

1 **Varying water deficit stress (WDS) tolerance in grain amaranths involves**  
2 **multifactorial shifts in WDS-related responses**

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28 **Highlight:** Differential water deficit stress tolerance in grain amaranths and their ancestor,  
29 *Amaranthus hybridus*, is a multifactorial process involving various biochemical changes  
30 and modified expression patterns of key stress-related genes.

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51 **Abstract**

52 In this study, water deficit stress (WDS)-tolerance in several cultivars of grain amaranth  
53 species (*Amaranthus hypochondriacus* [Ahypo], *A. cruentus* [Acru] and *A. caudatus*  
54 [Acau]), in addition to *A. hybridus* (Ahyb), an ancestral amaranth, was examined. Ahypo  
55 was the most WDS-tolerant species, whereas Acau and Ahyb were WDS-sensitive. Data  
56 revealed that the differential WDS tolerance observed was multifactorial. It involved  
57 increased proline and raffinose (Raf) in leaves and/ or roots. Higher foliar Raf coincided  
58 with induced *Galactinol synthase 1* (*AhGolS1*) and *Raffinose synthase* (*AhRafS*) expression.  
59 Unknown compounds, possibly larger RFOs, also accumulated in leaves of WDS-tolerant  
60 amaranths, which had high Raf/ Verbascose ratios. Distinct nonstructural carbohydrate  
61 (NSC) accumulation patterns were observed in tolerant species under WDS and recovery,  
62 such as: i) high Hex/ Suc ratios in roots coupled to increased cell wall and vacuolar  
63 invertase and sucrose synthase activities; ii) a severer depletion of starch reserves; iii) lower  
64 NSC content in leaves, and iv) higher basal hexose levels in roots which further increased  
65 under WDS. WDS-marker gene expression patterns proposed a link between amaranth's  
66 WDS tolerance and abscisic acid-dependent signaling. Results obtained also suggest that  
67 *AhTRE*, *AhTPS9*, *AhTPS11*, *AhGolS1* and *AhRafS* are reliable gene markers of WDS  
68 tolerance in amaranth.

69 **Keywords:** grain amaranth, water deficit stress tolerance, proline, raffinose family  
70 oligosaccharides, nonstructural carbohydrates, trahalose.

71 **Abbreviations:** ABA (abscisic acid), Ahypo (*Amaranthus hypochondriacus*), Acru (*A.*  
72 *cruentus*), Acau (*A. caudatus*), Ahyb (*A. hybridus*), CWI (cell wall invertase), CI  
73 (cytoplasmic invertase), Glu (glucose), Gol (galactinol), GolS (galactinol synthase), Fru  
74 (fructose), MWDS (moderate water deficit stress), NSC (nonstructural carbohydrate), Pro  
75 (proline), Raf (raffinose), RafS (raffinose synthase), RFO (Raffinose Family  
76 Oligosaccharides), Sta (stachyose), R (recovery), Suc (sucrose), RT (retention time), SuSy  
77 (sucrose synthase), SWDS (severe water-deficit stress), TF (transcription factor), T6P  
78 (trehalose-6-phosphate), TPS (trehalose-6-phosphate synthase), TPP (trehalose phosphate  
79 phosphatase), Tre (trehalose), TRE (trehalase), VI (vacuolar invertase), WDS (water deficit  
80 stress).

## 81 **Introduction**

82 Plants have evolved to avoid, escape or tolerate stress conditions using numerous  
83 mechanisms that include several morphological, physiological and metabolic adaptations  
84 (Golldack *et al.*, 2014). Plant drought and salt adaptation involves control of water flux and  
85 cellular osmotic adjustment via the regulation of stomatal aperture, biosynthesis of  
86 osmoprotectants and reestablishment of the cellular redox status via the removal of reactive  
87 oxygen species (ROS) (Golldack *et al.*, 2014). Gene expression is also profoundly modified  
88 upon salt and drought stress. Stress-related genes code for proteins involved in osmolyte  
89 biosynthesis, detoxifying processes and transport, as well as in regulatory processes.  
90 Transcription factors (TFs), protein kinases, and phosphatases are central players in the  
91 latter. Both abscisic acid (ABA)-dependent and ABA-independent signaling pathways are  
92 activated to cope with abiotic stress (Krasensky and Jonak, 2012; Golldack *et al.*, 2014).

93 The accumulation of compatible solutes constitutes a protective mechanism employed by  
94 plants to ameliorate the damaging effects of drought and other abiotic stresses. This diverse  
95 group includes proline (Pro), soluble non-structural carbohydrates (NSCs; i.e., sucrose,  
96 glucose and fructose), and raffinose family oligosaccharides (RFOs), among others. They  
97 can accumulate in many plants in response to different stresses, and may perform roles  
98 other than osmoprotection, such as ROS scavenging or protein stabilization. Pro  
99 accumulation has also been found to promote plant recovery from drought stress (An *et al.*,  
100 2013). Starch can be rapidly mobilized to provide soluble sugars. Thus, starch catabolism is  
101 accelerated in response to salt drought and other stresses usually as an osmotic adjustment  
102 response via increased soluble NSCs accumulation (Castrillón-Arbeláez *et al.*, 2012;  
103 Vargas *et al.*, 2013; Reguera *et al.*, 2013). These can maintain cell turgor or protect  
104 membranes and proteins from stress-related damage. Sucrose metabolism, via invertases,  
105 also regulates abiotic stresses responses by providing hexoses, as essential metabolites and  
106 signaling molecules (Ruan *et al.*, 2012) or promoting heat shock protein accumulation (Liu  
107 *et al.*, 2013). Likewise, glucose metabolism may prevent cell death via augmented reducing  
108 power and concomitant antioxidants biosynthesis (Bolouri-Moghaddam *et al.*, 2010). On  
109 the other hand, RFOs are extensively distributed in higher plants, functioning in carbon (C)  
110 storage and redistribution. (Ayre *et al.*, 2013). They are also known to accumulate during

111 seed desiccation and/ or in leaves of plants subjected to various abiotic stresses, although  
112 their precise role in plant stress tolerance acquisition is not fully understood (Nishizawa *et*  
113 *al.*, 2008; ElSayed *et al.*, 2014). Biosynthesis of RFO originates from galactinol (Gol)  
114 generated from myo-inositol (MI) and UDP-galactose by Gol synthase (GolS). Gol  
115 subsequently acts as a galactose unit donor to Suc to generate raffinose (Raf), stachyose  
116 (Sta) and higher order RFOs, via their respective glycosyltransferases (Peterbauer and  
117 Richter, 2001).

118 Trehalose (Tre) is a non-reducing disaccharide present in trace amounts in most plants. It is  
119 presumably involved in the regulation of plant development and abiotic stress resistance.  
120 Thus, targeted manipulation of trehalose-6-phosphate (T6P), Tre's precursor, also  
121 accumulating in trace amounts in most plants, has been found to improve abiotic stress  
122 tolerance and yield in some crop plants (Figuroa and Lunn, 2016). This phosphorylated  
123 precursor is synthesized by trehalose-6-phosphate synthases (TPSs) and may be  
124 subsequently dephosphorylated to Tre by trehalose-6-phosphate phosphatases (TPPs). Tre  
125 itself may be hydrolyzed to two glucose moieties by trehalase (TRE) (Lunn *et al.*, 2014).  
126 T6P's role as a sensor of C availability has been proposed to involve a negative interaction  
127 with sucrose non-fermenting related kinase-1 (SnRK1) a known inhibitor of plant growth  
128 (Liu *et al.*, 2013; Delorge *et al.*, 2014; Lunn *et al.*, 2014; Tsai and Gazzarrini, 2014;  
129 Figuroa and Lunn, 2016).

130 The genus *Amaranthus* consist of 60-70 species. Some are consumed as vegetables or are  
131 used as a source of grain. The latter (*Amaranthus hypochondriacus*, *A. cruentus*, and *A.*  
132 *caudatus*) possess desirable agronomic characteristics and produce highly nutritional seeds.  
133 Moreover, they adapt easily to drought and poor soils (Caselato-Sousa and Amaya-Farfán,  
134 2012). Domesticated grain amaranths presumably descend from wild *A. hybridus*, although  
135 their origin and taxonomic relationships are still uncertain (Sogbohossou and Achigan-  
136 Dako, 2014).

137 The physiological traits that enable amaranths to thrive in harsh conditions, such as  
138 drought, and be amenable for cultivation on marginal lands unsuitable for cereals, have  
139 been partly uncovered. In this work, we compared four amaranth species that differed in  
140 their tolerance to water-deficit stress (WDS) in order to identify common and/ or divergent

141 responses to this condition. The changes in the expression, in leaves and roots, of RFO-  
142 biosynthetic genes and of genes involved in Tre metabolism and signaling were also  
143 evaluated. The content of RFOs, NSCs and Pro as well as invertases, sucrose synthase and  
144 amylase activity was also determined. The combined results of this study demonstrated that  
145 the differential WDS tolerance detected in the amaranth species tested, was the result of a  
146 multifactorial response

## 147 **Materials and Methods**

### 148 2.1 Plant material

149 Three semi-domesticated grain amaranth species (*A. hypochondriacus* [Ahypo], *A. cruentus*  
150 [Acru] and *A. caudatus* [Acau]) (Sauer, 1967) together with an undomesticated vegetable  
151 amaranth (*A. hybridus* [Ahyb]), believed to be grain amaranths' ancestor (Stetter and  
152 Schmid, 2017), were employed in the greenhouse experiments here described. All plant  
153 materials were provided by Dr. Eduardo Espitia Rangel, INIFAP, México, curator of the  
154 Mexican amaranth germplasm collection. Approximately 3 week-old plants having 9-10  
155 expanded leaves were employed for experimentation. These were grown in 1.3 L plastic  
156 pots containing 250 g of a general substrate in a conditioned growth chamber, as described  
157 previously (Délano-Frier *et al.*, 2011). A total of 8 cultivars/ accessions of at least one of  
158 the above species was tested, as follows: Ahypo (“Gabriela”, “Revancha” and “DGTA”  
159 cultivars); Acru (“Amaranteca”, Dorada” and “Tarasca” cultivars), Acau (no classification  
160 available) and Ahyb (accession N°. 1330).

### 161 2.2 Water-deficit (WDS) stress experiments

162 All WDS experiments were performed in a commercial green house with zenithal and  
163 lateral type ventilation (Baticenital 850; ACEA S.A., Mexico) in May to August of 2015.  
164 The average temperatures in the greenhouse ranged between 15°C (night) and 38°C (day),  
165 with an average 55% R.H. The experiments were performed under natural light and  
166 photoperiod ( $\approx 1300 \mu\text{E}$ ,  $\geq 12$  h light). An initial experiment was performed to screen the 8  
167 cultivars/ accessions mentioned above for their tolerance to WDS. WDS was established by  
168 withholding watering for 7 or 10 days, time after which moderate to severe plant wilting  
169 was evident. WDS tolerance was scored by determining the leaf water potentials at the end

170 of the WDS treatments and the percentage of recovery one day after normal watering was  
171 restored following stress (results not shown). This led to the selection of the 4 materials for  
172 subsequent experimentation which were the following: Ahypo (var. “Gabriela”) and Acru  
173 (var. “Amaranteca”), classified as “WDS tolerant”, and Acau and Ahyb, as “WDS  
174 susceptible”.

175 Subsequently, two tandem experiments were performed in the above conditions to test  
176 WDS tolerance based on soil water depletion. Prior to the start of the WDS trials, each  
177 experimental 1.3 L pot was weighed individually until maximum soil water retention  
178 capacity (SWC) was attained. WDS trials were started when all pots were at 90% SWC.  
179 Control plants were kept in these conditions for the duration of the experiments, whereas  
180 WDS was established by withholding watering until the SWC in each pot reached either  
181 30% SWC (“moderate WDS, [MWDS]”) or 10% SWC (“severe WDS, [SWDS]”). These  
182 stress levels were reached approximately 5-6 and 9-10 days after regular watering was  
183 withheld, respectively. An additional group of plants was re-watered after reaching 10%  
184 SWC and was allowed to recover for 24 h (“recovery”, [R]). Weighing of the pots to  
185 determine water loss was done on a daily basis, taking care to ensure it was consistently  
186 done at approximately the same time of the day. Twelve plants having 10-to-12 expanded  
187 leaves were used per treatment. Once the desired conditions were reached, roots and leaves  
188 from 3 similarly treated plants were sampled and combined. Control plants were similarly  
189 sampled, generating four subsamples per experimental group. All pooled tissue samples  
190 were flash frozen in liquid N<sub>2</sub> and stored at -70°C until needed.

### 191 2.3 Extraction of total RNA and gene expression analysis by RT-qPCR

192 Quantitative gene expression analysis using SYBR Green detection chemistry (Bio-Rad,  
193 Hercules, CA, USA) was performed as described previously (Palmeros-Suárez *et al.*, 2015).  
194 Primers design for the amplification of the pertinent amaranth gene transcripts employed a  
195 published methodology (Thornton and Basu, 2011) and was based on recently published  
196 genomic data (Clouse *et al.*, 2016) (Table S1). Relative gene expression was calculated  
197 using the comparative cycle threshold method (Livak and Schmittgen, 2001) using the  
198 *AhACT7*, *AhEF1a* and *AhβTub5* genes for data normalization.

### 199 2.4 Determination of NSC and Pro

200 Leaf and root samples collected from control plants, and from plants subjected to MWDS,  
201 SWDS or R, were used to quantify soluble NSCs and Pro contents, according to Palmeros-  
202 Suárez *et al.* (2015).

#### 203 2.5 Determination of RFOs by HPAEC–PAD

204 Identification and determination of RFOs content in leaf and root samples was performed  
205 by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric  
206 Detection (HPAEC–PAD), according to Mellado-Mojica *et al.* (2016). All chemicals used  
207 for the optimization of the chromatographic separation conditions and for quantitation were  
208 acquired from Sigma (Sigma-Aldrich, St. Louis, MO, USA,). These were the following:  
209 MI, Gol, Raf, Sta, and verbascose (Ver).

#### 210 2.6 Determination of trehalose by GC/MS and thin layer chromatography (TLC) analysis

211 Tre levels were determined by GC/ MS using a ion selective method as described  
212 previously (Orona-Tamayo *et al.*, 2013). RFO analysis by TLC was performed using  
213 HPTLC silica gel 60 F254 plates as described previously (Waksmundzka-Hajnos *et al.*,  
214 2008).

#### 215 2.7 Determination of invertases, sucrose synthase and amylase activities

216 Vacuolar, cell wall, and cytoplasmic invertases and sucrose synthase (SuSy) activities were  
217 determined as described in Wright *et al.* (1998). Amylase activity was determined  
218 according to Bernfeld (1955). All assays were modified to fit a micro-plate format.

#### 219 2.8 Statistical analysis

220 All experiments were conducted using a randomized complete block design. One-way  
221 ANOVAs were utilized to evaluate differences between treatment means. For ANOVAs  
222 where the F test was significant at  $P \leq 0.05$ , the Tukey-Kramer test was applied. Statistical  
223 analysis was performed with R software (Development Core Team, [https://www.r-](https://www.r-project.org/)  
224 [project.org/](https://www.r-project.org/)).

### 225 **3. Results**



226 The initial screening to determine possible differences in WDS tolerance between amaranth  
227 species revealed that Ahypo cv. Gabriela, followed by Acru cv. Amaranteca were the most  
228 WDS tolerant species (with ca. 60% and 45% recovery after WDS, respectively). On the  
229 other hand, Acau was the most susceptible (with a 35% recovery rate to MWDS but unable  
230 to tolerate SWDS). Interestingly, completely desiccated Ahyb plants recovered from SWDS  
231 after watering was restored (results not shown).

232 A gene expression analysis in roots and leaves of the four amaranth genotypes was  
233 performed next. These were originally detected in a previous grain amaranth transcriptomic  
234 analysis (Délano *et al.*, 2011), and were later found to respond to severe defoliation in grain  
235 amaranth (Cisneros, 2016). Several genes involved in Tre biosynthesis and breakdown,  
236 including one class I TPS gene (*AhTPS1*), three TPP genes (*AhTPPA*, *AhTPPD* and  
237 *AhTPPI*) and one TRE gene (*AhTRE*) were analyzed. Also included were several non-  
238 catalytic class II TPS genes (*AhTPS5*, *AhTPS7*, *AhTPS8*, *AhTPS9*, *AhTPS10* and *AhTPS11*).  
239 Only the class I TPS gene (*AhTPS-AHYPO 004431*) and four additional *AhTPP* genes,  
240 annotated in the *A. hypochondriacus* genome (Clouse *et al.*, 2016), were not included in  
241 this study, whereas all 6 class II TPS genes were incorporated. All genes were named  
242 according to the closest homology shown with their respective *Arabidopsis thaliana*  
243 orthologs (Supplementary Fig. S1-S3). Also included were four genes involved in RFOs  
244 biosynthesis (Gol synthase [*AhGolS1* and *AhGolS2*], Raf synthase [*AhRafS*], and Sta  
245 synthase [*AhStaS*]) and a number of sucrose non-fermenting related kinases similarly  
246 shown to be affected by severe defoliation in grain amaranth (*SnRAK*, *SnRK1a*, *SnRK2.1*  
247 and *SnRK2.2*) (Cisneros, 2016). Finally, the expression of four ABA-related stress marker  
248 genes (*AhRAB18*, *AhABI5*, *AhDREB2C* and *AhLEA14*) were included as controls.

249 WDS had almost no effect on the expression of the class I *AhTPS1* gene in leaves (Table  
250 1A). Only limited induction was detected in Ahyb and Acau. Similarly, the expression of  
251 class II *AhTPS5*, *AhTPS7* and *AhTPS8* in leaves of all plants was predominantly unaffected  
252 by stress or downregulated. Downregulation of these genes by MWDS in Ahypo was  
253 prominent. In contrast, *AhTPS11* responded strongly to WDS and R in practically all plants  
254 tested. This response was particularly evident during SWDS. The expression of the other  
255 two class II TPS genes was also induced chiefly during SWDS, although differences

256 between species were observed in other conditions (Table 1B). Likewise, the *TPP* and *TRE*  
257 genes were, in general, unaffected or repressed by WDS in leaves. Noticeable exceptions  
258 were the induction, by WDS, of *AhTPPD* and *AhTPPI*, and the repression, in R, of  
259 *AhTPPA* and *AhTPPD*, in Ahypo. WDS also induced *AhTPPA*, *AhTPPI* and *AhTPPD* in  
260 Acru and Acau (Table 1C). Finally, *AhTRE* was exclusively induced by WDS in leaves of  
261 Ahypo (Table 1D).

262 The expression pattern of these genes changed in roots. The frequency with which they  
263 were induced in response to WDS or R was lower and their expression levels were reduced  
264 compared to those in leaves (Table 2). Thus, class I *AhTPS1* was repressed in WDS-tolerant  
265 species and unaltered in the other two (Table 2A). Likewise to leaves, class II *AhTPS5*,  
266 *AhTPS7* and *AhTPS8* genes generally remained unchanged or were repressed by WDS and/  
267 or R (Table 2B). *AhTPS10*, was also induced exclusively in Acru and Acau, whereas it was  
268 repressed by WDS in Ahyb. *AhTPS9* was induced by SWDS and R in all species tested  
269 with the exception of Ahyb, whereas *AhTPS11* was again induced universally by SWDS,  
270 but at lower levels. However, its expression in other conditions tested was more sporadic  
271 than in leaves. Importantly, the expression levels of *AhTPS9* during SWDS was  
272 significantly higher in roots of Ahypo and Acru, in concordance with their superior WDS  
273 tolerance. Also noticeable, was the widespread induction of *AhTPS9-AhTPS11* in Acau.  
274 Besides, the expression of all *AhTPP* genes tested was repressed or unaltered by WDS in  
275 roots (Table 2C), whereas *AhTRE* ceased to be induced in roots of Ahypo, similarly to the  
276 other species examined (Table 2D).

277 Tre levels were measured by GC-MS, considered more accurate than HPAEC (Quéro *et al.*,  
278 2013). The results show that a 2-to-3-fold Tre accumulation was induced by both MWDS,  
279 SWDS, and sometimes in R, in both leaves (Fig. 1A) and roots (Fig. 1B) of all amaranth  
280 species. However, the effect was more noticeable in roots and was stronger in WDS-  
281 susceptible species. Tre contents showed a poor correlation with the expression of Tre  
282 biosynthesis-related genes. This lack of synchronicity suggested that its accumulation may  
283 have involved a post-translational activation of class I TPS1 enzyme(s) (Delorge *et al.*,  
284 2015), an event that remains poorly understood (Rubio-Teixeira *et al.*, 2016). Tre  
285 accumulation could have also reflected the weak induction of Tre catabolism genes

286 observed, similar to related studies that connected Tre accumulation with TRE inactivation  
287 (Goddijn *et al.*, 1997; Müller *et al.*, 2001).

288 WDS and R induced the expression of *AhSnRAK* in leaves of WDS-susceptible plants and  
289 in roots of WDS-tolerant species. *AhSnRK1a* expression remained practically unchanged  
290 except for sporadic down- or up-regulated events in leaves and roots. Conversely,  
291 *AhSnRK2.1* and *AhSnRK2.2* were negatively affected in leaves of WDS-tolerant plants but  
292 induced by WDS in AHyb. Their expression remained unaffected in roots (Supplementary  
293 Tables S2, S3).

294 Regarding RFO-biosynthesis genes, *AhGols1* and *AhRafS* were almost universally induced  
295 by WDS in amaranth leaves, whose expression tended to be highest during SWDS (Table  
296 3). The induction of these genes was lower or returned to basal levels, in R. On the other  
297 hand, foliar expression of *AhGols2* was mostly unaffected by WDS. The expression of  
298 *AhStaS*, invariable in leaves of Ahypo and Ahyb, was intermittent in Acau and Acru.

299 *AhGols1* expression in roots of WDS-treated amaranth plants was intensely induced by  
300 SWDS, notably in Acru (Table 4). *AhGols1* expression in roots of Ahypo during SWDS  
301 was also high, being 1.8- to 3.6-fold higher than those detected in Acau and Ahyb,  
302 respectively. Thus, *AhGols1* expression pattern in roots of grain amaranth plants also  
303 coincided with their WDS tolerance. Conversely, *AhGols2* was almost universally induced  
304 in response to WDS in roots. Root *AhGols2* gene expression patterns in response to WDS  
305 were mirrored by those produced by the *AhRafS* and *AhStaS* genes, except for the  
306 occasional induction of the latter during R. The ca. 3- to 10-fold higher *AhRafS* expression  
307 levels detected in roots of Ahypo and Acru subjected to SWDS, compared to those in Acau  
308 and Ahyb, also agreed with their increased WDS tolerance.

309 The RFO accumulation pattern in leaves and roots (Fig. 2, 3) partially coincided the  
310 expression of RFO biosynthesis-related genes (Tables 3, 4). Raf accumulation in leaves  
311 could be likewise suggested as another contributing factor to the increased WDS tolerance  
312 observed in Ahypo and Acru. In contrast, practically no accumulation of Gol was detected  
313 in leaves of all species, irrespective of their treatment, suggesting an active utilization of  
314 this precursor for the synthesis of RFOs. On the other hand, MI content remained unaltered  
315 in leaves of Ahypo plants (Fig. 2A), but accumulated, particularly during SWDS, in Acru

316 and Acau (Fig. 2B, C). Sta content was minimal in leaves of all species and changes were  
317 small and sporadic (Fig. 2A, B, D). Similarly Ver content in Ahypo and Acru (Fig. 2A, B)  
318 was modest and static. However, Ver levels increased to ca. 5-fold higher levels than  
319 controls in response to WDS in Acau and Ahyp (Fig. 2C, D). The above results suggest that  
320 Raf/ Ver ratios in leaves could constitute a marker of WDS tolerance in amaranth.

321 The root RFO results differed and had a lower correspondence with WDS tolerance in  
322 amaranth. Raf did not to accumulate in response to WDS in Ahypo and Acru (Fig. 3A, B).  
323 In Ahyb, Raf content fluctuations in roots were similarly erratic than those in leaves (Fig.  
324 3D), whereas the ca. 2-fold higher basal Raf content in roots of Acau was drastically  
325 reduced by SWDS and R, similarly to Ahypo (Fig. 3C). SWDS conditions also induced the  
326 accumulation of MI in roots of Acru (Fig. 3B), while a significant increase occurred in  
327 Ahypo roots during R (Fig. 3A). Basal Gol contents in roots were ca. 2-fold lower than in  
328 leaves, and undetectable under certain conditions in roots of Ahyb (Fig. 3D). Sta contents  
329 remained low in roots and also showed a tendency to accumulate in response to SWDS.  
330 Contrary to leaves, root Sta accumulation was significantly increased by SWDS in all  
331 species tested (Fig. 3C). Likewise, Ver content increased in roots of all species in response  
332 to SWDS (Fig. 3A-D). Curiously, Ahypo and Ahyb accumulated almost identical Ver  
333 contents in response to WDS o R treatments (Fig. 3A, D).

334 The significantly higher foliar accumulation of Raf in Ahypo and Acru observed in  
335 response to MWDS and SWDS correlated with significantly augmented *AhGolSI* and  
336 *AhRafS* expression levels (Table 3). In roots this association was not found, although these  
337 genes were expressed to ca. 10-fold higher levels than those detected in leaves under  
338 similar conditions (Table 4). The reason(s) why the intense induction of these genes did not  
339 translate into high contents of Raf and perhaps other RFOs in roots remains unknown.  
340 Contrarily, changes in *AhStaS* expression in response to WDS and R agreed with root Sta  
341 levels (Table 4; Fig. 3). However, this correspondence was not detected in leaves (Table 3;  
342 Fig. 2). The lack of coincidence between RFO content and their correspondent gene  
343 expression in some amaranth species could be explained by the possibility that these were  
344 being converted to putatively larger RFO, whose structure is yet to be determined. In this  
345 respect, several unknown compounds having longer retention times (RTs), and perhaps

346 larger sizes, were detected (Supplementary Fig. S4-S7). Peaks with RTs of 16.1, 22.2 and  
347 33.8 min were abundant in leaves WDS tolerant Ahypo and Acru, particularly in the  
348 former. Thus, they could be considered as contributors to WDS tolerance in these species.  
349 Contrarily, two peaks with RTs of ca. 16.8 and 21.1 min accumulated in roots of most  
350 treated plants, noticeably during SWDS and R. Curiously, both compounds were more  
351 abundant in WDS susceptible species. Thin-layer chromatography traces of both leaf and  
352 root crude extracts (Supplementary Fig. S8) show bands having differential intensity that  
353 could correspond to these unknown compounds, whose nature remains to be determined  
354 experimentally.

355 WDS marker genes were not uniformly expressed in treated plants; they varied depending  
356 on the treatment applied, organ examined and species. In leaves, *AhABI5* and *AhLEA14*  
357 were the only genes induced almost uniformly across species by WDS (Table 5A), although  
358 *AhLEA14* expression was several-fold higher than *AhABI5*, and was induced in all  
359 conditions tested. Conversely, *AhRAB18* was sporadically induced by WDS in Acau and  
360 Ahyb, whereas it remained practically unchanged in Ahypo and Acru. Contrariwise,  
361 *AhDREB2C* was induced by all treatments in Ahypo only. All marker genes were more  
362 intensely induced in roots (Table 5B), distinctly in Ahypo, Acru and Acau, whereas they  
363 remained mostly unaltered in Ahyb. Importantly, marker genes reached their highest  
364 expression in both leaves and roots of treated Ahypo plants, in correspondence with their  
365 superior WDS tolerance.

366 Pro levels were significantly higher in leaves of WDS-tolerant species, where the highest  
367 Pro contents accumulated in response to SWDS (Fig. 4A). Contrariwise, Pro accumulation  
368 in roots (Fig. 4B), did not vary much between amaranth species, where a significant  
369 increase was only observed in SWDS (although ca. 2.5-fold lower than in leaves).  
370 Significantly higher root Pro levels were also detected in MWDS and R in Ahyb, whereas  
371 the lowest Pro accumulation occurred in Acau. In contrast, NSCs content fluctuations in  
372 leaves and roots were consistent with the contrasting WDS tolerance observed between  
373 amaranth species. Thus, all NSCs were significantly lower in leaves of WDS-tolerant  
374 species, distinctly during SWDS (Figs. 5). In roots (Fig. 6), the NSC content variation in  
375 Ahypo was manifestly different. Thus Glu and Fru were the highest detected and Suc and

376 starch levels the lowest. This occurred independently of the treatment analyzed. Lower  
377 hexose (Hex) content in leaves of Ahypo was in agreement with the WDS-unresponsive  
378 invertase activity observed (Fig. 7A-C), whereas consistently higher Glu and Fru contents  
379 in leaves of treated Acau and Ahyb plants coincided with increased cell wall invertase  
380 (CWI) (in most conditions tested; Fig. 7A), and to augmented vacuolar (VI) and  
381 cytoplasmic invertase activities (CI), mostly during R (Fig. 7A-C). In Acru, a gradual  
382 increase in Glu and Fru observed during WDS and R, could be attributed to an increased  
383 activity in all three invertases tested (Fig. 7A-C), mostly during SWDS. Nevertheless, its  
384 foliar Hex levels tended to be the lowest, together with Ahypo. Also intriguing was the Suc  
385 peak produced during SWDS in the latter species (Fig. 5A-C).

386 Conversely, the high Hex/ Suc ratio observed in roots of treated Ahypo plants was  
387 consistent with increased CWI and VI activity (Fig. 8A, B), and with a strong induction of  
388 SuSy activity in SWDS (Fig. 9). Lower SuSy activities, combined with repressed and/ or  
389 unchanged invertase activity in roots of Acau and Ahyb treated plants were consistent with  
390 their lower Hex/ Suc ratios. No SuSy activity was detected in leaves.

391 The above results indicated WDS had a different effect on the NSC content of leaves and  
392 roots in tolerant Ahypo and Acru, compared to susceptible Acau and Ahyb. Thus, leaves of  
393 tolerant amaranths tended to have lower Glu, Fru, and starch contents. The effect was  
394 drastic during SWDS, particularly for starch reserves, which were almost depleted (Fig. 5D,  
395 6D). In contrast, constitutive Glu levels in roots of Ahypo plants were significantly higher  
396 than those in all others and increased significantly in R (Fig. 5A), whereas constitutively  
397 high Fru levels, further increased after WDS treatment (Fig. 5B). Amylolytic activity was  
398 almost uniformly induced in leaves of all treated plants (Fig. 10A), whereas its induction by  
399 all treatments was observed only in roots of Ahypo (Fig. 10B). This contrast suggests that  
400 additional starch degradation mechanisms contributed to the starch depletion observed in  
401 leaves and roots of WDS-tolerant amaranths (Grennan, 2006; Turesson *et al.*, 2014).

#### 402 **4. Discussion**

403 It was previously shown that the WDS response in Ahypo roots included the accumulation  
404 of osmolytes and increased levels of ROS scavenging and heat shock proteins, together  
405 with the induction of certain TFs (Huerta-Ocampo *et al.*, 2011). Several other amaranth

406 genes have been subsequently proposed as possible contributing factors to increased  
407 tolerance against several (a)biotic stresses in grain amaranth, including an orphan gene  
408 (Massange-Sánchez *et al.*, 2015), a gene with an unknown function domain (Palmeros-  
409 Suárez *et al.*, 2017) and various TF genes (Palmeros-Suárez *et al.*, 2015; Massange-  
410 Sánchez *et al.*, 2016). The above genes were induced in grain amaranth by several stress  
411 conditions and frequently conferred stress tolerance when overexpressed in Arabidopsis  
412 plants.

413 The present study found, however, that WDS tolerance in grain amaranth varied within and  
414 between species. Ahypo and Acru tended to be tolerant, whereas Acau, an incompletely  
415 domesticated grain amaranth species (Stetter *et al.*, 2017), and Ahyb, an undomesticated  
416 species presumed to be their ancestor (Stetter and Schmid, 2017), were susceptible. A  
417 battery of molecular and biochemical tests were employed to identify the bases of such  
418 difference. Changes in Tre and RSOs accumulation, as well as in the expression of related  
419 genes, together with modifications in C mobilization and in Pro content during WDS and in  
420 R were monitored. The general unresponsiveness of *AhTPS1* and downstream targets (i.e.,  
421 *AhSnRK1*) (Tables 1, 2; Supplementary Tables S2, S3) to WDS suggest that the role of  
422 T6P-related signaling was probably not a defining factor of WDS tolerance in grain  
423 amaranth. A similar prediction could be proposed for Tre (Fig. 1). This was partly in  
424 agreement with a study showing that Tre did not protect yeast cells from desiccation  
425 (Petitjean *et al.*, 2015) and with others that found no link between increased Tre  
426 accumulation and stress tolerance. It was contradictory, however, to evidence connecting  
427 Tre accumulation with WDS tolerance (Figueroa and Lunn, 2016). Moreover, it may be  
428 suggested that increased foliar Tre levels, could have contributed to WDS susceptibility in  
429 Acau and Ahyb, similar to Arabidopsis *tre* null mutants that had increased Tre levels and  
430 were more sensitive to drought than WT plants (Van Houtte *et al.*, 2013). Such effect was  
431 ascribed to a proposed link connecting Tre metabolism, stomatal conductance and  
432 variations in stomata's responsiveness to ABA.

433 Moreover, gene expression assays established a poor correlation between other Tre-related  
434 genes and increased WDS tolerance in Ahypo, except for a few exceptions: i) the general  
435 downregulation of foliar *AhTPS5*, of several other *class II TPS* genes during R, and of the

436 *AhTPPD* and *AhTPPI* genes during WDS; ii) the high expression of *AhTPS9* and *AhTPS11*  
437 observed in leaves and roots (together with *Acru*) during SWDS, and iii) the induction of  
438 *AhTRE* during WDS (Tables 1, 2). Past studies have shown that class II TPS proteins have  
439 a differential sensitivity to Suc levels in plants (Schluepmann and Paul, 2009), which is  
440 important in the context of modified NSCs content observed in response to WDS in  
441 amaranth and other plants (Pinheiro and Chaves 2010). This property could explain the  
442 increased induction of the *AhTPS9* and *AhTPS11* in WDS-tolerant amaranth. However, the  
443 role of these genes in WDS amelioration remains to be determined. The above results were  
444 also consistent with the upregulation of *TPS11* and *TRE* detected in stomatal guard cells of  
445 sucrose-treated *Arabidopsis* (Bates *et al.*, 2012). Such coordinated effect was proposed to  
446 establish a connection between Tre metabolism, carbohydrate metabolism regulation, and  
447 stomatal movements via sugar sensing, and further supported the role of Tre in the  
448 regulation of stomatal behavior.

449 The significantly higher upregulation of *AhTPS9* and *AhTPS11* under SWDS in sucrose-  
450 depleted roots of *Ahyo* plants was also in accordance with studies showing that Suc-  
451 limiting conditions, led a the induction of the *AtTPS8-AtTPS11* genes in *Arabidopsis*  
452 (Baena-González *et al.*, 2007, Ramon *et al.*, 2009). Also relevant to the above results is the  
453 finding that the overexpression of *OsTPS9* in rice significantly increased tolerance toward  
454 cold and salinity stress through its proposed association with *OsTPS1* (Li *et al.*, 2011; Zang  
455 *et al.*, 2011). In contrast, the general WDS-unresponsiveness of *class II TPS*, *TPP* and *TRE*  
456 genes in *Acau* and *Ahyb* could have contributed to their WDS sensitivity.

457 Conversely, the differential Pro and RFOs accumulation observed during WDS and R  
458 strongly suggests their participation as WDS tolerance factors in amaranth. This proposal is  
459 supported by the known role of these compounds as osmoregulators, antioxidants, ROS  
460 scavengers, signaling molecules and/ or as C reservoirs for post-stress recovery (Reguera *et*  
461 *al.*, 2013; ElSayed *et al.*, 2014; Kaur *et al.*, 2015; Bascañán-Godoy *et al.*, 2016).

462 WDS was also observed to influence the expression levels of RFO biosynthetic genes in a  
463 differential way. The differences observed between WDS tolerant and susceptible  
464 amaranths were mostly quantitative and were of importance in roots, where the expression  
465 *AhGols1* and *AhRafs* was significantly higher in WDS-tolerant species, especially under



466 SWDS (Table 4). These results were consistent with findings in leaves of *Coffea canephora*  
467 clones with contrasting tolerance to WDS, where the expression of the *CcGolS1* gene  
468 differed between drought-tolerant and -sensitive clones, being strongly repressed in the  
469 latter (dos Santos *et al.*, 2015). Additionally, a related study in *C. arabica* reported that,  
470 similar to amaranth, the *CaGolS1* isoform was highly responsive to WDS (dos Santos *et al.*,  
471 2011). Likewise, the results in amaranth agreed with several other studies showing that the  
472 expression of *GolS* genes was congruous with abiotic stress tolerance in *Arabidopsis* (Taji  
473 *et al.*, 2002; Nishizawa *et al.*, 2008) and in transgenic tobacco plants (Kim *et al.*, 2008;  
474 Wang *et al.*, 2009). However, similar to observations in *C. canephora*, higher expression  
475 levels of these genes in amaranth leaves and roots did not always coincide with an  
476 accumulation of their respective RFOs. Such was the case of Gol, whose amounts were  
477 decreased or were undetectable in leaves of Ahypo and in roots of both WDS-susceptible  
478 amaranths. Likewise, decreased or unchanged Raf root content in SWDS, and the  
479 accumulation of Sta and Ver in leaves of stressed amaranth plants, were contrary to their  
480 corresponding gene expression patterns.

481 Nevertheless, WDS tolerance and RFOs accumulation in amaranth were in agreement with  
482 high leaf and root contents of Raf under MWDS and to foliar accumulation MI, Gol and  
483 Raf under SWDS, in Ahypo and Acru, respectively. Interestingly, the observed MI buildup  
484 may have supplied additional osmoregulatory and antioxidant activity, as previously  
485 reported (Ishitani *et al.*, 1996; Duan *et al.*, 2012). Similar results were reported in  
486 *Chenopodium quinoa*, an amaranth close relative (Downie *et al.*, 1997). Thus, an increase  
487 in MI and/ or Raf levels was observed in leaves of two contrasting *C. quinoa* genotypes  
488 subjected to WDS (Bascañán-Godoy *et al.*, 2016). However, contrary to Ahypo and Acru,  
489 Raf levels accumulated in R, which, in quinoa, was proposed to act as a C reservoir utilized  
490 for post-stress recovery (Karner *et al.*, 2004). Amaranth RFO accumulation patterns in  
491 response to WDS were also similar to those reported in a WDS tolerant alfalfa cultivar able  
492 to accumulate Raf and Gol in roots during stress (Kang *et al.*, 2011). A greater  
493 accumulation of shoot flavonoids and isoflavonoids was also proposed to contribute to  
494 higher WDS in alfalfa. The latter was in accordance with a previous report showing that  
495 WDS induced the accumulation of betacyanins and the induction of betacyanin-  
496 biosynthetic genes in vegetative tissues of Ahypo cultivars (Casique-Arroyo *et al.*, 2012).

497 The observed accumulation of Sta and Ver in roots of WDS-stressed amaranth plants was  
498 similar to that reported in leaves of *C. arabica* (dos Santos *et al.*, 2011), although it did not  
499 seem to affect WDS tolerance in amaranth. On the other hand, other results (Fig. S4-S9),  
500 suggest that putative RFOs with a higher degree of polymerization differentially  
501 accumulated in leaves of Ahyppo and Acru and may have, therefore, contributed to their  
502 WDS tolerance. This possibility remains to be determined. Nevertheless, it was in  
503 agreement with dos Santos *et al.* (2011, 2015) who argued that the drought-related increase  
504 in Gol biosynthesis in coffee was funneled to the generation of larger stress-protective  
505 RFOs by unidentified glycosyltransferases.

506 On the other hand, WDS tolerance in Ahyppo and Acru was also defined by significantly  
507 higher Pro contents in leaves, principally during SWDS. Significantly higher Pro amounts  
508 also accumulated in Ahyppo leaves during MWDS (Fig. 4A). On the other hand, Pro  
509 accumulation in roots in response to SWDS was, in general, similar in all species (Fig. 4B).  
510 Likewise to the behavior observed in alfalfa (Kang *et al.*, 2011), but contrary to the pattern  
511 reported in quinoa (Razzaghi *et al.*, 2015; Bascuñán-Godoy *et al.*, 2016), Pro levels  
512 declined during R in all species, except in roots of Ahyb. This display coincided with  
513 several studies reporting its rapid metabolism in order to provide N and reducing power  
514 during stress recovery processes (Hayat *et al.*, 2012; Kaur *et al.*, 2015). Conversely, the  
515 significantly higher Pro amounts additionally detected in Ahyb roots during R might partly  
516 explain the remarkable recovery observed when severely dehydrated Ahyb plants were re-  
517 watered. Pro accumulation was also found to be a contributing factor to WDS tolerance in  
518 quinoa (Razzaghi *et al.*, 2015; Bascuñán-Godoy *et al.*, 2016) and alfalfa (Kang *et al.*,  
519 2011).

520 The characteristic modifications in NSC contents that occur in plants under WDS, both in  
521 response to reduced photosynthesis and to the need to maintain water uptake and cell turgor  
522 (Seki *et al.*, 2007; Pinheiro and Chaves, 2010) were also observed in amaranth. However,  
523 the distinct patterns observed between species suggested that they might have contributed  
524 to their different WDS tolerance. Thus, tolerance in Ahyppo was associated with inherently  
525 low foliar starch levels than became even lower in stressed plants. On the other hand, it  
526 presented a basal high Hex/ Suc ratio in roots, which remained practically unchanged by

527 posterior treatments and also underwent a strong depletion of starch levels during WDS and  
528 R. The above also suggest that WDS-responsive root CWI, VI, SuSy and amylase enzymes  
529 may have been contributing factors to the WDS tolerance observed in Ahypo. Intermediate  
530 Acru shared with Ahypo the strong stress-related depletion of starch reserves in both leaves  
531 and roots, whereas sensitive Acau and Ahyb had NSC patterns that were essentially the  
532 opposite of those observed in Ahypo. Previous reports showing that severe defoliation led  
533 to a drastic reduction of C reserves and the induction of various sucrolytic and amyolytic  
534 enzyme genes (Cisneros, 2016; Castrillón Arbeláez *et al.*, 2012), including two of the four  
535 SuSy genes present in the grain amaranth genome (Clouse *et al.*, 2016) advocate this  
536 proposal.

537 The Ahypo NSC fluctuation observed in roots was consistent with the C flow from starch  
538 and/ or Suc to Hex triggered as an osmotic adjustment response to WDS in rice and other  
539 plants. It agreed, as well, with the increase in invertases and SuSy gene expression and  
540 activity that led to an accumulation of Hex in rice plants subjected to WDS (Reguera *et al.*,  
541 2013).

542 On the other hand, the opposite behavior was observed in WDS susceptible Acau and  
543 Ahyb, in which Suc levels tended to increase and starch reserves were less severely  
544 depleted during WDS and R. This supports the proposal that varying patterns of NSC  
545 accumulation are an additional WDS-tolerance contributing factor in amaranth. However,  
546 other aspects not explored in this study, such as fluctuations in N partitioning, have been  
547 found to be conducive to WDS tolerance in closely related species, such as quinoa  
548 (Bascuñán-Godoy *et al.*, 2016).

549 Another evident difference detected between WDS-tolerant and WDS-susceptible  
550 amaranths, was the variable expression of the ABA marker genes (Tables 5, 6). This  
551 suggests that differences in ABA content and/ or sensitivity could be additional factors  
552 contributing to the differential WDS tolerance observed in amaranth, as previously  
553 described in alfalfa (Kang *et al.*, 2011). In this respect, the general unresponsiveness to  
554 WDS of the SnRK2 subgroup of genes analyzed in this study was intriguing  
555 (Supplementary Tables S2, S3), considering that *SnRK2* genes are considered to play an

556 important role in stress amelioration, partly through their involvement in the ABA signaling  
557 pathway (Liu *et al.*, 2013; Lind *et al.*, 2015)

558  
559 In conclusion, this study revealed that differential WDS tolerance between grain amaranth  
560 species and leafy, undomesticated, Ahyb, was due to multiple factors. Contributing factors  
561 to the improved WDS tolerance observed in Ahybo and Acru, were augmented levels in  
562 leaves and/ or roots of Pro and Raf. The WDS-accumulation of Raf in leaves of these  
563 species was consistent with augmented *AhGols1* and *AhRafS* expression levels. Unknown  
564 compounds, possibly structurally related to RFOs, were also found to differentially  
565 accumulate in leaves of WDS-tolerant species. Additionally, high Raf/ Ver ratios in leaves  
566 were found to be a possible determinant of WDS tolerance in amaranth. Moreover, clearly  
567 contrasting NSC patterns of accumulation/ depletion in response to WDS and R were  
568 observed in leaves and roots of WDS-tolerant and WDS-susceptible amaranth plants. Thus,  
569 high Hex/ Suc ratio in roots correlated with superior WDS-tolerance in Ahybo, which was  
570 in accordance with the induced activity of CWI, VI and SuSy in response to WDS. A  
571 severer depletion of starch reserves, which coincided with significantly increased amylase  
572 activity in roots, together with lower soluble NSCs in leaves, also appeared to correlate  
573 with WDS-tolerance in amaranth. This, in addition to higher basal levels of Hex in roots of  
574 Ahybo, which became even higher in response to WDS. Also significant was the high  
575 expression levels of ABA-marker genes in Ahybo plants, which suggested that the WDS  
576 tolerance shown by this species could be linked to a higher responsiveness to ABA-related  
577 WDS-tolerance mechanisms. Finally, the induced expression of *AhTRE* expression in  
578 leaves and of *AhTPS9*, *AhTPS11*, *AhGols1* and *AhRafS* in roots could be employed as  
579 markers of WDS tolerance in amaranth.

#### 580 **Supplementary data**

581 **Fig. S1.** Phylogenetic analysis of amaranth class I and II trehalose phosphate synthase  
582 proteins.

583 **Fig. S2.** Phylogenetic analysis of amaranth trehalose phosphate phosphatase proteins.

584 **Fig. S3.** Phylogenetic analysis of the amaranth trehalase protein.

585 **Fig. S4.** Accumulation of unidentified RFO-like compounds during WDS and R in leaves  
586 of amaranth plants.

587 **Fig. S5.** Accumulation of unidentified RFO-like compounds during WDS and R in roots of  
588 amaranth plants subjected to WDS.

589 **Fig. S6.** TLC separation of soluble NSCs and RFOs accumulating in leaf and roots of  
590 amaranth plants subjected to WDS.

591 **Fig. S7.** HPAEC-PAD traces of Ahypo leaf extracts showing the presence of un-identified  
592 RFO-like compounds in control and stressed plants.

593 **Fig. S8.** HPAEC-PAD traces of Acau roots extracts showing the presence of un-identified  
594 RFO-like compounds in control and stressed plants.

595 **Table S1.** List of qPCR primers used in this study.

596 **Table S2.** Expression patterns of selected *SnRK1* and *SnRK2* genes in leaves of four  
597 amaranth species during WDS and R.

598 **Table S3.** Expression patterns of selected *SnRK1* and *SnRK2* genes in roots of four  
599 amaranth species during WDS and R.

600

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## FIGURE LEGENDS

831 **Figure 1.** Trehalose content was quantified by GC/ MS in (A) leaf and (B) root extracts of  
832 four species of amaranth plants (i. e., *Amaranthus hypochondriacus* [Ahypo], *A. cruentus*  
833 [Acru], *A. caudatus* [Acau] and *A. hybridus* [Ahyb]) growing in optimal conditions (Op),  
834 subjected to moderate or severe water deficit stress (MWDS and SWDS, respectively) or  
835 allowed to recover from SWDS by restoring watering for 1 day (R).or severe and 1 day  
836 after normal watering was restored (R). Different letters over the bars represent statistically  
837 significant differences at  $P \leq 0.05$  (Tukey Kramer test). Bars and error bars indicate mean  
838 values and ES, respectively (n = 3 pools of four plants each). The results shown are those  
839 obtained from a representative experiment that was repeated in the spring-summer and  
840 summer-autumn seasons of 2014, respectively, with similar results.

841 **Figure 2.** Raffinose family oligosaccharides (RFOs) were quantified by HPAEC-PAD in  
842 leaf extracts of four species of amaranth plants: (A) *Amaranthus hypochondriacus* [Ahypo],  
843 (B) *A. cruentus* [Acru], (C) *A. caudatus* [Acau], and (D) *A. hybridus* [Ahyb]) growing in  
844 optimal conditions (Op; empty bars), subjected to moderate (M) or severe (S) water deficit  
845 stress (gray and black bars respectively) or allowed to recover from S, 1 day after normal  
846 watering was restored (R; striped bars). The RFOs and their respective precursors analyzed  
847 were myo-inositol (MI), galactinol (Gol), raffinose (Raf), staquiose (Sta) and verbascose  
848 (Ver). Different letters over the bars represent statistically significant differences at  $P \leq$   
849 0.05 (Tukey Kramer test). Bars and error bars indicate mean values and ES, respectively (n  
850 = 3 pools of four plants each). The results shown are those obtained from a representative  
851 experiment that was repeated in the spring-summer and summer-autumn seasons of 2014,  
852 respectively, with similar results.

853 **Figure 3.** Raffinose family oligosaccharides (RFOs) were quantified by HPAEC-PAD in  
854 root extracts of four species of amaranth plants: (A) *Amaranthus hypochondriacus*  
855 [Ahypo], (B) *A. cruentus* [Acru], (C) *A. caudatus* [Acau], and (D) *A. hybridus* [Ahyb])  
856 growing in optimal conditions (Op; empty bars), subjected to moderate (M) or severe (S)  
857 water deficit stress (gray and black bars respectively) or allowed to recover from S, 1 day  
858 after normal watering was restored (R; striped bars). The RFOs and their respective  
859 precursors analyzed were myo-inositol (MI), galactinol (Gol), raffinose (Raf), staquiose

860 (Sta) and verbascose (Ver). Different letters over the bars represent statistically significant  
861 differences at  $P \leq 0.05$  (Tukey Kramer test). Bars and error bars indicate mean values and  
862 ES, respectively (n = 3 pools of four plants each). The results shown are those obtained  
863 from a representative experiment that was repeated in the spring-summer and summer-  
864 autumn seasons of 2014, respectively, with similar results.

865 **Figure 4.** Proline content quantified *in vitro* in (A) leaf and (B) root extracts of four species  
866 of amaranth plants (i. e., *Amaranthus hypochondriacus* [Ahypo], *A. cruentus* [Acru], *A.*  
867 *caudatus* [Acau] and *A. hybridus* [Ahyb]) growing in optimal conditions (Op; empty bars),  
868 subjected to moderate (M) or severe (S) water deficit stress (gray and black bars  
869 respectively) or allowed to recover from S, 1 day after normal watering was restored (R;  
870 striped bars). Different letters over the bars represent statistically significant differences at  
871  $P \leq 0.05$  (Tukey Kramer test). Bars and error bars indicate mean values and ES,  
872 respectively (n = 3 pools of four plants each). The results shown are those obtained from a  
873 representative experiment that was repeated in the spring-summer and summer-autumn  
874 seasons of 2014, respectively, with similar results.

875 **Figure 5.** Non-structural carbohydrates (Glucose [Glu], Fructose [Fru], Sucrose [Suc] and  
876 starch) content quantified *in vitro* in leaves of four species of amaranth plants (i. e.,  
877 *Amaranthus hypochondriacus* [Ahypo; thick continuous line], *A. cruentus* [Acru; thin  
878 continuous line], *A. caudatus* [Acau; short dash line] and *A. hybridus* [Ahyb; long chain  
879 line]) growing in optimal conditions (Op), subjected to moderate (M) or severe (S) water  
880 deficit stress, or allowed to recover from S, 1 day after normal watering was restored (R).  
881 Different letters over the lines represent statistically significant differences at  $P \leq 0.05$   
882 (Tukey Kramer test). Bars and error bars indicate mean values and ES, respectively (n = 3  
883 pools of four plants each). The results shown are those obtained from a representative  
884 experiment that was repeated in the spring-summer and summer-autumn seasons of 2014,  
885 respectively, with similar results.

886 **Figure 6.** Non-structural carbohydrates (Glucose [Glu], Fructose [Fru], Sucrose [Suc] and  
887 starch) content quantified *in vitro* in roots of four species of amaranth plants (i. e.,  
888 *Amaranthus hypochondriacus* [Ahypo; thick continuous line], *A. cruentus* [Acru; thin  
889 continuous line], *A. caudatus* [Acau; short dash line] and *A. hybridus* [Ahyb; long chain

890 line]) growing in optimal conditions (Op), subjected to moderate (M) or severe (S) water  
891 deficit stress, or allowed to recover from S, 1 day after normal watering was restored (R).  
892 Different letters over the lines represent statistically significant differences at  $P \leq 0.05$   
893 (Tukey Kramer test). Bars and error bars indicate mean values and ES, respectively ( $n = 3$   
894 pools of four plants each). The results shown are those obtained from a representative  
895 experiment that was repeated in the spring-summer and summer-autumn seasons of 2014,  
896 respectively, with similar results.

897 **Figure 7.** (A) Cell wall invertase (CWI), (B) vacuolar invertase VI), and (C) neutral  
898 cytoplasmic invertase (CI) activities determined *in vitro* in leaf extracts of four species of  
899 amaranth plants: *Amaranthus hypochondriacus* [Ahypo], *A. cruentus* [Acru], (C), *A.*  
900 *caudatus* [Acau], and (D) *A. hybridus* [Ahyb], growing in optimal conditions (Op; empty  
901 bars), subjected to moderate (M) or severe (S) water deficit stress (gray and black bars  
902 respectively) or allowed to recover from S, 1 day after normal watering was restored (R;  
903 striped bars). Different letters over the bars represent statistically significant differences at  
904  $P \leq 0.05$  (Tukey Kramer test). Bars and error bars indicate mean values and ES,  
905 respectively ( $n = 3$  pools of four plants each). The results shown are those obtained from a  
906 representative experiment that was repeated in the spring-summer and summer-autumn  
907 seasons of 2014, respectively, with similar results.

908 **Figure 8.** (A) Cell wall invertase, (B) vacuolar invertase, and (C) neutral cytoplasmic  
909 invertase activities determined *in vitro* in root extracts of four species of amaranth plants:  
910 *Amaranthus hypochondriacus* (Ahypo), *A. cruentus* (Acru), *A. caudatus* (Acau), and *A.*  
911 *hybridus* (Ahyb), growing in optimal conditions (Op; empty bars), subjected to moderate  
912 (M) or severe (S) water deficit stress (gray and black bars respectively) or allowed to  
913 recover from S, 1 day after normal watering was restored (R; striped bars). Different letters  
914 over the bars represent statistically significant differences at  $P \leq 0.05$  (Tukey Kramer test).  
915 Bars and error bars indicate mean values and ES, respectively ( $n = 3$  pools of four plants  
916 each). The results shown are those obtained from a representative experiment that was  
917 repeated in the spring-summer and summer-autumn seasons of 2014, respectively, with  
918 similar results.

919 **Figure 9.** Sucrose synthase determined *in vitro* in root extracts of four species of amaranth  
920 plants: *Amaranthus hypochondriacus* (Ahypo), *A. cruentus* (Acru), *A. caudatus* (Acau), and  
921 *A. hybridus* (Ahyb), growing in optimal conditions (Op; empty bars), subjected to moderate  
922 (M) or severe (S) water deficit stress (gray and black bars respectively) or allowed to  
923 recover from S, 1 day after normal watering was restored (R; striped bars). Different letters  
924 over the bars represent statistically significant differences at  $P \leq 0.05$  (Tukey Kramer test).  
925 Bars and error bars indicate mean values and ES, respectively (n = 3 pools of four plants  
926 each). The results shown are those obtained from a representative experiment that was  
927 repeated in the spring-summer and summer-autumn seasons of 2014, respectively, with  
928 similar results.

929 **Figure 10.** Amylase activity quantified *in vitro* in (A) leaf and (B) root extracts of four  
930 species of amaranth plants (i. e., *Amaranthus hypochondriacus* [Ahypo], *A. cruentus*  
931 [Acru], *A. caudatus* [Acau] and *A. hybridus* [Ahyb]) growing in optimal conditions (Op;  
932 empty bars), subjected to moderate (M) or severe (S) water deficit stress (gray and black  
933 bars respectively) or allowed to recover from S, 1 day after normal watering was restored  
934 (R; striped bars). Different letters over the bars represent statistically significant differences  
935 at  $P \leq 0.05$  (Tukey Kramer test). Bars and error bars indicate mean values and ES,  
936 respectively (n = 3 pools of four plants each). The results shown are those obtained from a  
937 representative experiment that was repeated in the spring-summer and summer-autumn  
938 seasons of 2014, respectively, with similar results.

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956 **Table 1.** Relative expression values<sup>1</sup> of genes involved in trehalose synthesis and degradation in leaves of four *Amaranthus* species subjected to  
 957 two levels of water-deficit stress (moderate [M] and severe [S]) and to subsequent recovery ([R]). Induced (normalized expression values  $\geq 2.0$ ; in  
 958 normal text) and repressed (normalized expression values  $\leq 0.5$ ; in italicized text) expression values are emphasized in bold.

Gene	<i>A. hypochondriacus</i>			<i>A. cruentus</i>			<i>A. caudatus</i>			<i>A. hybridus</i>		
	M	S	R	M	S	R	M	S	R	M	S	R
<i>AhTPS1</i>	1.348	1.088	0.886	0.764	0.788	0.899	1.100	1.205	<b>2.532</b>	1.315	<b>1.704</b>	<b>3.030</b>
<i>AhTPS5</i>	<i>0.233</i>	<i>0.268</i>	<i>0.272</i>	0.552	0.526	<b>0.343</b>	0.664	0.606	0.645	<b>0.423</b>	0.536	0.638
<i>AhTPS7</i>	<b>0.498</b>	0.768	0.748	0.879	1.208	0.806	0.673	1.172	<b>2.245</b>	0.659	1.076	1.199
<i>AhTPS8</i>	<b>0.250</b>	0.902	<b>0.235</b>	0.967	0.896	0.711	1.076	<b>1.835</b>	<b>1.621</b>	<b>0.386</b>	0.827	0.974
<i>AhTPS9</i>	0.796	<b>2.917</b>	<b>0.287</b>	0.893	<b>2.454</b>	0.781	<b>2.045</b>	<b>2.715</b>	<b>2.832</b>	1.115	<b>2.136</b>	1.021
<i>AhTPS10</i>	<b>0.291</b>	1.016	<b>0.264</b>	<b>1.890</b>	<b>2.589</b>	<b>6.992</b>	<b>2.382</b>	1.262	<b>2.892</b>	<b>0.438</b>	<b>1.651</b>	1.154
<i>AhTPS11</i>	<b>5.006</b>	<b>17.995</b>	1.449	<b>2.052</b>	<b>3.522</b>	<b>1.530</b>	<b>3.650</b>	<b>17.420</b>	<b>2.927</b>	<b>5.047</b>	<b>23.795</b>	<b>2.449</b>
<i>AhTPPA</i>	0.515	1.121	<b>1.971</b>	1.189	0.856	0.534	1.170	<b>1.514</b>	1.054	0.751	1.491	1.083
<i>AhTPPD</i>	<b>0.334</b>	<b>0.186</b>	<b>2.972</b>	<b>1.579</b>	0.824	0.730	<b>1.556</b>	0.876	0.580	0.532	1.318	<b>1.948</b>
<i>AhTPPI</i>	<b>0.297</b>	<b>0.279</b>	0.769	1.022	0.591	<b>0.369</b>	0.951	<b>1.573</b>	1.062	0.628	1.176	1.016
<i>AhTRE</i>	<b>2.047</b>	<b>2.170</b>	1.276	0.583	<b>0.324</b>	<b>0.474</b>	0.562	0.643	1.143	0.996	0.716	0.987

959 <sup>1</sup>Calculated according to the comparative cycle threshold method (Livak and Schmittgen, 2001) using the *AhACT7*, *AhEF1a* and *Ah $\beta$ Tub5* amaranth genes for  
 960 data normalization.

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962 **Table 2.** Relative expression values<sup>1</sup> of genes involved in trehalose synthesis and degradation in roots of four *Amaranthus* species subjected to two  
 963 levels of water-deficit stress (moderate [M] and severe [S]) and to subsequent recovery ([R]). Induced (normalized expression values  $\geq 2.0$ ; in  
 964 normal text) and repressed (normalized expression values  $\leq 0.5$ ; in italicized text) expression values are emphasized in bold.

Gene	<i>A. hypochondriacus</i>			<i>A. cruentus</i>			<i>A. caudatus</i>			<i>A. hybridus</i>		
	M	S	R	M	S	R	M	S	R	M	S	R
<i>AhTPS1</i>	0.648	0.783	<b>0.259</b>	<b>0.388</b>	1.162	<b>0.314</b>	0.653	0.639	1.013	1.272	1.265	0.565
<i>AhTPS5</i>	1.463	0.523	1.314	0.833	0.684	0.922	0.911	0.775	<b>1.615</b>	<b>0.423</b>	<b>0.294</b>	1.473
<i>AhTPS7</i>	<b>0.470</b>	0.935	<b>2.427</b>	<b>0.481</b>	0.977	1.440	0.621	0.635	1.444	0.817	0.668	0.841
<i>AhTPS8</i>	<b>1.715</b>	0.715	0.939	0.713	0.991	0.760	<b>1.877</b>	1.114	1.478	<b>0.384</b>	<b>0.285</b>	0.616
<i>AhTPS9</i>	1.371	<b>2.206</b>	<b>1.549</b>	1.004	<b>3.402</b>	<b>1.535</b>	1.392	<b>1.836</b>	<b>1.634</b>	<b>0.397</b>	<b>1.706</b>	<b>0.466</b>
<i>AhTPS10</i>	0.536	0.810	0.695	1.154	<b>2.562</b>	0.868	1.321	<b>1.512</b>	<b>2.212</b>	<b>0.325</b>	<b>0.356</b>	0.888
<i>AhTPS11</i>	1.189	<b>6.624</b>	1.202	0.662	<b>8.619</b>	<b>2.289</b>	<b>1.631</b>	<b>4.945</b>	<b>3.003</b>	0.614	<b>3.490</b>	0.570
<i>AhTPPA</i>	0.920	0.705	<b>0.250</b>	0.640	0.850	0.706	0.703	1.108	1.062	0.631	<b>0.314</b>	0.722
<i>AhTPPD</i>	1.452	<b>0.396</b>	<b>0.197</b>	1.188	0.750	0.776	1.035	1.329	0.744	<b>0.460</b>	<b>0.256</b>	0.594
<i>AhTPPI</i>	0.804	0.786	0.619	0.858	1.133	<b>0.469</b>	0.968	<b>0.441</b>	0.967	<b>0.310</b>	<b>0.253</b>	0.529
<i>AhTRE</i>	0.854	0.711	0.722	1.192	1.257	0.615	0.526	0.622	1.346	0.520	<b>0.495</b>	1.058

965 <sup>1</sup>Calculated according to the comparative cycle threshold method (Livak and Schmittgen, 2001) using the *AhACT7*, *AhEF1a* and *Ah $\beta$ Tub5* amaranth genes for  
 966 data normalization.

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968 **Table 3.** Relative expression values<sup>1</sup> of genes involved in the biosynthesis of raffinose family oligosaccharides in leaves of four *Amaranthus*  
 969 species subjected to two levels of water-deficit stress (moderate [M] and severe [S]) and to subsequent recovery ([R]). Induced (normalized  
 970 expression values  $\geq 2.0$ ; in normal text) and repressed (normalized expression values  $\leq 0.5$ ; in italicized text) expression values are emphasized in  
 971 bold.

Gene	<i>A. hypochondriacus</i>			<i>A. cruentus</i>			<i>A. caudatus</i>			<i>A. hybridus</i>		
	M	S	R	M	S	R	M	S	R	M	S	R
<i>AhGolS1</i>	<b>29.100</b>	<b>66.822</b>	1.311	<b>13.030</b>	<b>26.332</b>	<b>3.041</b>	<b>20.212</b>	<b>23.297</b>	<b>1.889</b>	<b>11.695</b>	<b>44.194</b>	<b>3.035</b>
<i>AhGolS2</i>	<i>0.129</i>	<i>0.308</i>	<i>0.476</i>	<b>1.714</b>	1.030	<i>0.432</i>	<b>4.607</b>	1.238	0.569	<i>0.430</i>	0.941	0.936
<i>AhRafS</i>	<b>4.674</b>	<b>11.415</b>	1.052	<b>2.373</b>	<b>7.810</b>	0.735	<b>2.863</b>	<b>2.351</b>	0.727	<b>4.179</b>	<b>4.526</b>	1.085
<i>AhStaS</i>	1.430	0.7346	0.595	1.389	<b>1.735</b>	<i>0.431</i>	<b>1.502</b>	0.862	<i>0.300</i>	1.000	0.885	0.548

972 <sup>1</sup>Calculated according to the comparative cycle threshold method (Livak and Schmittgen, 2001) using the *AhACT7*, *AhEF1a* and *AhβTub5* amaranth genes for  
 973 data normalization.

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983 **Table 4.** Relative expression values<sup>1</sup> of genes involved in the biosynthesis of raffinose family oligosaccharides in roots of four *Amaranthus*  
 984 species subjected to two levels of water-deficit stress (moderate [M] and severe [S]) and to subsequent recovery ([R]). Induced (normalized  
 985 expression values  $\geq 2.0$ ; in normal text) and repressed (normalized expression values  $\leq 0.5$ ; in italicized text) expression values are emphasized in  
 986 bold.

Gene	<i>A. hypochondriacus</i>			<i>A. cruentus</i>			<i>A. caudatus</i>			<i>A. hybridus</i>		
	M	S	R	M	S	R	M	S	R	M	S	R
<i>AhGols1</i>	<b>22.202</b>	<b>136.744</b>	0.819	<b>14.745</b>	<b>248.857</b>	<b>2.128</b>	<b>18.018</b>	<b>75.932</b>	1.088	<b>17.009</b>	<b>37.691</b>	<b>1.870</b>
<i>AhGols2</i>	0.673	<b>3.471</b>	0.261	<b>2.697</b>	<b>3.345</b>	1.198	<b>2.401</b>	<b>2.539</b>	1.124	<b>2.355</b>	<b>2.328</b>	0.696
<i>AhRafS</i>	<b>3.426</b>	<b>14.981</b>	1.270	<b>1.795</b>	<b>15.190</b>	1.008	<b>2.268</b>	<b>5.426</b>	0.539	<b>3.699</b>	1.407	0.510
<i>AhStaS</i>	<b>2.960</b>	<b>6.219</b>	1.378	<b>6.583</b>	<b>5.126</b>	<b>1.553</b>	<b>5.525</b>	<b>5.089</b>	<b>2.381</b>	<b>2.943</b>	<b>2.758</b>	1.034

987 <sup>1</sup>Calculated according to the comparative cycle threshold method (Livak and Schmittgen, 2001) using the *AhACT7*, *AhEF1a* and *Ah $\beta$ Tub5* amaranth genes for  
 988 data normalization.

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998 **Table 5.** Relative expression values<sup>1</sup> of abscisic acid (ABA) marker genes in leaves of four *Amaranthus* species subjected to two levels of water-  
 999 deficit stress (moderate [**M**] and severe [**S**]) and to subsequent recovery ([**R**]). Induced (normalized expression values  $\geq 2.0$ ; in normal text) and  
 1000 repressed (normalized expression values  $\leq 0.5$ ; in italicized text) expression values are emphasized in bold.

Gene	<i>A. hypochondriacus</i>			<i>A. cruentus</i>			<i>A. caudatus</i>			<i>A. hybridus</i>		
	M	S	R	M	S	R	M	S	R	M	S	R
<i>AhABI5</i>	<b>2.005</b>	<b>3.047</b>	<b>2.202</b>	<b>1.937</b>	<b>4.014</b>	0.726	<b>3.244</b>	1.469	1.317	<b>2.437</b>	<b>2.590</b>	<b>2.507</b>
<i>AhDREB</i>	<b>5.300</b>	<b>8.754</b>	<b>3.867</b>	0.858	1.251	0.631	<b>1.655</b>	0.921	0.739	0.665	<b>1.689</b>	1.159
<i>AhRAB18</i>	0.776	0.789	0.963	0.885	0.843	<i>0.350</i>	<b>1.642</b>	1.233	0.861	0.502	<b>1.815</b>	1.052
<i>AhLEA14</i>	<b>21.284</b>	<b>64.530</b>	<b>5.835</b>	<b>16.518</b>	<b>31.398</b>	<b>2.136</b>	<b>49.051</b>	<b>120.856</b>	<b>4.553</b>	<b>16.745</b>	<b>46.504</b>	<b>2.539</b>

1001 <sup>1</sup>Calculated according to the comparative cycle threshold method (Livak and Schmittgen, 2001) using the *AhACT7*, *AhEF1a* and *Ah $\beta$ Tub5* amaranth genes for  
 1002 data normalization.

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1013 **Table 6.** Relative expression values<sup>1</sup> of abscisic acid (ABA) marker genes in roots of four *Amaranthus* species subjected to two levels of water-  
 1014 deficit stress (moderate [**M**] and severe [**S**]) and to subsequent recovery ([**R**]). Induced (normalized expression values  $\geq 2.0$ ; in normal text) and  
 1015 repressed (normalized expression values  $\leq 0.5$ ; in italicized text) expression values are emphasized in bold.

Gene	<i>A. hypochondriacus</i>			<i>A. cruentus</i>			<i>A. caudatus</i>			<i>A. hybridus</i>		
	M	S	R	M	S	R	M	S	R	M	S	R
<i>AhABI5</i>	<b>13.870</b>	<b>22.754</b>	<b>18.979</b>	<b>5.903</b>	<b>8.446</b>	<b>1.855</b>	<b>2.307</b>	<b>1.632</b>	0.977	<b>0.426</b>	1.062	<b>0.392</b>
<i>AhDREB</i>	<b>9.951</b>	<b>16.009</b>	<b>16.034</b>	<b>2.607</b>	<b>5.077</b>	<b>1.793</b>	<b>3.055</b>	<b>2.007</b>	0.859	1.199	1.491	0.913
<i>AhRAB18</i>	<b>5.641</b>	<b>5.735</b>	<b>14.445</b>	1.477	<b>2.318</b>	1.016	0.978	1.093	0.729	0.548	<b>0.418</b>	1.121
<i>AhLEA14</i>	<b>4.464</b>	<b>12.829</b>	<b>1.629</b>	<b>2.356</b>	<b>6.916</b>	0.898	<b>1.761</b>	<b>7.408</b>	1.231	<b>3.880</b>	<b>5.183</b>	0.761

1016 <sup>1</sup>Calculated according to the comparative cycle threshold method (Livak and Schmittgen, 2001) using the *AhACT7*, *AhEF1a* and *Ah $\beta$ Tub5* amaranth genes for  
 1017 data normalization.

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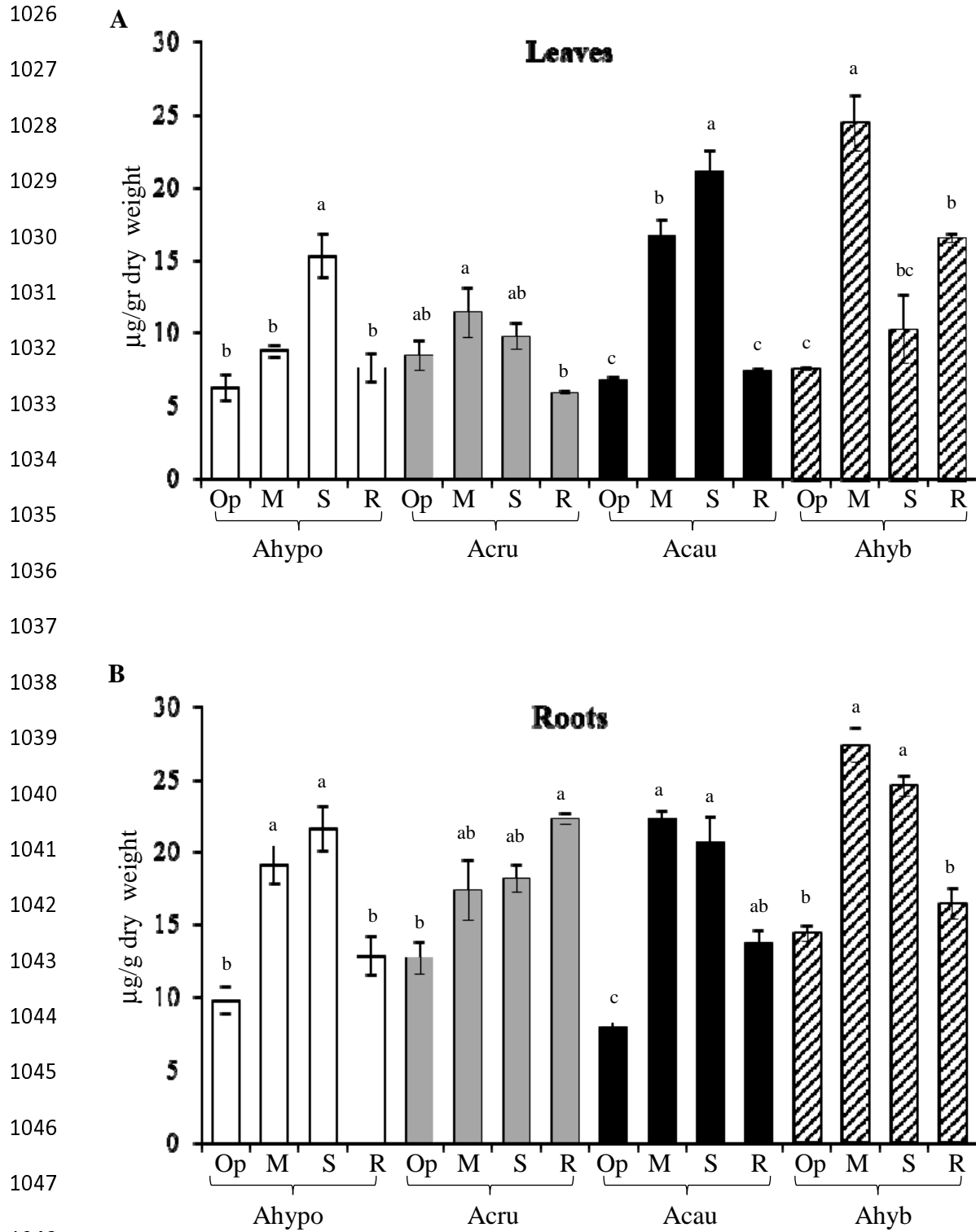


Fig. 1

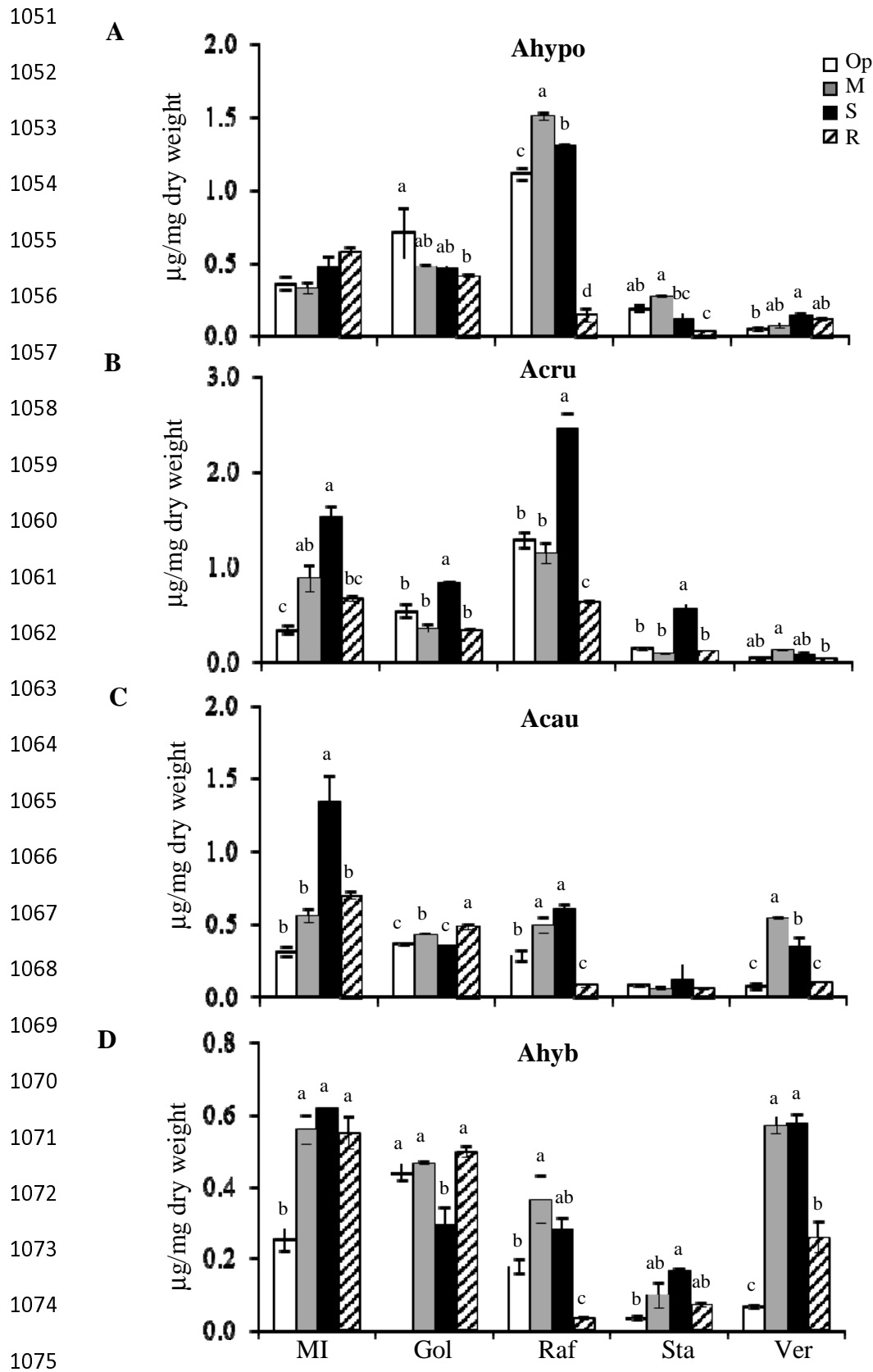


Fig. 2



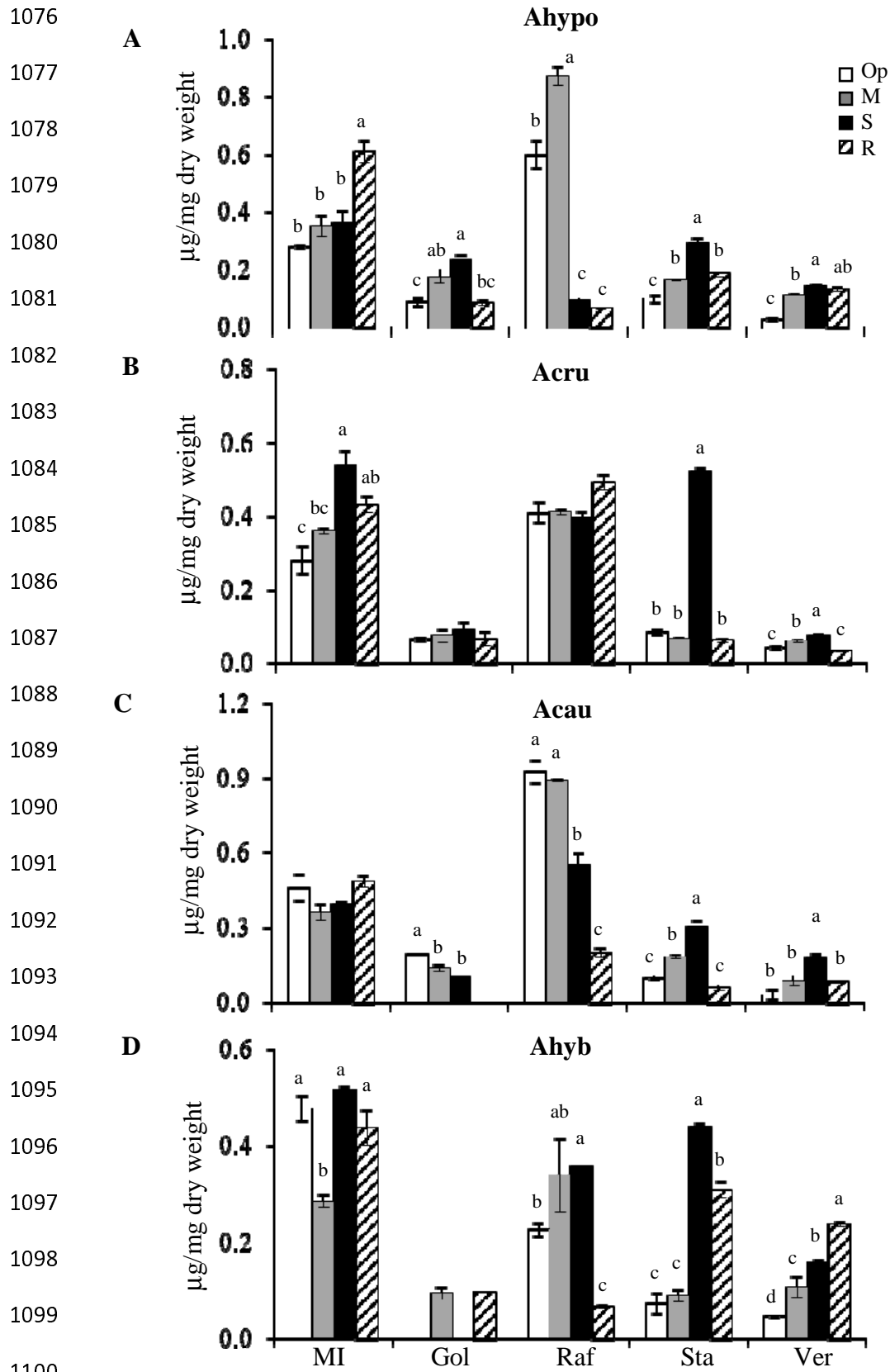


Fig. 3

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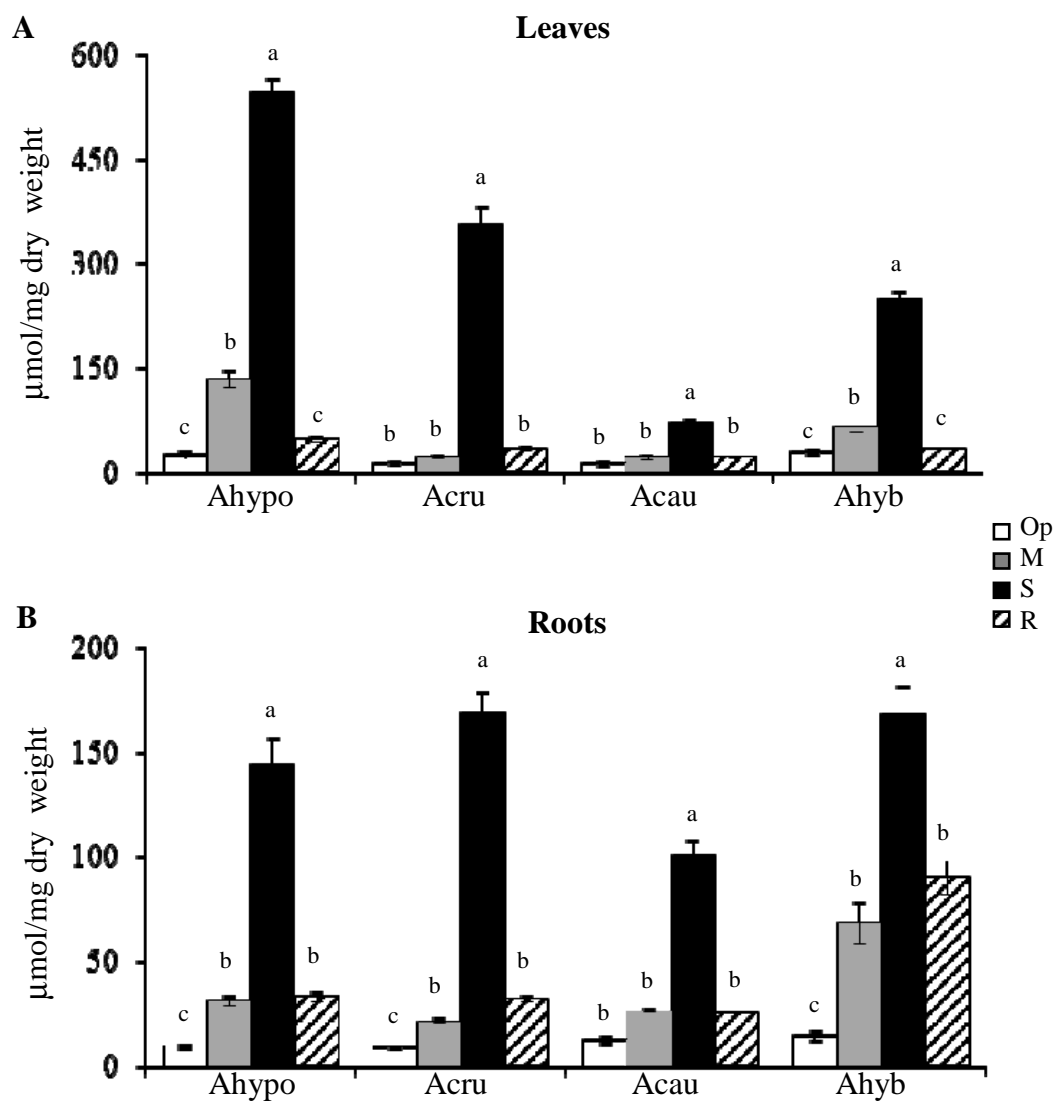


Fig. 4

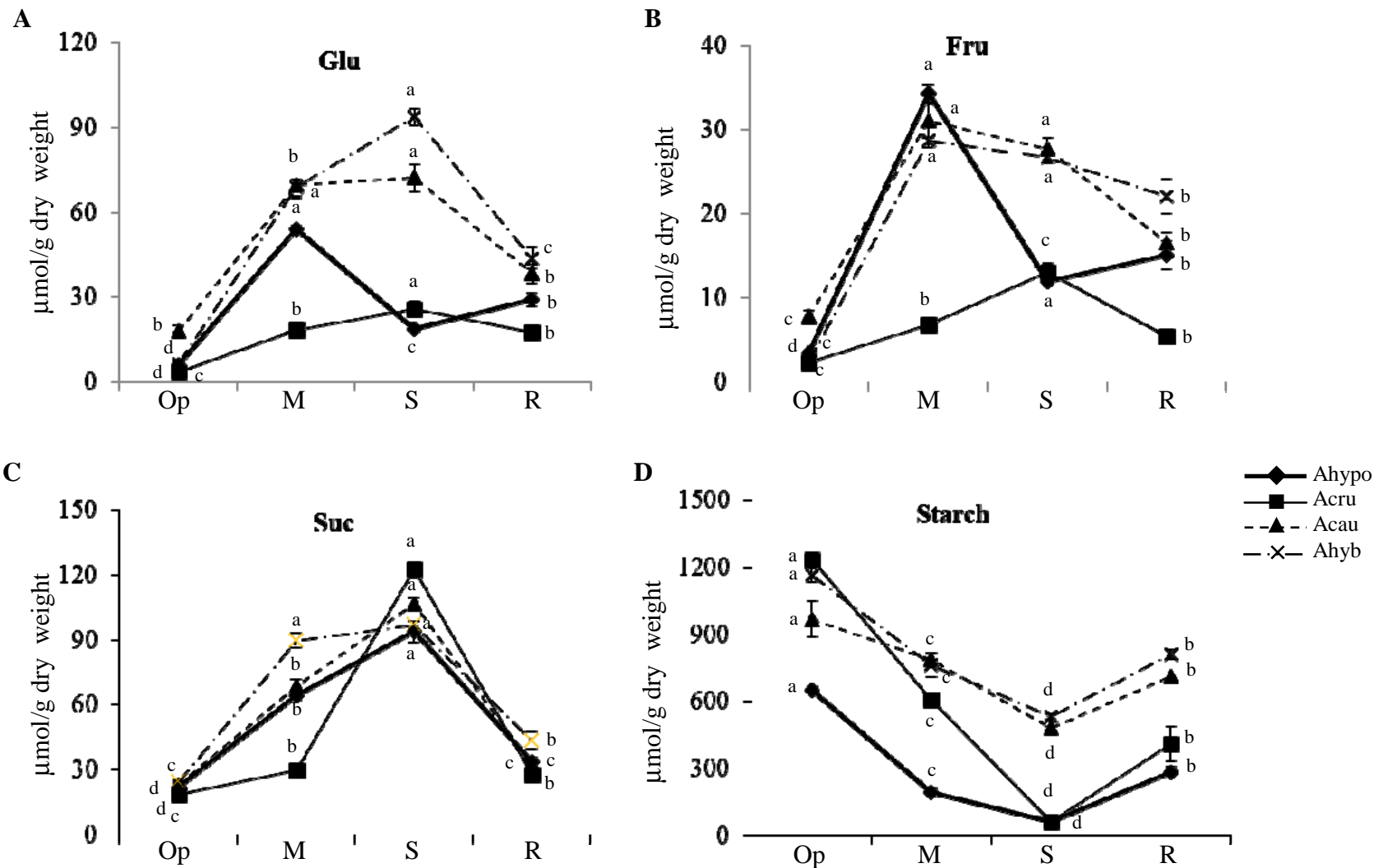


Fig. 5

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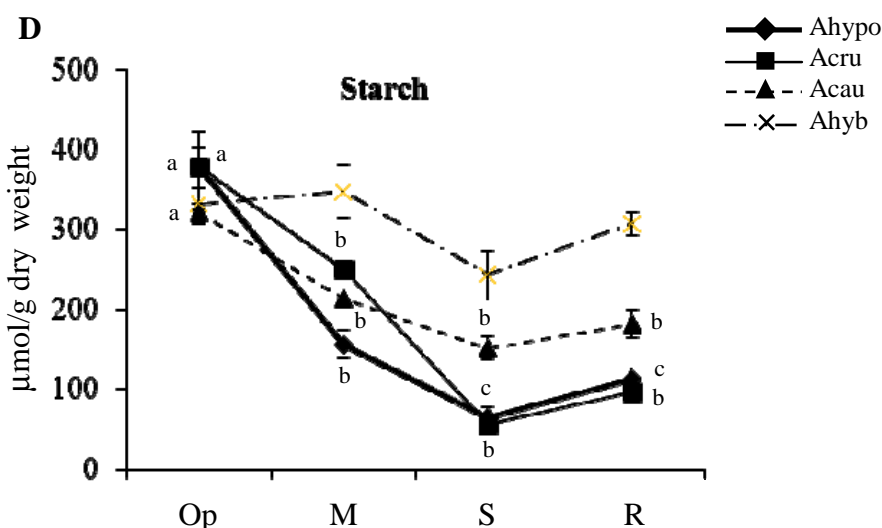
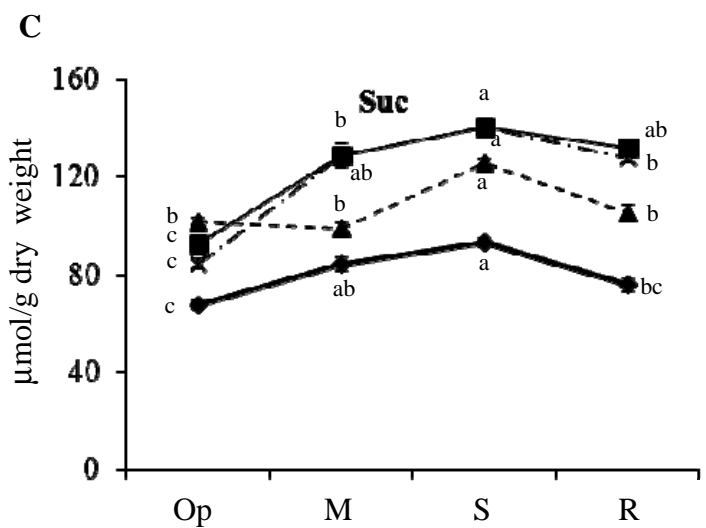
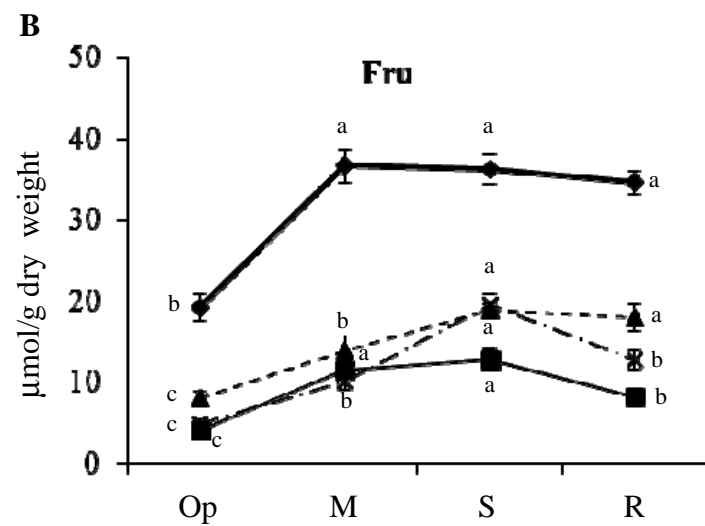
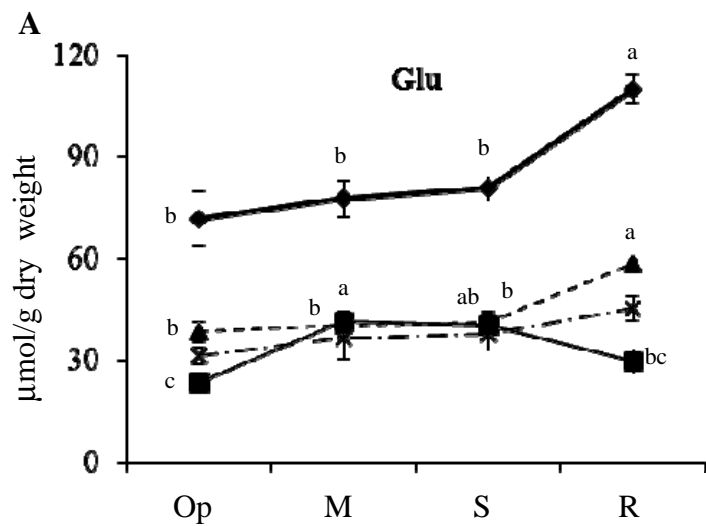


Fig. 6

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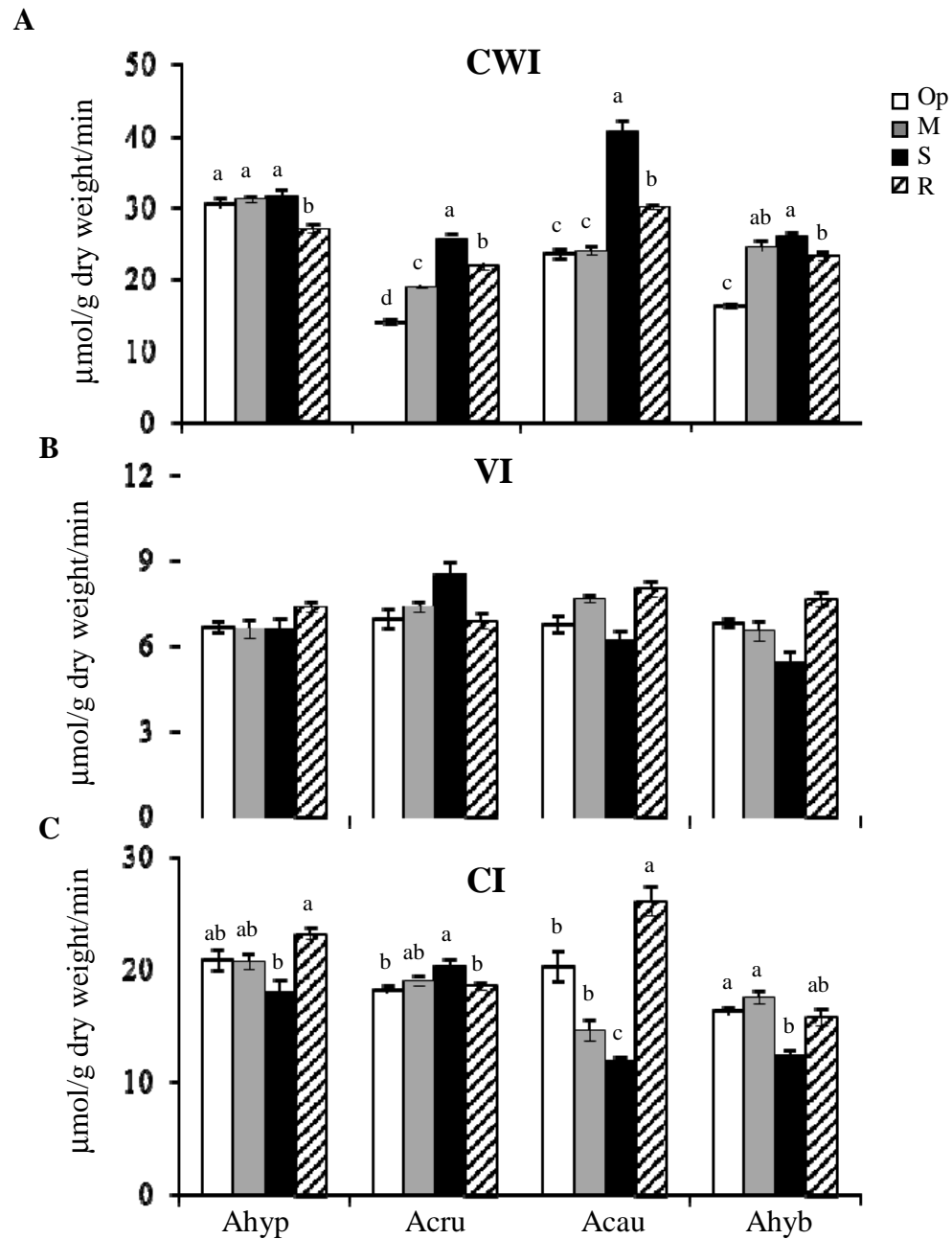


Fig. 7

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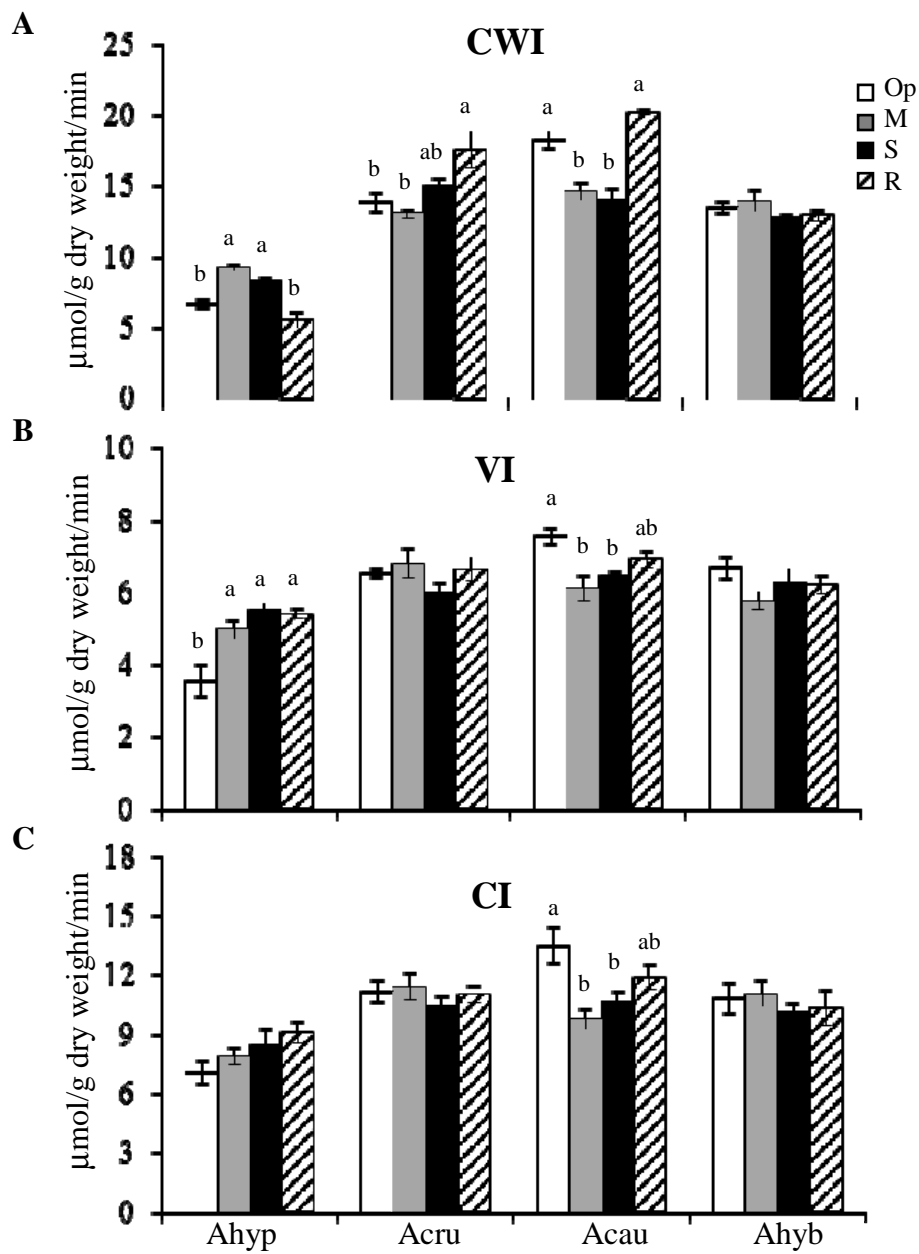


Fig. 8

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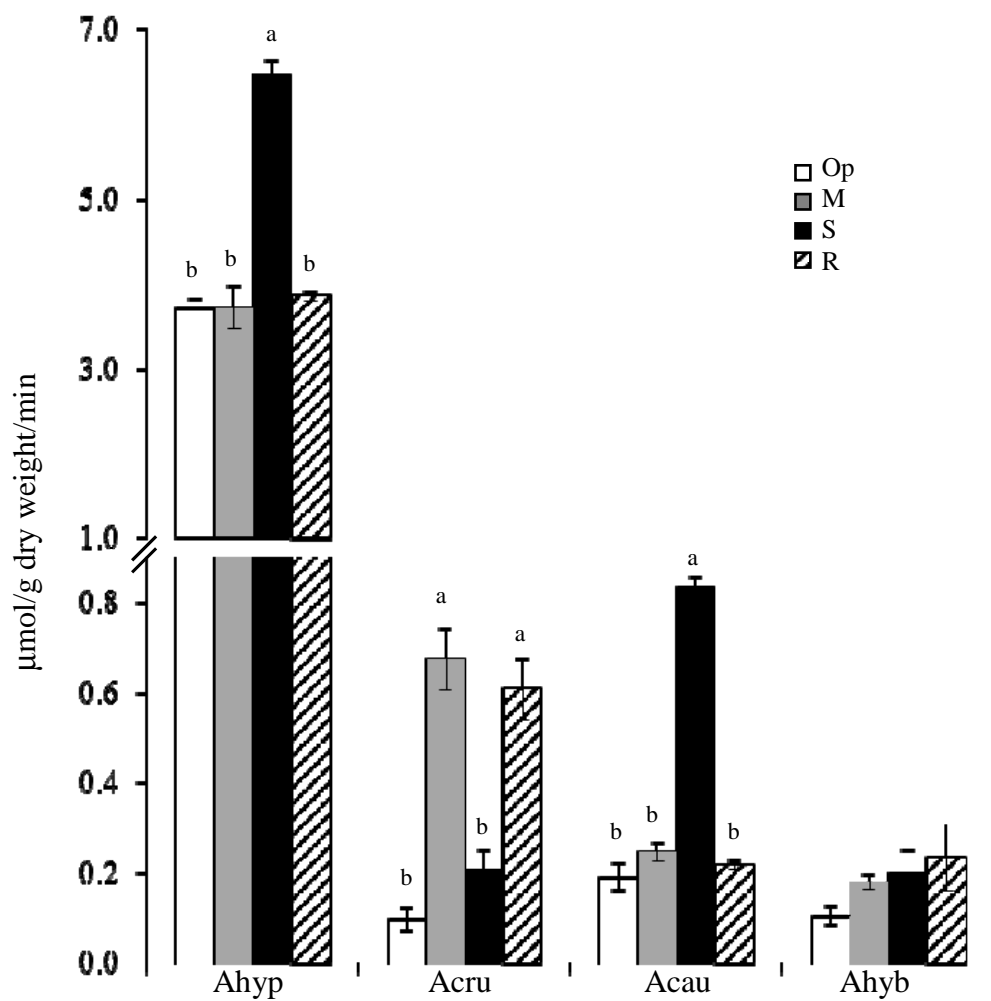


Fig. 9

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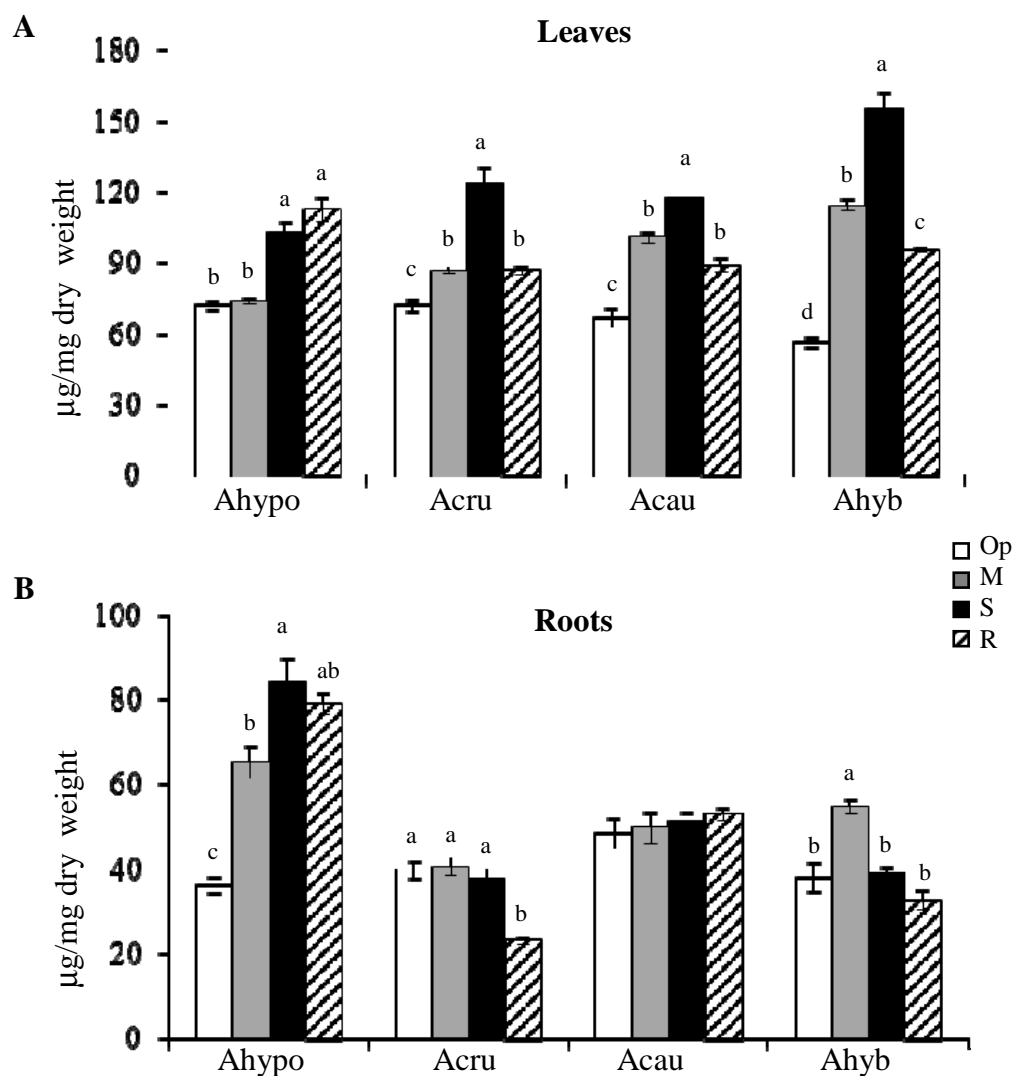


Fig. 10