyunsaturated fatty acids and minerals (Abugoch et al., 2009; E & Da, 2016; Vega-Gálvez et al., 2010). As a climate-resilient crop with great value, its genome has been recently sequenced (Jarvis et al., 2017). However, quinoa was lately classified as only particularly suited for biosaline agriculture because of genotypic differences in agro-physiological, biochemical and isotopic responses to salinity stress (Hussain, Al- Dakheel, et al., 2018) and various habitats (Bertero et al., 2004; Karyotis et al., 2003; Shabala, 2013; Zurita-Silva et al., 2014). At the global level, there are more than 6000 varieties of quinoa (Rojas et al., 2015) with different degrees of salt resistance. One reason of this variation may be their natural appearance in five ecotypes, based on geographic adaptation, 1) inter-Andean valleys, 2) Altiplano (highlands of the Andes), 3) Salares (edges of deserts and high-altitude salt lakes), 4) sea-level (coastal areas) and 5) subtropical wet areas (Yungas) (Bazile et al., 2016; Hinojosa et al., 2018). However, salinity resistance in quinoa does not seem to correlate with geographic distribution.

There is a high demand for the selection of Quinoa varieties growing with alternatives and, for instance, saline water resources. Therefore, salt-resistance mechanism by which quinoa plant cope with salt stress were extensively studied in the last decade. The majority of reports on quinoa salt-resistance revealed that this facultative halophyte had developed a network of several physiological, biochemical and molecular mechanisms to mitigate the deleterious effects of salinity (Adolf et al., 2013; Demidchik, 2015; Derbali et al., 2020; Eisa et al., 2012; Hariadi et al., 2011; Iqbal et al., 2017; Ruiz et al., 2016; Tewari et al., 2017). Most of the salt resistance mechanisms of quinoa were highly correlated to: (*i*) maintain convenient tissue water supply and

control of the transpiration rate. (*ii*) low level of sodium toxicity accompanied by high K^+/Na^+ selectivity, high K^+ and Mg^{2+} use-efficiency under high salinity. (*iii*) high stomatal conductance associated with high intercellular CO_2 concentration. (i*v*) limitation of membrane lipid peroxidation, oxidative stress and photorespiration. (*v*) Furthermore, genotypic variability of quinoa concerning the antioxidants system, oxidative stress and osmotic adjustment responses under salinity conditions was described (Eisa et al., 2012; Hussain, Hussain, et al., 2018; Koyro & Eisa, 2008; Shabala et al., 2013; Shabala & Mackay, 2011; Waqas, Kaya, et al., 2019).

Despite these studies on salt resistance mechanisms by which quinoa plants cope with salinity, there is a limited amount of other evidence regarding the genetic regulation and gene expression (Aloisi et al., 2016).

In consideration of this background, the present study aimed mainly at monitoring salt-induced responses of two *Chenopodium quinoa* genotypes, differing in salt resistance to moderate and hyperosmotic salinity and comparing their leaf protein profile using comparative proteomics analysis. Our intention was to identify stress-regulated proteins in quinoa and to provide, in combination with physiological and biochemical parameters, some novel insights into the molecular mechanisms and interactions enabling metabolic homeostasis of quinoa. We expected to identify possible gene candidates responsible for high resistance to abiotic stresses.

2. Materials and methods

2.1. Chemicals

The LC-MS-grade solvents, acetonitrile (ACN) and water (H_2O), were purchased from VWR chemicals (VWR international, Darmstadt, Germany). The in-gel tryptic digestion kit was purchased from Thermo Fisher Scientific (Pierce, Thermo Fisher Scientific, Bonn, Germany). Zip Tip C18 was purchased from Millipore (Bedford, MA, USA).

115 **2.1. Plant material and growth conditions**

116 Quinoa (*Chenopodium quinoa* Willd.) seeds collected from the south of Chile were
117 provided by the United States Department of Agriculture (USDA) and the seed bank of the
118 International Center for Biosaline Agriculture (ICBA). According to our previous report on the
119 screening of salt-resistant quinoa genotypes, two contrasting varieties were selected for this
120 study: Kcoito (Altiplano Ecotype),(Gómez-Pando et al., 2019) considered as salt-sensitive and
121 UDEC-5 (Sea-level Ecotype), Chilean lowland origin, affected by soil salinity, (Murphy &
122 Matanguihan, 2015) as salt-resistant genotype (Derbali et al., 2020). Seeds were disinfected for 5
123 min by soaking in a 20% (v/v) sodium hypochlorite solution and rinsed generously with distilled
124 water. Seeds were sown in a mixture of 2/3 commercial peat; 1/3 sand, and irrigated with
125 distilled water. The obtained seedlings (4 weeks old) were transferred to continuously aerated
126 hydroponic medium, with a half-strength Hewitt's nutrient solution (Hudson, 1967) containing:
127 3.5 mM $Ca(NO_3)_2$, 3.0 mM KNO_3, 1.5 mM $MgSO_4$, 1.6 mM KH_2PO_4, 0.6 mM K_2HPO_4, 3 µM
128 Fe–K-EDTA, 0.05 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.04 µM $CuSO_4$, 0.05 µM $ZnSO_4$, and 0.02 µM
129 $(NH_4)_6Mo_7O_{24}$. After two weeks for plant acclimation, the hydroponic medium was substituted
130 by full-strength nutrient Hewitt's solution (Hewitt et al., 1966). After seedlings establishment, at
131 the four fully expanded leaves stage (45 days after sowing), salt treatment was started. To
132 prevent the osmotic shock, the salinity level was daily increased stepwise by 50 mM NaCl, until
133 reaching the final concentration of 300 mM NaCl (equivalent to 60 % seawater salinity). Plants
134 were harvested after three weeks of salt treatment. Plant culture was conducted in an
135 environmentally-controlled greenhouse in Giessen, Germany, at the following day/night
136 conditions: room temperature of 25/18 °C, relative humidity of 70/85% and photoperiod of 16/8
137 hrs. The lighting intensity amounted in the daily mean to 200 ±20 µmol m^{-2} s^{-1} PAR.

138 **2.2. Determination of plant growth**

139 At the end of salt treatment, plants were harvested, fresh weight (FW) of root, stem and
140 leaf were recorded and dry weight (DW) was determined after desiccation at 80°C for 72h. Leaf
141 water content (WC, $ml.g^{-1}$ DW) was estimated using the following equation: WC =
142 (FW-DW)/DW. A portion of the fresh samples was also immediately frozen in liquid nitrogen
143 and stored at −80 °C and later used for the analysis of the antioxidant defense system and the
144 protein composition.

145 **2.3. Gas exchange and chlorophyll determination**

146 Before plant harvest, gas exchange measurements were performed with a LI-6400XT
147 Portable Photosynthesis System (PSC-2928) equipped with the 6400-04 fluorometer (LI-COR
148 Lincoln, Nebraska, USA) IRGA analyzer (LCi, Analytical Development Company Ltd,
149 Hoddesdon, UK). Net photosynthetic rate (An) and stomatal conductance (gs) were determined
150 on fully emerged leaf blades (third and fourth node) from 10:00 to 12:00 a.m. under the
151 following conditions: CO_2 partial pressure of 400 $mmol*mol^{-1}$, temperature of 28-32°C and
152 relative humidity of 50-60%. A light response curve of net photosynthesis (A_{net}) was measured
153 between 0 to 1500 µmol photon $m^{-2}s^{-1}$ PAR. Net photosynthetic rate (A_{net}), stomatal conductivity
154 (gs), transpiration (E), internal CO_2 (Ci) and water use efficiency (PWUE) (A/E) were measured
155 at saturating irradiation of each treatment.

156 **2.4. Hydrogen peroxide (H_2O_2) content**

157 The hydrogen peroxide (H_2O_2) concentration was measured according to the method of
158 Loreto and Velikova (2001). Frozen leaf samples (500 mg) were extracted with 3 ml TCA (0.1%,
159 w/v) in an ice bath and centrifuged at 12000 g for 15 min. Subsequently, 0.5 ml of supernatant
160 were mixed with 0.5 ml of potassium phosphate buffer (10 mM, pH 7.0) and 1.5 ml of potassium

iodide (1 M) in a ratio 2:1 (v/v). The absorbance was spectrophotometrically measured at λ = 390 nm using the M550 double beam scanning UV/VIS spectrophotometer (Camspec, UK). The hydrogen peroxide content was calculated using a standard curve using different concentrations of H_2O_2.

2.5. Enzyme extraction and assays

Total soluble protein content was measured according to the method of Bradford (1976). Fresh leaves (100 mg) were homogenized with ice-cold sodium phosphate buffer (50 mM, pH 7.2) containing 1 mM EDTA.Na_2 and 2% (w/v) PVPP and then centrifuged at 13.000 x g for 40 min at 4°C. The supernatant was collected and stored in small aliquots at −80°C. For protein quantification, the supernatant was mixed with Bradford reagent (B6916) and incubated thereafter in the dark for 5 min. The absorbance was spectrophotometrically measured at λ = 595 nm using the M550 double-beam scanning UV/VIS spectrophotometer (Camspec, UK). Soluble protein concentration in enzyme extract was estimated by using Bradford's method (Bradford, 1976) and bovine serum albumin (BSA) as the protein standard. The collected supernatant was also used to evaluate the antioxidants (SOD, CAT, APX, GR and GPOX) activities:

Total superoxide dismutase (EC 1.15.11) activity was estimated, following the method of Giannopolitis and Ries (1977). The activity was measured by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the *p*-nitro blue tetrazolium photoreduction as monitored at λ = 560 nm.

Total catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the rate of decomposition of H_2O_2 at λ = 240 nm, according to the method of Aebi (1984). The reaction was carried out in a final volume of 3 ml of the reaction mixture, containing 30% H_2O_2 (v/v) and 0.1

184 mM EDTA, suspended in sodium-phosphate buffer (50 mM, pH 7.2). Activity was calculated
185 using extinction coefficient (ε) 0.036 $mM^{-1}cm^{-1}$ and expressed as U mg^{-1} protein min^{-1}.

186 Total ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by monitoring the
187 decrease in absorbance at λ = 290 nm as ascorbate was oxidized according to the method of
188 Nakano and Asada (1981) and using a molar extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. The
189 enzymatic activity was expressed in micromoles ascorbate U mg^{-1} protein min^{-1}.

190 Glutathione reductase (GR, EC 1.6.4.2) activity was determined at 25°C as described
191 previously by Esterbauer and Grill (1978), by following the rate of NADPH oxidation at λ =
192 340 nm. Activity was calculated using the extinction coefficient for NADPH of 6.22 mM^{-1} cm^{-1}
193 and expressed as mmol NADPH oxidized mg^{-1} protein.

194 Total guaiacol peroxidase (EC 1.11.1.7) activity was assayed according to the method of
195 Upadhyaya et al. (1985) by monitoring the increase in absorbance at λ = 470 nm due to guaiacol
196 oxidation. Activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1} within 1
197 min and expressed as μmol mg^{-1} protein min^{-1}.

198 **2.6. Protein extraction**

199 Leaf proteins were extracted using a phenol extraction procedure Geissler et al. (2010).
200 About 0.5 g of the obtained fresh leaf powder were mixed with 750 μl of the extraction buffer pH
201 8.0 (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, 2% (v/v) β-mercapto-ethanol,
202 and 2 mM PMSF). After 10 min incubation in ice, 750 μl of water-saturated phenol (Amresco
203 Biotech Chemicals) was added and the mixture was vortexed before shaken at 300 rpm at room
204 temperature for 30 min (Mixer 5432, Eppendorf), and centrifuged for 10 min at 12,000 ×g and
205 4 °C. The centrifugation step was repeated after the upper phenolic phase, containing soluble
206 proteins, had been removed carefully and the initial sample volume had been restored by

addition of extraction buffer. The proteins extracted in the resulting phenolic phase were precipitated at -20 °C, over night by adding 100 mM ammonium acetate in methanol. This mixture was subsequently centrifuged for 3 min at 15,000 ×g and 4 °C and the pellet was re-suspended in 1 ml of the precipitating solution before re-centrifugation. The created pellet was rinsed with 80% (v/v) ice-cold acetone, re-centrifuged, and air-dried at room temperature for 10 min. Before 2-DE, proteins were solubilized in lysis buffer (8 M Urea, 2 % (w/v) CHAPS, 0.5 % (v/v) Triton X-100, 30 mM DTT, 1.2 % (v/v) pharmalytes pH 3-10) and protein concentration was measured according to the modified Bradford assay (Ramagli & Rodriguez, 1985).

2.7. Two-dimensional gel electrophoresis (2-DE)

About 300 µg of the protein pellet were re-suspended in 350 µl of the rehydration buffer before Isoelectric focusing (IEF) was performed (Geissler et al., 2010). The vortexed suspension was centrifuged for 5 min at 17,000 ×g and 4 °C. IEF was carried out for 12 h with 18 cm dry gel strips (IPG strips, pH 3–11 non-linear, GE Healthcare, Munich, Germany) using the IPGphor system (GE Healthcare, Munich, Germany) with a current limit of 50 µA/strip at 20 °C. Sample rehydration was performed for 12 h at 30 V, followed by focusing in four steps 500 V (1 h), 1000 V (1 h), 8000 V (1 h), and 8000 V (6 h). After migration, IEF strips were stored at -80°C or immediately incubated in equilibration buffer, respectively, for 20 min. A first equilibration was performed in a solution buffer containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT, and 2 mg bromophenol blue. A second equilibration was carried out using the same solution in which DTT was replaced by 2.5% (w/v) iodoacetamide to alkylate free thiol groups of the proteins.

Equilibrated IPG strips were then horizontally placed on a 12% tricine SDS-PAGE gel (Schägger et al., 1988), and sealed with a solution containing 0.5% agarose and 2–4 mg

bromophenol blue in 100 ml tricine gel buffer pH 8.45 (3 M Tris and 0.3% SDS). SDS-PAGE was carried out at 20 °C using Biorad Protean Plus Dodeca cell electrophoresis chamber (2 h at 70 V, 15 h at 100 V).

2.8. Protein staining, gel scanning and image analysis

The obtained gels were fixed using a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid for 2 h and stained overnight with colloidal Coomassie Blue (0.1% (w/v) CBB-G250, 10% (w/v) ammonium sulphate, and 2% ortho-phosphoric acid in 20% methanol) (Geissler et al., 2010). Gels subsequently were washed carefully with bi-distilled water to remove the background due to staining. Stained gels were scanned at 300 dpi resolution, and stored under the Tagged Image File Format (tif). For spot detection and volume quantification, tif-files were transformed into Maya Embedded Language (Mel) and analyzed using Imagemaster TM 2D Platinum software 6.0 (GE Healthcare, USA). Three images representing three independent biological replicates of either germinating seeds or leaves exposed to salinity were grouped to calculate the mean volume of all the individual protein spots. The spot abundance was normalized as relative volume according to the normalization method provided by the software to obtain the individual relative spot volume (%), i.e. the spot volume of one spot in relation to the sum of all detected spots on the gel. This method eliminates eventual protein loading differences (Führs et al., 2010).

2.9. Protein identification by mass spectrometry

In-gel digestion was performed for the targeted protein spots showing at least a 1.5-fold change in expression abundance under the NaCl treatment. The 24 spots were excised manually and de-stained two times with 200 µl of 50% ACN while shaking for 30 minutes. The gel pieces were reduced and alkylated with 100 mM dithiothreitol at 56 °C for 15 min, and 200 mM

253 iodoacetamide at room temperature (dark place) for 30 min, respectively. The digestion was
254 performed with mass spec grade Trypsin/Lys-C mix (1:25 enzyme to proteins ratio) at 37 °C.
255 The reaction was stopped after 16 h by adding concentrated formic acid and incubating at 37 °C
256 for 10 min prior to centrifugation. The peptide samples were desalted before MS measurements
257 by using ZipTip C18 and then concentrated by using Eppendorf Concentrator Plus (Eppendorf,
258 Hamburg, Germany) and finally stored at –80 °C for future use.

259 The peptides were separated using an UltiMate 3000 RSLC UHPLC system (Thermo
260 Fisher Scientific) on a Kinetex C18 (2.1 × 100 mm, 2.6 µm 100 A° particle size) column
261 (Phenomenex, CA, USA) coupled to a Q Exactive HF-X (Thermo Fisher Scientific, Bremen,
262 Germany). Chromatographic analysis was performed at 250 µl/min flow rate with water/0.1%
263 formic acid (mobile phase A) and acetonitrile/0.1% formic acid (mobile phase B). The gradient
264 elution of 90 min was applied as follows: isocratically (2% B) for 5 min, followed by 2-40% B
265 over 70 min, 40-50% B over 10 min, 50-98% B over 5 min, and re-equilibration in 2% B. The
266 mass spectrometer was operated in data-dependent acquisition (top-10 DDA) with the following
267 parameters in full MS scans: mass range of m/z 350 to 1800, mass resolution of 120,000, AGC
268 target of 3e6, IT of 50 ms, and MS/MS scans: mass range of m/z 200 to 2000, mass resolution of
269 30,000, AGC target of 1e5, IT of 120 ms, isolation window m/z 1.3 and dynamic exclusion of
270 60s.

271 The raw files were processed using Proteome Discoverer version 2.2 (Thermo Scientific)
272 with SEQUEST search engine against the UniProtKB databases, taxonomically set to the
273 Viridiplantae (taxon ID # 33090). The parameters were set to two missed cleavage sites of
274 trypsin digestion, minimum peptide length of 6, MS1 and MS2 tolerances of 10 ppm and 0.5 Da
275 respectively. Dynamic modification was set to oxidation (+15.995 Da [M]) and static

modification to carbamidomethyl (+57.021 Da [C]). Percolator node was used to validate identified PSMs (peptide-spectrum matches) and filter the data with parameters of a strict Target FDR (false discovery rate) of 0.01 and a relaxed Target FDR of 0.05. The cRAP contaminant database (https://www.thegpm.org/) was used to mark contaminants in the results file. Peptides and proteins were filtered with only high confidence and master proteins in the final results.

2.10. Statistical analysis

The statistical analysis was performed using 'Statistica' software (version 6.0). All means values and standard error (SE), of physiological and biochemical parameters were obtained from 6 replicates. Only differences with a P value < 0.05 were considered statistically significant according to Duncan's multiple range tests. For gel image analysis, one-way ANOVA was applied to detect variation on normalized spot volume from the three gel repeats with $P < 0.01$. On the deduced set of spots, a two-way ANOVA was performed to detect genotype, treatment, and interaction effects, a $P < 0.01$ was considered statistically significant.

3. Results and discussion

In the present work, we selected two varieties of quinoa with different degrees of salt resistance to illustrate their individual adjustment to salinity. A series of recent studies has indicated that several cellular processes and mechanisms involved in salt-resistance are highly complex and related not only to genotype resistance and genotype x environment interaction but also to salt stress duration and plant development stage (Ashraf & McNeilly, 2004; Manaa et al., 2011; Negrão et al., 2016).

It was our intention to identify and describe the specific role of stress-regulated proteins as an integral part of interacting physiological and biochemical processes of both quinoa

298 varieties under identical conditions in an advanced stage of development and after three weeks at
299 moderate or hyperosmotic salinity.

300 First of all, to mitigate the deleterious effects of salinity, we established a network of
301 information on various relevant processes such as CO_2/H_2O-gas exchange and enzyme activities
302 and their impact on plant growth and development (Manaa et al., 2013; Munns, 2002).

303 **3.1. Toxicity symptoms and plant growth**

304 One of the initial effects of salinity on plants is the reduction of the growth rate.
305 However, under moderate salinity (100 mM NaCl), plant growth remained constant for both
306 genotypes, as compared to control (Fig. 2A). Hyperosmotic salinity (300 mM NaCl) induced a
307 significant decrease of plant DW about 52% and 46%, as compared to control, respectively, for
308 Kcoito and UDEC-5 (Fig. 1 and 2A). Furthermore, plant growth reduction was more apparent in
309 Kcoito (Altiplano Ecotype), which showed in addition toxicity symptoms such as petiole
310 necrosis, leaf fall or leaf chlorosis.

311 Several investigators have demonstrated that shoot growth is more sensitive to salinity
312 than root growth and leads to an increased root/shoot ratio (Nirit et al., 2004; Qian et al., 2000).
313 Our results also showed that the shoot of Kcoito (an increase of about 36% in root/shoot dry
314 matter ratio) is more sensitive to salinity than the root system. Salt-stressed roots emphasize
315 elongation growth, which helps them to acquire water and minerals (McCarty & Dudeck, 1993).
316 Compared with the shoot growth, the root growth of the genotype UDEC-5 (sea-level Ecotype)
317 was less affected or even stimulated by salt stress. This was in agreement with the previous
318 reports on warm-season or more stress-resistant grasses/halophytes (Marcum et al., 2005;
319 Pessarakli & Kopec, 2004; Sagi et al., 1997).

All these observations suggest a high adaptation of both quinoa varieties, especially under moderate salinity, and qualified these quinoa genotypes as facultative halophytic plant, as previously demonstrated (Derbali et al., 2020; Jacobsen et al., 2003; Koyro & Eisa, 2007). However, all growth-related parameters indicate for UDEC-5 a significant higher salt resistance than for Kcoito.

3.2. Photosynthesis performance

Salinity affects plant growth via the perturbation of water uptake, photosynthetic rate (A_{net}), pigment synthesis and gas exchange (Derbali et al., 2020; Ghars et al., 2008; Goussi, Manaa, Derbali, Ghnaya, et al., 2018; Talbi Zribi et al., 2018). The present study demonstrated that both varieties maintained their water content (WC) and chlorophyll content under moderate salinity (Fig. 2G). However, under high salinity (300 mM NaCl), a significant decrease in water and chlorophyll contents was observed for both genotypes. It should be noted that UDEC-5 kept at all salinity levels higher water and chlorophyll contents compared to Kcoito genotype.

Regarding gas exchanges, moderate salinity (100 mM NaCl) had no significant effect on intercellular CO_2 concentration (C_i) for both genotypes, while this parameter decreased under high NaCl treatment (300 mM) (Table 1). At all salinity levels, transpiration rate (E), stomatal conductance (gs) and net CO_2 assimilation (A_{net}) showed a significant decrease when compared to control, for both genotypes. Similar adjustments to salinity were also found in various other quinoa varieties such as Utusaya, Titicaca, Achachino and Hualhuas (Adolf et al., 2012; Becker et al., 2017). The closure of stomata reduces water loss but also CO_2 uptake, thereby inhibiting photosynthesis (Dinneny, 2015).

Despite these perturbations on gas exchanges, UDEC-5 preserved a much higher level of transpiration rate (E), stomatal conductance (gs), net CO_2 assimilation (A_{net}) and (PWUE) especially, under 300 mM NaCl treatment when compared to Kcoito genotype (Table 1).

The difference in salt resistance of both studied genotypes UDEC-5 and Kcoito could be associated to the protection and control of CO_2 assimilation, as demonstrated by the photosynthetic light response curves. UDEC-5 exhibited a significantly smaller decrease in photosynthetic rate at all salinity levels than Kcoito (Table1 and Fig. 3).

Our data are in agreement with previous studies conducted on the model halophyte *Thellungiella salsuginea* which demonstrated that maintenance of intercellular CO_2 concentration (C_i) and transpiration rates (E) during moderate salinity reflects certainly an efficient strategy to ensure leaf gas exchange and nutrient supply in a saline root environment (M'rah et al., 2006; Mohamed Ali et al., 2008). However, extreme halophytes, such as *Arthrocnemum macrostachyum* cultivated under high salinity (Redondo-Gómez et al., 2010) and *Sarcocornia fruticosa* subjected to low salinity level (Redondo-Gómez et al., 2007) can even exhibit a salt stimulation of net photosynthesis (A_{net}).

3.3 Oxidative stress and ROS-scavenging systems

As consequence of salt stress, stomatal closure induces over-illumination (Kramer & Evans, 2011), leading to a decrease in the photosynthetic rate (Bethke & Drew, 1992). This excess of light leads to photo-inhibition and induces over–reduction of the electron transport chain inside the thylakoid membrane (Asada, 1987). All these changes are the main causes for the production of reactive oxygen species (ROS) which directly impair both PSI and PSII activities and their structures (Jaspers & Kangasjärvi, 2010; Nishiyama et al., 2011).

Ion toxicity in form of Na^+/Cl^- salinity can lead directly or indirectly to the generation of ROS in plants by the depletion of the oxidized $NADP^+$, the final acceptor of electrons in PSI. It also can increase the $O_2\bullet^-$ accumulation. This reactive oxygen species is a precursor of hydrogen peroxide (H_2O_2) generation in the apoplast and hydroxyl radical ($OH^\bullet$) (Asada, 2006). Our study demonstrated that Kcoito achieved in comparison to UDEC-5 a significantly higher accumulation of H_2O_2 in leaves at 100 or 300 mM NaCl (Fig. 4A).

Therefore, it can be supposed that the salt resistance of the genotype UDEC-5 observed in the present study is at least partially associated to the limitation of H_2O_2 accumulation and to the antioxidant system, which control the level of ROS production by synthesis of different antioxidants enzymes. In fact, as compared to Kcoito, the genotype UDEC-5 showed the highest level of SOD and APX activities under moderate salinity (100 mM NaCl) (Fig. 4C and E) and the highest GR and GPOX activities under high salinity (300 mM NaCl) (Fig. 4C, D and F).

The results are in agreement with studies of Derbali et al. (2020) on several genotypes of quinoa. The authors of this study recommended the study of antioxidant activities (especially; SOD, GR and GPOX) as appropriate physiological and biochemical markers for screening salt resistant quinoa genotypes at seedling stage.

3.4. Leaf proteomic analysis

During this study and in response to salinity, we detected overall the following differences between both genotypes: Toxicity symptoms, plant growth performance, photosynthesis performance and intensity of ROS-defense. We assume that these parameters contribute solely or in combination to the individual salt resistance of the studied genotypes. We expected a mirroring of these differences in the proteome of each genotype and indirectly the

385 identification of potential candidate genes or gene clusters responsible for the salt-resistance of
386 each genotype.

387 The high resolution of protein separation by using two-dimensional gel electrophoresis
388 (2-DE) coupled with protein identification by mass spectrometry and database research provide a
389 global protein expression overview (Ndimba et al., 2005). Such a proteomic approach has
390 become a powerful tool to study plant responses to salt stress (Azri et al., 2016; Belghith et al.,
391 2018; Jiang, Feng, et al., 2007; Koyro et al., 2013; Manaa et al., 2013). 2-DE patterns were
392 analysed by comparing control *vs*. salt-stress treatment for each variety to detect the proteome's
393 change under hyperosmotic salinity (Fig. 5). Our analysis added up 700 detected spots in the
394 mass range of 10 to 120 kDa by digital image analysis. Twenty-four protein spots were selected
395 for further analysis because they exhibited significant abundance variation (up- or down-
396 regulation with $P < 0.01$) at least in one of both genotypes under NaCl stress as compared to
397 control. These spots were excised, digested with trypsin and identified using LC-MS/MS
398 analysis (Table 2, Fig.6). Identified proteins were classified according to their function and
399 physiological processes, i.e., energy and carbon metabolism (8 spots), photosynthesis-related
400 proteins (4 spots), ROS (reactive oxygen species) scavenging and detoxification (5 spots), stress
401 defense and heat shock proteins (2 spots), enzyme activation (2 spots) and ATPases (3 spots). It
402 was found that four proteins were identified in two spots, although they were excised from the
403 same gel: Transketolase (spots 6, 7 and 8), 14 -3-3 domain-containing protein (spots 11 and 12),
404 and ATP synthase (spots 3, 4 and 5) (Fig.6a+b). Further examination of electrophoresis patterns
405 indicated that the inferred mass or isoelectric point values of these spots differed, due perhaps to
406 post-translational modification or degradation. Post-translational modifications such as

glycosylation, phosphorylation, etc. can change the molecular weight and/or charge of proteins (Manaa et al., 2013).

3.4.1. Carbon metabolism and energy-related proteins

The central carbohydrate and energy metabolism are known to be susceptible to salt stress with respect to species or genotypes, plant stage development, stress duration and degree of salt resistance (Benjamin et al., 2020; Jiang & Deyholos, 2006; Jiang, Yang, et al., 2007; Manaa et al., 2011; Manaa et al., 2019). Several previous studies demonstrated that the expression of some proteins from the Calvin cycle and glycolysis are affected in a different way by abiotic stresses according to the plant's ability to overcome the stress conditions (Chaves et al., 2009; Davidson et al., 2009). For example, the increase or the decrease in abundance of fructose 1,6-bisphosphate aldolase was highly correlated to both, genetic and environmental factors.

Our results showed that under control condition, the majority of proteins involved in the Calvin cycle and the glycolysis pathway such as putative triose-phosphate isomerase (spot 14), glyceraldehyde 3-phosphate dehydrogenase (spot 23), ribulose- phosphate-3-epimerase protein (spot 15), fructose-bisphosphate aldolase (spot 18) and malate dehydrogenase (spot 17), were more abundant in the salt-sensitive genotype Kcoito than in UDEC-5 (Fig.6a+b, Fig.7). However, the expression of these proteins increased under hyperosmotic salinity (300 mM NaCl) in the salt resistant genotype UDEC-5 and was down-regulated in Kcoito (Fig. 6b). It was shown that ROS production was enhanced by strong light and by deceleration of the Calvin cycle (Nishiyama et al., 2006). Plants respond to saline environments often with the reduction of stomatal conductance to avoid water loss (Flexas et al., 2002) followed by decrease of the internal CO_2 concentrations and slowdown of carbon assimilation by the Calvin cycle. As the

regenerative step of the cycle ceases to use NADPH under these conditions, the immediate consequence is $NADP^+$ depletion and delivery of the excess of energy and reducing equivalents to O_2, with concomitant formation of ROS in chloroplasts (Lodeyro et al., 2016). Therefore, reducing the Calvin cycle enzymes in the salt-sensitive genotype Kcoito could reflect the salt-induced decrease of carbon assimilation and the enhanced risk of ROS production. This interpretation is in agreement with the strong increase of H_2O_2 concentration (Figure. 4) and decrease of CO_2 fixation (Figure 3) in the leaves of Kcoito. It also explains the differences in salt resistance between the studied quinoa genotypes. Since the response of UDEC-5 was much more targeted than of Kcoito, Salinity in UDEC-5 in contrast to Kcoito led to an increase of the Calvin cycle enzymes (Table 2), a moderate decrease of CO_2 fixation and a much lower increase of H_2O_2 content.

Salinity facilitates the risk of oxidative stress and enhances the energy requirement (Koyro, 2002). Both quinoa genotypes responded moreover with the expression of transketolase, triose phosphate isomerase and cytosolic malate dehydrogenase (Table 2, Fig. 7).

Three isoforms of transketolase (spots 6, 7 and 8) were identified in this study, and their abundance increased under hyperosmotic salinity. One of these isoforms (spot 6) showed the same pattern of variation without any differences related to the degree of salt resistance of genotypes. Our results are in disagreement with previous studies conducted on the succulent annual halophyte *Halogeton glomeratus* (Wang, Meng, et al., 2015), *Salicornia europaea* (Wang et al., 2009), and wild halophytic rice (Sengupta & Majumder, 2009), which showed a down-regulation of transketolase under salt conditions. This enzyme is related to the pentose phosphate pathway which produces the cytosolic NADPH, required for different ROS-scavenging systems, especially under salt stress conditions (Rapala-Kozik et al., 2008). The up-regulation of this

453 enzyme in quinoa genotypes under saline condition might reflect a balanced oxidative stress
454 defense to scavenge toxic ROS.

455 Two proteins (triose phosphate isomerase and cytosolic malate dehydrogenase) were
456 identified in this study and are known to be involved in glycolysis, being essential for ATP
457 production required for many biosynthetic pathways in plant cells (Sobhanian et al., 2011). The
458 up-regulation of these proteins under salt treatment in the salt-resistant genotype UDEC-5 might
459 reflect a high capacity to provide high ATP demand as additional energy, required to maintain
460 ion homeostasis or to decrease damage caused by oxidative stress (Fig. 6a, Fig. 7). Indeed, the
461 decrease of abundance observed in the salt-sensitive genotype Kcoito could be associated with
462 its failing antioxidant defense system as described above (Fig. 6b). Similar results have been
463 reported on salt and/or drought treated rice (Abbasi & Komatsu, 2004; Dooki et al., 2006) ,
464 *Triticum durum* (Caruso et al., 2008) and *Thellungiella halophila* leaves (Gao et al., 2008) during
465 salt stress treatment.

466 **3.4.2. Photosynthesis-related proteins**

467 It is well known that photosynthesis is among the most severely affected processes in
468 saline environments (Stepien & Johnson, 2009; Sudhir & Murthy, 2004; Zhu et al., 2007). We
469 have just demonstrated above that hyperosmotic salinity affects photosynthesis by inducing
470 stomatal closure, declining CO_2 availability, and consequently overproduction of reactive oxygen
471 species (ROS). Moreover, previous studies on *Chenopodium quinoa* (Manaa et al., 2019) and
472 *Thellungiella salsuginea* (Goussi, Manaa, Derbali, Cantamessa, et al., 2018) demonstrated that
473 salinity induces a considerable change in photosystems efficiency, electron transport activity and
474 abundance of PSII proteins. In the present proteomic analysis, we identified response of three
475 proteins involved in photosynthesis: RuBisCO activase (spot 20), oxygen-evolving enhancer

476 protein (spot 21) and ribulose bisphosphate carboxylase large chain (spot 9)(Fig.6a+b). The
477 abundance of these proteins was significantly changed according to salt stress and genotype
478 considered (Fig. 7). There was a close relationship between salinity, genotype, biomass
479 production (Figure 2), photosynthetic rate (Figure 3) and expression of ribulose bisphosphate
480 carboxylase (spot 9, Table 2). In comparison with the salt-sensitive genotype Kcoito, the
481 resistant genotype UDEC-5 maintained a higher abundance level of this enzyme, a higher rate of
482 CO_2 assimilation, and finally a higher biomass production, see also Derbali et al. (2020). This
483 might be explained by the pivotal role of RuBisCO activase in maintaining the active
484 conformation of Rubisco (Salvucci & Ogren, 1996). It fits nicely with the above-mentioned
485 arguments that the up-regulation of this protein was previously reported as a good marker for
486 high photosynthetic performance on the halophyte *Spinacia oleracea* L. (Bagheri et al., 2015),
487 *Beta vulgaris* , *Halogeton* glomeratus (Wang, Wu, et al., 2015) and *Suaeda salsa* (Li et al.,
488 2012).

489 Another protein, the putative oxygen-evolving enhancer protein 1 (OEE1, spot 21),
490 stabilizes the manganese cluster, the primary water-splitting site (Bagheri et al., 2015). Sugihara
491 et al. (2000) demonstrated that OEE1 is the most important protein for oxygen evolution and
492 PSII stability in the Mangrove *Bruguiera gymnorrhiza*. It was considered that the recovery or
493 turnover of OEE1 is one of the mechanisms to maintain the capacity of PSII under NaCl
494 treatment. OEE 1 was found to be up-regulated in Kcoito in response to NaCl treatment, while it
495 remains constant for the UDEC-5 genotype (Fig. 6a+b). The high PSII efficiency of UDEC-5
496 was previously discussed (Manaa et al., 2019) . It seems that it was only in the salt-sensitive
497 genotype Kcoito necessary to enhance the expression of OEE1 at saline conditions to maintain
498 the capacity of PS-II. However, the adjustment of OEE1 expression is not generally a sign of salt

499 sensitivity. Previous studies have shown that the level of OEE1 was also upregulated under salt
500 stress conditions in the halophyte *Halogeton glomeratus* (Wang, Li, et al., 2015). This report also
501 showed an enhancement of transcript levels of two other OEEs (OEE2 and OEE3), leading to an
502 acceleration of association with PSII complex to repair protein damage caused by dissociation
503 and to keep oxygen evolving.

504 **3.4.3. ROS scavenging, detoxification and stress defense**

505 As shown before, plants often respond to saline environments with the reduction of
506 stomatal conductance, followed by decrease of the internal CO_2 concentrations and a decrease in
507 the photosynthetic rate (Flexas et al., 2002). Latter one leads to a reduced demand of light energy
508 and a high probability for an over-production and accumulation of molecules containing
509 activated oxygen (harmful reactive oxygen species, ROS) (Demidchik, 2015; Jaspers et al., 2010;
510 Kramer & Evans, 2011; Nishiyama et al., 2011; Vass & Cser, 2009). Oxidative stress may be
511 caused not only by an imbalance between ROS generation and detoxification (Fulda et al., 2011),
512 but also by ROS biosynthesis as a constituent part of stress signaling and immunity response
513 needed for defense and adaptation. Plants have evolved antioxidant systems to control the level
514 of ROS production by the synthesis of various scavenging enzymes (Gill & Tuteja, 2010; Valko
515 et al., 2006).

516 In this study, four proteins involved in ROS scavenging and detoxification were
517 identified because they exhibited significant abundance variation in saline environment:
518 glutamine synthetase (spot 24), ascorbate peroxidase (spot 13), thioredoxin (spot 22) and
519 lactoylglutathione lyase (spot 10). The salt-resistant genotype UDEC-5 showed a significantly
520 higher expression than Kcoito of all four antioxidant enzymes under salt treatment (Fig. 6a+b).
521 This high expression of different antioxidant enzymes in UDEC-5 can be associated with a

522 highly regulated cellular redox state because it corresponds with a limited H_2O_2 accumulation
523 (Figure 4) and (except for catalase) high enzymatic antioxidant activities (Figure 4).

524 Our results are consistent with previous studies showing that salt stress stimulates the
525 accumulation of antioxidant enzymes (glutamine synthetase and ascorbate peroxidase) in the root
526 of the halophyte *Cakile maritime* (Belghith et al., 2018) and the shoots of *Salicornia europaea*
527 (Wang et al., 2009).

528 However, there were also similarities between both quinoa genotypes in the expression of
529 enzymatic antioxidants in saline environments. An identified protein called "lactoylglutathione
530 lyase" (spot 10) related to oxidative processes (in glycolysis, lipid peroxidation, protein
531 degradation and photosynthesis) was found to be up-regulated under NaCl treatment in both
532 genotypes (Fig. 6a+b, Fig. 7). Since, stress leads to increased level of glycolysis, hence
533 spontaneous production of at higher concentration detrimental methylglyoxal via glycolysis is an
534 unavoidable consequence such as its detoxification with lactoylglutathione lyase (Hossain,
535 2009). Subsequently, the link with D-lactate dehydrogenase (D-LDH) catalyzes the breakdown
536 of, D-lactate, into D-pyruvate which enters into TCA cycle for energy production. Therefore,
537 lactoylglutathione lyase is discussed as the most important gene for providing tolerance in
538 salinity stress (Jain et al., 2018). The antioxidant enzyme thioredoxin, which acts as a major
539 defense system against oxidative damage by reducing the disulfide bonds of oxidized proteins,
540 was also detected, and its abundance increased under salt stress (spot 22). Thioredoxin regulates
541 by this way confirmation and activity of catalytical center, which controls the function of
542 chloroplasts from biogenesis and assembly of chloroplast machinery to light and carbon fixation
543 reactions as well as photoprotective mechanisms (Nikkanen & Rintamäki, 2019). Both proteins

were previously considered as very important in salt adaptation because they stabilize the function of other proteins in plants (Apel & Hirt, 2004; Askari et al., 2006).

There is some indication that the salt-sensitive genotype Kcoito has an increased problem with the overall stability of proteins under elevated salt conditions (Fig. 6b). In addition to the increased abundance of protein disulfide isomerases (spot 2), salinity only in Kcoito induced an increase of the expression of HSP70 (spot 1) as compared to UDEC-5. HSP70 plays a pivotal role in various cellular processes by enabling protein folding and stabilization of the enzymatic homeostasis by preventing protein aggregation (Sung et al., 2001). However, Kcoito did not seem to undergo severe stress conditions since upregulation of other stress induced HSPs like HSP100 and small stress proteins could not be detected (Jacob et al., 2017). Contrastingly, the HSP-response was not affected in UDEC-5 (Fig. 6a). This is in line with the observation that in more salt resistant plants salinity might even lead to a reduction in HSP redundance, as described for *Puccinellia ciliate* (Jenkins et al., 2010). Mostly, however, salt tolerating plants showed no significant effect on the abundance of the HSP70, like for instance *Cakile maritima* (Debez et al., 2012). In summary, data currently available about heat shock proteins HSP are far from complete and allow no simple conclusions.

3.4.4. ATPases

Several subunits of the F-type chloroplast ATP synthase (spots 3, 4, 5 and 18) were identified in our study (fig. 5). It is well known that ATP synthase activity in the chloroplast is influenced by ROS, especially by H_2O_2, which oxidizes in particular methionine residues within the γ-subunit (Buchert et al., 2012). This leads to a substantial increase of the ΔpH between stroma and grana (Kanazawa & Kramer, 2002) providing a molecular mechanism to increase Non-Photochemical Quenching (NPQ) as a detoxifying element under high light conditions and

567 other stressors which induce ROS. ATP synthase activity also plays a pivotal role in response to
568 several stresses (Schöttler & Tóth, 2014). Earlier reports conducted on the halophyte *Cakile*
569 *maritima* (Debez et al., 2018), and *Sesuvium portulacastrum* (Peng et al., 2019) demonstrated
570 that moderate salinity (100 mM NaCl) induced an increase in abundance of 2 subunits of ATP
571 synthase (α, and β). The chloroplastic ATP synthase subunit α has also been identified in both
572 species *Suaeda maritima* (L.) and *Salicornia brachiata* and increased in their abundance under
573 200 mM NaCl, whereas it was weakly down-accumulated at 500 mM NaCl (Benjamin et al.,
574 2020) . The remarkable increase of ATPase subunits in our study in the salt-resistant genotype
575 UDEC-5 (Fig. 6a) might be explained by the fact, that the both bigger units of ATPase are able
576 to connect PSII with LHCP and stabilize granum stacks (Koyro 2002). We assume that the
577 inhibition of distortion of grana could contribute to the maintenance of the photosynthetic
578 activity under high-salinity level in tolerant varieties. Moreover, the increased abundance of
579 these chloroplastic ATP synthases in salt-treated UDEC-5 was accompanied by an increase in
580 the abundance of some proteins involved in carbohydrate metabolism and glycolysis (Fig.7 and
581 tab 2 spots 14, 15, 17, 18 and 23). This may reflect that energy transfer from chloroplasts to the
582 cytosol is mediated by the DHAP/GAP– 3-P-glycerate shuttle (Hampp et al., 1982; Parker et al.,
583 2006; Yi et al., 2014). For that reason, we observed in the salt-resistant genotype UDEC-5 an
584 increase in the abundance of the Glyceraldehyde-3-phosphate dehydrogenase (spot 23) under
585 salinity condition. This might reflect together with the high level of ATP synthase and
586 lactoylglutathione lyase, a high metabolic demand for NADPH and ATP as supplementary
587 energy, essential to surmount oxidative damages (KOHZUMA et al., 2009; Schöttler et al.,
588 2007). However, this is not the case in Kcoito (Fig 6b). It can be assumed that NaCl inhibits in
589 this variety the export of glyceraldehyde-3-phosphate at the carrier of the chloroplast

590 (competitive inhibition of Pi by Cl^-) leading to an enrichment of NADPH and consequently an
591 inhibition of the electron transport.

592 **4. Conclusion**

593 Physiological and proteomic states of two quinoa genotypes differing in their salt
594 resistance to salinity were investigated in the present study. A suited adjustment of both quinoa
595 genotypes was recorded under moderate salinity, which could be linked to the maintaining of
596 high-water uptake and maintaining of photosynthetic rate.

597 However, plant performance, growth and physiological results indicate that UDEC-5 had
598 an enhanced capacity to withstand salinity stress compared to Kcoito. The obtained results
599 revealed that the application of hyperosmotic salinity could affect plant growth and development,
600 especially in the Kcoito genotype, which showed some toxicity symptoms under high salinity. In
601 fact, a relatively high photosynthetic activity was maintained in the salt-resistant genotype
602 UDEC-5 even under hyperosmotic salinity and could be associated with the high stability of the
603 antioxidant system (via accumulation of APX, SOD, GR and GPOX), which controls the level of
604 ROS production. The proteomic data enhanced the validity and confirmed the results of eco-
605 physiological and biochemical analysis and demonstrate a high salt resistance of UDEC-5
606 genotype associated with: (***i***) high metabolic activity to balance the supplementary demand for
607 energy and intermediates (*ii*) high photosynthesis efficiency via maintaining the structure of
608 Rubisco, up-regulation of some photosynthesis-related enzyme and ATP synthase accumulation
609 and (***iii***) high stability of the antioxidant system via accumulation of ROS scavenging enzymes,
610 especially under high salinity.

611 Studies need to be extended on the basis of the scientific findings in order to obtain a
612 survey about salt resistances of more quinoa varieties, with the aim to identify possible gene

candidates responsible for high resistance to abiotic stresses in this genus. This study raises expectations that the adaptation of agriculture to changing climatic conditions and dietary needs through the optimization of growth conditions and the use of suitable crops is a practicable way.

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