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Different proline responses of two Algerian durum wheat cultivars to in vitro salt stress --Manuscript Draft--

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Abstract:	<p>Durum wheat, <i>Triticum turgidum</i> subsp durum (Desf.) Husn., is one of the most salt-sensitive cereal crops, but the physiological responses of different cultivars to salt stress vary. Cultivars that are suited to arid conditions like in Algeria may not necessarily be tolerant of increased salinity. When 10-day seedlings of Algerian durum wheat varieties Hedba 3 (HD3) and Mohamed Ben Bachir (MBB) were subjected to salt stress, they accumulated proline and expressed stress-related and proline metabolism genes in a classic salt-stress response. Expression of the selective sodium transporter genes HKT1;4-1 and -2 was found to be organ-specific and modulated by salt stress in both cultivars. Adding proline to the salt-containing growth medium alleviated some salt stress effects such as the decrease in water content, ion leakage and expression oxidative stress markers while growth parameters were partially rescued to different extents in the two cultivars. Durum wheat seedlings accumulated sodium ions (Na⁺) at the expense of potassium ions (K⁺) under salt stress which lowered the in planta K⁺/Na⁺ ratio. The two durum wheat cultivars studied here respond differently to salt stress in terms of responsiveness to proline, HKT1;4 gene expression, and Na⁺ and</p>						

K⁺ accumulation. Notably, salt stress can be partially alleviated by proline in the drought-resistant cultivar MBB, even though it is relatively salt-sensitive. Testing for the proline alleviation in vitro during salt stress could be a useful test prior to large-scale field experiments.

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1 **Different proline responses of two Algerian durum wheat cultivars to *in vitro* salt stress**

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Summary

Durum wheat, *Triticum turgidum* subsp *durum* (Desf.) Husn., is one of the most salt-sensitive cereal crops, but the physiological responses of different cultivars to salt stress vary. Cultivars that are suited to arid conditions like in Algeria may not necessarily be tolerant of increased salinity. When 10-day seedlings of Algerian durum wheat varieties Hedba 3 (HD3) and Mohamed Ben Bachir (MBB) were subjected to salt stress, they accumulated proline and expressed stress-related and proline metabolism genes in a classic salt-stress response. Expression of the selective sodium transporter genes *HKT1;4-1* and *-2* was found to be organ-specific and modulated by salt stress in both cultivars. Adding proline to the salt-containing growth medium alleviated some salt stress effects such as the decrease in water content, ion leakage and expression oxidative stress markers while growth parameters were partially rescued to different extents in the two cultivars. Durum wheat seedlings accumulated sodium ions (Na⁺) at the expense of potassium ions (K⁺) under salt stress which lowered the *in planta* K⁺/Na⁺ ratio. The two durum wheat cultivars studied here respond differently to salt stress in terms of responsiveness to proline, *HKT1;4* gene expression, and Na⁺ and K⁺ accumulation. Notably, salt stress can be partially alleviated by proline in the drought-resistant cultivar MBB, even though it is relatively salt-sensitive. Testing for the proline alleviation *in vitro* during salt stress could be a useful test prior to large-scale field experiments.

Introduction

Durum wheat, *Triticum turgidum* subsp *durum* (Desf.) Husn., is an important crop both economically and nutritionally. It is especially cultivated in Mediterranean regions, which like others around the world are currently subject to progressive soil salinization (Munns and Tester, 2008). On farms where the sole water supply for agriculture is from irrigation, the salinity of soil tends to increase because there is not enough rainfall to leach salt away (Corwin et al., 2007). Salinity limits the growth of many salt-sensitive (or glycophyte) crops, lowering yield (Horie et al., 2012). Rice (*Oryza sativa*) is one of the least salt-tolerant cereal species (Dionisio-Sese and Tobita, 2000; Colmer et al., 2006; Munns and Tester, 2008; Munns et al., 2010), while barley (*Hordeum vulgare*) is relatively salt-tolerant being able to grow in the presence of up to 250 mM NaCl. Amongst wheat species, *Triticum monoccocum* is salt-resistant, *Triticum aestivum* (bread wheat) is moderately salt-tolerant, and durum wheat is the least salt tolerant (Munns and James, 2003; Munns and Tester, 2008; Wu et al., 2018). Relative salt tolerance does however depend on the cultivar as much as the species of wheat (Plazek et al., 2013).

The impact of salinization on plant growth results from the combination of hyperosmotic stress and ionic toxicity caused by the accumulation of salts, mainly NaCl, in plant organs (Munns and Tester, 2008; Almeida et al., 2017; Waters et al., 2013). Plants growing on salinized soil accumulate high concentrations of sodium ions (Na⁺) that can damage the cell

66 membrane, alter levels of growth regulators, inhibit enzymes, disrupt photosynthesis,
67 interfere with ionic homeostasis, produce harmful reactive oxygen species (ROS), and thus
68 lead to plant death (Munns and Tester, 2008; Julkowska and Testerink, 2015). High Na⁺
69 concentrations also have inhibitory effects on the absorption of major nutrients with similar
70 physicochemical properties, such as K⁺, by the root (Almeida et al., 2017; Hamamoto et al.,
71 2015).

72 The control of Na⁺ transport and exclusion of Na⁺ from leaf tissues are important processes
73 protecting plants from sodium toxicity (Hanin et al., 2016). Durum wheat and rice both have
74 a low capacity for Na⁺ exclusion (Dionisio-Sese and Tobita, 2000; Colmer et al., 2006; Munns
75 and Tester, 2008; Munns et al., 2010). Sodium transporters have been found to be important
76 for salt tolerance, such as the *SOS1* and *SOS4* genes in durum wheat (Feki et al., 2011, 2013,
77 Ramenzani et al., 2011). *HKT* genes encoding high-affinity K⁺ transporters (HKT) are
78 important for sodium tolerance in *Triticum species* (Huang et al., 2006, 2008; James et al.
79 2006, 2011; Byrt et al., 2014). A proposed mechanism for Class-I HKT is where HKT activity in
80 xylem parenchymal cells pumps Na⁺ out of xylem, lowering the Na⁺ concentration in the
81 circulating xylem sap, which prevents Na⁺ from accumulating in the leaf blade (Horie et al.,
82 2009; Byrt et al., 2014). Class-II HKT are involved in nutritional sodium uptake during
83 potassium deficiency (Horie et al., 2009). Salt sensing and exclusion is also attributed to
84 *SOS1* type protein (Shi et al., 2000, 2002; Wu et al., 2018) and Na⁺/H⁺ exchangers (Apse et al.,
85 1999; Shabala et al., 2015). Interestingly, the introduction of some genetic characteristics
86 from the relatively salt-resistant *T. monoccocum* led to improved salt tolerance in durum
87 wheat (Huang et al., 2006; Huang et al., 2008; James et al., 2006, 2011). The *T. monoccocum*
88 loci *Nax1* and *Nax2* responsible for the improved salt tolerance trait encode HKT sodium
89 transporters (Platten et al., 2006; Horie et al., 2009; Byrt et al., 2014; Almeida et al., 2017).

90 The deleterious consequences of salt stress on the plant can in some cases be counteracted
91 by the accumulation of solute compounds, such as the amino acid proline (Zhang et al.,
92 2016; Rana et al., 2016, Annunziata et al., 2017). The accumulation of proline in plants
93 growing under saline conditions may contribute to stress protection through a number of
94 mechanisms, for example, as an osmotic agent, as a ROS quencher, as a stabilizer of
95 membranes and macromolecules, or as an inducer of the expression of salt-stress responsive
96 genes (Hayat et al., 2012). The processes of proline metabolism and catabolism contribute to
97 balancing redox potential (Szabadoz and Saviouré, 2010). Proline biosynthesis occurs via two
98 pathways either from glutamate or from ornithine, but the glutamate pathway probably
99 predominates under stress conditions (Hu et al., 1992; Delauney and Verma, 1993). The
100 enzymes Δ^1 -pyrroline-5-carboxylate (P5C) synthetase (P5CS) and P5C reductase (P5CR),
101 respectively, catalyse the first two steps of proline biosynthesis from glutamate (Verbruggen
102 et al., 1993; Amini et al., 2015). Proline catabolism involves the sequential action of proline
103 dehydrogenase (PRODH), which converts proline to P5C, and P5C dehydrogenase (P5CDH),
104 which converts P5C to glutamate (Elthon and Stewart, 1981; Hare and Cress, 1999;
105 Verbruggen and Hermans, 2008; Zhang and Becker, 2015). In most plant species, the
106 metabolism of proline is upregulated by stress, often by transcriptional activation of *P5CS*,

107 resulting in high intracellular proline concentrations (Mi Zhang and Becker, 2015; Liang et al.,
1 108 2013; Verslues and Sharma, 2010; Silva-Ortega et al., 2008). However, stress-induced proline
2 109 accumulation is variable in crops and depends on the species, the growth stage and the salt
3 110 concentration (Annunziata et al., 2017). Proline can be applied exogenously to salt-stressed
4 111 plants to increase endogenous levels *in planta*, thus minimizing damage, re-establishing salt
5 112 tolerance (Roy et al., 1993; Hoque et al., 2007), and improving water retention, growth, and
6 113 antioxidant defences. Several studies have shown a beneficial effect of exogenous proline on
7 114 durum wheat and other crops subject to moderate stress induced by up to 100 mM NaCl
8 115 (Mahboob et al., 2016), although relative salt tolerance does not correlate to endogenous
9 116 proline content in durum wheat (Plazek et al., 2013).

10 117 Drought resistance is a desirable trait in durum wheat, but it is not necessarily associated
11 118 with salt-stress resistance. Here we **studied** two durum wheat cultivars from Algeria that
12 119 perform differently under drought stress (Dionisio-Sese and Tobita, 2000; Colmer et al.,
13 120 2006; Munns and Tester, 2008; Munns et al., 2010). The Hedba 3 (HD3) cultivar has been
14 121 characterized as being relatively drought sensitive while the Mohamed Ben Bachir (MBB)
15 122 cultivar is relatively drought tolerant (Ali Dib and Monneveux, 1992, Monneveux et al., 1986,
16 123 Mekhlouf et al., 2006). We aimed to evaluate the physiological and gene expression
17 124 responses of the cultivar seedlings grown *in vitro* with a harsh salt stress (10 g.L⁻¹, 171 mM
18 125 NaCl), including the impact of exogenous proline on stress relief.
19 126

127 **Material and Methods**

129 **Plant material**

130 *Triticum turgidum* subsp *durum* (Desf.) Husn. (durum wheat) seeds were obtained from the
131 Crops Technical Institute (ITGC), Algiers, Algeria (<http://www.itgc.dz/>). Hedba 3 (HD3) and
132 Mohamed Ben Bachir (MBB) are cultivars chosen for their contrasting responses to water
133 stress. HD3 is relatively sensitive to water stress (Ali Dib and Monneveux, 1992) while MBB is
134 relatively resistant to it (Monneveux and Nemmar, 1986; Mekhlouf et al., 2006).
135

136 **Plant growth conditions**

137 Seeds were surface-sterilized for 20 min in 6% sodium hypochlorite, then rinsed five times in
138 sterile water, washed once in 70% ethanol (v/v) for 1 min, and rinsed in sterile pure water.

139 Seeds were germinated on 0.7% agar solid MS medium (Murashige and Skoog, 1962) for 3
140 days (d). To ensure homogeneity of samples, only seedlings that were already 2-3 cm long
141 were transferred to glass tubes containing 10 mL of 0.35% agar solid MS medium (to allow
142 for seedling growth and facilitate handling) and plugged with sterile cotton wool. For stress
143 conditions the medium was supplemented with either 10 g.L⁻¹ (171 mM) NaCl or 20 mM
144 proline, or both. The chosen NaCl and proline concentrations are within the ranges of those
145 used to evoke a strong stress response *in vitro* or in aquaponics (Wu et al., 2018; Per et al.,
146 2017). Seedlings were grown for a further 10 d in a growth chamber at 22 °C with a

147 photoperiod of 16 h of neon light, averaging 90 μmoles of photons $\text{m}^{-2} \text{sec}^{-1}$ of
148 photosynthetically active radiation at the level of seedlings, and 8 h of dark. Seedlings grew
149 satisfactorily in these conditions, the control reaching 25 cm on average.

150 Leaves and roots were harvested and frozen in liquid N_2 then stored at -80°C until further
151 analysis.

152 When short-term responses to stress were studied, 10-d seedlings grown on control MS
153 medium were carefully up-rooted then placed in a beaker with the roots in MS liquid
154 medium, either with or without NaCl and/or proline.

156 **Observation of root tips**

157 Root tips (1 cm) from 10-d plants were excised and immediately incubated in Hoyer's
158 solution (Anderson, 1954) at 4°C for 6 d, then observed using a Zeiss Axioskop microscope
159 equipped with DIC optics and a $\times 10$ magnification objective. Images were recorded using an
160 AxioCam camera MR (Zeiss) and processed and archived with AxioVision software (Zeiss).

162 **Measurement of physiological parameters of seedlings**

163 After 10 d, seedlings were uprooted and the maximum lengths of shoots and roots were
164 measured. Fresh weight (FW) was recorded. To determine relative water content (RWC),
165 tissues were allowed to fully hydrate on the surface of pure water for 1 d at 4°C in the dark
166 and their turgid weight (TW) was recorded. Tissues were allowed to dry for 2 d at 80°C then
167 weighed to determine the dry weight (DW). Relative water content (RWC) was calculated as
168 $100 \times (\text{FW} - \text{DW}) / (\text{TW} - \text{DW})$.

170 **Quantification of proline, malondialdehyde and electrolyte leakage**

171 Proline content was determined using a colorimetric assay adapted from Bates et al. (1973).
172 Powdered frozen seedling tissue (50 mg FW) was homogenized in 1.5 mL of 3% sulfosalicylic
173 acid. The homogenate was centrifuged at 14000 rpm at 4°C for 10 min. Ninhydrin buffer
174 (2.5% ninhydrin, 60% acetic acid in 2.5 M phosphoric acid) and 100% acetic acid were added
175 to 0.4 mL of supernatant (1:1:1, v/v/v). The proline-ninhydrin reaction was allowed to
176 continue for 60 min at 95°C . After cooling on ice, 0.8 mL of toluene was added to each
177 sample to extract the coloured proline-ninhydrin complex. The optical density at 520 nm of
178 the upper organic phase was determined. Proline was quantified by comparison with known
179 concentrations of L-proline up to 20 mg/L (0.174 mM).

180 To estimate the amount of lipid peroxidation, malondialdehyde (MDA) resulting from lipid
181 peroxidation, can be used as markers of salt stress (Hodges et al., 1999; Pang and Wang,
182 2008). MDA was quantified using the thiobarbituric acid colorimetric reaction according to
183 Hodges et al. (1999).

184 Electrolyte leakage was quantified as a way to estimate the degree of membrane integrity.
185 Ten 1-cm long leaf fragments were immersed in 20 mL of distilled water at room
186 temperature for 15 minutes, rinsed thoroughly then left for 1 h in fresh 20 mL of distilled
187 water, which was found sufficient to ensure reliable measurement of ion leakage. The initial

188 electrical conductivity (EC1, $\mu\text{S}/\text{cm}$) was measured. The samples were boiled for 5 min then
189 cooled to room temperature and the conductivity was measured again (EC2, $\mu\text{S}/\text{cm}$).
190 Electrolyte leakage (EL) was computed as: $\text{EL} = (\text{EC1}/\text{EC2}) \times 100$ (Dionisio-Sese and Tobita,
191 1998).

193 **Superoxide dismutase (SOD) activity assay**

194 Superoxide dismutase (SOD) is detoxifying enzyme SOD used as a marker of oxidative stress
195 (Miller et al., 2010; Saibi and Brini, 2018; Joseph and Jini, 2010). Enzymatic extracts were
196 prepared by homogenizing 0.5 g of powdered frozen shoots in cold phosphate buffer (50
197 mM KPO_4 buffer pH 7.0, 1 mM EDTA, Triton X100, and 1% PVP). Insoluble material was
198 pelleted by centrifugation at $16000 \times g$ for 30 min at 4°C . The supernatant was loaded onto a
199 PD10 Sephadex G25 column (GE Healthcare). Soluble proteins were eluted with 100 mM
200 potassium phosphate buffer pH 7.8 and quantified (Bradford, 1976) against bovine serum
201 albumin as standard. SOD activity was determined spectrophotometrically at 560 nm based
202 on the capacity of SOD to inhibit the photochemical reduction of nitro blue tetrazolium
203 (NBT) to formazan by riboflavin in the light (Beyer and Fridovich, 1987). The assay mixture
204 consisted of 1.5 mL of reaction buffer (50mM KPO_4 buffer pH 7.8, 1 mM EDTA, 13 mM
205 methionine, 2.25 mM NBT and 2 mM riboflavin) containing 20 μl of enzyme extract (20 to 60
206 μg protein). The reaction, started by illumination, lasted 13 min. SOD activity was expressed
207 in relative units (U mg^{-1} protein) where 1 U caused a 50% decrease in NBT reduction at 25°C .

209 **Measurement of Na^+ and K^+ content of tissues**

210 Frozen plant tissues were lyophilized under vacuum. Powdered dry tissues (50 mg) were
211 suspended in 5 mL of 0.5 M nitric acid for 1 h at 80°C , as described in Munns et al. (2010).
212 After centrifugation for 10 min at $3000 \times g$ the supernatant was used to quantify Na^+ or K^+
213 using a Sherwood M410 flame ionization spectrophotometer (Sherwood Scientific Limited,
214 UK). Ranges of NaCl and KCl dilutions were used for calibration.

216 **Analysis of gene expression by reverse transcription polymerase chain reaction (RT-PCR)**

217 All gene sequence identifiers are given in Table 1. *PRODH*, *P5CS*, *P5CR*, the durum wheat
218 dehydrin (*DHN*) (Rampino et al., 2006), and tubulin (*TUB*) gene sequences are available in
219 the NCBI GenBank (National Center for Biotechnology Information,
220 <https://www.ncbi.nlm.nih.gov>). Durum wheat *HKT1;4-1* and *HKT1;4-2* sequences were
221 retrieved from the wheat genome database (<https://wheat-urgi.versailles.inra.fr>).

222 The Multalin program was used to align and compare multiple sequences
223 (<http://multalin.toulouse.inra.fr/multalin/>) to identify highly similar gene regions on which
224 to base the design of specific primers. Primer sequences used in this study are shown in
225 Table 1. Gene-specific primer pairs were designed and selected using Primer3 software,
226 tested on Virtual PCR software (bioinformatics.org/sms2/pcr_products.html) and
227 synthesized by Eurogentec (Belgium).

228 RNA was extracted from 100 mg of frozen powdered tissue homogenized in 0.5 mL of
1 229 extraction buffer (0.2 M Tris-HCl pH 7.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS) then
2 230 extracted twice with a mixture (1:1, v/v) of phenol-citrate pH 4.3 (Sigma-Aldrich) and
3 231 chloroform. The suspension was centrifuged 5 min at 14000 × *g*. RNA was selectively
4 232 precipitated twice from the upper aqueous phase with 2 M LiCl (final concentration) for 8 h
5 233 to 16 h at 0°C. After 10 min of centrifugation at 14000 × *g*, the RNA pellet was rinsed with
6 234 70% ethanol (v/v), air dried and suspended in 30 µL of pure water. RNA was quantified by
7 235 measuring UV absorbance at 260 and 280 nm using a Nanovue spectrophotometer (ND1000
8 236 UV-VIS). After a DNase treatment, RNA integrity was checked by electrophoresis through a
9 237 1% agarose gel in Tris-acetate-EDTA buffer.

10 238 For reverse transcription 1.5 µg of RNA was used with Revert Aid Reverse Transcriptase
11 239 according to the manufacturer's instructions (Life Technologies). Complementary DNA
12 240 samples were diluted fourfold with ultrapure water. PCR was done using Dream Taq Green
13 241 DNA polymerase (Life Technologies). For each PCR reaction, 2 µL of cDNA was used as a
14 242 template, 0.8 µM of both forward and reverse primers, 0.2 mM dNTP and 1 unit of
15 243 DreamTaq in 1 × GreenTaq Buffer. PCR conditions were: 5 min at 94°C; then 28 cycles of 30s
16 244 at 94°C, 30s at 55°C and 30s at 72°C; followed by 10 min at 72°C. The number of PCR cycles
17 245 was adapted for each gene, so that the amount of PCR product amplified allowed semi-
18 246 quantitative estimation of the level of expression compared to control gene expression.
19 247 Quantification of PCR samples was performed using ImageJ software
20 248 (<https://imagej.nih.gov>) image analysis. Each sample was quantified first relative to the
21 249 endogenous *TUB* gene expression and then to the corresponding control sample. Only
22 250 expression ratios from measurable gene expression are calculated, otherwise data is
23 251 indicated as not determined (nd).

252 253 **Results**

254 **Proline accumulates preferentially in leaves of salt stressed durum wheat seedlings grown** 255 ***in vitro***

256 In the control condition, free proline content was similar in roots and leaves of both durum
257 wheat cultivars studied, ranging from 33 to 36 µmol g⁻¹DW (Figure 1A, B). In response to salt
258 stress, proline content mainly increased in leaves, threefold in HD3 and fourfold in MBB
259 (Figure 1A, B). When proline was present in the growth medium, proline accumulated in
260 both leaves and in roots, as might be expected if it were taken up by the roots and
261 transported to the shoots (Figure 1A, B). In MBB roots 60% more proline accumulated than
262 in HD3 roots (244 compared to 150 µmol g⁻¹DW, Figure 1B). In both cultivars, the
263 combination of salt stress and proline increased proline content even more, particularly in
264 leaves (Figure 1A, B), such that leaves contained tenfold as much proline as control leaves,
265 whilst HD3 roots contained fivefold and MBB roots sevenfold as much as control roots
266 (Figure 1A, B). The observed differences in proline accumulation between HD3 and MBB
267 suggest that proline metabolism and transport are not the same in the two cultivars.

268

269 **Effect of salt stress and proline on expression of stress and proline-related genes**

270 Using semi-quantitative reverse-transcription PCR, we measured the effect of salt stress on
271 expression of *TdDHN15.3* (shortened to *DHN* here), a durum wheat gene known to be
272 responsive to water stress and salt stress that encodes a dehydrin (Rampino et al., 2006).
273 We observed an increase in *DHN* transcripts in leaves and roots of seedlings exposed to salt
274 stress from 8 h to 10 d (Figure 2A).

275 The expression of proline biosynthesis gene *P5CS* was up-regulated in leaves of both
276 cultivars after 8 h of salt stress, but this increase diminished after 10 d of salt stress. In roots
277 *P5CS* gene expression was less obviously modulated by stress but the basal level of
278 expression was higher than in leaves. Adding proline did not change *P5CS* gene expression
279 pattern. However, in leaves of seedlings subjected to proline and salt stress, *P5CS* expression
280 ratio lowers after 8 hours in HD3 and after 10 days in MBB (Figure 2B).

281 *P5CR* expression was high in control seedlings and was not influenced by the stress
282 conditions (Figure 2B). Here, we found that a short period of salt stress lowered *PRODH*
283 expression in roots (Figure 2B), but this effect was not observed when proline was present,
284 especially for MBB. *PRODH* gene expression was slightly upregulated in the presence of
285 proline alone, most noticeably in HD3 leaves after 10 d of exposure to proline.

286 Overall the stress-related and proline metabolism genes studied are regulated in a salt-stress
287 and organ-specific manner. Salt stress can transiently upregulate the expression of *P5CS* and
288 repress the expression of *PRODH*. However, the durum wheat cultivars differ in the fine
289 regulation of these genes.

290

291 **Differences in proline alleviation of stress marker expression in MBB and HD3**

292 Relative water content (RWC) of leaves was similar in the two cultivars in control conditions.
293 Under salt stress, leaf RWC was significantly lower (81%) for the MBB cultivar indicative of
294 hyperosmotic stress (Figure 3A). When proline was present during salt stress the RWC of
295 MBB remained similar to that of non-stressed controls (Figure 3A). The proline alleviation
296 effect on RWC is therefore cultivar specific.

297 Here we found that SOD activity increased around threefold in salt-stressed HD3 (Figure 3B)
298 and more than fourfold when proline and salt stress were combined (Figure 3B). In MBB the
299 basal SOD activity was lower than in HD3, did not increased in salt stress, and lowered with
300 proline alone (Figure 3B). MDA content increased under salt stress in the MBB cultivar, but
301 not in the HD3 cultivar (Figure 3C). MDA content in MBB remained low, to control level,
302 when both salt and proline were present (Figure 3C).

303 Ion leakage from leaf tissues is a measure of membrane integrity which is affected by
304 oxidative stresses and lipid peroxidation. We found that salt stress increased ion leakage
305 while added proline counterbalanced the effect of salt stress in both cultivars (Figure 3D).
306 Proline therefore has different effects on antioxidative activity in MBB and HD3 under salt
307 stress, with MBB generally being more responsive.

308

309 **Proline rescues the detrimental effects of salt stress on seedling growth**

1 310 Plant growth was evaluated by measuring the length of the longest leaves and roots (Figure
2 311 4A-C) and the fresh weight (FW) and dry weight (DW) of whole shoots and roots. In the
3 312 absence of salt stress, proline stimulated growth of the HD3 cultivar, with an increase in DW
4 313 of 24% in leaves and 14% in roots (Figure 4B, C). Proline stimulation of growth was negligible
5 314 in MBB.
6 315

7 316 In both cultivars NaCl had a negative impact on both leaf and root growth compared to the
8 317 non-stressed controls (Figure 4A-C). It was noted that the appearance of salt-stressed roots
9 318 was altered near the meristem zone and near the root tip (Supplementary Figure 1). Organ
10 319 growth was differentially inhibited in the presence of NaCl as roots were more sensitive than
11 320 shoots. Roots of both cultivars were similarly sensitive to salt stress with decreases in DW of
12 321 53% for MBB and 57% for HD3 (Supplementary Table 1). MBB leaves however were more
13 322 sensitive to salt as they lost 32% of DW compared to 21% lost from HD3 leaves
14 323 (Supplementary Table 1).

15 324 When proline was present during salt stress, a beneficial effect was observed as growth was
16 325 partially restored in both durum wheat cultivars (Figure 4A-C). The two durum wheat
17 326 cultivars responded differently though. Proline reduced the inhibitory effects of salt stress
18 327 on root growth by 39% in HD3 and 9% in MBB and on leaf growth by 5% in HD3 and 16% in
19 328 MBB (Supplementary Tables 2 and 3). While proline can partially alleviate the negative
20 329 effects of salt stress in durum wheat, each cultivar has specific salt and proline sensitivities in
21 330 leaves and roots.

22 331 **Salt stress regulates sodium transporter gene expression**

23 332 We compared the relative expression of two durum wheat *HKT1* genes *HKT1;4-1* and -2
24 333 (Figure 5). *HKT1;4-1* was expressed at a low level in leaves (Figure 5). *HKT1;4-1* was
25 334 expressed in roots of control seedlings but this expression was repressed by salt stress
26 335 (Figure 5). The *HKT1;4-2* gene was expressed mainly in leaves where it was induced by salt
27 336 stress in both durum wheat cultivars (Figure 5). Weaker salt-stress induction of *HKT1;4-2*
28 337 expression was also observed in MBB roots. The difference in expression between leaves
29 338 and roots suggests that the transporters encoded by *HKT1;4-1* and -2 have organ-specific
30 339 regulation and roles. Although up or down regulation of *HKT1;4* genes was pronounced after
31 340 a few hours of salt stress, longer exposure to salt stress durum wheat seedlings did not
32 341 sustain the same levels of expression (Figure 5). Proline interfered with *HKT1;4-2* gene
33 342 expression in HD3 cultivar leaves solely, after 8 hours in the absence of stress and after 10
34 343 days under salt stress (Figure 5).
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37 346 **Sodium and potassium levels in durum wheat seedlings under salt stress**

38 347 Sodium content is very low, not more than 15 μ moles per g DW, in control seedlings with or
39 348 without proline in the growth medium (Figure 6A). The consequences of salt stress on
40 349 sodium content were dramatic (Figure 6B). Sodium content was a hundredfold higher in salt-
41 350 stressed leaves (more than 1100 μ moles per g DW, Figure 6B). Sodium was distributed
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350 differently in the two salt-stressed cultivars studied here. In HD3 sodium content in leaves
351 and roots was similar. In MBB more sodium accumulated in leaves (Figure 6B). Adding
352 proline to salt stress resulted in 50% less sodium in MBB leaves, but 30% more in roots,
353 compared to the salt-stress sample (Figure 6B). These results suggest that sodium
354 accumulation is sensitive to proline in the MBB cultivar but not in the HD3 cultivar.

355 Here the HD3 and MBB cultivars respectively contained 712 and 987 μ moles per g DW of
356 potassium in control conditions (Figure 6C). Surprisingly, when proline was present without
357 salt stress, the potassium content of leaves was much higher, respectively 976 and 1887
358 μ moles per g DW (Figure 6C). Salt stress effects on potassium levels differed according to the
359 organ and the cultivar (Figure 6C). Salt stress caused a 37% reduction in the amount of
360 potassium in MBB leaves but no change in potassium occurred in HD3 leaves. Potassium
361 levels in roots are more affected by salt stress, with roots containing 55% to 69% less
362 potassium than control. Proline modulates potassium in roots but only slightly (Figure 6C).

363 Here we found that in control conditions the K^+/Na^+ ratios of the two varieties were
364 different, HD3 having lower ratios in both roots and leaves than MBB (Figure 7). Under salt
365 stress the K^+/Na^+ ratio was at least a hundredfold lower than in the control condition and
366 proline did not significantly improve the K^+/Na^+ ratio under stress (Figure 7).

367 DISCUSSION

368 Salt stress affects durum wheat seedling growth

369 Durum wheat is one of the most salt-sensitive cereal crops. Compared to bread wheat or
370 other *Triticum* species, durum wheat has a relatively low ability to exclude sodium (James et
371 al., 2006; Munns et al., 2006; Rampino, 2006), it does not efficiently store sodium in cellular
372 compartments (Wu et al., 2018), and its root meristem is less perceptive to salt stress (Wu et
373 al., 2018). The decrease in leaf RWC in seedlings grown in the presence of NaCl indicates
374 that a hyperosmotic stress is occurring in leaf tissues. The accumulation of sodium ions
375 would also cause ionic stress, which is often also associated with oxidative stress. The
376 deleterious effect of salt stress on durum wheat seedling growth was mitigated by proline,
377 as also reported for bread wheat (Talat et al., 2013), rice (Sobahan et al., 2009), barley (Lone
378 et al., 1987), and others plant species (Butt et al., 2016; Szabados and Savouré, 2010; Khedr
379 et al., 2003; Dawood et al., 2014; Medeiros et al., 2015; Nasseem et al., 2007; reviewed by
380 Per et al., 2017).

381 We found that durum wheat root tissues became disorganized in the presence of salt.
382 Wheat root cells from the division and transition zone have been described as being severely
383 altered by salt stress (Annunziata et al., 2017). Proline might act as a signal molecule by
384 modulating the cell division in root in *Arabidopsis thaliana* (Biancucci et al., 2015). Here
385 proline effect on root is weak.

386 Durum wheat tolerance to salt varies greatly according to the cultivar **studied** and the
387 method used, including aquaponics and *in vitro* (Wu et al, 2018). Comparing the growth of
388 the two cultivars **studied** here, HD3 might be considered to be more salt-tolerant *in vitro*.
389 Our results suggest that the responses of MBB leaves and of HD3 roots to added proline can

390 minimize the harmful effects of NaCl stress. The salt-stress alleviation effect of proline
391 therefore also depends on the cultivar studied (Per et al., 2017).

392 393 **Salt stress and exogenous proline increase proline content and modulate proline** 394 **biosynthesis gene expression**

395 Proline is a compatible osmolyte with cellular protective properties, and proline
396 accumulation is an indicator of stress such as hyperosmotic and ionic stress (Verslues and
397 Sharma, 2010; Szabados and Saviouré, 2010; Mansour et al., 2017). The protection provided
398 by accumulated proline was shown to vary according to the genotype and stress intensity
399 (Plazek et al., 2013, Rana et al., 2016). Salt tolerance does not correlate with endogenous
400 proline in durum and bread wheat (Plazek et al., 2013). However, higher levels of proline
401 accumulation have been reported in more salt-tolerant durum wheat genotypes (Rana et al.,
402 2016). Here the highest proline concentration was found in the MBB genotype, which is
403 relatively more sensitive to salt stress, suggesting that the accumulated proline, is a marker
404 of the perceived intensity of stress (Almansouri et al., 1999; Munns, 2002). In this case,
405 endogenous proline accumulation might not have provided cells with sufficient protection
406 against salt stress.

407 We observed that proline metabolism gene expression was modulated by stress, with *P5CS*
408 mRNA abundance upregulated after 8 h of salt stress in leaves. Salt-induced *P5CS* gene
409 expression in durum wheat may therefore lead to increases in P5CS enzyme and proline
410 synthesis (Annunziata et al., 2017; Amini et al., 2015). *P5CR*, another gene involved in
411 proline biosynthesis, was not as obviously modulated as *P5CS*, as already shown in durum
412 wheat (Mattioni et al., 1997) and contrasting with the modulation reported in bread wheat
413 (Ma et al., 2008). We found that expression of proline catabolism gene *PRODH* was
414 downregulated by salt stress, as occurs in many plant species (Peng et al., 1996; Servet et al.,
415 2012). *PRODH* activity itself is lowered by salt stress (Mattioni et al., 1997) including in
416 durum wheat (Soccio et al., 2010). *PRODH* gene expression is known to be up-regulated by
417 proline (Verslues and Sharma, 2010; Yoshiba et al., 1997; Servet et al., 2012; Cabassa-
418 Hourton et al, 2016), but here only a slight *PRODH* up-regulation was observed. Proline
419 accumulates in leaves as the combined result of salt-stress induced synthesis inhibition of
420 catabolism. Our results suggest that while proline synthesis is upregulated in durum wheat,
421 it is not sufficient to overcome the deleterious effects of the severe salt stress imposed.

422 The durum wheat seedlings studied were able to take up exogenous proline provided in
423 medium. Increases in free proline content resulting from uptake of exogenous proline has
424 indeed been observed in plants including wheat, rice and sugarcane (Hur et al., 2004;
425 Mahboob et al., 2016; Bhusan et al., 2016; Medeiros et al., 2015; Mervat et al., 2015).
426 Proline accumulated *in vivo* from an exogenous source can complement the low level of
427 proline produced endogenously to counterbalance salt-stress effects.

428 We observed a correlation between proline accumulation and less salt stress, as shown by
429 oxidative stress marker levels. The combination of salt and proline had different effects on

430 ROS detoxification, membrane integrity protection and growth according to the organ and
1 431 cultivar **studied**.

432 4 433 **Tissue specific and differential regulation of *HKT1;4-1* and *-2* genes by salt stress**

6 434 Sodium transporters contribute to lowering otherwise toxic sodium levels in plant tissues
7 435 (Huang et al., 2006, 2008; James et al. 2006, 2011; Byrt et al., 2014; Wu et al., 2018). High
8 436 affinity potassium transporters (HKT) class 1 transporters have also been associated with salt
10 437 stress tolerance in durum wheat (Horie et al., 2009; Byrt et al., 2014), and *HKT* genes are
11 438 important for sodium tolerance in *Triticum species* (Huang et al., 2006, 2008; James et al.
13 439 2006, 2011; Byrt et al., 2014). Here we found that *TdHKT1;4-1* and *TdHKT1;4-2* expression is
14 440 organ specific and is responsive to short-term salt treatments. Basal expression of *TdHKT1;4-*
16 441 *1* in roots is repressed by salt which suggests *TdHKT1;4-1* may be important in regulating
17 442 ionic balance in the absence of salt stress. By contrast, *TdHKT1;4-2* was induced in response
19 443 to short-term salt stress. The pattern of *TdHKT1;4-2* expression is similar in both cultivars,
20 444 with long-term induction observed in HD3 leaves. *TdHKT1;4-2* could be important for
22 445 regulating salt and balance at early stages of salt stress. The differences in regulation of
23 446 *TdHKT1;4-1* and *-2* genes in leaves and roots and by salt stress suggest that durum wheat
25 447 class-I HKT have different physiological roles.

26 448 The introduction in durum wheat of *HKT* genes from *T. monococcum* (*TmHKT1;4 A1* and *A2*)
28 449 confers salt tolerance (James et al., 2006, 2011). Expression of *TmHKT1;4* is salt responsive in
29 450 *T. monococcum* leaves (Tounsi et al., 2016). *TmHKT1;4-A1* is predominantly expressed in
31 451 leaves. *TmHKT1;4-A2* is more strongly expressed than *TmHKT1;4-A1* in roots and in leaves
32 452 (Tounsi et al., 2016). Sodium conductance of *TMHKT1;4-A2* is also higher than that of
34 453 *TMHKT1;4-A1*. *TmHKT1;4-A2* gene may have a predominant role, possibly representing the
35 454 active part of the *Nax1* salt tolerance locus (Tounsi et al., 2016). In our study *TdHKT1;4* gene
37 455 expression was relatively low in prolonged salt stress, suggesting that any role in sodium
38 456 exclusion is limited. Comparing *HKT1;4* gene expression in *T. durum* and *T. monococcum* thus
40 457 helps us understand why durum wheat is relatively salt intolerant. Externally added proline
41 458 can to some extent protect durum wheat seedlings from the harmful effects of salt, and can
43 459 modulate gene expression, including *HKT1;4* genes, but it is not sufficient to provide full
44 460 protection against salt stress. A recent study on hydroponic grown durum wheat stressed
46 461 with 200 mM NaCl (Wu et al., 2018) suggested that the combination of sodium sensing, root
47 462 sodium exclusion and sodium accumulation in vacuoles might be key to explaining the
49 463 difference in salt tolerance between bread wheat and durum wheat (Wu et al., 2018). The
50 464 *NHX1* sodium transporter might be involved in this process (Wu et al., 2018). Possibly,
52 465 regulation of *HKT1* gene expression and HKT activity might also be involved in salt tolerance.

53 466 55 467 **Na⁺ and K⁺ accumulation is disturbed by salt stress**

56 468 Plant tissues readily accumulate potassium (Ashley et al., 2006). The K⁺/Na⁺ ratio can be
58 469 used as an indicator of the level of salt tolerance in durum wheat cultivars (James et al.,
59 470 2006). Both durum wheat cultivars accumulated Na⁺ in shoots and roots under salt stress. K⁺

471 decreased in roots resulting in an ion imbalance, that is a low K^+/Na^+ ratio, as seen in
1 472 numerous salt stress studies in plants (Zhe-Yong et al., 2004; Cuin et al., 2008). Leaves
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3 473 accumulated more Na^+ than roots, which reflects the long-distance transport of Na^+ cations
4 474 from root tissues to photosynthetic tissues. This result confirmed that durum wheat has
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6 475 limited capacity to control and minimize sodium transport to the shoots compared with
7 476 other *Triticaceae*, such as *T. aestivum* and *T. monococcum* (Tounsi et al., 2016). Durum wheat
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9 477 cultivar HD3 was able to maintain a slightly higher K^+ content in leaves which might allow for
10 478 better cell protection under NaCl stress (Horie et al., 2009). Our results can be compared
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12 479 with those of *T. aestivum* (Talat et al., 2013) and salt-sensitive rice (Siddique et al., 2015)
13 480 where added proline leads to a decrease in shoot Na^+ content by lowering apoplastic uptake
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15 481 of Na^+ (Nounjan et al., 2012; Sobahan et al., 2009). Positive impacts of proline on ion content
16 482 have also been observed in other monocot species such as sugarcane (Medeiros et al., 2015)
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18 483 and maize (Nassem et al., 2007).

19 484 Proline in combination with salt stress led to a decrease in the Na^+ content of MBB leaves,
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21 485 whose growth was the most responsive to proline (Supplementary Table 2). In MBB roots,
22 486 which were less responsive to proline, Na^+ content increased. However, no effect of proline
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24 487 on Na^+ accumulation was observed in the HD3 cultivar. Proline can help maintain ion
25 488 homeostasis by limiting K^+ efflux in several species (Cuin and Shabala, 2005, 2007). The
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27 489 proline effect on salt sensitivity therefore depends on species and genotype (Plazek et al.,
28 490 2013, Per et al., 2017; Wu et al., 2018).
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30 491 Conclusion

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32 492 The two Algerian durum wheat cultivars studied here have different sensitivities to salt
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34 493 stress and to proline alleviation of this stress. The *in vitro* methods used here could be used
35 494 to test durum wheat genotypes for their salt and proline sensitivities before field tests for
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37 495 breeding or large-scale experiments.
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1 499 **Figure legends**

2 500 **Figure 1. Effect of salt and proline on proline accumulation in seedlings.**

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4 501 Durum wheat seedlings of HD3 and MBB cultivars were grown for 10 d on medium in the
5 502 presence of NaCl and proline (see Materials and Methods). **A**, Proline accumulation in leaves
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7 503 and roots of **A**, HD3 and **B**, MBB. Data are averages of at least three replicates, of 12
8 504 seedlings each, with bars indicating standard errors. In each panel, histograms marked with
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10 505 different letters indicate values that are significantly different ($p < 0.05$) in a two-way
11 506 ANOVA Tukey's test.

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14 508 **Figure 2. Stress and proline metabolism gene expression in durum wheat seedlings.**

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16 509 Durum wheat seedlings of HD3 and MBB cultivars subjected to salt stress in the presence or
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18 510 absence of proline in the growth medium (see Materials and Methods) for 8 h or 10 d. RNA
19 511 was extracted from leaves and roots. Gene expression was analysed by RT-PCR. Expression
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21 512 ratio relative to control is indicated below each panel. Nd: not determined (see methods). **A**,
22 513 Transcripts of the stress-related gene dehydrin (DHN). White arrow points to a double PCR
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24 514 product. **B**, Transcripts of the proline metabolism genes P5C synthase (P5CS), P5C reductase
25 515 (P5CR) and proline dehydrogenase (PDH). Numbers under gels in **A** and **B** indicate fold
26 516 differences in transcript abundance relative to the control for each organ/cultivar set. **C**,
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28 517 control. Pro, proline. Na, NaCl. **C**, Transcripts of the control gene tubulin (TUB) whose
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30 518 expression is not affected by proline or stress.

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33 520 **Figure 3. Effect of salt and proline on stress physiology of durum wheat seedlings.**

34 521 Durum wheat seedlings of HD3 and MBB cultivars were grown for 10 d on medium
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36 522 containing NaCl and/or proline (see Materials and Methods). **A**, Relative water content
37 523 (RWC), **B**, superoxide dismutase (SOD) activity, **C**, malondialdehyde (MDA) content, and **D**,
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39 524 ion leakage of leaves. In each panel, histograms marked with different letters indicate values
40 525 that are significantly different ($p < 0.05$) in a two-way ANOVA Tukey's test. Data are averages
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42 526 of at least three replicates, of 12 seedlings each,

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45 528 **Figure 4. Effect of salt and proline on growth of durum wheat seedlings.**

46 529 Durum wheat seedlings of HD3 and MBB cultivars were grown for 10 d on medium
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48 530 containing NaCl and/or proline (see Materials and Methods). **A**, Maximum length, **B**, fresh
49 531 weight, and **C**, dry weight of leaves and roots. Data are averages of $n = 12$ plants with error
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51 532 bars indicating standard errors. Within each panel, bars marked with different letters
52 533 indicate significantly different values ($p < 0.05$) in two-way ANOVA Tukey's test.

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55 535 **Figure 5. Sodium transporter gene expression in durum wheat seedlings.**

56 536 Durum wheat seedlings of HD3 and MBB cultivars were subjected to salt stress in the
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58 537 presence or absence of proline in the growth medium for 8h or 10d (see Materials and
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60 538 Methods). RNA was extracted from leaves and roots. Gene expression was analysed by RT-

539 PCR by amplifying transcripts of sodium transporter genes HKT1;4-1 and HKT1;4-2 and
1 540 control gene tubulin. Gene expression was analysed by RT-PCR. Expression ratio relative to
2 541 control is indicated below each panel. Nd: not determined (see methods). Arrows with
3 542 question marks indicate spurious bands that do not correspond to the transcript of interest.
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8 547 **Figure 6. Sodium and potassium levels in durum wheat seedlings.**

9 548 Durum wheat seedlings of HD3 and MBB cultivars were grown on medium in the presence or
10 549 absence of NaCl and/or proline (see Materials and Methods). **A**, Sodium ion (Na^+) levels in
11 550 leaves and roots of control seedlings. **B**, Sodium ion (Na^+) levels in leaves and roots of salt-
12 551 stressed seedlings. **C**, Potassium ion (K^+) levels of control and salt-stressed seedlings. In each
13 552 panel, histograms marked with different letters indicate significantly different values. Data
14 553 are averages of $n = 12$ plants with error bars indicating standard errors. Within each panel,
15 554 bars marked with different letters indicate significantly different values ($p < 0.05$) in two-way
16 555 ANOVA Tukey's test. C, control. Pro, proline. Na, NaCl.
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25 564 **Figure 7. Potassium to sodium ratio in durum wheat seedlings.**

26 565 Ratio of potassium to sodium ion concentrations in leaves and roots of control (left panel)
27 566 and salt-stressed (right panel) seedlings (grown as in Figure 6). Note the different scales on
28 567 the vertical axes. Data are averages of at least three independent measurements with error
29 568 bars indicating standard errors. In each panel, bars marked with different letters indicate
30 569 significantly different values ($p < 0.05$) in a two-way ANOVA Tukey's test. C, control. Pro,
31 570 proline. Na, NaCl.
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Gene	Genebank Ref	Sequence	Amplicon	Cycles
<i>TUB</i>	U76558.1	F: TGAAGAAGTTGGTGCTGAGT R: ACCACAAAGCAAACGTTCAA	162 bp	27
<i>HKT1.4-2</i>	KF443079	F: CCATCTTTGTCATCGCCATC R: GAATGAGGATGAGTTTGCCG	228 bp	33
<i>HKT1.4-1</i>	KF443078	F: TGACTGTCCTCATGTTTCGTC R: CGAAGGTCCACATGTTTCAG	402 bp	33
<i>P5CR</i>	AY880317.1	F: GATTTGCAGGATTGGTCTGG R: TACCACTCAAGCCAGTAACC	208 bp	27
<i>P5CS</i>	FK827071.1	F: GGGTATGAGAGTGCTTTGGT R: CCATTACCACTTCGAATGGC	248 bp	28
<i>PRODH</i>	AK332189.1	F: TCGACTACTTCACCTTCGTG R: TTGTAGCAGTCGTGGGTG	259 bp	30
<i>DHN</i>	AM180931.1	F: GAGTACCAGGGACAGCAG R: ATGCCATCATCCTCAGACG	203 bp	28

Table 1

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852 **Supplementary tables 1 to 3**

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2 853 Sensitivity index was calculated as the ratio of dry weights of control condition compared to
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4 854 test condition.

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	Sensitivity index (%)	
	Leaves	Roots
HD3	-21.26	-57.57
MBB	-32.04	-53.87

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19 858 **Supplementary table 1: Salt sensitivity (NaCl / Control)**

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Proline/ Control	Sensitivity index (%)	
	Leaves	Roots
HD3	24.12	14.42
MBB	4.59	0.71

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31 860 **Supplementary table 2: Proline sensitivity (Proline / Control)**

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NaCl + Proline / NaCl	Sensitivity index (%)	
	Leaves	Roots
HD3	5.61	39.27
MBB	16.49	9.30

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47 868 **Supplementary table 3: Proline sensitivity to salt stress (NaCl + Proline / NaCl)**

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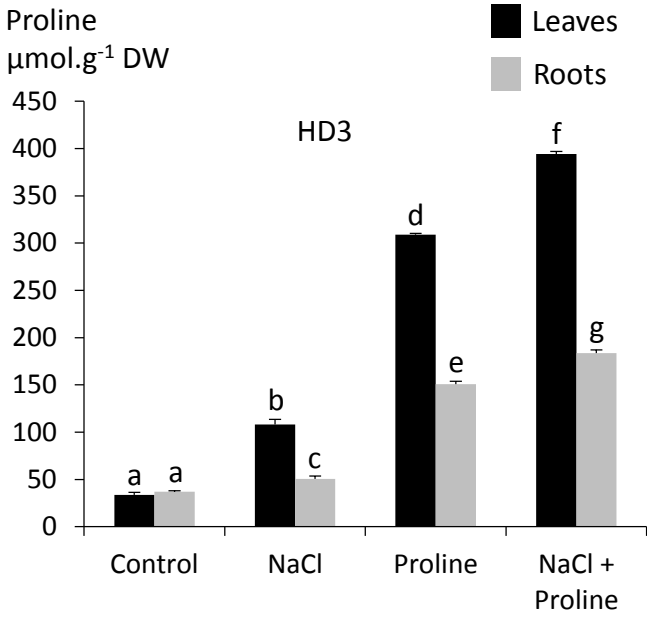
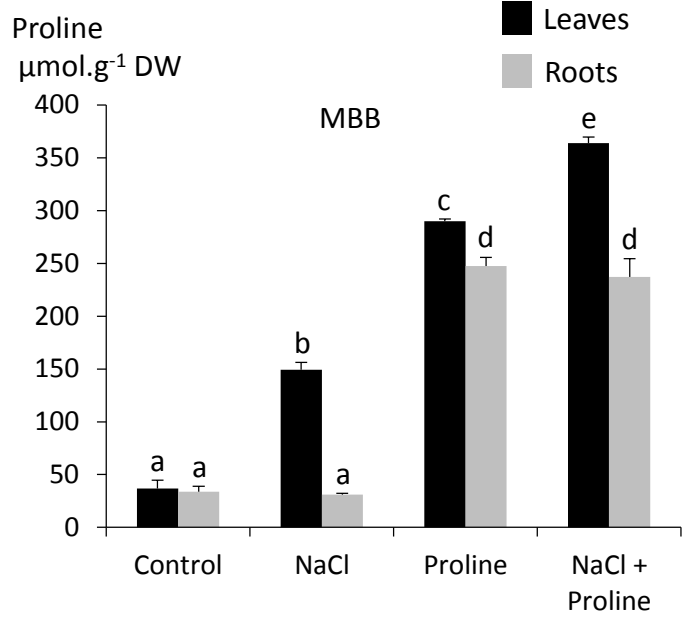
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A**B**

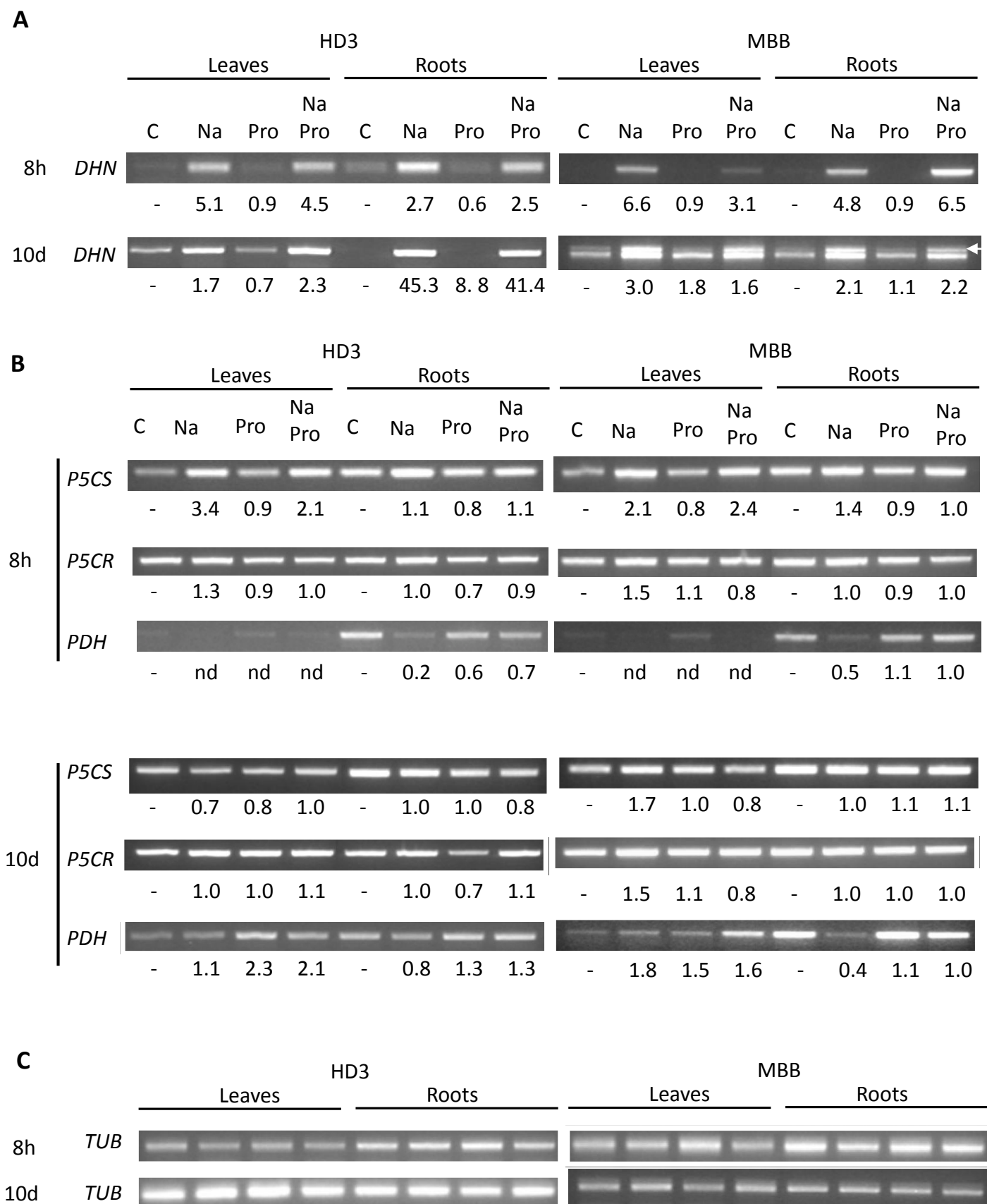


Figure 2

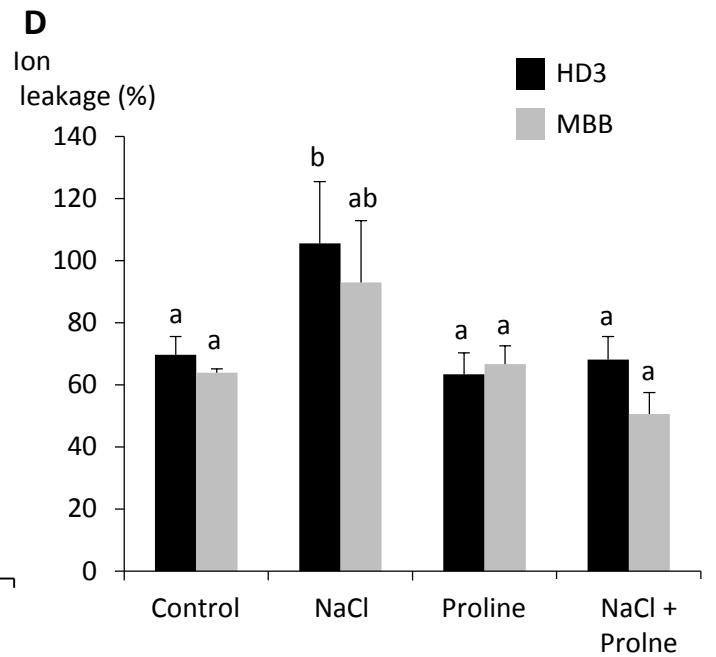
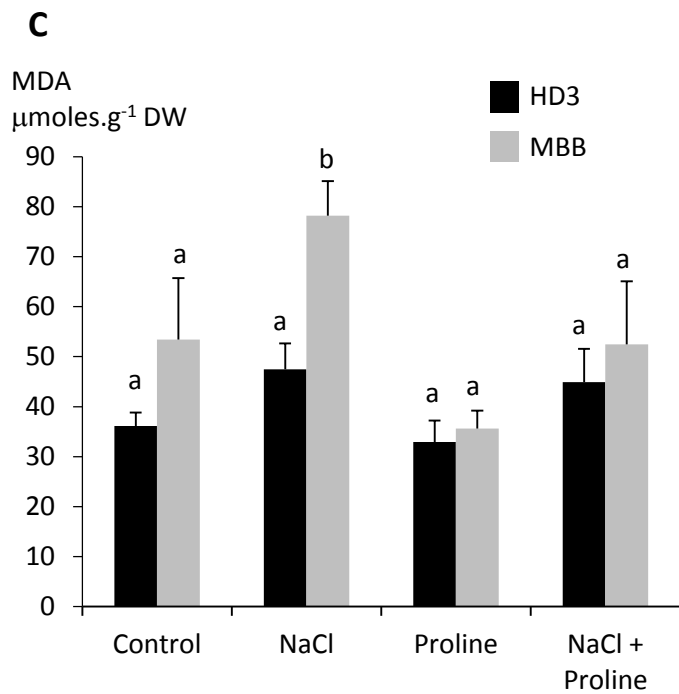
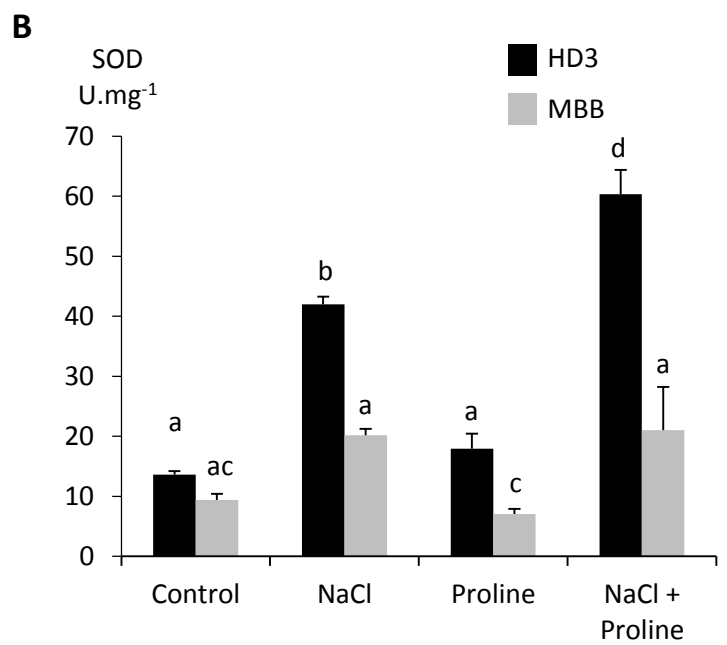
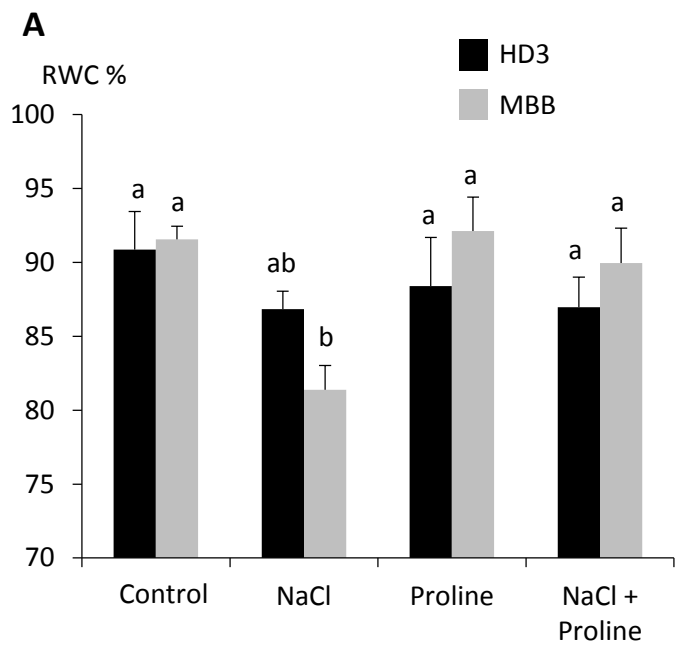


Figure 3

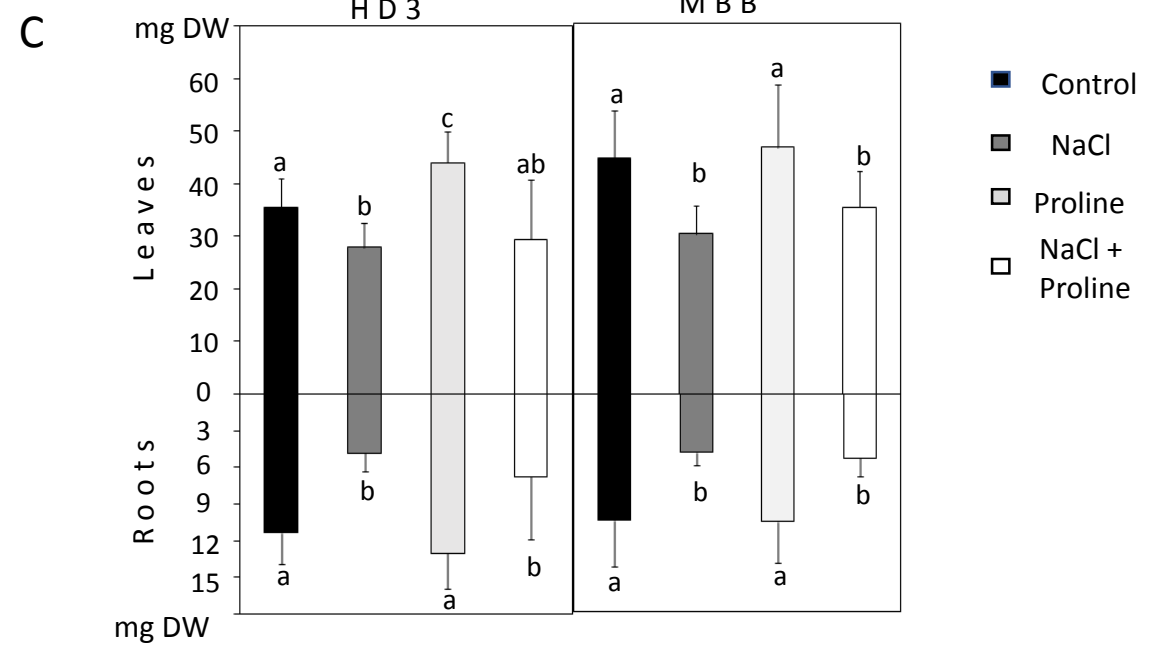
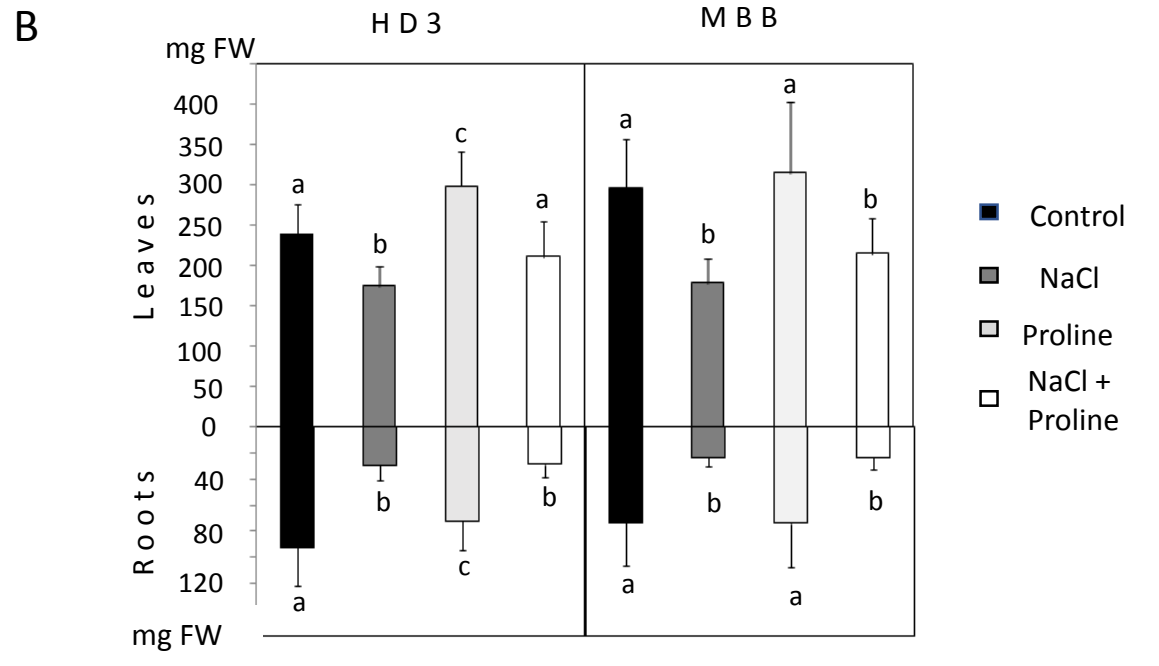
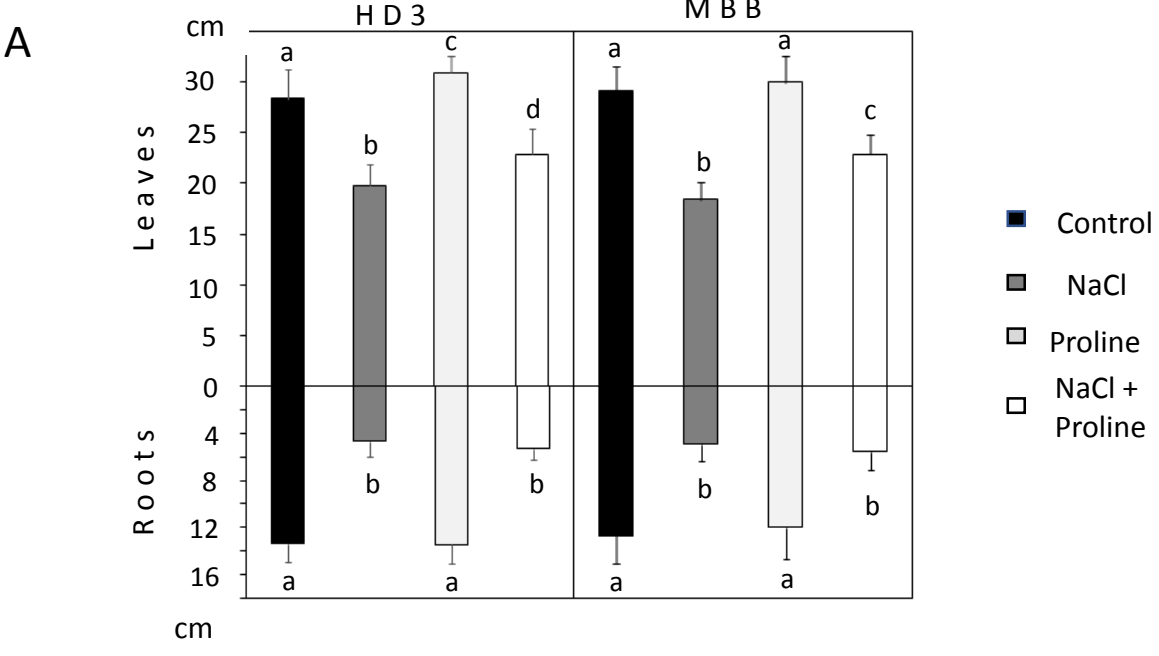


Figure 4

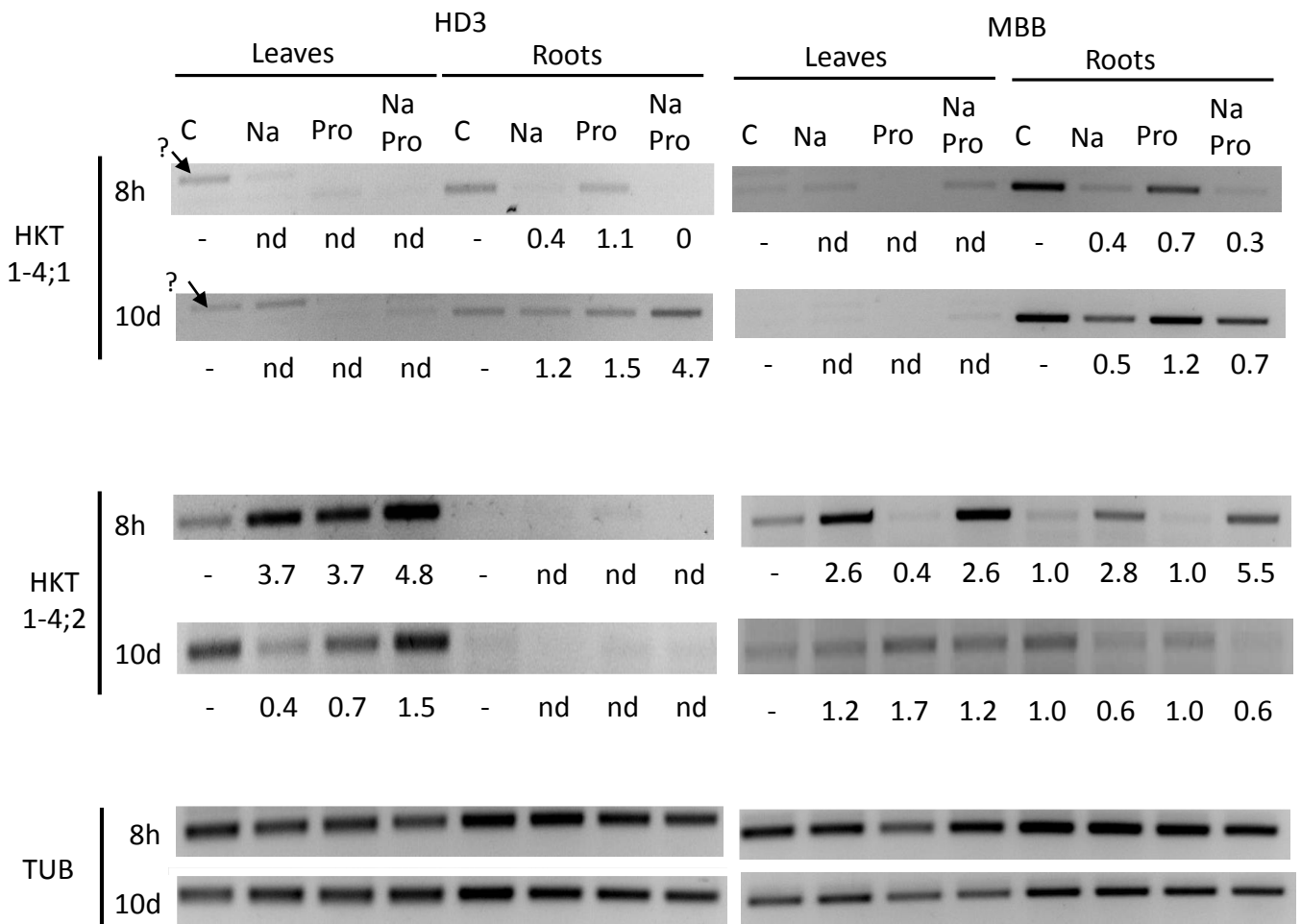


Figure 5

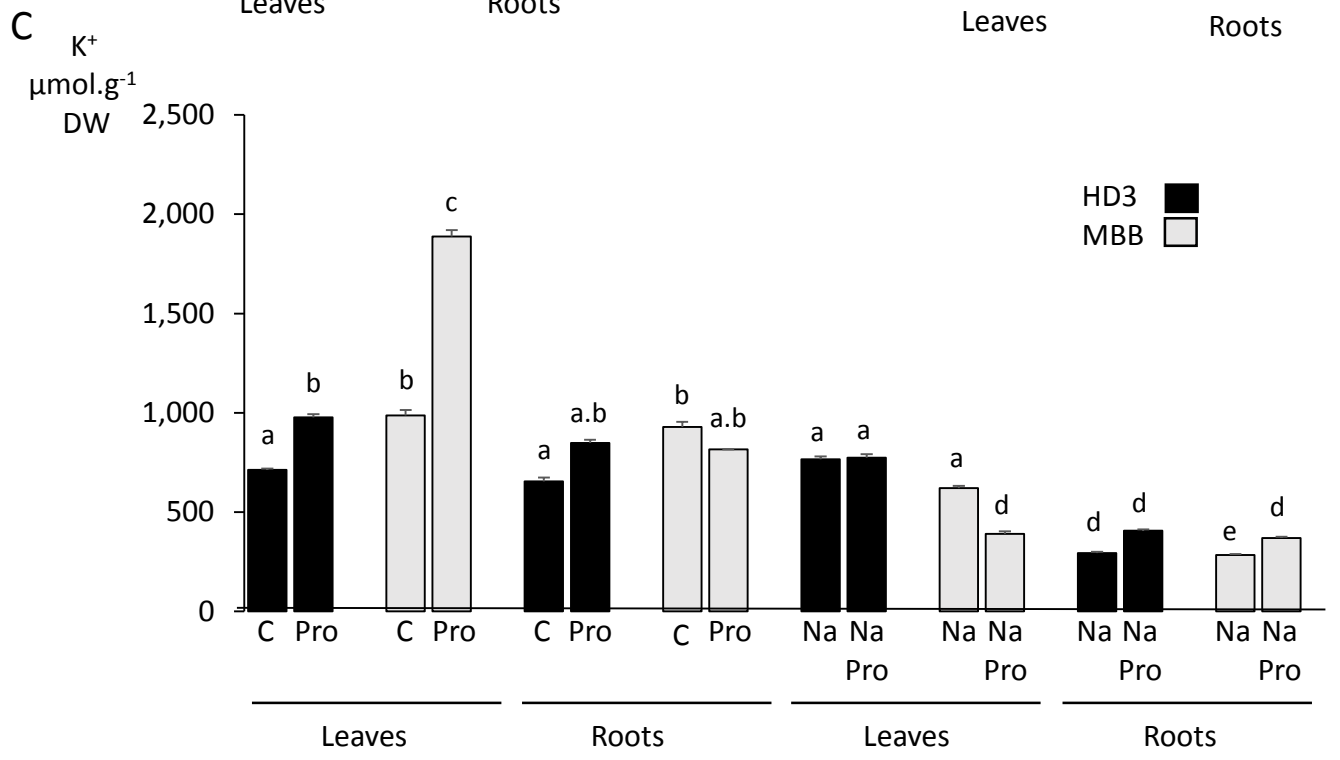
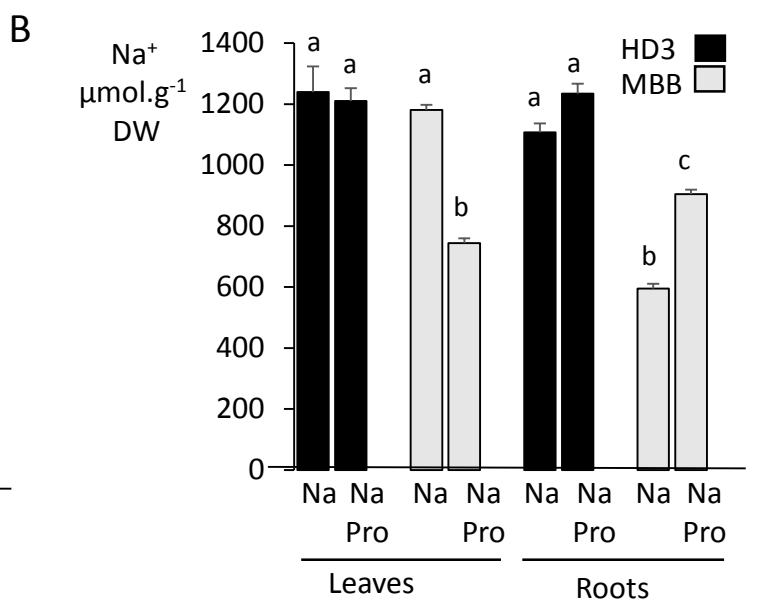
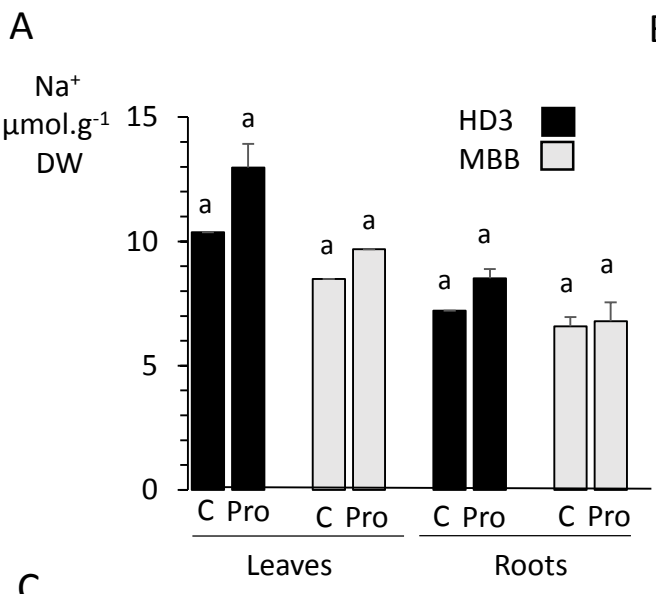


Figure 6

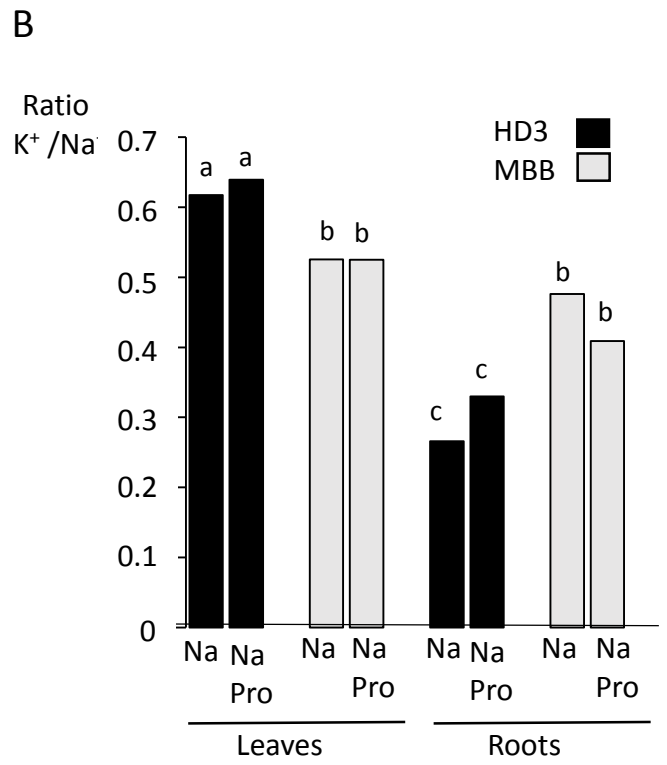
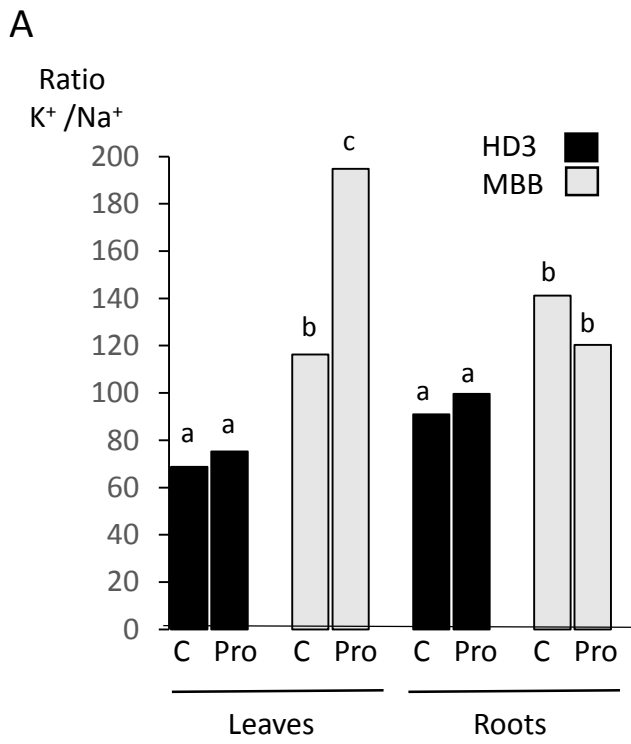
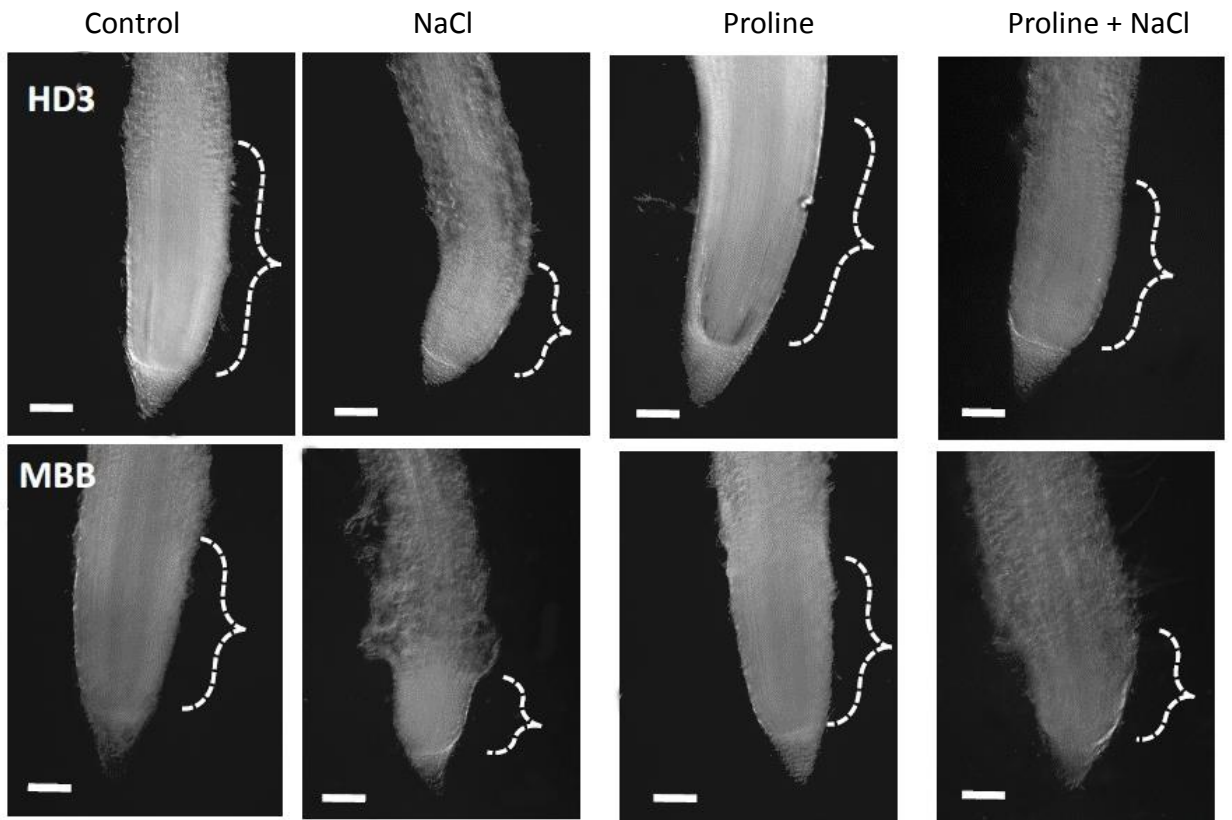


Figure 7



Supplementary figure 1: root tip of durum wheat seedlings grown for ten days on MS medium (control), 10 g l⁻¹ salt stress (NaCl), 20 mM proline (Proline), or the combination of both (NaCl + Proline). Brackets indicate the cell division area of the roots. Bar: 0.2 mm

This paper is a new version of a previous submission to *Acta Physiologiae Plantarum* (Ref.: Ms. No. ACPD-D-18-00829) for which we have been kindly asked to re-submit an improved version. The title is modified, the text and figures have been improved according to the reviewer's comments and suggestions, the language has been corrected by a professional scientific English editing service. We hope that you will consider our manuscript for review in your journal.

Title: Different proline responses of two Algerian durum wheat cultivars to *in vitro* salt stress

Authors: Katia AMI, Séverine PLANCHAIS, Cécile CABASSA, Anne GUIVARC'H, Anne-Aliénor VERY, Majda KHELIFI, Réda DJEBBAR, Ouzna ABROUS-BELBACHIR, Pierre CAROL^{1*}

Keywords : durum wheat, *Triticum turgidum*, salt stress, proline, HKT, *in vitro*

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Authors role:

KA: did most of the experimental work, suggested experiments, analysed data and contributed to the manuscript writing and revising

SP: contributed to most of the experiments, set-up and supervised the molecular biology, contributed to the manuscript writing and revising

CC: contributed to experiments and set-up and supervised the biochemistry, contributed to the manuscript writing and revising

AG: performed the roots anatomy observations contributed to the manuscript revising

A-AV: designed the gene expression studies of HKT genes, co-wrote, revised and improved the manuscript

MK: designed the experimental project and revised the manuscript

RD: analysed the data, co-wrote and revised the manuscript

OAB: designed the experimental project, funded the project, analysed the data, co-wrote and revised the manuscript

PC: supervised the project, set up some experiments, analyzed data, wrote, revised and submitted the manuscript

Short description:

This paper results from a collaboration between Algiers (Algeria) and Paris (France) universities. A common interest in plant response to abiotic stress led us to set up a collaborative research. We focused on *Triticum turgidum durum* (durum wheat) a species that is of agricultural importance. We studied growth, biochemical and molecular responses of seedlings under stress. Salt impairs durum wheat growth more than other wheat species.

We aimed to evaluate the responses of durum wheat seedlings by comparing two cultivar seedlings grown *in vitro* with a harsh salt stress. *In vitro* NaCl stress on seedlings was chosen for practical reasons. Proline is an osmolyte with known beneficial role in stressed plants. Endogenous or exogenously added proline might have a beneficial to stressed durum wheat seedlings. Typical physiological responses to the harsh salt stress were observed in both cultivars with some quantitative differences. Partial alleviation of stress effects by proline were also found cultivar dependent as well as gene expression, especially encoding proline biosynthesis and sodium transport (*HKT*) genes.

We are extremely grateful for the in-depth analysis and comments on our manuscript. The reviewers' comments were all extremely accurate and allowed us to rewrite a better manuscript. Please find below the reviewers' comments and our answers (in bold letters) of our previous submission.

Ref.: Ms. No. ACPP-D-18-00829

Differential alleviating effects of proline on two *in vitro* salt stressed durum wheat cultivars
Acta Physiologiae Plantarum

Comments:

Reviewer #1: It is an interesting paper on the evaluation of proline to alleviate salt stress. However, the manuscript requires some drastic changes. First, the authors should read carefully the manuscript and edit it. The introduction require some improvement (see comments below). The discussion is very poorly written, the flow needs to be improved, I suggest to rewrite the entire discussion. Another concern is the way the data on gene relative expression are presented. Some figures needs to be revised. See below for details.

Abstract

L36-38: "like water content" be more specific, is it "decrease water content"?

Text modified according to your recommendation: "such as decrease of water content"

Introduction

L58-59: This sentence is not really connected to the other sentences

L54-61: The sentences have been rewritten for to improve the logical flow: "Rice (*Oryza sativa*) is one of the least salt-tolerant cereal species (Dionisio-Sese and Tobita, 2000; Colmer et al., 2006; Munns and Tester, 2008; Munns et al., 2010), while barley (*Hordeum vulgare*) is relatively salt-tolerant being able to grow in the presence of up to 250 mM NaCl. Amongst wheat species, *Triticum monoccocum* is salt-resistant, *Triticum aestivum* (bread wheat) is moderately salt-tolerant, and durum wheat is the least salt tolerant (Munns and James, 2003; Munns and Tester, 2008; Wu et al., 2018). Relative salt tolerance does however depend on the cultivar as much as the species of wheat (Plazek et al., 2013)."

L62-63: I do not understand the meaning of this sentence
This section has been rewritten for better clarity (see below).

L63: Define HKT: **we added the following sentence to define HKT**
“HKT genes encoding high-affinity K⁺ transporters (HKT) are important for sodium tolerance in *Triticum species* (Huang et al., 2006, 2008; James et al. 2006, 2011; Byrt et al., 2014).”

L62-71: this part requires some improvement. It should be located later on after line 82. Please combine with the paragraph l84-92 talking about Na transport.

We followed the reviewer recommendation to restructure this section (line 62 to 77):
“Plants growing on salinized soil accumulate high concentrations of sodium ions (Na⁺) that can damage the cell membrane, alter levels of growth regulators, inhibit enzymes, disrupt photosynthesis, interfere with ionic homeostasis, produce harmful reactive oxygen species (ROS), and thus lead to plant death (Munns and Tester, 2008; Julkowska and Testerink, 2015). High Na⁺ concentrations also have inhibitory effects on the absorption of major nutrients with similar physicochemical properties, such as K⁺, by the root (Almeida et al., 2017; Hamamoto et al., 2015).

The control of Na⁺ transport and exclusion of Na⁺ from leaf tissues are important processes protecting plants from sodium toxicity (Hanin et al., 2016). Durum wheat and rice both have a low capacity for Na⁺ exclusion (Dionisio-Sese and Tobita, 2000; Colmer et al., 2006; Munns and Tester, 2008; Munns et al., 2010). Sodium transporters have been found to be important for salt tolerance, such as the *SOS1* and *SOS4* genes in durum wheat (Feki et al., 2011, 2013, Ramenzani et al., 2011).”

L117-130: This part needs to be improved. State clearly how your experiment is different from what has already been done. Do not report your results.

We rewrote this section in order to show the aim of our study relative to previous ones:
“Drought resistance is a desirable trait in durum wheat, but it is not necessarily associated with salt-stress resistance. Here we studied two durum wheat cultivars from Algeria that perform differently under drought stress (Dionisio-Sese and Tobita, 2000; Colmer et al., 2006; Munns and Tester, 2008; Munns et al., 2010). The Hedba 3 (HD3) cultivar has been characterized as being relatively drought sensitive while the Mohamed Ben Bachir (MBB) cultivar is relatively drought tolerant (Ali Dib and Monneveux, 1992, Monneveux et al., 1986, Mekhlouf et al., 2006). We aimed to evaluate the physiological and gene expression responses of the cultivar seedlings grown *in vitro* with a harsh salt stress (10 g.L⁻¹, 171 mM NaCl), including the impact of exogenous proline on stress relief.”

Methods

L146: Why using semi-solid medium?

We removed “semi-solid” as it is misleading since all media are gels of agar, we added: “to ensure seedling growth and easy root manipulation”

Why only one salt concentration? We justify the choice of these concentrations: **“NaCl and proline concentration are within range used for a strong stress *in vitro* or aquaponics experiments (Wu et al., 2018; Per et al., 2017).”**

L151: the light intensity is very low

These conditions were satisfactory for seedling growth. Light was measured at the seedling level.

L185: why using only 1 h? it is usually 5h.

We added the following precision: “rinsed thoroughly then left for 1 h in fresh 20 mL of distilled water, which was found sufficient to ensure reliable measurement of ion leakage.”

L242: data analysis?

We added gel quantification to the figure in order to appreciate the difference in PCR product accumulation with an explanation of how this was measured and calculated in Materials and Methods.

Results

L254-256, L295-208, L303-305, L338-341: not part of the results

These paragraphs are now included in the discussion section

L289: there was no significant difference (see figure 3A). It is important to follow the results of the statistical analysis.

Thank you for pointing this mistake.

“Under salt stress, leaf RWC was significantly lower (81%) for the MBB cultivar indicative of hyperosmotic stress (Figure 3A).”

L341-342: Where are the data for the relative expression of the different genes? Pictures are not sufficient.

RT-PCR quantification was included in Material and Methods and in relevant figures

Discussion

L382-389: This part needs some work. It is not clear why the authors talk about stress intensity (L386) as only one concentration of salt was used in the experiment. Rephrase L 387-389. Why proline might not provide sufficient protection?

This section has been rewritten we hope the new version is clearer now : better clarity: “Proline is a compatible osmolyte with cellular protective properties, proline accumulation is an indicator of stress such as hyperosmotic stress and ionic stresses (Verslues and Sharma, 2010; Szabados and Savouré, 2010; Mansour et al., 2017). Protection provided by accumulated proline was shown to vary according to the genotype and stress intensity (Plazek et al., 2013, Rana et al., 2016). Salt tolerance does not correlate with endogenous proline in durum and bread wheat (Plazek et al., 2013). However, higher levels of proline accumulation have been reported in more salt-tolerant durum wheat genotypes (Rana et al., 2016). Here the highest proline concentration was found in the MBB genotype, which is relatively more sensitive to salt stress, suggesting that accumulated proline, is a marker of the perceived intensity of stress (Almansouri et al., 1999; Munns, 2002). In our experiment, endogenous proline accumulation might not provide sufficient cell protection to salt stress.”

L396-403: This part needs to be edited. This part is not clear, what is the point that the authors want to make?

This section has been rewritten for better clarity (now lines 413-418:

“We found that expression of proline catabolism gene *PRODH* was downregulated by salt stress, as occurs in many plant species (Peng et al., 1996; Servet et al., 2012). *PRODH* activity itself is lowered by salt stress (Mattioni et al., 1997) including in durum wheat (Soccio et al., 2010). *PRODH* gene expression is known to be up-regulated by proline (Verslues and Sharma, 2010; Yoshida et al., 1997; Servet et al., 2012; Cabassa-Hourton et al, 2016), but here only a slight *PRODH* up-regulation was observed.”

L404-405, L413-415: short paragraphs

Paragraph modified

L417: why talking about the effects of salt stress now? Why not starting with this?

This section has been moved to the beginning of the discussion

L421: Trigger salt responses from sensing areas such as root meristem: meaning?

Modified in: and its root meristem is less sensitive to salt stress (Wu et al., 2018).

L422: the decrease in leaf RWC was not significantly different (see figure 3A, it should be stated that it is the leaf RWC)

The sentence has been modified: “The decrease in leaf RWC in seedlings grown in the presence of NaCl indicates that hyperosmotic stress is occurring in leaf tissues.”

L430-434: Where are your results to support this?

It is in the result section and shown on Supplementary Figure 1.

L503-507: the conclusion should be more specific

The conclusion has been refocused.

Page 19: 2 times figure 4

corrected

Figure 3: SOD should be expressed in U/mg of protein

corrected

Figure 4: the presentation of units and values on the Y axis should be improved

corrected

Fig 6: why having control on one figure and salt treated plants on the other?

In this figure, we showed the effect of added proline to Na accumulation. On Figure 6A, we compared Control to Proline and on Figure 6B we compared salt-stress with salt stress and added proline. We show that proline had very little effect on sodium accumulation in durum wheat in absence of added salt (Figure 6A). On the other hand, we show that in the presence of salt stress proline significantly affects sodium accumulation in MBB cultivar on Figure 6B). Separating Figure 6A and 6B allow the use of a single Y axis as the measured sodium quantities vary one hundred times when comparing Control and salt stress conditions.

Fig 7: Be consistent when reporting the results of the statistical analysis (letters a, b and c are used differently on fig A and B)

We used the same sentence in figures 6 and 7 to explain the meaning of the lettering. The letters only refer to the histogram in which they appear: “In each panel, bars marked with

different letters indicate significantly different values ($p < 0.05$) in a two-way ANOVA Tukey's test. C, control. Pro, proline. Na, NaCl."

The format of table 1 is not a suitable format for publication.

The table has been improved

References: The number of references is too high. They should be in alphabetical order

The alphabetical order has been corrected. Some references have been removed as the text was edited.

Reviewer #2: Manuscript concerns an important problem of salt resistance of durum wheat. The experiment was correctly planned and studied parameters relating to plant response to salinity were well chosen. Manuscript is worth to publish, however with major corrections. Abstract presents too much preliminary information, while not enough results, which are enigmatic for a reader. Authors should write names of studied cultivars.

We have taken into account these advices: names of cultivars are now introduced directly in the Abstract and then at the end of the introduction. The cultivars are now referred to (though not by name) in the title. The context of the challenge of growing/developing crops for arid regions like Algeria where these cultivars originate from is now introduced immediately in the Introduction and recapitulated at the end of the Discussion.

Line 32: "10-d-old durum wheat seedlings".... should be replaced by "10-day seedlings of durum wheat".

Altered as suggested.

Line 43: "tested" should be replaced by "studied" in whole text

Altered as suggested.

Line 119: It is not true, please read article of Płazek et al. 2013. Acta Physiologiae Plantarum 35: 2513-2523

In order to follow the reviewer comment, we included the following sentence: "although, relative salt tolerance does not correlate with endogenous proline in durum wheat (Plazek et al., 2013)".

Material and methods

Line 147: Authors missed information about control medium without proline and NaCl as well as medium containing NaCl plus proline.

This has been corrected to be more precise.

Line 154: Please inform about goal of this part of the experiment.

We rewrote this sentence so that the goal of this method is obvious to the reader: "When short-term responses to stress were studied, 10-d seedlings grown on control MS medium

were carefully up-rooted then placed in a beaker with the roots in MS liquid medium, either with or without NaCl and/or proline.”

Results

In this part of the manuscript no references should be given. They should be transferred to Discussion.

References and sentences leading to discussion were moved to the discussion section except where it was necessary to introduce essential details for the first time. E.g. specific details of stress markers required for understanding the experimental approach, where only practical details were previously mentioned in Materials & Methods or overarching concepts in Introduction.

The legend lacks a description of figure 7.

The mistake in figure legend numbering has been corrected

I could not find any description of results relating to microscope observations of roots under salt stress. It must be added to the Results.

Supplementary material should be included to the tables and figures of main text. .

The following sentence has been added: “A deleterious effect of salt on root tip growth was also observed (Supplementary Figure 1).”

Discussion

Line 462 and 463: TmHKT1;4-A2 and TmHKT1;4A1 - these are the names of genes or proteins? When genes - they should be written with italics.

Thank you for pointing-out the mistake in nomenclature, the protein names that were in lower case are now in upper case letters.

Conclusion

This chapter is poor. Please write the conclusions more precisely.

The conclusion has been rewritten and edited by a professional native English language editor

References: Line 611: Shabal et al. 2015 must be moved alphabetically to S....

Line 803: the same mark for Saibi and Brini 2018

Sorry for the mistake, the references have been put alphabetically.

Associate Editor

The reviewers appreciated an important research problem, i.e. the assessment of resistance to stress of durum wheat, at using proline. The reviewers emphasize the proper planning and conducting of the experiment. However, the manuscript itself requires in-depth improvement, due to numerous errors, irregularities and inaccuracies. All detailed comments are included in both reviews.

