Short title: Sugarcane drought tolerance: from mothers to daughters

Stressed mothers, tolerant daughters: a case study about the physiological responses and growth of sugarcane plants under water deficit

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Abstract

Drought stress can imprint marks in plants after a previous exposure, leading to a permissive state that facilitates a more effective response to subsequent stress events. Such stress imprints would benefit plants obtained from progenitors previously exposed to drought. Herein, our hypothesis was that daughter plants obtained from mother plants previously exposed to water deficit will perform better under water deficit as compared to those obtained from mothers that did not face stressful conditions. Sugarcane mother plants were grown under well-hydrated conditions or subjected to three cycles of water deficit by water withholding. Then, daughter plants produced through vegetative propagation were subjected to water deficit. Leaf gas exchange was reduced under water deficit and daughters from mothers that experienced water deficit presented a faster recovery of CO_2 assimilation and higher instantaneous carboxylation efficiency after rehydration as compared to daughters from mothers that did not face water deficit. Plants obtained from mother plants that faced water deficit showed the highest leaf proline concentration under water deficit as well as higher leaf H_2O_2 concentration and leaf ascorbate peroxidase activity regardless of water regime. Under well-watered conditions, daughters from mothers that faced stressful conditions presented higher root H_2O_2 concentration and root catalase activity than ones from mothers that did not experience water shortage. Such physiological changes were associated with improvements in leaf area and shoot and root dry matter accumulation in daughters from stressed mothers. Our results suggest that root H_2O_2 concentration is a chemical signal associated with stress memory and improved sugarcane growth. Such

findings bring a new perspective to sugarcane production systems, in which stress memory can be explored for improving drought tolerance in rainfed areas.

Key words: drought, memory, photosynthesis, propagation, resistance.

Introduction

As a semi-perennial species, sugarcane plants face seasonal drought under field conditions, where water deficit causes reduction in photosynthesis and accumulation of carbohydrates, changes in antioxidant metabolism, and finally impairment of plant growth and sucrose yield [1, 2]. However, recurrent cycles of drought followed by rehydration are known to improve plant performance during a new stressful event [3-5]. Such phenomenon indicates that plants are able to change their metabolism and growth after an external stimulus, improving recovery of photosynthesis, increasing intrinsic water use efficiency [4] and photoprotection [6] and reducing the negative impact of drought on yield [7].

Improved plant response induced by previous exposure to a limiting factor is an evidence of stress memory, a way to storage information of stressful events [3, 8, 9]. In fact, such stress memory can assist plants in future stresses [9] and one important issue is the site (plant tissue) in which information is stored within plants. Plants do not have a specific region to store information and they can sense the environment with all their body and the intricate cell signaling system. Then, plants can perceive one stimulus in one site and the respective response be found in a different organ due to signaling [10].

One important requirement for retaining information is that stress-induced signals are still present when the stressor is no longer affecting plants [11].

In nature, plant phenotype is also defined by transgenerational regulation, which occurs when internal changes persist in the next generation through epigenetic marks such as DNA methylation [8]. There is reasonable evidence for assuming that plants can sense changes in the environment during growth and modify the phenotype of their progeny to be more adapted to growing conditions [8, 12]. The stress-induced memory can be transferred to subsequent generations by seeds and through vegetative propagation [13]. In the first case, plants can pass epigenetic information through the meiosis process and produce seeds with stress memory [12, 14]. For instance, Boyko et al. [14] showed that *Arabidopsis thaliana* exposed to cold, heat and flooding had increased global genome methylation and higher tolerance to stress as compared to progeny from plants that never faced stressful conditions. However, stress-induced signals may be erased or diminished during meiosis, reducing stress memory. On the other hand, clonal plants produced by vegetative propagation have apparently better ability to recover signals acquired during stress events than non-clonal plants [15].

Considering stress memory, plant propagation and drought-induced effects on plants, we hypothesized that plants obtained from others previously exposed to drought will perform better under water deficit as compared to plants obtained from mother plants that never faced water shortage. Through vegetative propagation, information about previous stresses (memory) can be stored in sugarcane buds, which will sprout and produce new plants. Sugarcane is an important crop for ethanol and bioenergy production – a clean alternative for energy production – and its expansion to rainfed

areas needs more drought tolerant plants. Then, stress memory would be an interesting tool for improving crop establishment and initial growth in such new areas.

Materials and methods

Plant material and growth conditions

Sugarcane (*Saccharum* spp.) plants cv. IACSP94-2094 were obtained from mini-stalks containing one bud and grown in plastic pots (0.5 L), with commercial substrate composed of sphagnum peat, expanded vermiculite, limestone dolomite, agricultural gypsum and NPK fertilizer (Carolina Soil®, Vera Cruz RS, Brazil). Thirty-four days after planting, plants were transferred to larger pots (20 L) containing typical red-yellow Latosoil [16], fertilized with urea (equivalent to 300 kg N ha^{-1}), superphosphate (equivalent to 300 kg P_2O_5 ha^{-1}) and potassium chloride (equivalent to 260 kg K_2O ha^{-1}) according to Dias and Rossetto [17]. During the experiment, other three fertilizations were performed at 30, 60 and 150 days after planting, with the same amount of urea, superphosphate and potassium chloride as the first fertilization. The plants were grown under greenhouse conditions, where the average air temperature was 24.4±6.6 °C, relative humidity was 76±17% and the maximum photosynthetic photon flux density (PPFD) was approximately 1,200 µmol m^{-2} s^{-1}. Plants were irrigated daily and grown under well-hydrated conditions until they were six-month old.

Inducing water deficit to mother plants

When plants were 6-month old, one group of plants was maintained under daily irrigation (W) and another group was subjected to three cycles of water deficit (D) by water withholding. Each cycle of water deficit lasted nine days and soil moisture was monitored with soil moisture-sensors model Water Scout SM100 (Yara ZimTechnology, Berlin, Germany). While soil volumetric water content (VWC) reached 20% during cycles of water deficit, it was higher than 60% in well-watered pots. After nine days of water deficit, plants were irrigated and maintained under well-watered conditions for six days before the new cycle of water deficit, with leaf gas exchange being measured daily. After three cycles of water deficit, we evaluated the number of tillers, number of green and senescent leaves, total leaf area and dry matter of leaves, stems and roots. Then, daughter plants were produced through vegetative propagation from those mother plants that experienced or not cycles of water deficit, as cited in the previous section.

Inducing water deficit to daughter plants

After sprouting in commercial substrate (Carolina Soil®, Vera Cruz RS, Brazil), one-month old plants were placed in plastic boxes (12 L) with nutrient solution and transferred to a growth chamber (PGR15, Conviron, Winnipeg MB, Canada) under air temperature of 30/20 °C (day/night), with 12 h photoperiod, air relative humidity of 80% and PPFD of 800 µmol m^{-2} s^{-1}. Only the root system was immersed in modified Sarruge [18] nutrient solution (15 mmol L^{-1} N [7% as NH_4^+]; 4.8 mmol L^{-1} K; 5.0 mmol L^{-1} Ca; 2.0 mmol L^{-1} Mg; 1.0 mmol L^{-1} P; 1.2 mmol L^{-1} S; 28.0 µmol L^{-1} B; 54.0 µmol L^{-1} Fe; 5.5 µmol L^{-1} Mn; 2.1 µmol L^{-1} Zn; 1.1 µmol L^{-1} Cu and 0.01 µmol L^{-1} Mo). Nutrient solution was

renewed in week intervals and pH was maintained at 5.8±0.2 and electrical conductivity at 1.72±0.18 mS cm^{-1}. The osmotic potential of nutrient solution was -0.12 MPa. Two boxes containing plants obtained from irrigated mother plants and two boxes containing plants from those mothers subjected to three cycles of water deficit were prepared.

Forty-eight days after transferring plants to the hydroponic system, one group of plants was subjected to water deficit by adding PEG-8000 (CarbowaxTM PEG-8000, Dow Chemical Comp, Midland MI, USA) to the nutrient solution for nine days. We added PEG-8000 gradually to prevent osmotic shock. Then, the osmotic potential of nutrient solution was reduced to -0.27, -0.57 and -0.77 MPa in three consecutive days. After nine days, the plants were recovered by supplying them with a nutrient solution with osmotic potential of -0.12 MPa (control condition) for five days. At the end, four treatments were defined taking into account the plant origin and also the water regime plants were facing: plants obtained from mother plants grown under well-watered conditions and then maintained under well-watered conditions (W/W); plants obtained from mother plants grown under well-watered conditions and then subjected to water (W/D); plants obtained from mother plants that experienced water deficit and then maintained under well-watered conditions (D/W); plants obtained from mother plants that faced water deficit and then subjected to water (D/D).

Leaf gas exchange and photochemistry

Leaf gas exchange was measured daily with an infrared gas analyzer (LI-6400, LICOR, Lincoln NE, USA) attached to a modulated fluorometer (6400-40 LCF, LICOR, Lincoln NE, USA). The measurements were performed between 10:00 and 13:00 h under PPFD of 2,000 µmol m^{-2} s^{-1} and air CO_2 concentration of 380 µmol mol^{-1}. CO_2

158 assimilation (A), stomatal conductance (g_S), intercellular CO_2 concentration (C_i),
159 transpiration (E), intrinsic water use efficiency (A/g_S), and the instantaneous
160 carboxylation efficiency ($k=A/C_i$) were evaluated in fully expanded leaves. A and E values
161 were integrated throughout the experimental period to estimate the total CO_2 gain (A_i),
162 the total H_2O loss through transpiration (E_i), and the integrated water use efficiency
163 (WUE=A_i/E_i). The integrated values were estimated assuming that the values measured
164 between 10:00 and 13:00 h were constant during the 12 hours of photoperiod.
165 Chlorophyll fluorescence was measured simultaneously to leaf gas exchange and the
166 apparent electron transport rate (ETR) was estimated as ETR=$\phi_{PSII}\times$ PPFD × 0.85 × 0.4, in
167 which ϕ_{PSII} is the effective quantum efficiency of photosystem II (PSII), 0.85 is the light
168 absorption and 0.4 is the fraction of light energy partitioned to PSII [19, 20]. Additionally,
169 the non-photochemical quenching of fluorescence (NPQ) was evaluated and ETR/A
170 calculated. In leaf tissues adapted to darkness (30 min), the potential quantum efficiency
171 of photosystem II (F_V/F_M) was estimated [20].
172

173 Leaf water potential and relative water content

174 Leaf water potential (ψ) was evaluated at the predawn with a pressure chamber
175 (model 3005, Soilmoisture Equipment Corp., Santa Barbara CA, USA). The leaf relative
176 water content RWC) was calculated using the fresh (FW), turgid (TW) and dry (DW)
177 weights of leaf discs according to Weatherley [21]: $\mathrm{RWC} = 100 \times$
178 $(\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW})$. Both variables were measured in daughter plants at the
179 maximum stress condition (9^{th} day of water deficit) and recovery period.
180

Carbohydrates and proline

The extraction of total soluble carbohydrates (SS) was done with methanol:chloroform:water solution [22] and quantified by the phenol–sulfuric acid method [23]. Sucrose content was quantified following van Handel [24] and starch (Sta) was determined by the enzymatic method proposed by Amaral et al. [25]. The concentration of nonstructural carbohydrates (NSC) in leaves and roots was calculated as NSC=SS+Sta. Total NSC was calculated considering the dry matter of each plant (mg plant^{-1}). Plant nonstructural carbohydrates were calculated by the sum of leaf and root carbohydrates and carbohydrate partitioning among sugar types was also evaluated in both organs.

Leaf proline content was determined in test tubes by the reaction with the ninhydrin reagent (ninhydrin, acetic acid and orthophosphoric acid), glycine and acetic acid for 35 minutes at 100°C. The reaction mixture was extracted with toluene and the proline concentration was determined from a standard curve [26].

Hydrogen peroxide and antioxidant enzymes

Evaluation of hydrogen peroxide (H_2O_2) was performed in 0.16 g fresh tissue (leaves and roots) ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v) [27]. The extract was centrifuged at 12,000 *g*, 4°C for 15 min. The crude extract was added to the reaction medium (1.2 mL of KI 1 mol L^{-1}, potassium phosphate buffer pH 7.5 and 0.1 mol L^{-1}) in microtubes and incubated on ice under dark for 1 h. After this

period, the absorbance was evaluated at 390 nm. The calibration curve was done with H_2O_2 and results were expressed as µmol g^{-1} FW.

Enzymes were extracted from 0.2 g of fresh tissues of leaves and roots grounded in liquid nitrogen, with 1% of PVPP and 2 mL of extraction medium composed by 0.1 mol L^{-1} potassium phosphate buffer (pH 6.8), 0.1 mmol L^{-1} ethylenediaminetetraacetic (EDTA) and 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF). This homogenate was centrifuged at 15,000 *g* for 15 min and 4°C and the supernatant was collected and preserved on ice.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was evaluated in a reaction medium with 3 mL of 100 mmol L^{-1} sodium phosphate buffer (pH 7.8), 50 mmol L^{-1} methionine, 5 mmol L^{-1} EDTA, deionized water, crude extract, 100 µmol L^{-1} riboflavin and 1 mmol L^{-1} nitro blue tetrazolium chloride (NBT). A group of tubes was exposed to light (fluorescent lamp of 30 W) for 15 min, and another group remained in darkness. The absorbance was measured at 560 nm and one unit of SOD is the amount of enzyme required to inhibit the NBT photoreduction in 50% [28]. SOD was expressed as U g^{-1} FW min^{-1}.

Catalase (CAT, EC 1.11.1.6) activity was assayed in a reaction medium of 3 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.8), deionized water, 125 mmol L^{-1} H_2O_2 and crude extract. The decrease in absorbance at 240 nm was measured and CAT activity was estimated using a molar extinction coefficient of 36 M^{-1} cm^{-1} and expressed as nmol g^{-1} FW min^{-1} [29].

For ascorbate peroxidase (APX, EC 1.11.1.11) activity, the reaction medium was composed by 3 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.0), deionized water, 10 mmol L^{-1} ascorbic acid, 10 mmol L^{-1} H_2O_2 and crude extract. The decrease in

227 absorbance at 290 nm was measure and we used a molar extinction coefficient of 2.8
228 M^{-1} cm^{-1} to estimate APX in nmol g^{-1} FW min^{-1} [30].
229

230 ## Biometry

231 The total leaf area was measured using the LI-3000 leaf area meter (LICOR,
232 Lincoln NE, USA), and shoot and root dry matter were evaluated after drying samples in
233 a forced air oven at 65 °C. Measurements were taken at the end of the experimental
234 period.
235

236 ## Statistical analysis

237 The experimental design was in randomized blocks and the causes of variation
238 were water conditions (two levels) and material origin (two levels). The data were
239 subjected to ANOVA procedure and the mean values (n=4) were compared by the Tukey
240 test at 5% probability level.
241

242 # Results

243

244 ## Mother plants under water deficit

245 Herein, mother plants are defined as those ones that provided vegetative
246 material for propagation, i.e., small stalk segments with buds. Mother plants were
247 subjected to three cycles of water deficit and leaf gas exchange was measured during
248 dehydration and rehydration stages (S1 Fig). There was a significant reduction in leaf

249 CO_2 assimilation after four days of water withholding in all cycles of water deficit (S1
250 Fig), with net photosynthesis reaching null values or even negative ones (respiration).
251 Full recovery of leaf CO_2 assimilation was noticed in all cycles and the negative impact
252 of water deficit was reduced from the first to the third cycle (S1 Fig). After three cycles
253 of water deficit, there was a significant reduction of biomass production (S2 Fig), with
254 reductions in the number, dry matter and area of green leaves as well as decreases in
255 root and stem dry matter (S1 Table).

256 Then, small stalk segments (around 3 cm) with one bud were obtained from
257 those mother plants and planted in individual recipients to produce new plants, i.e.,
258 daughter plants. Buds from mother plants subjected to water deficit had higher
259 sprouting (*ca.* 95%) than buds from mother plants maintained under well-watered
260 conditions (*ca.* 74%). Thirty days after planting, daughter plants were placed in plastic
261 boxes with nutrient solution and four treatments were done after 18 days: plants from
262 mother plants grown under well-watered conditions maintained under well-watered
263 conditions (W/W) or subjected to water deficit (W/D); and plants from mother plants
264 grown under cycles of water deficit maintained under well-watered conditions (D/W) or
265 subjected to water deficit (D/D).

266

267 Daughter plants under water deficit

268 Water deficit reduced leaf CO_2 assimilation, stomatal conductance and the
269 instantaneous carboxylation efficiency, regardless of the plant origin (Fig 1).
270 Interestingly, plants originated from mother plants that experienced water deficit (D/D)
271 presented a faster recovery of leaf CO_2 assimilation and carboxylation efficiency as
272 compared to W/D plants (Fig 1A,C). Integrated leaf CO_2 assimilation and transpiration

were reduced by water deficit in a similar way when comparing W/D and D/D treatments (Fig 2A,B). However, recovery of photosynthesis was favored in D/D plants and then integrated water use efficiency was improved under water deficit (Fig 2C).

Fig 1. Time course of leaf gas exchange in daughter plants under water deficit. Leaf CO_2 assimilation (*A*), stomatal conductance (*B*), and instantaneous carboxylation efficiency (*C*) in sugarcane plants grown under well-watered conditions (W/W and D/W) or subjected to water deficit (W/D and D/D). Daughter plants were obtained from mother plants previously exposed to water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). The gray area indicates the water deficit period. Each symbol is the mean values ± s.d. (n=4).

Fig 2. Integrated leaf gas exchange in daughter plants during and after water deficit. Integrated CO_2 assimilation (*A*), transpiration (*B*) and water use efficiency (*C*) in sugarcane plants grown under well-watered conditions (W/W and D/W) or subjected to water deficit (W/D and D/D). Daughter plants were obtained from mother plants previously exposed to water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Integration was done during the water deficit (stress) and recovery (gray area) periods, as shown in Fig 1. Each histogram is the mean values + s.d. (n=4). Different letters mean statistical differences among treatments ($p<0.05$).

After nine days of water deficit, pre-dawn leaf water potential was reduced and D/D plants showed the lowest values (Fig 3A). Regarding the leaf relative water content, there was a similar response to water deficit and both W/D and D/D plants exhibited the

297 lowest values (Fig 3B). While the pre-dawn leaf water potential was fully recovered, leaf
298 relative water content was partially recovered after four days of plant rehydration (Fig
299 3).
300
301 **Fig 3. Water relations in daughter plants under water deficit.**
302 Predawn leaf water potential (*A*) and relative water content (*B*) in sugarcane plants
303 grown under well-watered conditions (W/W and D/W) or subjected to water deficit
304 (W/D and D/D). Daughter plants were obtained from mother plants previously exposed
305 to water deficit (D/W and D/D) or grown under well-watered conditions (W/W and
306 W/D). Measurements were done during the water deficit (stress) and recovery (gray
307 area) periods, as shown in Fig 1. Each histogram is the mean values + s.d. (n=4). Different
308 letters mean statistical differences among treatments ($p<0.05$).
309
310 Water deficit caused decreases in the potential quantum efficiency of PSII (F_v/F_m)
311 and also in the apparent electron transport rate (ETR) of W/D and D/D plants (Fig 4A,B).
312 Although D/D plants had shown the lowest ETR values, the ratio ETR/*A* was similar
313 between W/D and D/D plants, increasing in more than three times due to water deficit
314 (Fig 4C). Non-photochemical quenching was increased by water deficit only in W/D
315 plants (Fig 4D). All photochemical indices were recovered after plant rehydration, with
316 W/W *vs.* W/D and D/W *vs.* D/D plants showing similar values.
317
318 **Fig 4. Photochemistry of daughter plants during and after water deficit**
319 Potential quantum efficiency of photosystem II (*A*), the apparent electron transport rate
320 estimated (*B*), ETR/A ratio (*C*), and the non-photochemical quenching of fluorescence

(D) in sugarcane plants grown under well-watered conditions (W/W and D/W) or subjected to water deficit (W/D and D/D). Daughter plants were obtained from mother plants previously exposed to water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were done during the water deficit (stress) and recovery (gray area) periods, as shown in Fig 1. Each histogram is the mean values + s.d. (n=4). Different letters mean statistical differences among treatments ($p<0.05$).

Leaf proline content was increased under water deficit and D/D plants presented the highest values. After the recovery period, W/D plants presented higher proline content than D/D plants (Fig 5).

Fig 5. Leaf proline concentration in daughter plants under water deficit.
Leaf proline concentration in sugarcane plants grown under well-watered conditions (W/W and D/W) or subjected to water deficit (W/D and D/D). Daughter plants were obtained from mother plants previously exposed to water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were done during the water deficit (stress) and recovery (gray area) periods, as shown in Fig 1. Each histogram is the mean values + s.d. (n=4). Different letters mean statistical differences among treatments ($p<0.05$).

Leaf sucrose content was also increased by water deficit but only in plants originated from mother plants maintained under well-watered conditions, i.e. W/W *vs.* W/D (Fig 6A). Curiously, D/W plants had higher leaf sucrose content than W/W ones, suggesting an influence of mother plants. Such influence was also found in roots, with

D/W plants presenting lower sucrose, soluble total sugars and total non-structural carbohydrates than W/W plants (Fig 6). Reductions in root concentrations of sucrose, soluble total sugars and total non-structural carbohydrates due to water deficit were found only in plants obtained from those ones that did not face drought (Fig 6E-H). When considering the total amount of non-structural carbohydrates in plants (Figure 6I), D/W plants had higher values than W/W plants and the carbohydrate partitioning between leaves (86% to 91%) and roots (9% to 15%) was similar among treatments (Fig 6J).

Fig 6. Leaf and root carbohydrates and their partitioning in daughter plants under water deficit.

Sucrose (*A*, *E*) soluble sugars (*B*, *F*), starch (*C*, *G*) and non-structural carbohydrates (*D*, *H*) in leaves (*A-D*) and roots (*E-H*), amount of total non-structural carbohydrates in the entire plant (*I*) and their partitioning among plant organs (*J*) in sugarcane plants grown under well-watered conditions (W/W and D/W) or subjected to water deficit (W/D and D/D). Daughter plants were obtained from mother plants previously exposed to water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were taken after 9 days of water deficit (maximum water deficit). Each histogram is the mean values + s.d. (n=4). Different letters mean statistical differences among treatments ($p<0.05$).

Regarding the antioxidant metabolism, leaf SOD and CAT activities were not affected either by water regime or plant origin (Fig 7A,D), while leaf H_2O_2 concentration and leaf APX activity increased due to water deficit (Fig 7B,C). The highest leaf APX

369 activity was found in W/D plants (Fig 7C). In roots, non-significant changes were found
370 for SOD and APX activities (Fig 7E,G). Root H_2O_2 concentration and CAT activity increased
371 due to water deficit in daughter plants originated from well-watered mother plants (Fig
372 7F,H). On the other hand, root H_2O_2 concentration was reduced and root CAT activity
373 did not change under water deficit when considering daughter plants originated from
374 plants grown under cycles of water deficit (Fig 7F,H). Interestingly, D/W plants had
375 higher root H_2O_2 concentration and higher root CAT activity than W/W plants (Fig 7F,H).
376

377 **Fig 7. Antioxidant metabolism in daughter plants under water deficit.**
378 Activities of SOD (*A*, *E*), APX (*C*, *G*), CAT (*D*, *H*) and H_2O_2 concentration (*B*, *F*) in leaves (*A-*
379 *D*) and roots (*E-H*) of sugarcane plants grown under well-watered conditions (W/W and
380 D/W) or subjected to water deficit (W/D and D/D). Daughter plants were obtained from
381 mother plants previously exposed to water deficit (D/W and D/D) or grown under well-
382 watered conditions (W/W and W/D). Measurements were taken after 9 days of water
383 deficit (maximum water deficit). Each histogram is the mean values + s.d. (n=4).
384 Different letters mean statistical differences among treatments ($p<0.05$).
385

386 Water deficit reduced shoot biomass production regardless plant origin, but D/D
387 plants had higher shoot biomass than W/D plants (Fig 8A). While daughter plants
388 obtained from well-watered mother plants presented increases in root biomass under
389 water deficit, the opposite was found in daughter plants obtained from mothers that
390 experienced cycles of water deficit (Fig 8B). In general, root biomass of D/W plants was
391 about four times higher than one of W/W plants, with D/D plants showing similar root
392 biomass as compared to W/D plants. Leaf area was also reduced by water deficit (Fig

393 8C), but D/D plants had higher leaf area than daughter plants obtained from well-
394 watered mothers, despite the water regime.
395
396 **Fig 8. Biomass accumulation by daughter plants under water deficit.**
397 Leaf (*A*) and root (*B*) dry matter and leaf area (*C*) in sugarcane plants grown under well-
398 watered conditions (W/W and D/W) or subjected to water deficit (W/D and D/D).
399 Daughter plants were obtained from mother plants previously exposed to water deficit
400 (D/W and D/D) or grown under well-watered conditions (W/W and W/D).
401 Measurements were taken at the end of experiment. Each histogram is the mean values
402 ± s.d. (n=4). Different letters mean statistical differences among treatments ($p<0.05$).
403

404 # Discussion

405 Herein, we induced transgenerational stress memory through vegetative
406 propagation of sugarcane by inducing cycles of water deficit to mother plants.
407 Epigenetic changes caused by varying environmental conditions help plants to adapt and
408 have advantageous growth and acclimation under unstable environments [13]. Such
409 epigenetic changes may manifest in the future generations, a transgenerational stress
410 memory if mother plants were previously stressed [14]. Although sugarcane
411 propagation does not involve meiotic recombination, mitotic alterations during
412 vegetative propagation may also produce a source of epigenetic variation that helps
413 plants to persist and succeed in environmental colonization [13].
414 Our findings indicate that propagules obtained from plants growing in areas with
415 low water availability would be more tolerant to drought as compared to propagules of

the same genotype grown under irrigation of in areas without occurrence of water deficit. Interestingly, daughters of mother plants that faced water deficit produced more biomass than ones from mother plants maintained well-watered, regardless water regime (Fig 8). This suggest that plants have increased their efficiency in using natural resources such as water and sunlight through the transgenerational stress memory. We have shown recently that stress memory is induced in sugarcane plants after three cycles of water deficit, with plants showing higher photosynthesis and improved growth under water limiting conditions [5]. Those previous results together with ones reported herein indicate that drought tolerance of sugarcane could be improved by water management and by selecting the propagation material for planting new crop fields.

When exposing mother plants to water deficit, stress memory was induced and the information likely stored in bud meristems, as suggested by improved performance of daughter plants obtained by vegetative propagation. Besides causing decreases in photosynthesis (S1 Fig) and biomass production (S2 Fig; S1 Table), cycles of dehydration and rehydration are able to create a number of chemical signals, such as increases in concentration of abscisic acid (ABA), a hormone that alter the expression pattern of many genes linked to drought response [31]. Changes in gene expression patterns might be stored through epigenetic changes such as DNA methylation and acetylation and induce stress memory [32]. In spite of a large decrease in biomass production of mother plants under water deficit (S2 Fig; S1 Table), daughter plants had faster sprouting and higher biomass than ones obtained from well-hydrated mother plants (Fig 8). Such improved plant growth due to the transgenerational stress memory was reported previously and it is likely linked to changes in DNA methylation [8], a research topic that

439 should be further investigated for revealing the molecular bases of stress memory and
440 tolerance in sugarcane.

441 The ability of clone plants in recovering the stored environmental information
442 [15] can explain both morphological and physiological responses of D/D plants. D/D
443 plants exhibited higher photosynthesis than W/D plants at recovery and this was caused
444 by higher instantaneous carboxylation efficiency (Fig 1A,C). Regarding primary
445 photochemistry, non-photochemical quenching was lower in D/D plants than in W/D
446 plants, indicating less dissipation of energy as heat in the former ones (Fig 4D). Another
447 interesting index suggesting stress memory is the water use efficiency [31], which
448 indicates an optimization of CO_2 assimilation per unit of H_2O transpired in D/D plants
449 under water limiting conditions (Fig 2C).

450 Interestingly, D/D plants were able to maintain metabolic activity and produce
451 more biomass than W/D plants (Fig 8) even presenting lower leaf water potential (Fig
452 3A). As RWC was similar in W/D and D/D plants (Fig 3), our data indicate the occurrence
453 of more intense osmotic adjustment in D/D plants. This can be explained by higher
454 concentration of proline in leaves (Fig 5), an osmotic and osmoprotectant molecule [33].
455 During stressful conditions, high proline levels in D/D plants suggest that these plants
456 have synthesized this osmolyte for adjusting the osmotic equilibrium and cell
457 homeostasis, one form of memory according to [34]. After rehydration, there was a large
458 degradation of proline in D/D plants, which would increase the remobilization of
459 nitrogen to assimilatory pathways for resuming plant growth. Evidence of
460 transgenerational stress memory was found even at the last day of rehydration, when
461 D/D plants had higher photosynthesis (25.7±2.7 *vs.* 15.7±3.8 μmol m^{-2} s^{-1}) and integrated
462 water use efficiency (7.2±0.3 vs. 6.3±0.4 μmol mol^{-1}) than W/D plants.

463 Plants respond to abiotic stresses by altering their metabolism and accumulating
464 substances such as sugars, amino acids and other metabolites with important roles in
465 stress tolerance [35]. Maintenance of high sucrose concentration even under well-
466 watered conditions may be another evidence of stress memory [36], as found in D/W
467 plants (Fig 6A). In addition, plants obtained from mother plants that faced drought did
468 not present any change in both leaf and root sucrose concentrations under water deficit
469 (Fig 6A,E). Sucrose accumulation would help plants under water deficit by improving
470 osmoregulation and protecting proteins and then maintaining photosynthesis under low
471 water availability.

472 Low concentrations of ROS in plants previously exposed to stressful conditions
473 could be an indication of stress memory [37]. However, our data indicate that exposure
474 of mother plants to water deficit caused higher root H_2O_2 concentration in plants
475 maintained under well-watered conditions (Fig 7F). In addition to its role in plant
476 signaling [38], ROS accumulation is also associated with modifications in DNA
477 methylation pattern [39], an epigenetic change that would store information and induce
478 faster stress response. The presence of ROS in controlled amounts is important for plant
479 growth, with plants showing higher H_2O_2 concentration in the region of root elongation
480 [37]. In this way, high root H_2O_2 concentration in D/W plants (Fig 7F) would explain high
481 root biomass (Fig 7F and 8B). In fact, H_2O_2 is produced by mitochondria during the
482 synthesis of NADH and ATP for supplying aerobic plant metabolism in active growing
483 regions [40].

484 Based on results reported herein, the next step towards the improvement of
485 drought tolerance in sugarcane plants would be the evaluation of field-grown plants,

considering the persistence of stress memory and its consequences for crop yield and biomass production as well as the genotypic variation within *Saccharum* complex.

Conclusion

Our findings clearly show that sugarcane growth is improved in daughter plants obtained from mother plants that faced water deficit. The bases of such transgenerational stress memory should be further studied taking into account possible epigenetic markers. Our data also revealed that bud meristems of sugarcane are able to store information acquired from previous stressful events. Accumulation of H_2O_2 in roots is a possible chemical signal related to stress memory, being associated with improved root growth in well-watered plants. Benefits of such stress memory were noticed in leaf gas exchange and plants showed improved photosynthetic water use efficiency and faster recovery of photosynthesis after rehydration. As consequence, daughter plants obtained from stressed mothers exhibited improvements in biomass production, regardless of water conditions. Finally, our results bring a new perspective for the management of sugarcane fields as plant performance could be improved under field conditions due to a large root system and faster recovery of photosynthesis after facing water shortage.

Author contributions

Conception and experimental design - Fernanda C C Marcos and Rafael V Ribeiro; Data collection - Fernanda C C Marcos, Neidiquele M Silveira and Paulo E R Marchiori; Data analysis and interpretation - Fernanda C C Marcos, Eduardo C Machado, Gustavo M

509 Souza and Rafael V Ribeiro; Drafting of the article - Fernanda C C Marcos and Rafael V
510 Ribeiro; Critical revision and final approval of the article - all authors.
511

512 Conflict of interest statement

513 The authors declare that this research was conducted in the absence of any commercial
514 or financial relationships that could be construed as a potential conflict of interest.
515

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643
644

Supporting information

S1 Fig. Time course of leaf gas exchange in mother plants under water deficit.

Leaf CO_2 assimilation of mother-plants maintained well-watered (W) or subjected to three cycles of water deficit (D). The grey area represents water withholding (nine days) and the dotted line indicates null photosynthesis. Each symbol represents the mean values ± s.d. (n = 4).

S2 Fig. General view of mother plants after water deficit.

Visual aspect of mother plants grown under cycles of water deficit (left) or under well-watered conditions (right).

S1 Table. Biomass accumulation by daughter plants under water deficit.

Biometry of mother plants grown under well-watered (reference) conditions or subjected to cycles of water deficit. Measurements were taken after 80 days of treatment. Different letters mean statistical differences between treatments ($p<0.05$).

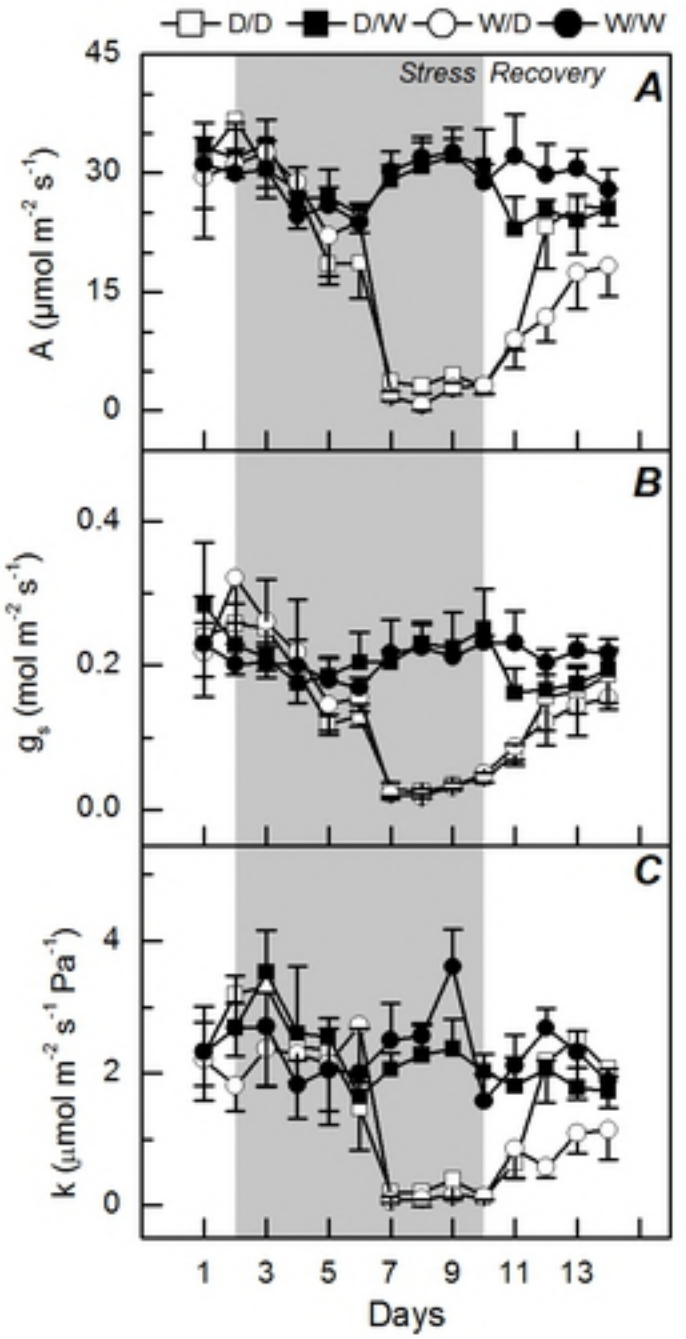
D/D
D/W
W/D
W/W
Stress
Recovery
A
B
C
A (µmol m-2 s-1)
gs (mol m-2 s-1)
k (µmol m-2 s-1 Pa-1)
45
30
15
0
0.4
0.2
0.0
4
2
0
1
3
5
7
9
11
13
Days

Figure 1

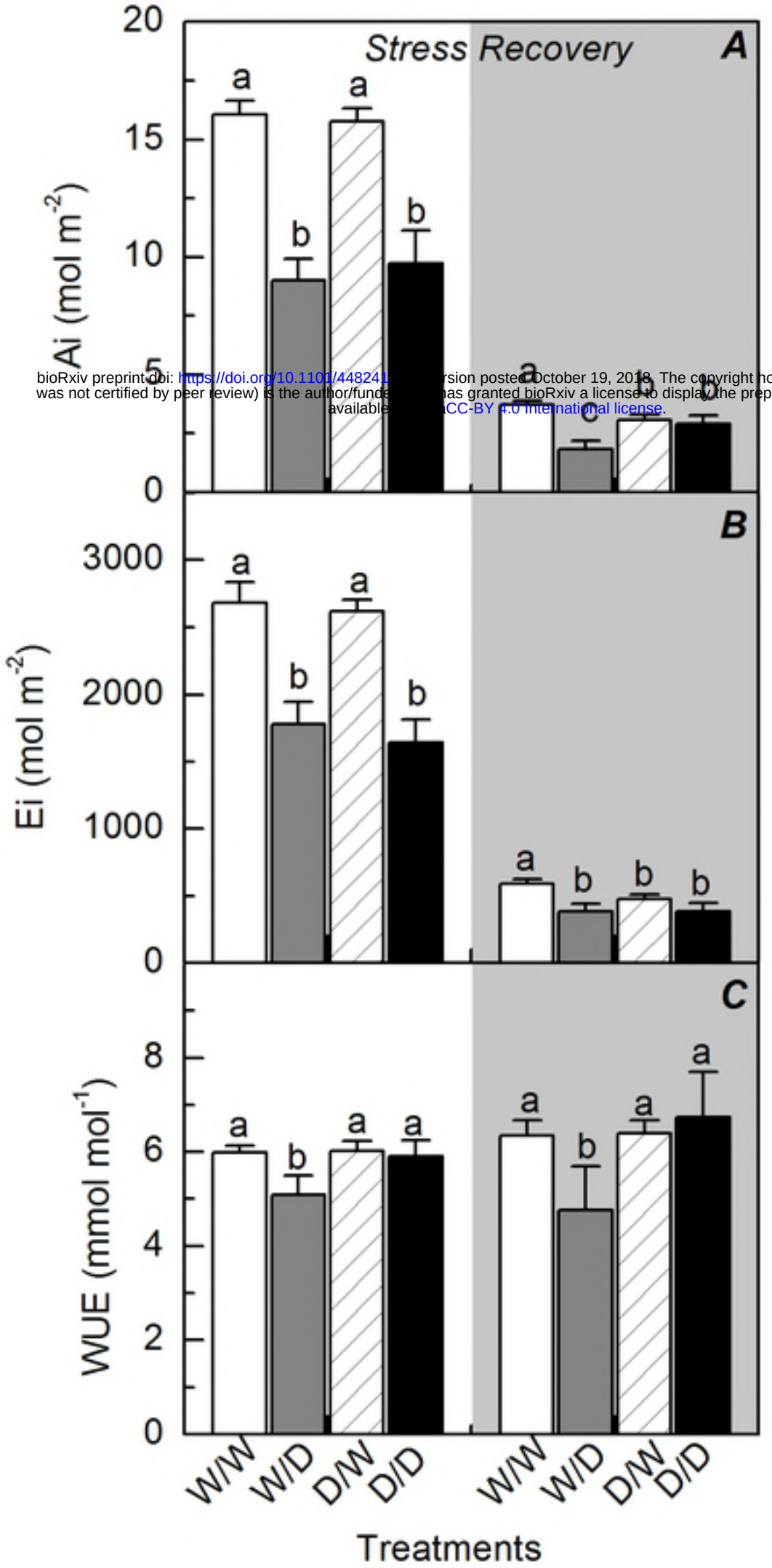

Figure 2

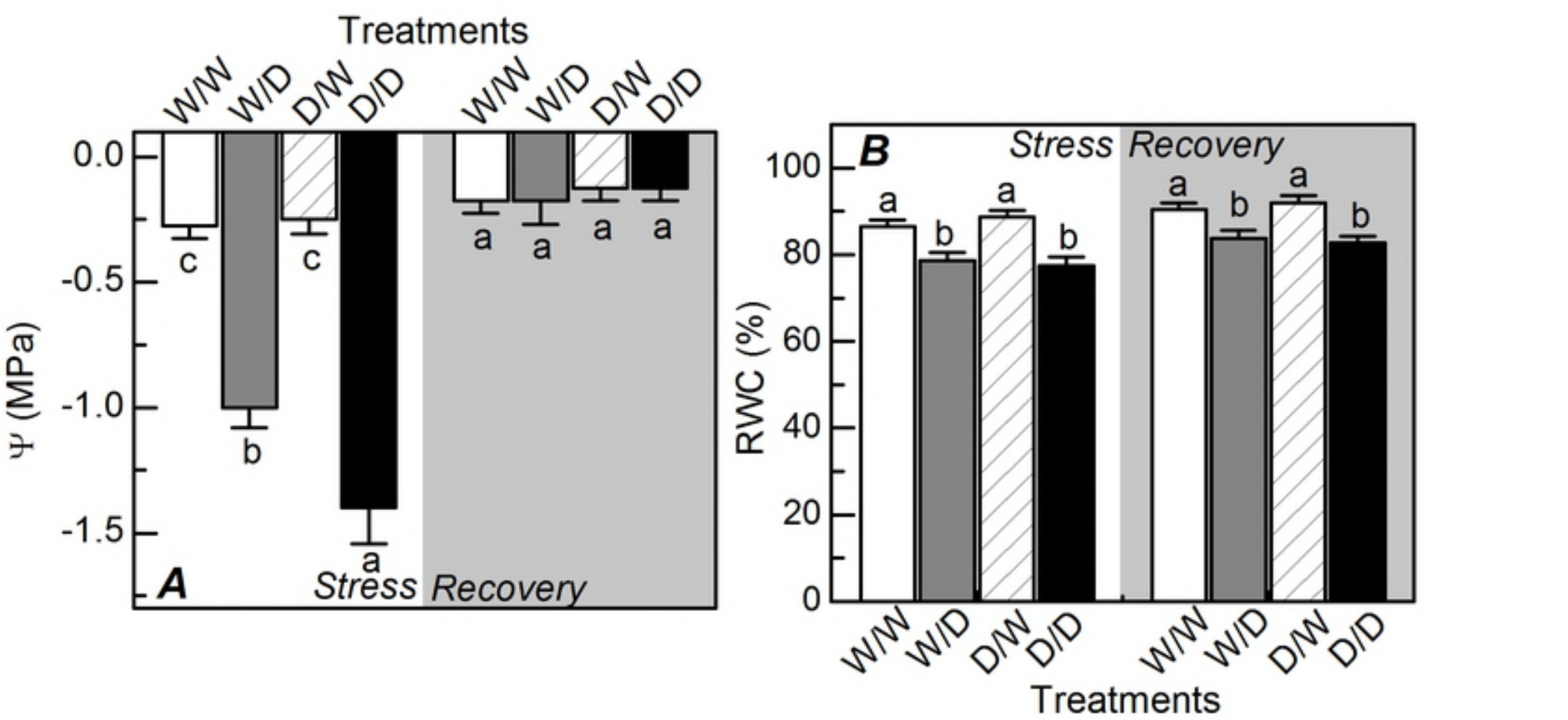
Treatments
W/W
W/D
D/W
D/D
0.0
-0.5
-1.0
-1.5
Ψ (MPa)
c
b
c
a
a
a
a
A
Stress Recovery
B
RWC (%)
100
80
60
40
20
0
a
b
a
b
a
b
a
b
Stress Recovery
Treatments

Figure 3

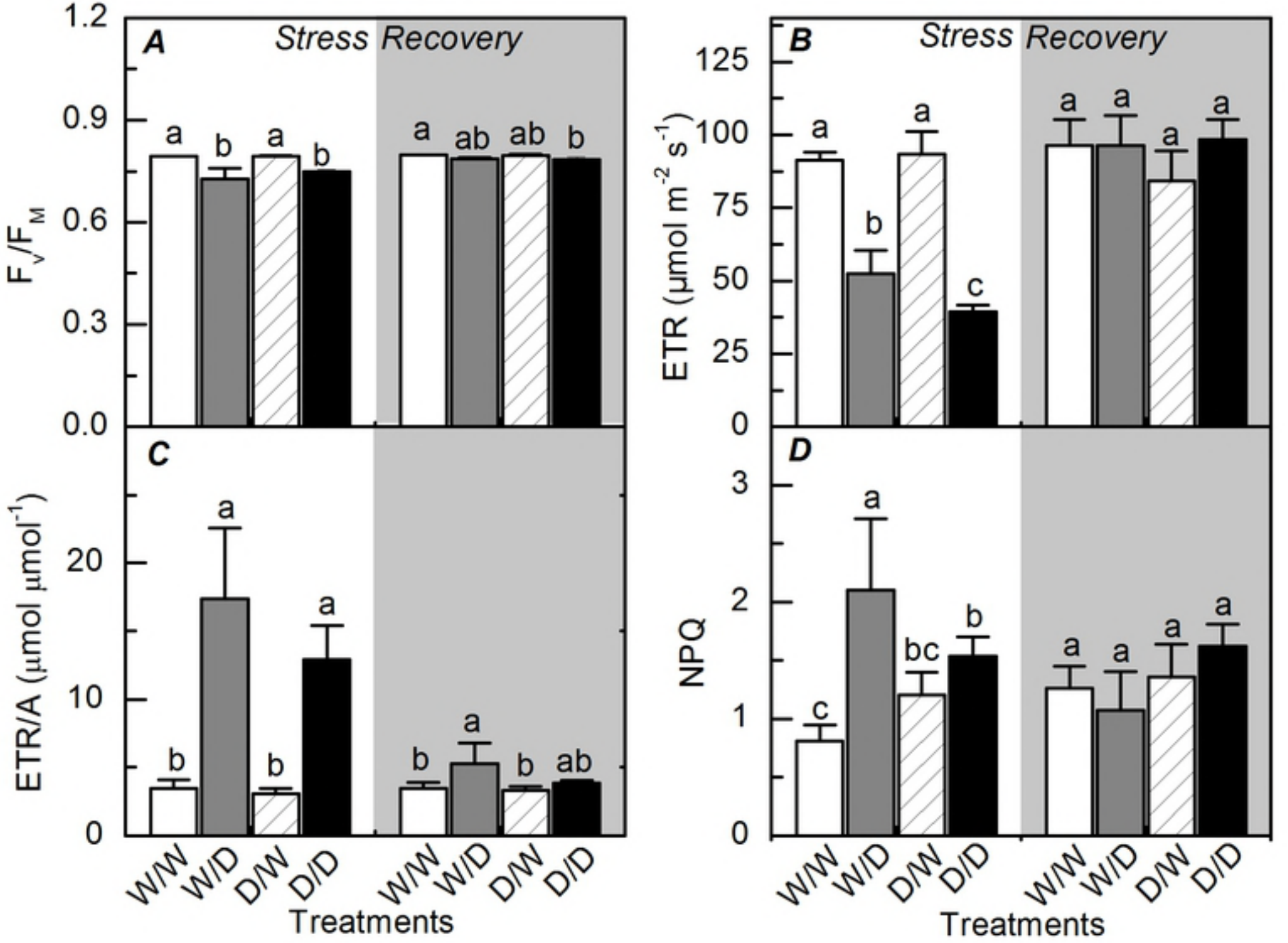
A
Stress
Recovery
F_v/F_M
1.2
0.9
0.6
0.3
0.0
a
b
a
b
a
ab
ab
b
B
ETR (µmol m^{-2} s^{-1})
125
100
75
50
25
0
C
ETR/A (µmol µmol^{-1})
20
10
0
D
NPQ
3
2
1
0
W/W
W/D
D/W
D/D
Treatments

Figure 4

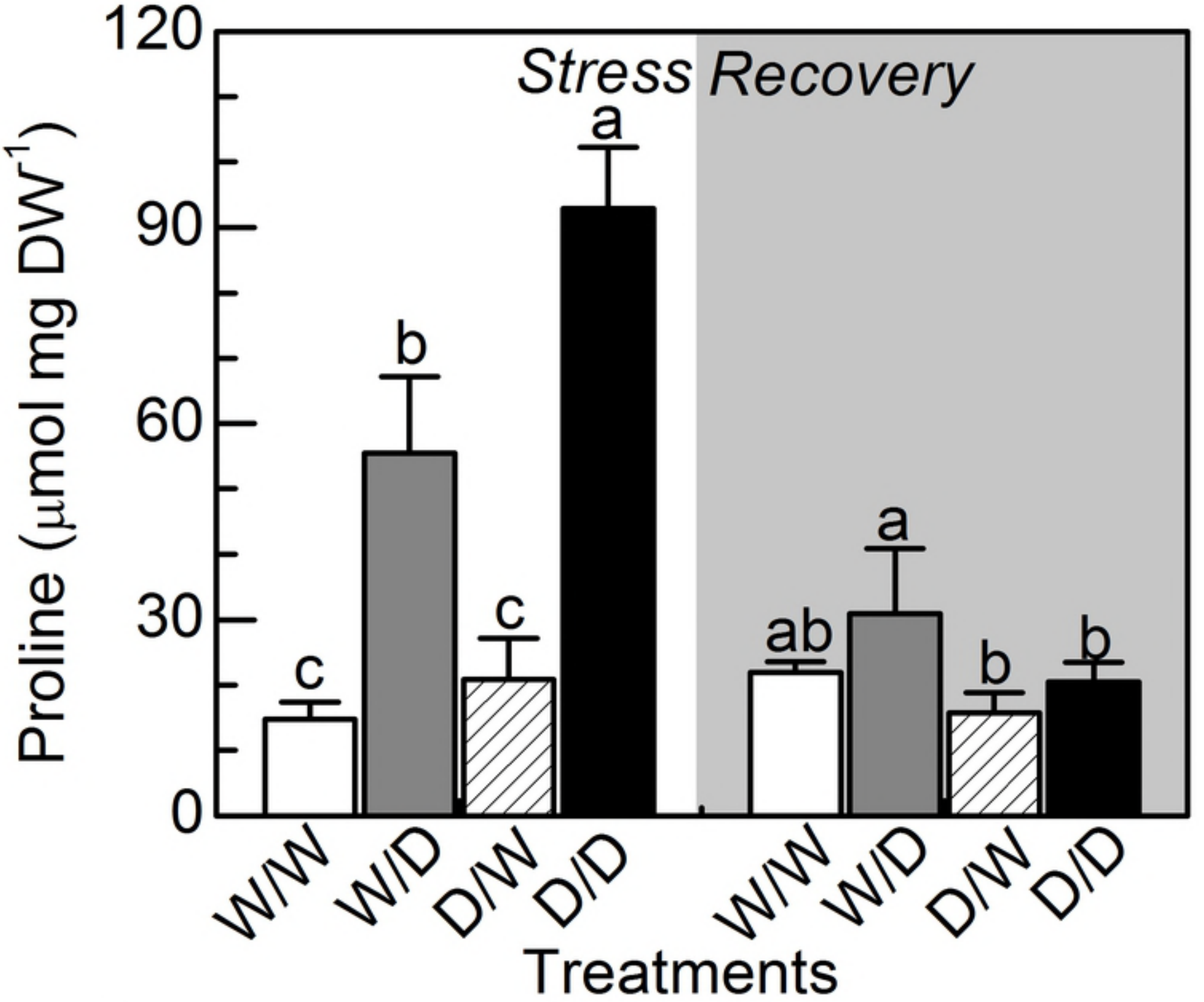
Stress
Recovery
Proline (µmol mg DW^{-1})
120
90
60
30
0
a
b
c
c
ab
a
b
b
W/W
W/D
D/W
D/D
W/W
W/D
D/W
D/D
Treatments

Figure 5

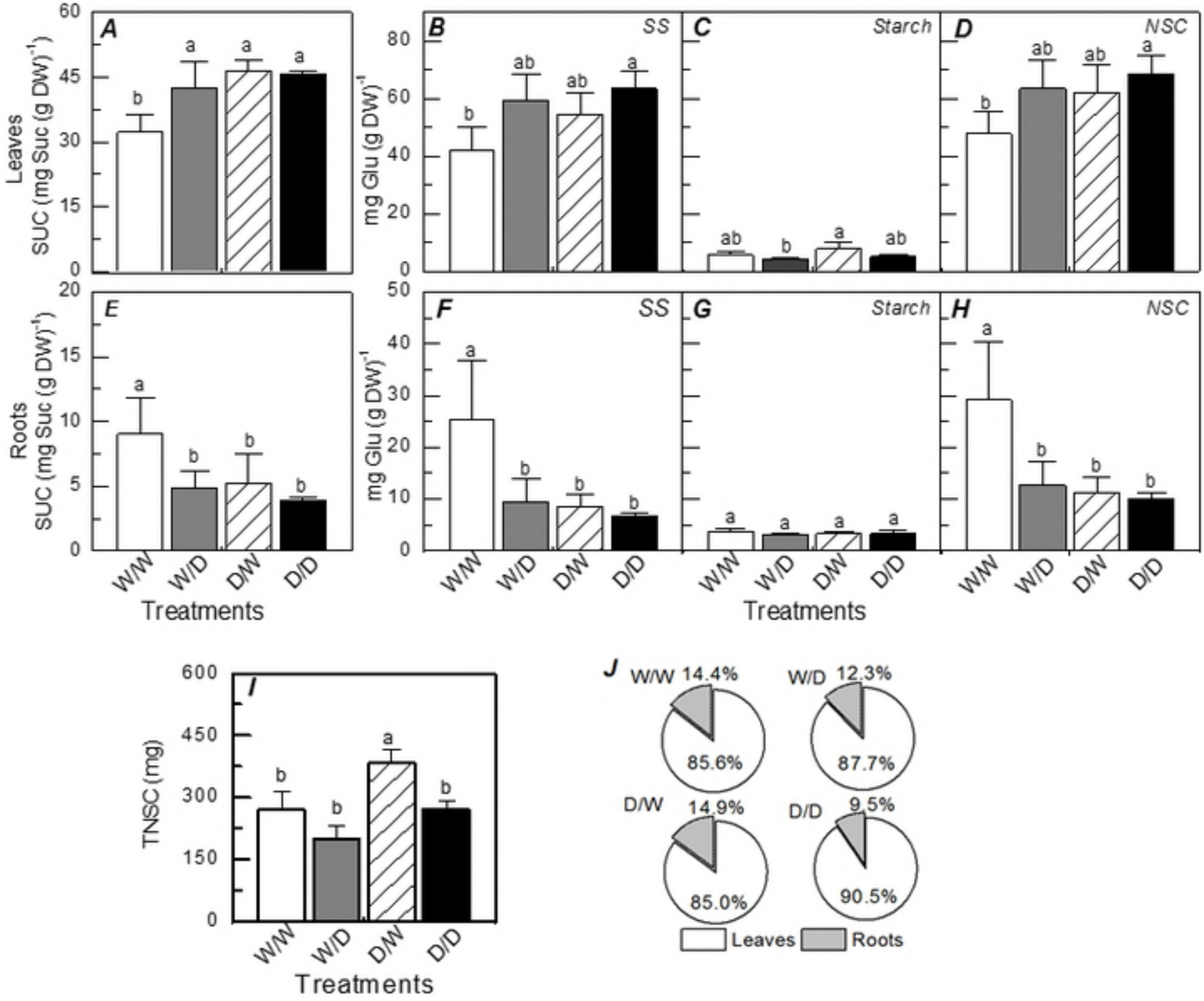
A
B
SS
C
Starch
D
NSC
Leaves
SUC (mg Suc (g DW)-1)
mg Glu (g DW)-1
E
F
G
H
Roots
Treatments
W/W
W/D
D/W
D/D
I
TNSC (mg)
J
W/W 14.4%
85.6%
W/D 12.3%
87.7%
D/W 14.9%
85.0%
D/D 9.5%
90.5%
Leaves
Roots

Figure 6

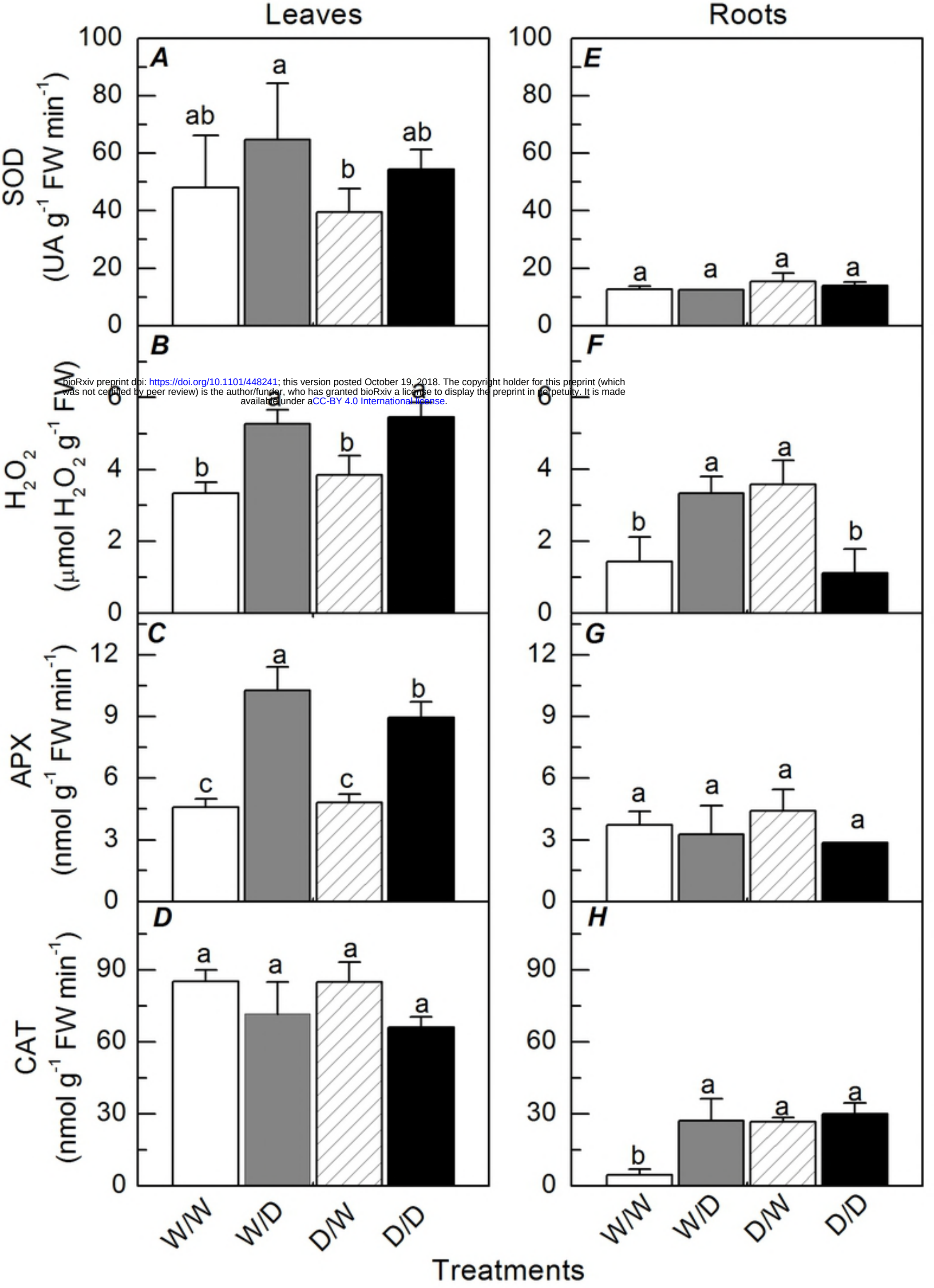

Figure 7

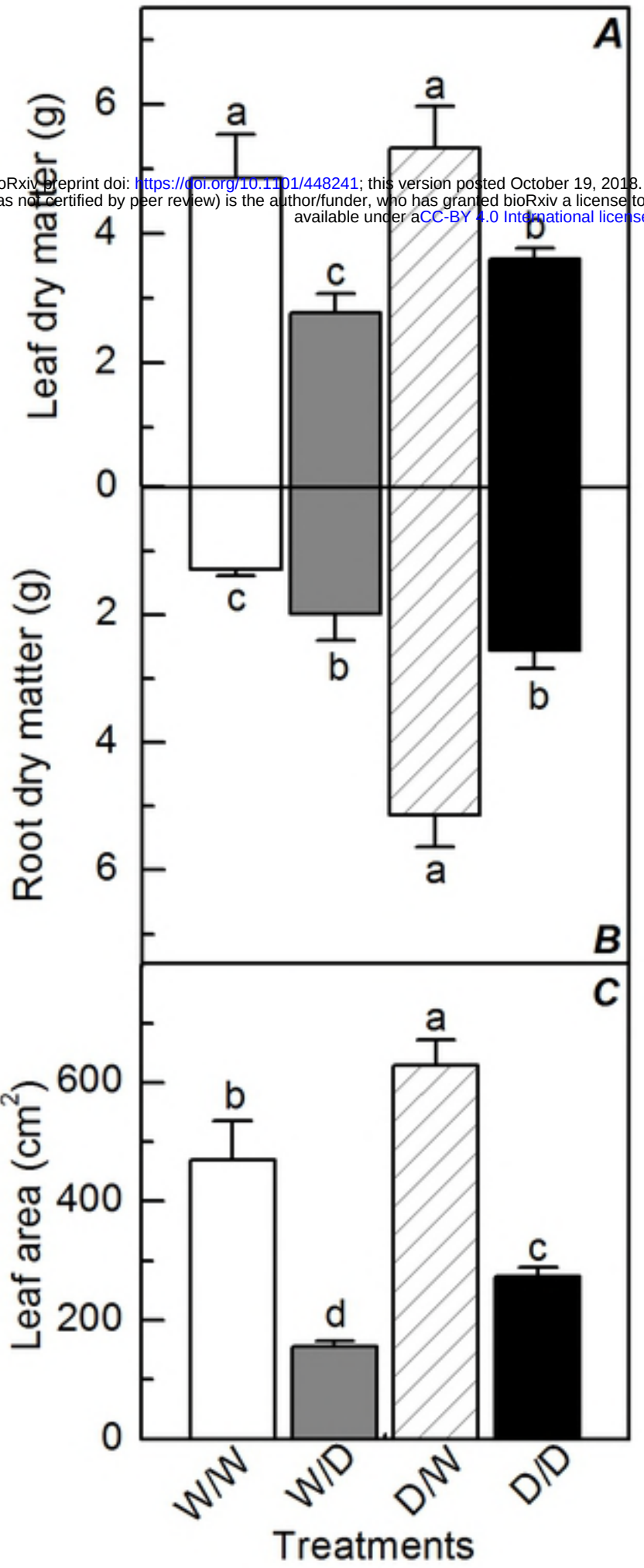

Figure 8