MORPHO-PHYSIOLOGICAL TRAITS ASSOCIATED WITH DROUGHT RESPONSES IN SOYBEAN (*Glycine max* L.)

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/csc2.20314.

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Received _____.

ABSTRACT

Drought is currently a major constraint to soybean production worldwide and is becoming more widespread due to increased aridity and warmer temperatures in the context of global climate change. In this context, breeding for soybean varieties more tolerant to drought stress is critical and requires efficient screening techniques. To find traits associated with drought-tolerance in the vegetative stage which are still present at the reproductive stage, we evaluated morphological, physiological, and biochemical traits, in two soybean genotypes contrasting in their response to drought stress. Under drought stress at the vegetative stage, the tolerant A 5009 RG genotype showed higher proline and chlorophyll contents, and early activation of the enzymatic antioxidant system compared with well-watered plants. On the other hand, the sensitive ADM 50048 genotype increased malondialdehyde (oxidative damage marker) and non-enzymatic antioxidant response under stress. Manipulative field assays under contrasting levels of water availability at the reproductive stage mimicked the biochemical patterns observed in the greenhouse tests for the sensitive and tolerant genotypes. A principal component analysis of parameters from vegetative and reproductive stages revealed proline and chlorophyll contents as drought-tolerance traits in soybean. We found those traits useful to classify 14 genotypes from the INTA germplasm bank, identifying two new drought-tolerant genotypes (PI548510 and PI200492). We propose proline and chlorophylls as a useful tool to classify soybean genotypes according to its drought responses in early developmental stages, potentially reducing breeding times.

KEYWORDS

Water deficit; Legumes; Abiotic stress; Proline; Chlorophylls

ABBREVIATIONS

a/b Chl, *a* and *b* chlorophylls; APX, ascorbate peroxidase activity; ASC, ascorbate; BIO, biomass; C, control; SC, control sensitive ADM 50048 genotype; CAT, catalase activity; D, drought-treated; DW, dry weight; F-FRAP, FRAP on field-grown plants; F-MDA, MDA on field-grown plants; F-Pro, proline on field-grown plants; FRAP, ferric reducing ability of plasma; FW, fresh weight; LA, individual leaf area; MDA, malondialdehyde; Pro, proline; RWC, relative water content; SOD, superoxide dismutase activity; SWC, soil water content; SD, drought-treated sensitive ADM 50048 genotype; TC, control tolerant A 5009 RG genotype; TD, drought-treated tolerant A 5009 RG genotype; U, units.

1. INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is the fifth grain crop considering yield (tones per hectare, Ritchie and Roser, 2013) and an important source of high-quality vegetable oil and protein in the world (Maestri et al., 1998). Given the health benefits associated with soybean products and its use in the livestock feed industry, the demand for soybean products is continuously increasing (Mwenye et al., 2016). Drought is the major constraint for soybean production worldwide (~40 % reduction, Specht et al., 1999; Pareek et al., 2020) since most soybeans are produced in rain-fed fields. Additionally, this situation is going to worsen as climate change simulations predict more frequent and intense drought events in the future (Godfray et al., 2010; Blum, 2011).

In this context, the development of new genetic drought-tolerant genotypes becomes critical for sustainable soybean production. Direct classification of soybean genotypes by

assessment of yield and other quantitative traits on the field is highly time-consuming and also unspecific due to weather variability (Mitra, 2001; Manavalan et al., 2009). A possible approach to improve the selection efficiency is to screen the genotypes in early developmental stages, by monitoring specific physiological traits associated with drought tolerance (Khan et al., 2016; Tommasino et al., 2018). Our main objective was to find drought-response traits that could be measured at vegetative stages and that could still be detected at reproductive stages. These traits will allow us to classify soybean materials according to different drought-responses in shorter times compared to full-season screenings.

Among several physiological traits associated with drought responses, the evaluation of osmotic and oxidative balances has been described as a potentially successful approach to identify drought tolerance (Gill and Tuteja, 2010; Singh et al., 2015; Osmolovskaya et al., 2018). Osmotic stress happens in plant tissues when the substrate water potential is reduced by a lack of water or an increase of solute concentration. Drought and other stress, trigger the accumulation of compatible solutes such as sugars and amino acids, which in turn allows keeping the water uptake and metabolic functions (Blum, 2011). The amino acid proline has protective roles such as stabilization of proteins, membranes, and subcellular structures, adjustment of the redox potential, and reduction of cell acidity (Hare and Cress, 1997; Fang and Xiong, 2015; Forlani et al., 2019). Proline is known to accumulate under drought in soybean tissues (Hossain et al., 2014; Tripathi et al., 2016; Buezo et al., 2019). That accumulation is associated with soybean's ability to tolerate such stress (Akitha Devi and Giridhar, 2015; Du et al., 2020), and it is a heritable trait (Esack et al., 2015; Hanson et al., 1979) which is useful to screen genotypes for drought tolerance.

On the other hand, oxidative stress is a massive redox alteration derived from primary stress such as drought, salinity, and chilling. In general, oxidative stress is a major imbalance between the generation and scavenging of reactive oxygen species (ROS). Under oxidative

stress, high levels of ROS are produced on several subcellular compartments damaging membranes, proteins, and nucleic acids (Miller et al., 2010). These reactive molecules cause lipid peroxidation increasing membrane permeability. This damaging process can be assessed in plant tissues by measuring malondialdehyde content (MDA) (Gill and Tuteja, 2010). Previous works have demonstrated that foliar MDA correlates with tolerance to saline and heat stress in genotypes of *Chloris gayana* (Luna et al., 2000) and *Cenchrus ciliaris* (Griffa et al., 2010; Lanza Castelli et al., 2010; Tommasino et al., 2012). MDA was also useful for selecting cross-tolerance to drought and heat stress in genotypes of *C. ciliaris* (Tommasino et al., 2018).

To cope with oxidative stress, plant tissues have complex enzymatic and non-enzymatic antioxidant systems (Mittler, 2017). The antioxidant enzymes include superoxide dismutase (SOD), peroxidases (POX), and catalase (CAT), while the non-enzymatic system includes compounds such as ascorbic acid, glutathione, and tocopherols. The Ferric Reducing Ability (FRAP) is often used to assess the non-enzymatic antioxidant capacity in plant tissues (Avramova et al., 2017). Several species (including soybean) with high antioxidant levels, either constitutive or induced, usually have greater tolerance to oxidative damage and therefore to drought stress (Rychter, 2006; Akitha Devi and Giridhar, 2015).

On the other hand, one critical consequence of drought stress is photosynthesis inhibition usually join by chlorophyll degradation, which happens in several species (Flexas et al., 2006). Chlorophyll degradation is a trait that has been used to classify maize genotypes under drought stress, associating a smaller reduction of chlorophyll content with drought tolerance (Khayatnezhad and Gholamin, 2012; Xiang et al., 2013).

To demonstrate that the characterization of soybean at the vegetative stage allows classifying genotypes accordingly to their tolerance, we selected two genotypes previously identified as sensitive (ADM 50048) and tolerant (A 5009 RG). These genotypes were

categorized as tolerant and sensitive based on their contrasting ability to maintain relative water content and growth under drought stress (Silvente et al., 2012). Additionally, the tolerant genotype has been the most planted in the semiarid region of Argentina for the last 12 years ("Informe Sistema de Informacion Simplificado Agricola," 2018).

Here, we characterized the response in the vegetative stage and selected specific traits related to growth, osmotic balance, oxidative stress, and antioxidative response. We validated the vegetative stage classification testing these materials in field conditions and evaluated these biochemical markers at reproductive stages. Among all evaluated traits, the most accurate and useful to detect drought-tolerance in these two genotypes were proline and chlorophyll contents. To test the validity of this method in a larger genotype pool, we tested those two traits, together with relative water content, to classify additionals 14 soybean genotypes accordingly to their drought response.

2. MATERIALS AND METHODS

2.1. Plant material and experimental treatments

We evaluated soybean (*Glycine max* L.) genotypes ADM 50048, A 5009 RG (formerly DM50048 and NA5009, respectively), A5409, A5417, A5520, Alim 3.44, Alim 5.9, Champaqui 5.7, DM 4800, Essex, Himeshirazu, J032998, PI 200492, PI 548510, PI 548558, and RAE 514 (Table S1) provided by the EEA Marcos Juarez soybean germplasm bank (INTA), Córdoba, Argentina. The genotypes ADM 50048 and A 5009 RG were selected as checks due to their contrasting response to water deficit stress (Silvente et al., 2012).

2.2. Greenhouse experiments

Seeds were germinated in plastic trays on sterile wet filter paper. After emergence, seedlings were transplanted to 0.5 L pots containing soil mixture (soil and sand proportion 1:1 v/v). 10

pots per genotype were grown under a 16:8 light: dark photoperiod, at 25 ± 5 °C, and 720 μmol m^{-2} s^{-1} maximum radiation intensity.

Soybean plants were fully watered until the first trifoliate leaf emerged (more than 2 cm length, day 0). At day 0, pots were watered until saturation and the next day were weighted to calculate maximum water holding capacity of the soil (100% water content). Water irrigation was withheld to progressively reduce the water content until it reached 30% (at day 7) and it was kept in that moisture level for one week (Fig. S1). To ensure all the plants were exposed to the same stress intensity and dry-down slope, the water content was monitored daily by pot weighing, adding the water lost every day.

2.3. Field experiment

The field experiment was planted at the spring of 2013 and harvested in the fall of 2014, at Campo Experimental Villarino, located in Zavalla, Santa Fe, Argentina (33°1′ S, 60°53′ W). Soil type was silty clay loam, Vertic Agiudoll, Roldan series. The drought stress treatment was imposing using an automatic rain-out shelter.

The growth conditions are fully described in Di Mauro et al. (2019). Briefly, this system prevented rainfall to enter the plots and therefore water availability was managed by drip-irrigation. The irrigated control was located 10 m from the rain-out shelter. Irrigation applied to the shelter environment and daily rainfall were recorded. Water availability (mm) was estimated as the sum of available soil water content at sowing and in-season precipitation (from sowing to physiological maturity), and irrigation (from sowing to physiological maturity). Total water availability for control treatment was 820 mm, and 540 mm for the drought stress condition. Between R3 and R7 well-watered and water-restricted sites showed a difference of 414 mm of accumulated water in the soil (50% of reduction) which in turn produced 38% of yield reduction in the water-stressed plants (average of the two genotypes).

Within each water level treatment, genotypes were arranged in a completely randomized block design with four replications. Plots were four rows, 0.35 m apart, and 3 m long. Final plant density was adjusted to 30 plants m^{-2} after manual thinning at V1 (Fehr and Caviness, 1977). Weeds were chemically controlled, and hand removed whenever necessary. Pests and diseases were controlled by spraying commercially recommended soybean products.

2.4. Sampling strategy

For greenhouse experiments, the second trifoliate leaf was measured and/or harvested at 0, 5, 7, and 14 days of treatment, for all measurements. For each sample time, 5 individual pots (individual replications) were harvested. For the 14 additional soybean genotypes, proline, chlorophylls, and relative water content (RWC) were evaluated on day 7, when the soil reached 30% of water content. For all the biochemical analyses, the leaves were flashed frozen into liquid N_2 and stored at -80 °C until further analysis.

For the field experiment, samples for biochemical determinations were taken from the second youngest completely expanded leaves at the R5 developmental stage. For each sample time, 10 leaves from different plants were harvested. Leaves per plot were combined and flashed frozen into liquid N_2 and stored at -80 °C until further analysis.

2.5. Individual leaf area, height, and total biomass

The individual leaf area (LA) was measured with a portable caliber. Leaf length (LL) and leaf width (LW) were measured daily and the next equation was used for calculations:

$$LA = \frac{LL}{2} \times \frac{LW}{2} \times 3.14$$

Height was measured with a portable ruler, from cotyledonal node to the apical meristem base. Total aerial fresh weight (FW) was measured at the final harvest. Then the same samples were dried at 65 ℃ for 72 h and weighted to determine the dry weight (DW).

2.6. Relative Water Content (RWC)

RWC was measured according to Barrs and Weatherley, (1962). Leaves were cut near noon and weighted (less than 5 min after the cut) to obtain fresh weight (FW). They were incubated in a dark humid chamber at 4 °C for 18 h, carefully dried and weighted (saturated weight, SW), and dried at 60 °C until constant weight (dry weight, DW). RWC was calculated as

$$RWC\ (\%) = \frac{FW - DW}{SW - DW} \times 100$$

At least 4 leaves were weighted for each sample time.

2.7. Proline

Proline was quantified using the ninhydrin assay (Bates et al., 1973). Grounded plant tissue was resuspended in 3 % (w/v) sulfosalicylic acid, which precipitated proteins, and centrifuged at 14000 *g* at 4 °C for 10 min. The colored reaction product was extracted with one volume (respect total reaction volume) toluene and the supernatant was quantified by spectrophotometry at 520 nm. The proline values were related to the L-proline (Sigma, USA) calibration curves.

2.8. Antioxidant determinations

Total superoxide dismutase activity (SOD; EC 1.15.1.1) was measured based on SOD ability to inhibit the nitro blue tetrazolium (NBT) reduction by superoxide radicals generated photochemically (van Rossum et al., 1997). The blue formazan produced by NBT photoreduction was measured as the increase in absorbance at 560 nm. One SOD unit was defined as the enzyme amount required to inhibit 50 % of the NBT photoreduction in comparison with tubes lacking the plant extract and expressed as enzyme activity units (U) g-1 FW min-1.

Ascorbate peroxidase activity (APX; EC 1.11.1.11) was measured in a 1 ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, and 0.5 mM

ascorbate. The reaction was started by the H_2O_2 addition and the decrease in absorbance at 290 nm was recorded for 1 min to determine the ascorbate oxidation rate (Amako et al., 1994).

Catalase (EC 1.16.1.6) activity was assayed using H_2O_2 as the substrate by following the decrease in absorbance at 240 nm, according to (Chance and Maehly, 1955) with minor modifications. The decrease in absorbance at 240 nm was measured for 2 min. H_2O_2 concentration was calculated using the absorbance extinction coefficient (43.6 mM^{-1} cm^{-1}). Catalase activity was calculated as H_2O_2 µmol consumed per min^{-1} per mg^{-1} and expressed as units (U) per mg of FW, being 1 U, the enzyme needed to consume 1 µmol of H_2O_2 in 1 min.

The Ferric Reducing Ability Potential (FRAP) assay was used to measure the total non-enzymatic antioxidant capacity (Benzie and Strain, 1996). FRAP was determined in 50 mg of fresh biomass, homogenized with 1 ml of 80 % v/v ethanol, and centrifuged at 14000 g for 10 min. Serial TROLOX (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Sigma, USA) dilutions were used for the calibration curve. FRAP content was expressed as TROLOX equivalent in µmol per

mg.

2.9. Lipid peroxidation

Membrane damage was assessed by measuring malondialdehyde (MDA) generation by lipid peroxidation according to (Hodges et al., 1999), with minor modifications. Approximately 50 mg leaf segments were homogenized with 1 ml 80 % v/v ethanol and centrifuged at 14000 *g* for 10 min. After centrifugation, the supernatant (0.25 ml) was mixed with 0.5 ml 0.65 % (w/v) thiobarbituric acid (TBA) in 20 % (w/v) trichloroacetic acid (TCA) and incubated in hot water (90 °C) for 20 min. Then, it was cooled immediately on an ice bath to stop the reaction and centrifuged at 14000 *g* for 10 min. Absorbance at 532 and 600 nm was determined, and MDA concentration was estimated by subtracting the non-specific

absorption at 600 nm from the absorption at 532 nm, using an absorbance extinction coefficient (155 mM^{-1} cm^{-1}). MDA content was expressed as nmol per mg.

2.10. Total chlorophylls

Total chlorophyll content was measured according to (Lichtenthaler, 1987). Briefly, grounded plant tissue was solubilized in 95 % v/v ethanol, heated 20 min at 80 °C, centrifuged 5 min at 5000 r.p.m. Pellet was extracted two more times with a final proportion of 1 ml ethanol to 10 mg fresh biomass. All supernatants were pulled and diluted 10 times in ethanol 95% v/v and measured at 649 and 664 nm. Total chlorophyll (*a* and *b*) was calculated according to the following formulas:

$$\text{chlorophyll } a \;=\; 13.36\, A_{664.2} - 5.19\, A_{648.6}$$

$$\text{chlorophyll } b \;=\; 27.43\, A_{648.6} - 8.12\, A_{664.2}$$

2.11. Yield and biomass

At maturity, 2 linear meters from the two central rows were hand clipped, bagged, and dried at 65°C in an air forced oven for obtaining dry total aboveground biomass (kg. ha^{-1}). After weighing, samples were threshed, and seeds were weighed to calculate seed yield (kg. ha^{-1}). The yield was reported on a dry basis.

2.12. Statistical Analyses

For the greenhouse experiment with the two main genotypes, a two-way ANOVA was conducted and a total of 7 treatments was defined as the combination of water availability and the day after the watering withholding onset. For the field experiments, a two-way ANOVA was conducted with water availability and genotype as the main factors. For the 14 genotypes (plus the checks) one-way ANOVA was used to check if there was variability in the responses among genotypes for Drought Sensitivity Index (DSI) ($p<0.001$, for the three parameters). In supplementary data, we expressed the data as the natural logarithm (*ln)* of the ratio tolerant/sensitive or stressed/control, and statistical analyses were carried out over the

raw data. Overall ANOVA with the post hoc Tukey's test was carried out with $p<0.05$ using Infostat software (Di Rienzo et al., 2011).

To explore associations between vegetative and reproductive traits, a principal component analysis (PCA) was performed combining the parameters from greenhouse and field experiments. For greenhouse measurement, we selected the sample time which showed the highest contrast between the tolerant and the sensitive for each parameter (RWC, proline, FRAP, MDA, APX, SOD at 5 days, Chl at 7 days, and the rest at 14 days). The results were visualized through a biplot graph constructed from the first and second principal components (PC 1 and PC 2) derived from the PCA (Ergo et al., 2018). We also compared only drought-responses for the check tolerant and sensitive genotypes, using the same sample times.

To compared the responses of the 14 soybean genotypes (plus the two tolerant and sensitive checks), we generated the Drought Stress Index (DSI, Liu et al., 2015) for each parameter in each genotype:

$$\mathrm{DSI} = \frac{\mathrm{drought}_{\,i}}{\mathrm{control}_{ave}}$$

being 'i' each soybean individual from each genotype under drought, and "ave" the control average for the well-water individuals from that genotype.

To classify the genotypes according to their drought response, we carried out a PCA with the DSI for each parameter (proline, chlorophylls, and RWC), and generated the ranking according to Liu et al. (2015), using the eigenvectors from the PC1 (84% of the variance), for each variable:

$$\mathrm{PC1}_{\,n} = (-0.87 \times \mathrm{RWC}_n) + (-0.92 \times \mathrm{Pro}_n) + (-0.94 \times \mathrm{Chl}_n)$$

Then, a numeric value was attributed to each ranked genotype ("Numeric Rank" in Table 1).

3. RESULTS

3.1 Relative water content at the vegetative stage

First, we studied drought effects in two soybean genotypes at the vegetative stage in greenhouse experiments. The soil water content was monitored daily and registered as a percentage of the maximum water holding capacity of the soil (Fig. S1). The relative water content (RWC) was measured in a drought-sensitive (ADM 500048) and a drought-tolerant (A 5009 RG) soybean genotypes. RWC in the sensitive plants was affected from day 7 of drought stress and it reached 60 % RWC of control leaves at 14 days (Fig. 1). On the contrary, in the tolerant plants, RWC was only slightly reduced at 5 days, then tissues recovered to maximum hydration levels.

3.2 Morphological traits at the vegetative stage: height, leaf area, and biomass

Morphological characterization included plant maximum height, individual leaf area (in the second trifoliate leaf), and total fresh- and dry-biomass (Fig. 2, Fig. S2). Under full irrigation, the sensitive genotype was longer and with larger individual leaf area than the tolerant one (Fig. 2A, B); however, water deficit shortened the sensitive genotype with respect to well-water control by almost 20 and 30 % (at 14 days, in height and leaf area, respectively), while tolerant genotype was not affected. Fresh and dry biomass (FW and DW, respectively) in well-watered plants were increasing with the age but similar between genotypes. Under drought stress, both FW and DW were reduced in the sensitive genotype (around 50 %) while they were only marginally reduced in the tolerant genotype (Fig. 2C, D).

3.3 Biochemical traits at the vegetative stage: proline, FRAP, MDA, antioxidant activities, and chlorophylls

Among the biochemical parameters, we measured proline, non-enzymatic antioxidant capacity (FRAP, ferric reducing activity), membrane damage marker (MDA) (Fig. 3, Fig S3), enzymatic antioxidant activities (Fig. 4, Fig S4), and chlorophylls (Fig. 5, Fig S3) in both

genotypes. Relative change ratios between genotype and between treatments are shown in the supplemental material (Fig. S3-4).

Proline content increased with drought stress severity in both genotypes, although the dynamic and intensity of those increments were different between genotypes. The tolerant genotype showed a higher concentration (1.7 times), earlier (at day 5 vs. day 14 in the sensitive), and more pronounced increment (5.1 vs. 3.4 times compared to well-watered controls) in proline content than the sensitive one (Fig. 3A). Total non-enzymatic antioxidant capacity measured as FRAP was increased in both sensitive and tolerant genotypes under drought stress, but the highest increase (more than 2-fold change compared to the control) was observed in the sensitive genotype at 5 and 7 days (Fig. 3B).

The oxidative damage, evaluated as MDA content, was increased in the sensitive genotype at all-time points under stress. In contrast, tolerant plants showed almost a constant and stress-insensitive MDA levels (Fig. 3C).

Among enzymatic antioxidant activities, superoxide dismutase activity (SOD_{act}) was slightly higher in the tolerant genotype (Fig. 4A). Catalase activity (CAT_{act}) was affected only at later times of stress, with a larger change in the tolerant genotype at 14 days (Fig. 4B). Ascorbate peroxidase activity (APX_{act}) in the tolerant genotype was overall higher than the sensitive genotype (40 to 70% higher), in both well-watered and water-restricted conditions (Fig. 4C).

Well-watered plants showed slightly higher chlorophyll content in the sensitive genotype. Surprisingly, drought stress induced an increase in chlorophylls in both genotypes (Fig. 5, Fig. S3). In the sensitive plants, chlorophylls (*a* and *b,* Fig. 5A) were slightly increased at day 5 of water restriction, and only chlorophyll *a* was increased at 14 days with respect to the irrigated control (Fig. 5B). In the tolerant genotype, both chlorophylls (*a* and *b*)

were moderately increased at day 5 and highly increased at day 7 (more than 2-fold over their control).

3.4 Reproductive stage traits: proline, FRAP, MDA, biomass, and yield

To confirm the results obtained in the greenhouse, we carried out an on-field experiment (spring 2013 to fall 2014) imposing moderate drought stress during the whole soybean crop cycle. Controls were kept in well-watered conditions (rain-fed, Fig. S5). As biochemical parameters, we evaluated proline, FRAP, and MDA at the seed filling stage (R5), in leaves completely expanded under stress, equivalent to the ones evaluated in our greenhouse experiments (Fig. 6; Fig. S4). Proline increased in the tolerant genotype almost 40 % in response to water restriction (Fig. 6A). Total non-enzymatic antioxidant capacity and MDA only increased in the sensitive genotype under drought stress, with no changes in the tolerant genotype (Fig. 6B, C). Overall, the biochemical parameters measured at the reproductive stages showed similar responses under drought to those measured at the vegetative stage in the greenhouse.

We also evaluated the total biomass and yield at the end of the soybean crop cycle (harvest). Under the rain-fed condition, the sensitive genotype showed lower dry biomass and yield than the tolerant genotype (Fig. 7) and drought stress reduced total dry biomass and yield in both genotypes. Overall, the tolerant genotype showed higher biomass and grain yield in both under rain-fed and rain-out conditions.

3.5 Principal Component Analysis

To visualize the relationships among relevant variables in the vegetative and reproductive stages, we applied the Principal Component Analysis (PCA) (Fig. 8). We selected the times showing the highest contrast between genotypes for each parameter at the vegetative stage and all the parameters measured at the reproductive stage. The analysis revealed the data structure according to their variance, expressed as eigenvector values. The first two principal

components (PC 1 and PC 2) explained 77.7 % of the total variability in the data. The PCA allowed us to separate soybean genotypes and treatments. The greatest separation was found between well-watered and water-stressed plants in PC1 (49.2 %). Additionally, acute and obtuse vector angles indicate positive and negative correlation, respectively, whereas right angles denote no correlation between traits for that component. The PC1 revealed treatment differences (control [C] vs drought [D]) and showed a positive association of proline, chlorophylls, and antioxidant activities (SOD, CAT, and APX) with drought-treated plots. On the contrary, LA, FW, DW, and RWC (vegetative stage), and biomass and yield (reproductive stage) were negatively associated with drought tolerance, being these vectors oriented towards control plots. The PC2 revealed genotype differences under drought (SD vs TD) and allows identifying parameters associated with drought tolerance: RWC, APX_{act}, and proline (vegetative and reproductive stages) were positively associated with drought tolerance since these vectors were orientated to the drought-treated tolerant genotype. On the contrary, MDA and FRAP (vegetative and reproductive stages) were negatively associated with tolerance since these vectors are orientated towards the sensitive genotype under drought. Additionally, to confirm the selected traits were able to separate the genotypes under drought, we carried out a second PCA with the drought-stressed measurements only (Fig. S6). The first two principal components (PC 1 and PC 2) explained 85.6 % of the total variability and the PC1 explain the separation between genotypes (72%), and, overall, the associations among traits and drought responses were similar to the previous PCA.

3.6 Using proline and chlorophylls to classify soybean genotypes at the vegetative stage

To validate the selected parameters, we evaluated additional 14 genotypes from the soybean germplasm bank (INTA, Argentina), together with the two tolerant and sensitive genotypes (previously characterized here), as the respective checks (Table 1).

Plants were exposed to drought stress in early stages of development in the greenhouse, and proline and chlorophylls, together with RWC (as a direct water deficit response) were monitored at day 7. To compare the responses among genotypes, we calculated the Drought Sensitivity Index (DSI, Liu et al., 2015) for each parameter (Fig. 9).

Then we used the DSI to carry out a PCA (Fig. S7). PC1 and PC2 explained 84 and 11 % of the variance, respectively. We classified the genotypes by a drought-response ranking using the eigenvectors and PC1 contribution to weight each parameter in all the soybean genotypes (Liu et al., 2015). Two genotypes were identified as more tolerant than the tolerant check (A5009RG), and 7 genotypes were classified as more sensitive than the sensitive check (ADM50048) (Table 1). In figure 9 the genotypes are shown according to the mentioned ranking.

4 DISCUSSION

Drought stress affects crop productivity by the reduction of plant growth and yield. Breeding crops to develop new drought-tolerant genotypes is a critical demand from agriculture. However, choosing the experimental system and growth conditions to classify the genotypes and find new traits is a key step for success. Plant breeders usually classify genotypes on the field since that is the condition where the crops grow, but it is not stress-specific and very time-consuming since it implies to wait for the whole cycle of the plant. On the contrary, studying plants on the field is only moderately useful for plant physiologists due to the exposition of the plants to several other environmental factors, in addition to the stress under study. So, for the physiologists using only field conditions hinders the elucidation of drought-specific responses. Then, more controlled conditions (as chambers and greenhouses) are useful to deeper understand the drought-tolerance physiology and to find new specific traits using less time and funds. Altogether, those limitations highlight the importance to

complement both approaches to increase the knowledge of drought-tolerance physiology and to develop new tolerant genotypes. Additionally, it is important to assure the same stress is imposed on all the genotypes, assuming they could have different water usage. One possible approach is to grow individuals of different genotypes combined in the same container, randomly distributed (Osmolovskaya et al., 2018). But, as we evaluated 16 genotypes, that was not viable, and we decided to monitor and adjust the soil water content every day, which was more time-consuming but allowed us to evaluate more genotypes at the same time.

Here we deeper characterized two genotypes previously proposed as tolerant and sensitive (Silvente et al., 2012). The most robust and useful traits we found associated with drought-tolerance were proline and chlorophyll contents (Fig. 3, 5, 8, S3, S6). Traits assessing enzymatic and non-enzymatic antioxidant capacities (Fig. 3, 4, 8, S3, S4, S6) showed less conclusive trends, although membrane damage (assessed as MDA content) can be useful to detect sensitivity to drought stress. We showed the classification of soybean genotypes using proline and chlorophylls traits at the vegetative stages is possible and effective. Also, proline and MDA responses still manifested when the stress occurred at the reproductive stage (R5), critical for yield (Fig. 6, S4).

Morphological and physiological parameters showed differential responses under drought stress, as expected. Overall morphological parameters showed the sensitive genotype was more affected by drought, while the tolerant genotype exhibited only minor reductions, in agreement with previous reports for these two genotypes (Fig. 1, 2; Silvente et al., 2012). It is important to mention that Silvente et al. (2012) did not find a significant increase in proline or chlorophylls but the plants were exposed to mild water stress (RWC of 85% in the sensitive genotype under stress). Consistently with a minor stress intensity, their responses were not opposite but more attenuated than ours, since they found a marginal increase in foliar proline in the tolerant genotype, suggesting the intensity of drought they imposed may

not induce the changes reported here. On the other hand, morphological traits evaluated in the greenhouse were consistent with on-field measurements: higher biomass productivity and grain yield were observed in the tolerant genotype under drought (Fig. 7), however, biochemical parameters seem to be only partially responsible for the yield differences. These results highlight the importance to evaluate other responses, such as those related to stomatal conductance and carbon assimilation to better understand the physiological base of the drought-response in the field.

Among physiological traits, the RWC (assessing water balance) was not affected by drought in the tolerant genotype (Fig. 1). Consistently, proline content (related to osmotic balance) increased (from earlier and to a higher level) in the tolerant genotype, both at the vegetative and reproductive stages (Fig. 4, 6, S3, S4). These results suggest proline accumulation contributes to maintaining the water balance under stress in the tolerant genotype. Similarly, higher proline content under drought was shown in roots and leaves of other soybean genotypes at vegetative and reproductive stages (Angra et al., 2010; Hossain et al., 2014; Akitha Devi and Giridhar, 2015; Tripathi et al., 2016). In agreement, studies in transgenic soybeans (overexpressing different transcription factors which increased drought tolerance), showed transcriptional activation of proline synthesis limiting step (*P5CS* gene) and a higher water use efficiency (de Paiva Rolla et al., 2014; Fuganti-Pagliarini et al., 2017). Altogether those results support the regulation of proline metabolism as a drought-tolerance response in soybean. Nonetheless, in other sensitive and tolerant soybean genotypes, Hossain et al. (2014) found similar proline levels under stress. The authors attributed drought-tolerance to the better performance of photosynthesis, highlighting that drought-tolerance responses are not always associated with an increase of proline, and other mechanisms, independent of proline synthesis, could be causing the tolerance. Yet, when present, proline

accumulation is indicative of drought tolerance, and as we supported here, it as a useful trait for the identification of drought tolerance in soybean genotypes.

Among the traits related to the redox balance, the enzymatic antioxidant response (SOD, CAT, and APX activities) was induced faster and with no changes in lipid peroxidation (assessed as MDA content) in the tolerant genotype at the vegetative stage (Fig. 3, 4). These results indicate the earlier antioxidant response in the tolerant genotype might contribute to its drought tolerance and reduce oxidative membrane damage in the stressed tissues. In contrast, in the sensitive plants, higher membrane damage was observed, suggesting the early increase in the non-enzymatic antioxidant capacity and the late activation of the enzymatic antioxidant system were not sufficient to overcome oxidative stress in the sensitive genotype, reducing its tolerance. Then, although the antioxidant traits showed an overall consistent trend, associating a faster response with drought tolerance, we do not recommend using the antioxidant activities individually since it may have low accuracy for classifying soybean genotypes. However, the measurement of MDA to identify the sensitive ones could be useful to select tolerant genotypes caused by unknown mechanisms (showing lower levels of MDA), which will not be identified using proline and chlorophylls.

Otherwise, since photosynthesis is usually inhibited and chlorophylls degraded under drought, the most unexpected result was the increase of chlorophyll (*a* and *b*) content in both genotypes under stress (Fig. 5). Also, and this response was associated with drought tolerance (Fig. 8). Similar responses (the increase in chlorophylls plus the photosynthesis inhibition) were previously described in maize and wheat leaves under drought (Zaefyzadeh et al., 2009; Xiang et al., 2013; Avramova et al., 2015). Additionally, chlorophylls retention maximizes photosynthesis recovery when water and, consequently, CO_2 are restored (Avramova et al., 2015). Then, the higher increase of chlorophyll observed in the tolerant soybean genotype (A 5009 RG) suggests this genotype may have a better performance also on recovery stages,

although this hypothesis must be challenged in soybean. Overall, since chlorophylls increased more in the tolerant genotype A 5009 RG and this trait was associated with drought tolerance by the PCA, we proposed the assessment of chlorophyll content under drought as a relevant trait for drought tolerance screening in soybean genotypes.

Furthermore, analyzing the data on the light of physiological relevance for drought tolerance, we found two interesting associations with proline accumulation: the antioxidant system activation and the chlorophyll accumulation. First, we observed the simultaneous accumulation of proline with the faster antioxidant system activation in the drought-tolerant genotype (Fig. 3, 4, 6). The endogenous accumulation of proline was associated with the activation of the antioxidant system and reduction of membrane damage in other drought-tolerant soybean genotypes (Angra et al., 2010; Masoumi et al., 2011; Akitha Devi and Giridhar, 2015; Iqbal et al., 2019) and transgenics (De Ronde et al., 2004; Ning et al., 2017; Wang et al., 2017) (Table 2). Also, the manipulation of proline content (by exogenous supply or transgenesis) consistently affects antioxidant activity and/or protects from membrane damage. As representative examples, there is two different kinds of evidence reported in other species under drought stress which also support that proline protection is mediated by changes in the redox balance (Table 2): *a*) the higher endogenous proline accumulation differentially increased SOD, CAT, and APX activities in transgenics of *Citrus paradise* (de Carvalho et al., 2013); and *b*) the exogenous proline application protected from peroxidative damage in Arabidopsis (Moustakas et al., 2011). Second, the accumulation of proline was associated with the protection of the photosynthetic machinery in soybean (Table 2), and with the retention of chlorophylls in maize under water deficit (caused by PEG; Altuntaş et al., 2020). Here, we showed the co-occurrence of both proline and chlorophyll accumulation (Fig. 3, 5, and 9) and a high correlation (Pearson =0.69 Fig. S7b) in the 16 classified genotypes. The positive association of both traits suggests that proline may act as chlorophyll

protector also in soybean, although that assumption must be further study. Also, this protective effect could be mediated by the antioxidant system activation previously discussed. For example, SOD activity was associated with chlorophyll protection in a tolerant wheat genotype under drought stress (Zaefyzadeh et al., 2009). Altogether these results support a protective role of proline metabolism activating the antioxidant system and protecting chlorophylls from degradation, directly or indirectly, under drought stress.

Among the traits we have measured at the vegetative stage, proline and chlorophylls were the most accurate to identify the tolerant genotype. However, we highlight the advantage to continue seeking other traits including different organizational levels which will make it possible to identify tolerant genotypes with a different physiological base. It is also important to consider that the method to measure proline is cheap but time-consuming, while chlorophyll measurement is not only cheap but also fast to carry out. However, the main advantage we propose for this strategy is the possibility to measure the traits in an early plant developmental stage which allows us to do several experiments per year, potentially reducing breeding times.

5. CONCLUSION

We propose proline and chlorophyll contents as useful biochemical traits to be measured at vegetative stages to screen for drought tolerant. MDA could be monitored as a sensitivity trait. Two new drought-tolerant genotypes (PI548510 and PI200492) were identified using this strategy and the utilization of those traits at vegetative stages could contribute to accelerating breeding for drought tolerance in soybean.

We are currently classifying another 90 soybean genotypes from the INTA Marcos Juárez germplasm bank and seeking new traits to be incorporated into our approach.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

CONTRIBUTION

MCG developed methodology, performed conceptualization, (lead) investigation, visualization, and writing the paper; CC conducted investigation, MSS conducted investigation; JLR performed conceptualization, field investigation, and writing the manuscript; MIM performed conceptualization, investigation, visualization, and lead writing the paper; CML developed methodology, performed conceptualization, supervision, funding acquisition, and writing the paper.

ACKNOWLEDGMENTS

Authors thank Javier Gilli for providing the seeds; Daniela Gomez, Yohana Ruartes, Carlos Torres, and René Vittar for technical support at laboratory and greenhouse; Dannae Serri for technical support about INFOSTAT software, M. Veronica Bianco for assistance with graphics edition, and Agustin Carbajal and Veronica Beligni for manuscript critical reading. Funding from MINCYT (PICT 2012-0339) and INTA (PNAGUA-1133032 and PNCYO-1127045) are gratefully acknowledged. The National Council of Scientific and Technical Research (CONICET) awarded the scholarship to M. S. S. and M. I. M.

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FIGURE CAPTIONS

Fig. 1. The relative water content of soybean leaves under drought stress at the vegetative stage. Drought stress was generated by dry-down after irrigation withholding on day 14 after emergence. Relative water content (RWC) of sensitive (ADM 50048) and tolerant (A 5009 RG) genotypes leaves were evaluated. Values in panel B and C are mean ± standard error from 3 independent experiments (n=15). Different letters indicate significant differences (ANOVA, $p<0.05$).

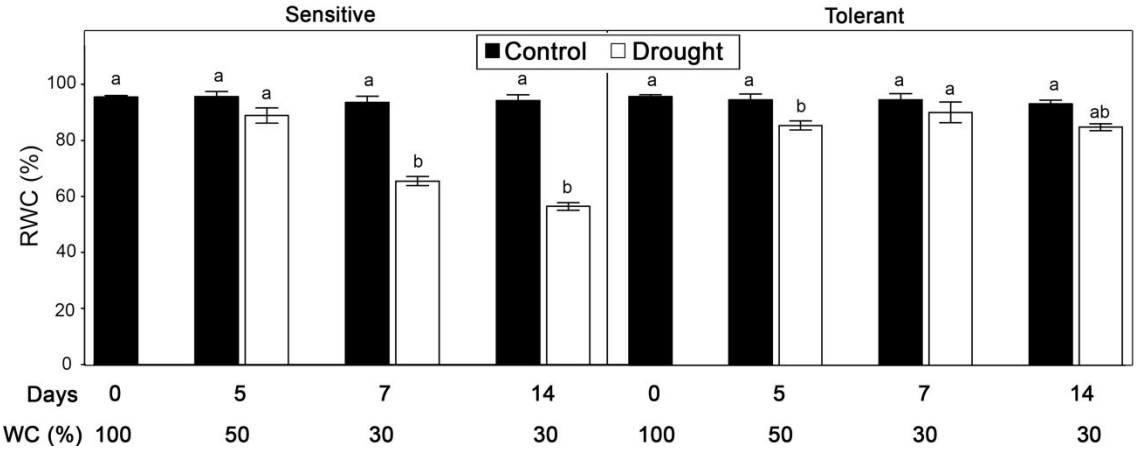

Fig. 2. Height (A), leaf area (B), fresh (C), and dry weights (D) of soybean genotypes under drought stress at the vegetative stage. Sensitive (ADM 50048) and tolerant (A 5009 RG) genotypes were evaluated. Drought stress was generated by irrigation suspension and gravimetric monitoring of pots every day. Values are the mean ± standard error, from 3 independent experiments. In each experiment, 5 plants were sampled per genotype per treatment. Different letters indicate significant differences (ANOVA, $p<0,05$). DW, dry weight; FW, fresh weight.

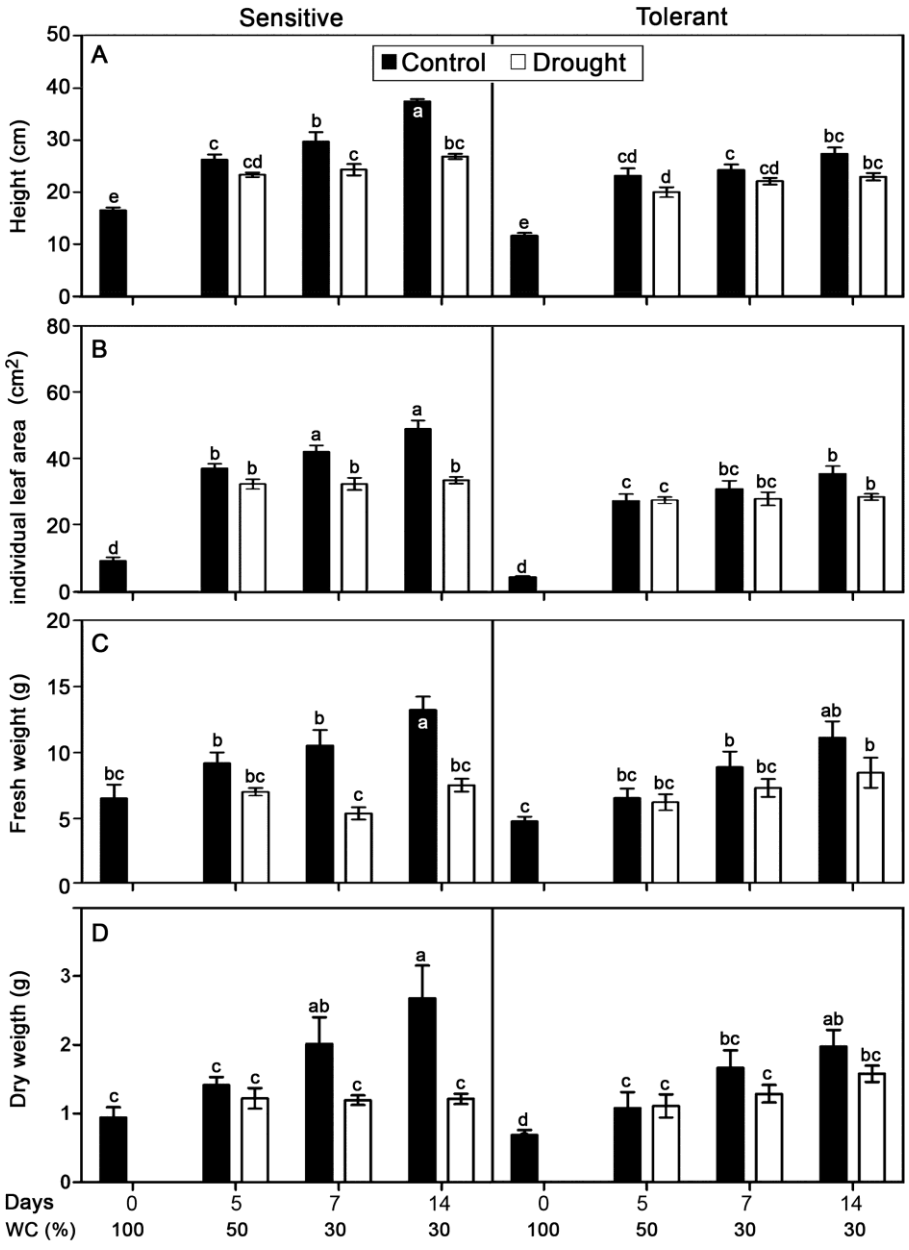

Fig. 3. Proline (A), total non-enzymatic antioxidant capacity (FRAP) (B), lipid peroxidation (as MDA content) (C) of soybean genotypes under drought stress at the vegetative stage. Sensitive (ADM 50048) and tolerant (A 5009 RG) genotypes were evaluated. Drought stress was generated by dry-down after irrigation withholding on day 14 after emergence. Gravimetric water was monitored daily. Values are mean ± standard error from 3 independent experiments (n=15). Different letters indicate significant differences (ANOVA, $p<0.05$). FRAP, ferric reducing ability; FW, fresh weight; MDA, malondialdehyde.

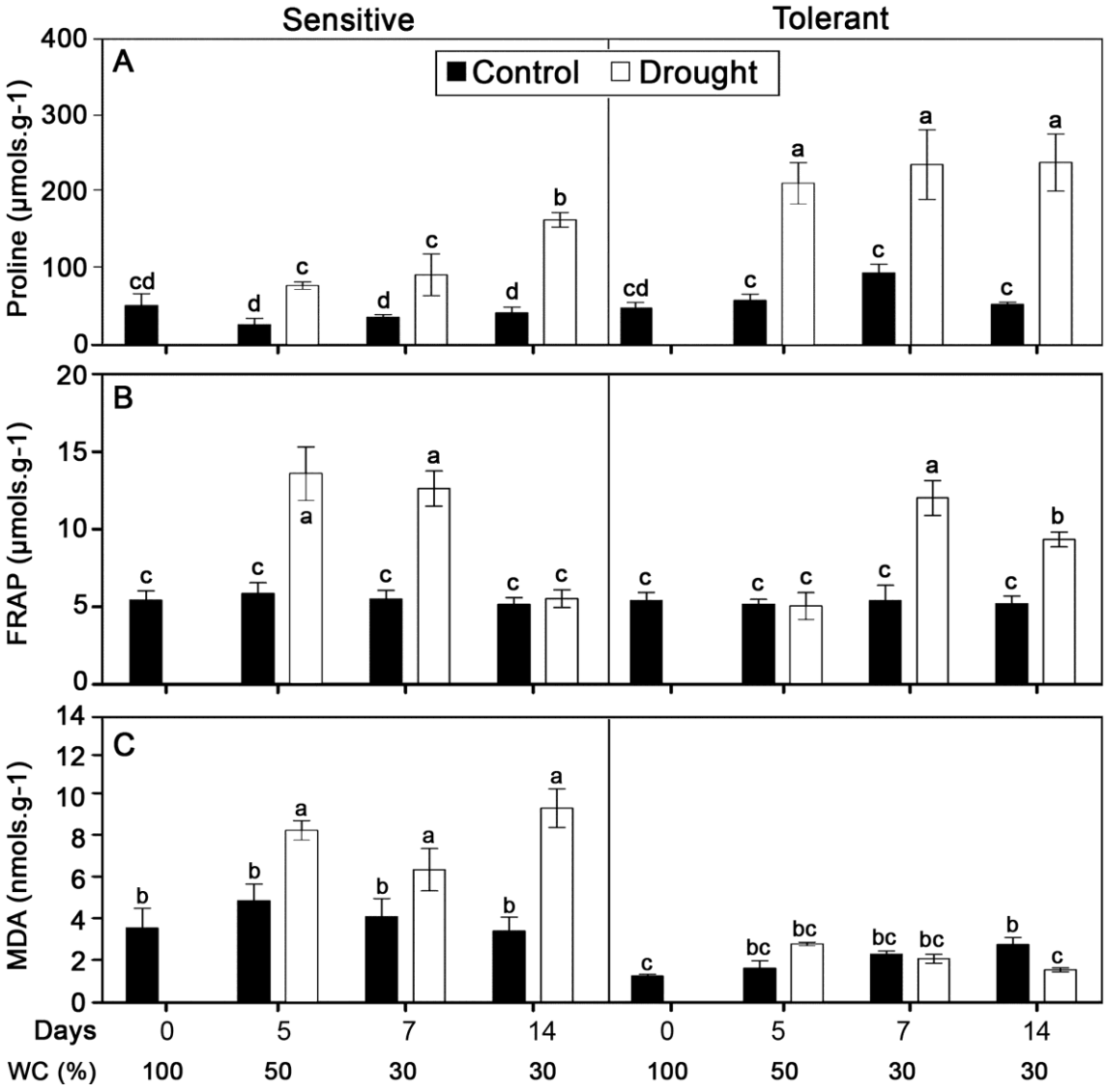

Fig. 4. Superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) activities of soybean genotypes under drought stress at the vegetative stage. Sensitive (ADM 50048) and tolerant (A 5009 RG) genotypes were evaluated. Drought stress was generated by dry-down after irrigation withholding on day 14 after emergence. Gravimetric water (% SWC) was monitored daily. Values are mean ± standard error from 3 independent experiments (n=15). Different letters indicate significant differences (ANOVA, $p<0.05$). ASC, ascorbate; APX, ascorbate peroxidase activity; CAT, catalase activity; FW, fresh weight; SOD, superoxide dismutase activity; U, units.

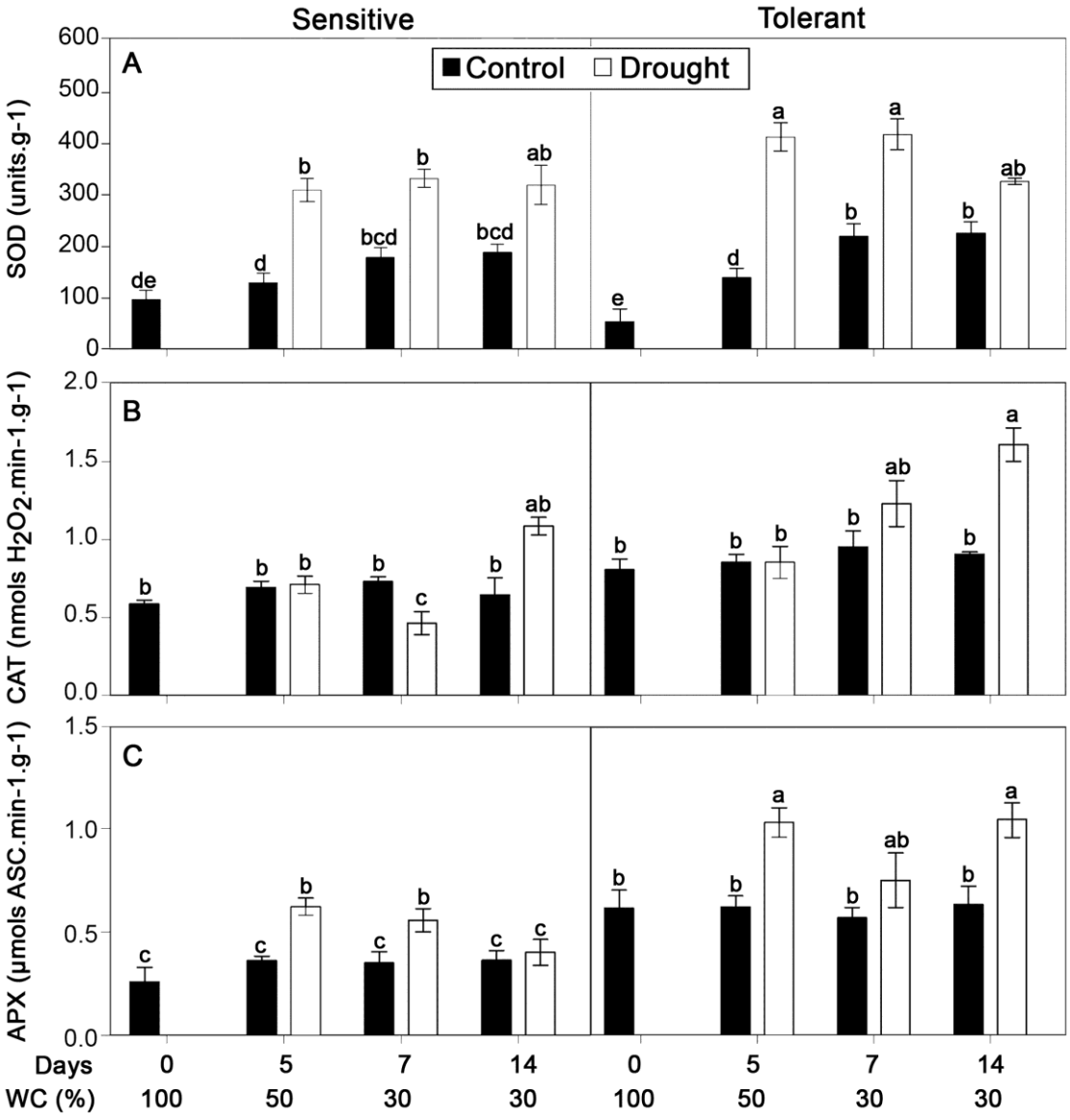

Fig. 5. Chlorophyll *a* (A) and *b* (B) contents of soybean genotypes under drought stress at the vegetative stage. Sensitive (ADM 50048) and tolerant (A 5009 RG) genotypes were evaluated. Drought stress was generated by dry-down after irrigation withholding on day 14 after emergence. Gravimetric water was monitored daily. Values are mean ± standard error from 3 independent experiments (n=15). Different letters indicate significant differences (ANOVA, $p<0.05$). FW, fresh weight.

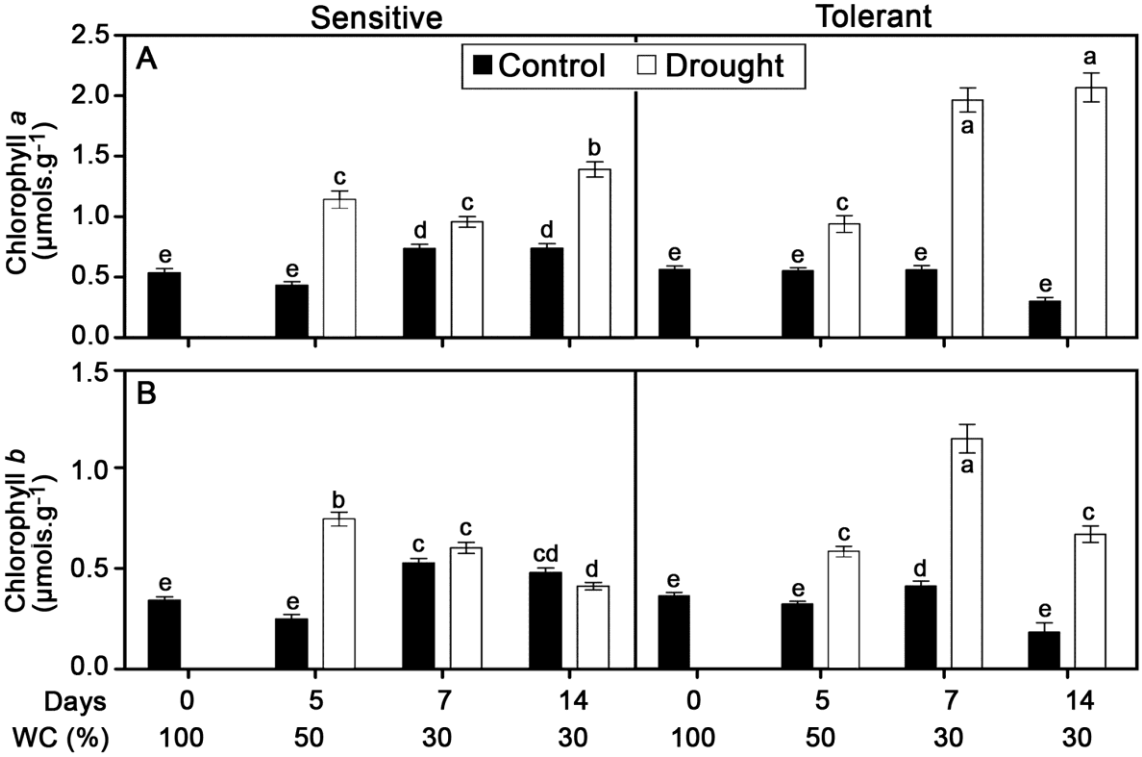

Fig. 6. Proline (A), total non-enzymatic antioxidant capacity (FRAP) (B), lipid peroxidation (as MDA content) (C) of soybean genotypes under drought stress at the reproductive stage. Sensitive (ADM 50048) and tolerant (A 5009 RG) genotypes were evaluated. The drought was imposed by a rainout shelter. Values are the mean ± standard error (n=3). Different letters indicate significant differences (ANOVA, $p<0.05$). C, control; D, drought stress; FRAP, ferric reducing ability; FW, fresh weight; MDA, malondialdehyde.

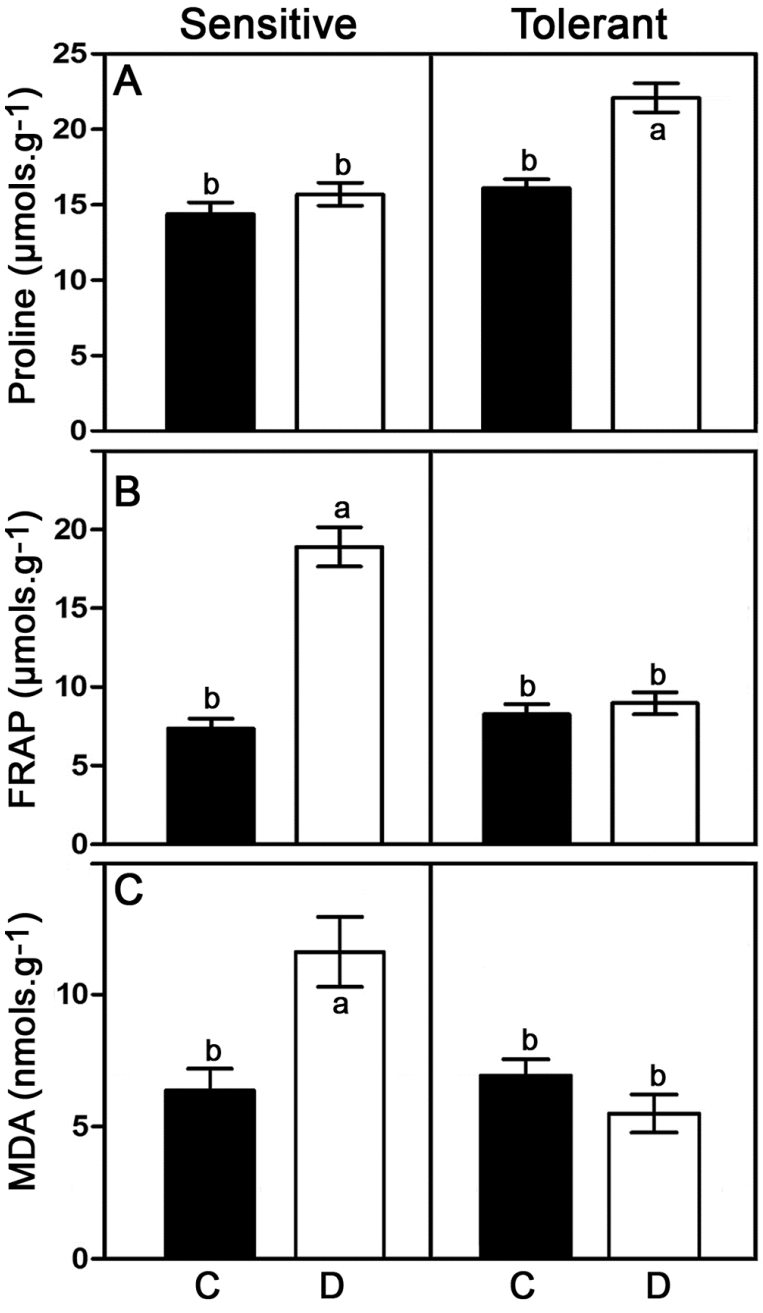

Fig. 7. Yield (A), and total biomass (B) of soybean genotypes under drought stress at the reproductive stage. Sensitive (ADM 50048) and tolerant (A 5009 RG) genotypes were evaluated. The drought was imposed by a rainout shelter. Values are the mean ± standard error (n=3). Different letters indicate significant differences (ANOVA, $p<0.05$). C, control; D, drought stress.

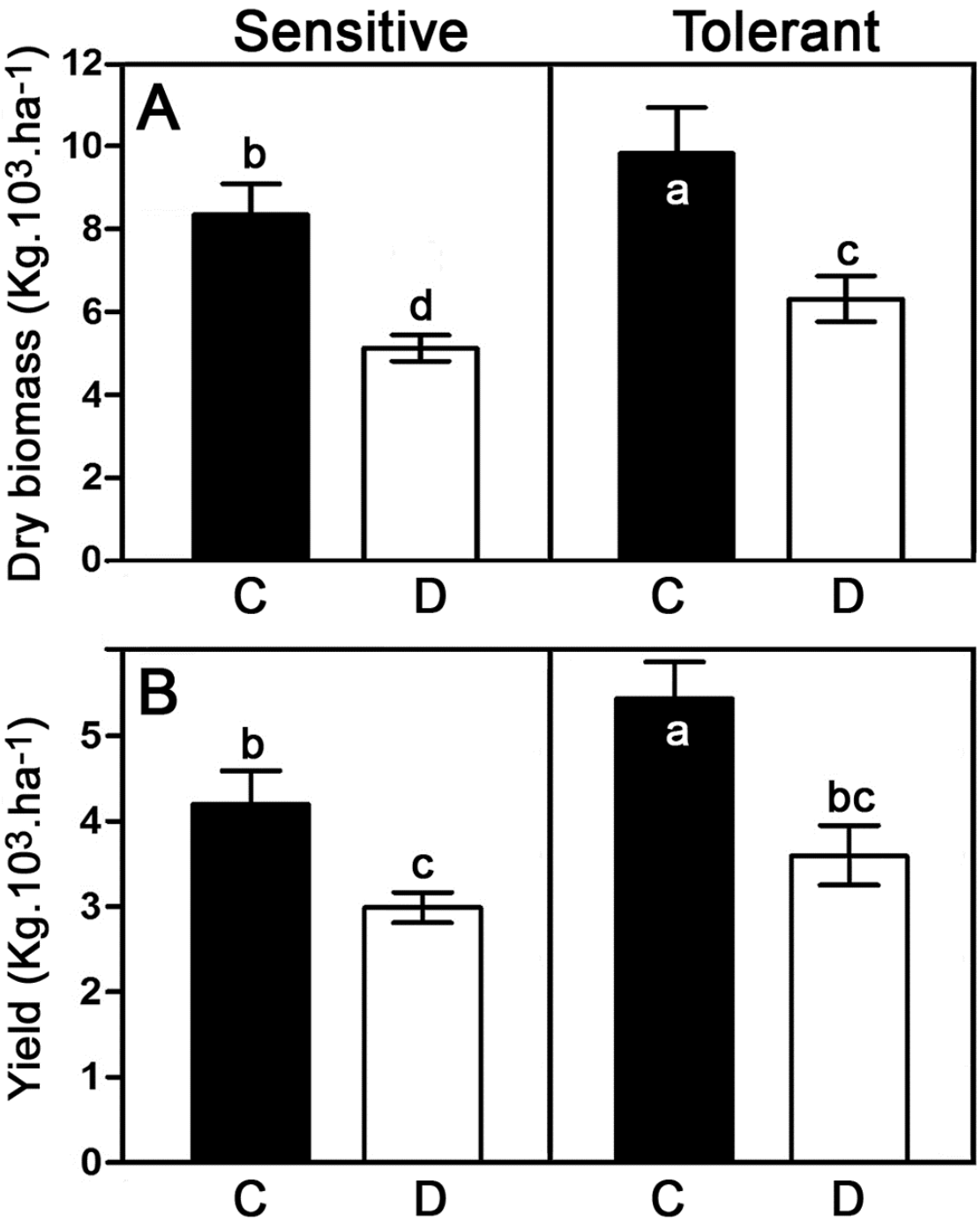

Fig. 8. Biplot from the first and second principal components (PC 1 and PC 2) of principal components analysis (PCA) at the vegetative and reproductive stages. The PCA shows the relationship between all measured variables (grey circles) and soybean genotypes (triangles, sensitive ADM 50048 genotype; circles, tolerant A 5009 RG genotype) grown under control (green) and water stress (orange) conditions. Variables and experimental conditions are a/b Chl, a and b chlorophylls (7 days); APX, ascorbate peroxidase activity (5 days); BIO, dry biomass from field-grown plants; CAT, catalase activity (14 days); DW, dry weight (14 days); F-FRAP, FRAP on field-grown plants; F-MDA, MDA on field-grown plants; F-Pro, proline on field-grown plants; FRAP, the ferric reducing ability of plasma (5 days); FW, fresh weight (14 days); height (14 days); LA, individual leaf area (14 days); MDA, malondialdehyde (5 days); Pro, proline (5 days); SOD, superoxide dismutase activity (5 days); RWC, relative water content (5 days); yield on-field. SC, control sensitive ADM 50048 genotype; SD, drought-treated sensitive ADM 50048 genotype; TC, control tolerant A 5009 RG genotype; TD, drought-treated tolerant A 5009 RG genotype.

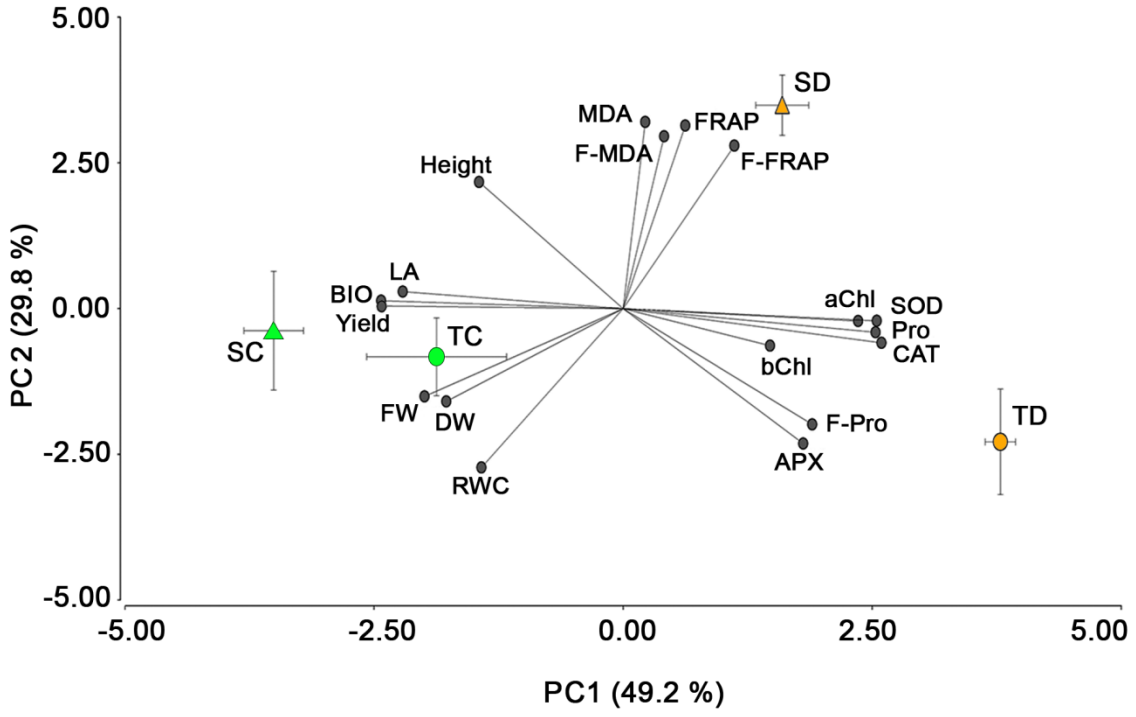

Figure 9. Relative Water Content (RWC), and proline and chlorophylls levels in 16 soybean genotypes from EEA Marcos Juarez germplasm Bank (Argentina) at the vegetative stage under drought stress. Drought stress was generated by dry-down after irrigation withholding on day 7 after emergence. Gravimetric water was monitored daily. Values are mean ± standard error from 3 independent experiments (n=10, in each experiment). Different letters indicate significant differences (ANOVA, $p<0.05$).

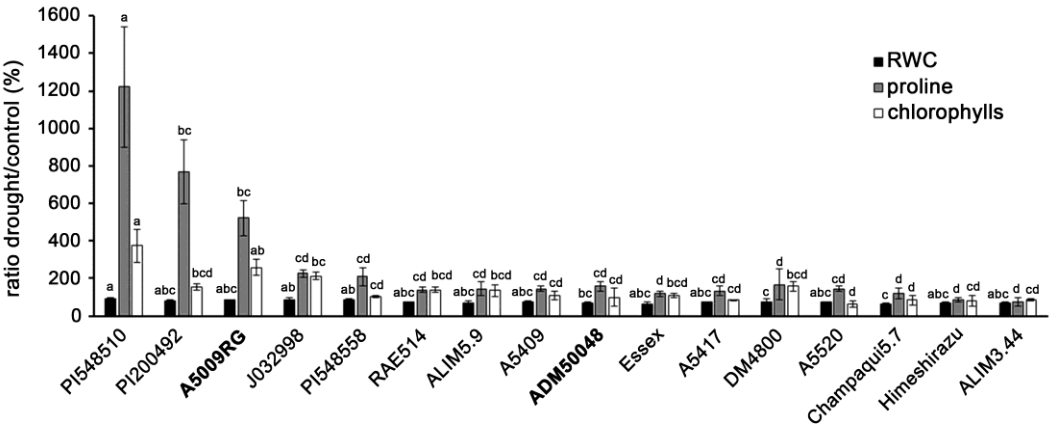

Table 1. Drought tolerance raking of 16 soybean genotypes after 7 days of drought stress at the vegetative stage. The checks (the tolerant A5009RG and the sensitive ADM50048 genotypes) are highlighted in bold.

Soybean genotypes	PC1 Ranking	Numeric Rank
PI548510	-39.5076	1
PI 200492	-23.5082	2
A5009RG	-20.2266	3
J032998	-12.2528	4
PI548558	-9.31811	5
RAE514	-8.21496	6
Alim_5.9	-8.19902	7
A5409	-7.51969	8
ADM50048	-7.49822	9
Essex	-7.04481	10
A5417	-6.69929	11
DM4800	-6.43179	12
A5520	-6.40909	13
Champaqui_5.7	-6.15955	14
Himeshirasu	-5.38636	15
Alim_3.44	-5.22567	16

Table 2. Evidence about the protecting role of proline metabolism under drought stress through the activation of the antioxidant system in soybean and other species

Proline response	Experimental approach	Redox response	Species	Reference
High endogenous accumulation	Tolerant genotype	Lower lipid peroxidation and PSII damage	*Glycine max, drought tolerant*	Iqbal et al., 2019
		Higher CAT activity and lower MDA		Masoumi et al., 2011
		Higher CAT and APX activity		Angra et al., 2010
Higher endogenous accumulation	Overexpression of *P5CR* (proline synthesis gene)	Lower PSII damage	*Glycine max, drought tolerant*	De Ronde et al., 2004
	Overexpression of *WRKY20* or *MYB84*	Higher SOD and CAT activities, and lower MDA		Ning et al., 2017; Wang et al., 2017
	P5CS promoter insensitive to proline	Higher SOD, CAT, and APX activities	*Citrus paradise*	de Carvalho et al., 2013
Exogenous proline application		Lower MDA	*Arabidopsis thaliana*	Moustakas et al., 2011

APX, ascorbate peroxidase; CAT, catalase; MDA, malondialdehyde; P5CS, pyrroline-5-carboxylate synthase; P5CR, pyrroline-5-carboxylate reductase; PSII, photosystem II, SOD, superoxide dismutase.