Drought tolerance response of high-yielding soybean varieties to mild drought: physiological and photochemical adjustments

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Soybean is a crop of agronomic importance that requires adequate watering during its growth to achieve high production. In this study, we determined physiological, photochemical and metabolic differences in five soybean varieties selected from the parental lines of a Nested Association Mapping (NAM) population during mild drought. These varieties have been described as high-yielding (NE3001, HY1; LD01-5907, HY2) or drought tolerant (PI518751; HYD1; PI398881, HYD2). Nevertheless, there has been little research on the physiological traits that sustain their high productivity under water-limited conditions. The results indicate that high-yielding varieties under drought cope with the shortage of water by enhancing their photoprotective defences and invest in growth and productivity, linked to a higher intrinsic water use efficiency. This is the case of the variety N-3001 (HY1), with a tolerance strategy involving a faster transition into the reproductive stage to avoid the drought period. The present study highlights the role of the physiological and biochemical adjustments of various soybean varieties to cope with water-limited conditions. Moreover, the obtained results underscore the fact that the high phenotypic plasticity among soybean phenotypes should be exploited to compensate for the low genetic variability of this species when selecting plant productivity in constrained environments.

Abbreviations – aa, amino acids; A_N, CO_2 assimilation; Chl, chlorophyll; Chl *a+b*, total chlorophylls; Chl *a/b*, Chl *a* to *b* ratio; δ13C, carbon isotope composition; E, transpiration rate; ETR, electron transport rate; DW, dry weight; δ13C, carbon isotope discrimination; $\delta^{15}N$,

isotope composition; g_s, stomatal conductance; LMA, leaf mass area; LWC, leaf water content; Lut, lutein; ΔF´/Fm', fluorescence-based photochemical yield of photosystem II; Fo, minimum level of fluorescence; Fm, maximum level of fluorescence; Fv/Fm, maximal quantum efficiency of photosystem II; FW, fresh weight; HI, harvest index; NPQ, non-photochemical quenching; OJIP, chlorophyll *a* fast fluorescence transients; NAM, Nested Association Mapping; qP, photochemical quenching; Pi_{Abs}, performance index; PS, photosystem; QTL, quantitative trait locus; var, variety; Vi, relative variable Chl fluorescence at 30 ms (at the I-step); Vj, relative variable Chl fluorescence at 2 ms (at the J-step); $Vc_{max,}$ maximum carboxylation velocity of rubisco; RC, reaction centres; Treat, treatment; V+A+Z, total xanthophyll pool; $_T$Car,. total carotenoids; $_T$Toc; total tocopherols; WUE, water-use efficiency; φ_{CO2}; quantum efficiency of CO_2 uptake; $\varphi_{PSII.}$, the actual quantum efficiency of PSII photochemistry.

Introduction

Soybean (*Glycine max* L.) is a crop of great economic and social importance worldwide with a cultivated area of 121 MHa and a production of 334 Mt ("FAOSTAT" 2016). The yield of this species has increased significantly since the Green Revolution as a result of investment in infrastructure, market development, breeding advances and improved management (Pingali 2012). Currently, crop yields face reductions due to global climate change, where widespread droughts are predicted to increase in the next 30-90 years and average temperatures are on the rise (Dai 2013). Therefore, to meet future food demands, the challenge facing breeding programs will not only be to increase current yields, but also to boost their tolerance to drought.

Soybean susceptibility to drought is conditioned by the duration of the stress and the developmental stage when the stress occurs. During vegetative growth (V3-V4), moderate drought periods have been observed to reduce soybean height and relative growth, but if the stress ends in that stage the plant will not suffer yield reductions and may acquire more tolerance to drought in other developmental stages (Desclaux et al. 2000, Kron et al. 2008). However, soybean growth is very sensitive to drought during the flowering and pod filling periods. Drought during these periods can reduce soybean yield between 30 to 80% (Brown et al. 1985, Desclaux et al. 2000, Eck et al. 1987). Although it is well known that soybean varieties show genotypic differences to drought and that there are soybean cultivars that show drought tolerance, research on physiological targets for crop improvement under these conditions is lacking. For example, Gilbert et al. (2011) and Hossain et al. (2014) documented different photosynthetic and stomatal conductance responses to drought among different soybean genotypes known for their yield tolerance under drought. In particular, Gilbert et al. (2011) highlighted that the intrinsic water-use efficiency (WUE, the ratio between photosynthesis and stomatal conductance) can be used in breeding due to its stability under drought. However,

other authors have pointed out that in grapes the intrinsic WUE measure in a single leaf does not represent the whole plant WUE (Escalona et al. 2013, Medrano et al. 2015). The problem arises when one tries to calculate the whole plant WUE because of the difficulty of measuring this parameter in the field or even in pot experiments. The carbon isotope composition ($\delta^{13}C$) signatures of plant biomass or seed samples have been demonstrated as being good surrogate measures of WUE in several crop species, showing a positive relationship between WUE and $\delta^{13}C$ (Farquhar et al. 1989, Ehleringer 1990, El-Sharkawy and De Tafur 2007). In addition, determination of plant tissue carbon isotope discrimination ($\Delta^{13}C$) has been also described as being advantageous over physiological measurements, such as leaf-level photosynthesis or stomatal conductance. Compared to physiological approaches, $\delta^{13}C$ integrates photosynthesis and transpiration responses over long periods of time and it can be readily determined in a large number of tissue samples (Farquhar et al. 1989).

The primary processes affected by water deficiencies include impairment of photosynthesis (which is mainly due to a reduction in stomatal and mesophyll conductance changes; Chaves et al. 2009), and the increase in WUE (Farooq et al. 2012). At this point, the proportion of light energy used by plants for photochemistry declines, increasing the excess energy dissipated as heat via non-photochemical quenching (NPQ). The energy excess that cannot be dissipated may lead to oxidative stress. In order to avoid this situation, plants have developed mechanisms that include an integrated system response of enzymatic and non-enzymatic antioxidants, together with xanthophyll cycle activation (Jahns and Holzwarth 2012). Tolerance responses also involve the accumulation of osmoprotectants to avoid water loss, such as proline (Aranjuelo et al. 2011). However, there are multiple events and metabolic cross-talks triggered by drought, such as hormone regulation, sugar synthesis and redox signals (Pinheiro and Chaves 2011).

Studying the physiological and biochemical responses of different varieties to drought enables the identification and characterization of traits that assure crop production in an unpredictable climatic conditions future. With the purpose of finding which genes code for a specific trait, researchers have used quantitative trait locus (QTL) mapping in order to associate genomic regions with specific traits in soybean such as seed yield (Palomeque et al. 2009), morphological traits (Lee et al. 2015) and abiotic stress (Lee et al. 2004). Nested Association Mapping (NAM) was created with the objective of increasing the resolution and power of QTL mapping (Yu et al. 2008, Yu and Buckler 2006). Unlike traditional bi-parental QTL mapping, which only uses the phenotypic and genotypic variation of two parental lines, NAM increases variability by using several parental lines of different origin, increasing genetic resolution and stability (Rafalski 2010). The soybean NAM population has been created by crossing a common 'hub' parent (IA3023) with 40 soybean cultivars selected for their high-yielding capacities, diverse ancestry and drought tolerance (Song et al. 2017). Although much knowledge has been

gained on the genetic control of yield, maturity, pest resistance, and agronomic characteristics of soy NAM parents and NAM populations (https://soybase.org/), no physiological studies targeted towards finding useful drought tolerance characteristics have been performed in the same populations.

The aim of the present study was to gain insight into the mechanisms that determine differences in the tolerance of soybean varieties to drought, comparing 5 cultivars that belong to the NAM soybean parent population (https://soybase.org/): 2 that are high-yielding under yield potential conditions (NE3001 HY1; LD01-5907, HY2), 2 that are high-yielding under drought (PI518751, HDY1; PI398881, HYD2) and the 'hub' parent cultivar (control: IA3023). Here we present a complete analysis of the main physiological mechanisms involved in drought (production traits, photosynthesis, and metabolites). These results will help target future phenotyping experiments by describing physiological attributes associated with soybean resistance to water deficit stress.

Materials and methods

Plant material and growth conditions

Soybean (*Glycine max* L.) cultivars were selected from SoyNAM population parents (https://www.soybase.org/SoyNAM/index.php) according to their high yield traits selected under yield potential conditions (NE3001, HY1*;* LD01-5907, HY2*;* Abney and Crochet, 2009) and under drought conditions (PI518751, HYD1; PI398881, HYD2; Arocho 2017, Prince et al. 2017), and for being the 'hub' parent of the NAM population as a control (IA3023). Seeds of each cultivar were obtained from the US Soybean Germplasm Repository at Urbana-Champaign (Illinois). Experiments were conducted in the Agrobiotechnology Institute greenhouse facility (42º47´N; 01º37´W, Navarre, Spain) between March and June 2016. The seeds were germinated in a dark and humid environment for 4 days at 25ºC. After germination, two seedlings of each species were transplanted to 10-l pots filled with a mixture of peat: vermiculite: perlite (1:2.5:2.5, v: v). After one week, seedlings were thinned to only one seedling per pot. We employed a randomized complete block design for the experimental plot layout, with fourteen replicates per variety. Plants were allowed to grow for two months in a greenhouse at average temperature (ºC) of 25.52 ± 2.58 (day) and 20.82 ± 2.76 (night). The average relative humidity (RH; %) during the experiment was 61.37 ± 4.28 % (day) and 83.79 ± 2.19 % (night), whereas average vapour pressure deficit (mbar) values were 12.62 ± 3.59 (day) and 3.96 ± 1.04 (night). The weekly watering schedule consisted of watering the plants every day with 200 ml of distilled water (to avoid salt accumulation in pots) and 240 ml of a Hoagland nutrient solution. When the plants were 60-day-old (when all of the plants were at the R2 stage), half of the plants (randomly assigned to a drought treatment by a randomized list for each variety) were exposed to drought conditions (with water withheld to maintained 30% of field capacity) whereas the

others were maintain in optimal water availability conditions (maintained at field capacity). Pot maximum soil volumetric water content values of fully irrigated plants were ~ 0.40 cm^3 cm^{-3}, whereas in the case of drought plants such values reached ~0.12 cm^3 cm^{-3}. Sample size was seven pots per treatment per variety. All the measurements were carried out on expanded leaves when all the plants were at the R5 stage and the leaf water content (LWC) at this point was in the following values: 80.0±0.03% LWC for control and 68±0.6% LWC for mild drought condition.

Growth, biomass and water state measurements

Harvested samples were dried in an oven at 60ºC for 48 h after which the dry weight was determined. The plants were divided into leaves, shoots and grains. The total biomass determination (g DW $plant^{-1}$) was calculated as the sum of the leaves, shoots and seeds. The harvest index (HI) was obtained with the ratio of seed yield/total biomass. The leaf mass area (LMA, g^1 DW cm^{-2}) was measured in ten selected leaves from five different plants for each treatment. Leaf area was measured using digital images and ImageJ software (Schneider et al. 2012). Plant water status was evaluated by measuring the LWC, calculated as (FW–DW)/FW, where FW refers to fresh weight and DW to dry weight.

Gas exchange, chlorophyll fluorescence, and chlorophyll *a* fluorescence kinetics measurements

A fully expanded developed leaf was enclosed in a Li-Cor 6400XT portable photosynthesis gas exchange system (Li-Cor, Lincoln, NE). The light-saturated rate of CO_2 assimilation (A_N) was measured under growth light conditions (1000 µmol m^{-2} s^{-1} PPFD), with 400 µmol s^{-1} air flow rate, 25ºC and 60% RH. Photosynthetic parameters were obtained using the equations of von Caemmerer and Farquhar (1981). Estimation of the maximum carboxylation velocity of Rubisco (Vc_{max}) was made using A_N/Ci curve method of Sharkey et al. (2007) at saturating light conditions (1500 µmol $m^{-2}s^{-1}$). The maximal quantum efficiency of photosystem (PS) II (Fv/Fm) and the actual quantum efficiency of PSII photochemistry (φ_{PSII}) were simultaneously measured with a fluorescence chamber (LFC 6400-40; Li-COR) coupled to the Li-Cor 6400 portable photosynthesis system. For Fv/Fm determinations, leaves were dark-adapted for 30 min. Non-photochemical quenching (NPQ) was calculated as described by Bilger and Björkman (1990). Photochemical quenching (qP) was calculated according to Murchie and Lawson (2013).

Measurements of chlorophyll *a* fast fluorescence transients (OJIP) were performed in soybean leaves with a FluorPen FP 100 fluorometer (Photon Systems Instruments, Brno, Czech Republic). This technique allows an estimation of photosynthetic performance and denotes the flow of energy through PSII, which is a highly sensitive signature of photosynthesis (for

detailed reviews, see Strasser et al. (2000) and Stirbet and Govindjee (2011)). Prior to measurements, leaves were dark-adapted for a night period (14 h) to allow the complete relaxation of oxidation of reaction centres in order to determine the minimum level of fluorescence (Fo). Excitation via blue light emitting diodes (455nm), optically filtered to provide a light intensity of 3000 µmol photons m^{-2} s^{-1} at the leaf sample, allowed to record fluorescence transients during 2 s at a frequency of 10 µs, 100 µs, 1 ms and 10 ms for the time intervals of 10-600 µs, 0.6-14 ms, 14-100 ms and 0.1-2 s, respectively. The fluorescence values at 40 µs (Fo, step 0, all reaction centres of the PSII are open), 100 µs (F_{100}), 300 µs (F_{300}), 2ms (step J), 30 ms (step I) and maximal (maximum level of fluorescence, Fm, step P, closure of all reaction centres) were taken into consideration. Cardinal points of the OJIP curve and derived parameters were calculated with the Fluorpen 2.0 software, based on the theory of energy fluxes in biomembranes by the formulas derived from Strasser et al. (2000). In this paper we have considered fluorescence parameters derived from the extracted data and normalised signals as (i) relative variable Chl fluorescence at time J-step, V_j and at time I-step, V_i, (ii) quantum yields and efficiencies, (iii) and the specific fluxes per active reaction centre (RC). We have also analysed the performance index, Pi_{Abs}, which is the potential performance index for energy conservation from photons absorbed by photosystem II to the reduction of intersystem electron acceptors. This parameter provides a useful tool to study the responses of the photosynthetic apparatus under stressful conditions, allowing in vivo evaluation of plant performance in terms of biophysical parameters that quantify photosynthetic energy conservation (Strasser et al. 2000). In this paper, we do not analyse the events relative to PSI (Zubek et al. 2009). The formulas used to calculate the above parameters plus more detailed information are provided in Appendix SI, Supplemental information.

C and N isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) and content

A fully expanded apical leaf (third or fourth apical leaf and similar to the one used for leaf photosynthesis measurements) was collected, dried at 60ºC for 48 h and then grinded; 1.5 mg samples were used for total organic matter analyses, and three biological replicates were analysed for each sample. Determinations were conducted with an elemental analyser (EA1108, Series 1, Carbo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen, Germany) operating in continuous flow mode. Air $\delta^{13}C$ samples were analysed by gas chromatography (Agilent 6890 Gas Chromatograph, Agilent Technologies, Barcelona, Spain) coupled to a Deltaplus isotope ratio mass spectrometer via a GC–C Combustion III interphase (ThermoFinnigan, Thermo, Barcelona, Spain).

The $^{13}C/^{12}C$ ratio (R) in plant material was expressed in δ notation ($\delta^{13}C$) with respect to Vienna Pee Dee Belemnite calcium carbonate (V-PDB), and measured with an analytical precision of 0.1‰:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} \right) - 1$$

The $\delta^{13}C$ accuracy was monitored using international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA-CH7 polyethylene foil, IAEA-CH6 sucrose and USGS-40 glutamic acid; IAEA, city, Austria).

The $^{15}N/^{14}N$ ratios (*R*) of plant material was expressed in δ notation ($\delta^{15}N$) using international secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N_1 and IAEA N_2 ammonium sulfate and IAEA NO_3 potassium nitrate) referred to N_2 in air, with analytical precision at about 0.2‰:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} \right) - 1$$

where: R_{sample} is the ratio of ^{15}N to ^{14}N in the sample and $R_{standard}$ is the ratio of ^{15}N to ^{14}N in the air.

Free amino acid determinations by GC-MS

Frozen leaves were ground to a fine powder in liquid nitrogen and a sub-sample was lyophilised. Lyophilised plant tissue (20 mg) was homogenised in 400 µl of 80% ethanol and mixed using a vortex, incubated at 80ºC for 1 h and centrifuged at 14 000 *g* and 4ºC for 10 min and the pellet was completely dehydrated. The pellet was re-suspended in 100 µl of milli-Q water, centrifuged at 14 000 g and 4ºC for 10 min and the supernatant was collected. The amino acid content in the supernatant was determined by HPLC (Waters Corporation, Barcelona, Spain) after derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Cohen and Michaud 1993).

Pigments and tocopherols

Pigments were extracted using a Tearor 985370 electric tissue homogeniser (BioSpec, Bartlesville, Okla., USA) with 1 ml of acetone (100%) with 0.5 g/l of $CaCO_3$ at ≤ 4ºC using cold racks (IsoPack, Eppendorf IsoTherm®, Madrid, Spain) in order to avoid acid traces that might change pigment composition. Once homogenised, samples were centrifuged at 16 000 *g* for 20 min at 4ºC and syringe-filtered through a 0.22 µm PTFE filter (Teknokroma, Barcelona, Spain). Extracts were injected (15 µl) on a reversed-phase C18 column (Waters Spherisorb ODS1, 4.6 × 250 mm, Milford, MA) HPLC system following the method of Garcia-Plazaola and Becerril (1999, 2001). The 717 plus autosampler was equipped with a thermostat, which maintains a constant temperature of 4°C avoiding pigment degradation or alteration. Photosynthetic pigments were measured with a PDA detector (Waters model 996) in the range 250-700 nm. Peaks were detected and integrated at 445 nm for carotenoid and chlorophyll content. Pigments

were identified by comparing spectral characteristics obtained by the PDA detector and retention times with those of standard materials (DHI, Hørsholm, Denmark). Retention times and conversion factors for pigments were the same as those described by García-Plazaola and Becerril (1999, 2001). For tocopherols, detection was carried out with a fluorescence detector (Waters model 474) set to λexc = 295 nm and λem = 340 nm and calibrated with tocopherol standards (Calbiochem, San Diego, CA).

Photochemical reflectance index (PRI)

The PRI was measured in the adaxial side of ten leaves selected for each condition from five different plants with a PRI-meter (PlantPen PRI 200, PSI, Brno, Czech Republic). This index was calculated as (Reflectance570 – Reflectance531)/(Reflectance531+Reflectance570).

Starch content determination

Lyophilised plant tissue (25 mg) was homogenised in 1ml of 80% ethanol and mixed using a vortex, incubated at 70ºC for 90 min and centrifuged at 14 000 *g* for 10 min and the pellet collected. The pellet was resuspended with 1 ml of 80% ethanol and mixed using a vortex and centrifuged at 14 000 *g* for 10 min and the pellet collected and dehydrated completely. The pellet was again resuspended in 400 µl of 0.2N KOH, then mixed and incubated at 95ºC for 90 min, after which ~220 µl of 1N acetic acid was added until the pH was adjusted to ~4.7 and the suspension was centrifuged at 14 000 g for 10 min. The supernatant was collected and subjected to starch analysis. Starch samples were prepared by using an amyloglucosidase-based test kit (Boehringer Mannheim, Germany) and determined with a spectrophotometer, measuring the absorbance of the samples at 340 nm.

Superoxide Dismutase Activity (SOD)

SOD activity of roots and shoots of soybean was measured in gel as described by Beauchamp and Fridovich (1971) and Asensio et al. (2011, 2012). Mitochondrial antioxidant manganese superoxide dismutase (MnSOD) isoform identification was achieved according to known mobility of SODs on native gels and based on the differential inhibition of SOD activity on gels pre-incubated with either 3 mM KCN, which inhibited the CuZnSODs, or 5 mM H_2O_2 for 1 h, which inhibited FeSOD (Asensio et al. 2012, 2011). The in-gel SOD activity assays were performed at least three times to ensure the consistency of the results.

Statistics

Differences among well-watered and drought treatments were evaluated with two way Analyses of Variance (ANOVA) considering variety (var.) and treatment (Treat) as fixed factors. All data were tested for normality (Kolmogorov–Smirnof test) and homogeneity of variances (Cochran test) and log-transformed if necessary. When this failed to meet ANOVA assumptions, they were analysed using the nonparametric Mann–Whitney test. The resulting *P*-values were considered to be statistically significant at $\alpha = 0.05$. Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 24.0. (IBM Corp. Armonk, NY). Asterisks indicate significant differences: *P<0.05, **P<0.001, ***P<0.0001

Results

Drought induced contrasting responses in physiological and production traits between varieties of soybean plants. This fact is exemplified in the production traits (Table1). Seed yield was significantly reduced by drought in the control variety (IA3032). Although not significant, seed yield was also reduced in HY2 by almost 50%, in contrast with no effect of drought observed in HY1. In addition, drought also reduced seed yield of HYD2. Soybean biomass was significantly reduced in all varieties as a result of the drought treatment, with the exception of HY1 and HYD1. Under well-watered conditions, HY2, HYD2, and control cultivars showed the highest biomass accumulation. The varieties HY1, HYD1, and HYD2 showed the highest values of LMA under well-watered status. In general, LMA was higher in water-limited plants than in the well-watered ones (Table 1).

Figures 1-4 are bisector plots that represent the relationship of the parameters under drought against well-watered conditions for each of the cultivars. Dotted lines represent the regression with slope 1, and data points above the line indicate that drought-affected plants showed a higher response in that parameter compared to well-watered plants. Drought significantly reduced the A_N in all the varieties, due to stomatal closure (g_s decreased under drought) with the correlation between both parameters being significant ($r^2 = 0.476$; $P \leq 0.001$). Interestingly, the HY1 variety exhibited a high A_N (≈30 μmol CO_2 m^{-2} s^{-1}) under normal irrigation, and also maintained quite a high rate during drought (≈20 μmol CO_2 m^{-2} s^{-1}), giving this cultivar the highest rate of photosynthesis in both conditions (Fig. 1A). In addition, HY1 showed the highest g_s and transpiration rate (E) under drought conditions and a moderate g_s under well-watered conditions (Fig. 1B, E). A higher intrinsic WUE under drought for all the varieties was found (Fig. 1C); however, HY1 showed similar WUE under both water treatments and had the greatest WUE in well-watered conditions. This variety also showed the highest ETR under drought, but the same ETR as the other varieties under well-watered conditions (Fig. 1G). The Vc_{max} did not decrease under drought, with HY1 showing the highest Vc_{max} under well-watered conditions and a similar rate to HYD1 and HYD2 under drought (Fig. 1D). The intercellular to atmospheric ratio of the CO_2 mole fraction (C_i/C_a) showed an interaction

between the cultivars and the water treatment, with HYD1, HYD2, and control having the highest Ci/Ca values under well-watered conditions, while HY1 and HY2 have the highest values under drought conditions (Fig. 1F). As shown in Fig. 1H, the ETR/A_N data highlighted that while this parameter was not affected by water availability, significant differences were detected between varieties. Moreover, while higher ETR/A_N values were detected in control and HY1 varieties, no water stress effects were observed in HYD1 and HYD2. On the other hand, the quantum efficiency of CO_2 uptake (φCO_2; Fig. 1I) revealed that both water treatments and especially variety factor significantly modified this parameter. The values for φCO_2 were higher in well-water than in drought conditions, with HY1 having the highest values.

The cultivars selected for their high yields under drought (HYD1 and HYD2) showed high Fv/Fm under this condition, which confirms their tolerance. Interestingly, not only did these drought-tolerant soybean plants show high values of Fv/Fm under drought, all the rest of the varieties (HY1, HY2, and control) also had high Fv/Fm values, which indicates no down-regulation of PSII (Fig 2A). The variety HY1 showed a high photochemical yield of PSII ($\Delta F'/Fm'$; Fig 2B) and high qP (Fig. 2D) under drought, but also the lowest $\Delta F'/Fm'$ and NPQ values under well-watered conditions (Fig. 2B, C). Differences in the way that varieties respond to drought were revealed by parameters derived after further analysis of the OJIP curves, such as the relative variable chlorophyll fluorescence at 30ms (Vi). This parameter showed lower values under drought for HY1 and HYD2. The quantum yield for energy dissipation (φ_{Do}) was higher under drought for HY1, HY2 and control varieties. The parameter Pi_{Abs} was significantly reduced under drought in HY2 and the control. Interestingly, both HYD1 and HYD2 showed low Pi_{Abs} under well-watered conditions (Table 2). Regarding the C and N isotope analyses, drought increased $\delta^{13}C$ and $\delta^{15}N$ significantly in all the cultivars without any differences between cultivars (Fig. 3A, B).

In the case of free amino acids in the leaves, many of them did not vary significantly between water treatments or among cultivars (Asp, Asn, Glu, Gln, Ser, Met, Arg; Table 3). However, Ala was the most affected amino acid under drought treatment. Proline had significantly higher values under drought only in HY2, due to the interaction between genotype and drought treatment. Some of the amino acid contents were affected by the interaction between water treatment and variety. For example, under drought conditions, the cultivar HY2 showed high Gly, Tyr, Val, Ile, and His values, whereas HY1 showed lower values of Ala, Lys, and GABA.

Total chlorophyll content in leaves (Chl *a*+*b*) decreased in all the varieties under the shortage of water, with the exception of HYD2 (Fig. 4A). The ratio of Chl *a/b* showed significant differences between treatment and varieties, the ratio being highest in HY1 and HYD2 under well-watered conditions and lowest under drought in the same varieties. In contrast, HYD1 showed one of the lowest ratios of chlorophyll *a* to *b* (Chl *a/b)* values under

well-watered conditions, but the highest ratio in the drought treatment (Fig. 4B). The total xanthophyll pool size on a chlorophyll basis (V+A+Z) was significantly enhanced in drought in HYD1 due to the interaction of the water treatment and variety assayed (Fig 4C). In addition, the lutein (Lut) pool size on a chlorophyll basis, which is the most stable carotenoid, also showed an increase in all the varieties under drought, except for HYD1 (Fig 4D). The total carotenoid pool size on a chlorophyll basis ($_{T}$Car) also increased under drought for all the varieties, except for HY2 (Fig 4E). Drought increased the total tocopherol levels on a chlorophyll basis ($_{T}$Toc, mainly α-tocopherol) in all cultivars with the exception of HY2. In addition, the control cultivar (IA3032) showed the lowest tocopherol content in both treatments (Fig. 4F).

Figure 5 summarises the biomass, physiological and biochemical strategies of each of the varieties under drought in relation to well-watered conditions (value 1). Deviation from value 1 indicates the impact of drought on the parameters, with values lower than 1 negatively affecting a given parameter, and with values higher than 1 positively affecting that given parameter. For example, the control variety response (green line) involved increased LMA, starch, WUE, and $_{T}$Toc, with a parallel decrease in seed yield, A_N, and Pi_{Abs}. A similar strategy seems to apply for HYD2, but with higher Vc_{max}, total amino acid pool, V+A+Z, and an increase in Pi_{Abs}. On the other hand, HYD1 showed an enhancement of seed yield, Vc_{max}, NPQ, starch, and V+A+Z (and therefore PRI, whose value was quite high). Surprisingly, HY1 showed an increased yield under drought that was accompanied by increases in the isoenzyme MnSOD alongside increased E, NPQ, WUE, and $_{T}$Toc. The variety HY2 showed a reduction in yield with an increase in WUE, total amino acid, and PRI (Fig. 5).

Discussion

Leaf traits adjustments to drought

Within the context of current and near future global climate change, selection of drought-tolerant varieties and understanding the physiological mechanisms that underpin this tolerance is gaining prominence (Beebe et al. 2013, Blum 2005, Chaves et al. 2009). The current study provides an integrated characterisation of water shortage on traits, including gas exchange, fluorescence, growth and biochemical analyses. One of the anatomical traits that were affected by drought was LMA, which reflects photosynthesis adaptation to the prevailing environmental conditions (Tosens et al. 2012) and it is closely linked to climate parameters (Wright et al. 2004). In response to drought, leaves with higher LMA are produced (more robust leaves probably due to the thickening of cuticles and an epidermis with more tightly packed mesophyll cells; Galmés et al. 2011, Poorter et al. 2009), as exemplify by cultivars showing high yields under drought (HYD1, HYD2) which tended to have an increased LMA tended to increase when water availability decreased. This trait was also significant in the control soybean

variety, which could indicate acclimation to a smaller transpiration surface (Poorter et al. 2009). Indeed, the obtained data revealed that drought decreased CO_2 assimilation as a consequence of the stomatal closure (g_s lower values; Fig. 1), which in turn decreased the available internal CO_2. This reduction on internal [CO_2] pushed the plant to fix any available CO_2 molecule leading to an enrichment in ^{13}C under drought conditions. However, the varieties HY1, HYD1, and HYD2 showed high A_N, without showing a stronger stomatal response, which was also confirmed by the lack of a variety effect on $\delta^{13}C$. According to previous publications, the lack of variety effect on $\delta^{13}C$, may indicate that the studied varieties did not differ in WUE (Ehleringer 1990, Farquhar et al. 1989). However, the data regarding instantaneous WUE (the ratio between A_N and g_s) showed variety variation. This discrepancy between $\delta^{13}C$, that estimates the whole plant WUE, and instantaneous WUE has been reported before in several plant species (Fullana-Pericàs et al. 2017, Medrano et al. 2015), and it can be linked to the fact that, in gas exchange measurements, instantaneous WUE only represents the fitness of the plant in a short window of time. Meanwhile, the $\delta^{13}C$ signature is able to integrate the plant's fitness and its relation with the environment from the moment that the plant starts photosynthesizing until the time of the sampling (Araus et al. 2003, 2002). Therefore, in this study, we can conclude that drought increased WUE, but that there were no differences in WUE between the varieties.

On the other hand, although A_N and biomass decreased in most of the varieties, the drought treatment only affected the Fv/Fm values slightly (Fig. 2), indicating a lack of alterations in fluorescence parameters associated with PSII activity and no down-regulation of photochemistry. This lack of effect has been repeatedly associated with drought (Flexas et al. 2012). Besides, no significant changes were observed in the energy dissipated as heat under either of the conditions. Only HY1 demonstrated a significant increase in the proportion of energy utilised by the photochemical reactions driving photosynthesis. A detailed analysis of the kinetic transients of chlorophyll *a* fluorescence using the Pi_{Abs} parameter (integrating the density of the reactive RC per PSII antenna chlorophyll, the maximal quantum yield of PSII, and the electron transport beyond QA; Strasser et al. 2000) showed lower values for HY2 and control varieties under drought. This indicates that HY1, HYD1, and HYD2, which maintains similar Pi_{Abs} under both treatments, had a similar dose-dependent improvement in energy conservation from absorbed photons to reduction under both well-watered conditions and drought (Table 2).

Metabolic adjustments to drought

In the context of plant acclimation to stressful growth conditions, previous studies have shown the relevance of multiple feedback processes between chloroplast metabolism and factors such as leaf carbohydrate at the whole plant level (Demmig-Adams et al. 2014). Within this context, our study showed that drought stress lowered the amount of carbohydrates accumulated in leaves during vegetative growth (Marcaida et al. 2014). In contrast, we found that starch increased in all the varieties under drought (Fig. 5), which indicates more stored carbohydrates.

The current data support the fact that plants might favour carbohydrate biosynthesis and storage metabolism of reserves (including starch) and repress the processes associated with photosynthesis and reserve mobilisation (Ho et al. 2001). Under drought, metabolites such as proline and other compounds play important roles in increasing osmotic balance and maintaining cell turgor, which are fundamental physiological traits for reducing the negative effects of drought (Aranjuelo et al. 2011). Indeed, proline is the amino acid that is usually accumulated under such situations (Shaw and Hossain 2013) and serves as an indicator of stress tolerance (Claussen 2005). However, in our study and also observed by Silvente et al. (2012), drought did not trigger proline synthesis (with the exception of HY2 under drought; Table 3). This suggests that this variety may have an early response to water withholding, being more sensitive to drought than the other varieties. The drought has been described as reducing the total Chl *a*+*b* pool (Esteban et al. 2015), which occurred for all the varieties with the exception of HYD2, demonstrating its tolerance. Interestingly, the ratio Chl *a*/*b* increased under drought in the other high yielding variety, HYD1. As both the PSI and PSII reaction centers are devoid of Chl *b*, the Chl *a/b* ratio reflects the reduction in the size of light harvesting complex II (Evans 1988). This ratio responds substantially to changes in the environment as a result of changes in the structure of the PS (Anderson et al. 2008). Therefore, this supports the fact that this variety adjusted its photosynthetic apparatus and acclimated to the condition of water shortage. All the varieties, with the exception of the control one (IA3032), showed higher total tocopherol contents (mainly due to α-tocopherol) both in drought and the well-watered state. Higher α-tocopherol contents have been correlated with higher tolerance to drought (Munné-Bosch 2005; Fig. 4). The HYD1 variety also showed an increase in the total V+A+Z pool. The rest of the varieties increased their total Lut pools. Interestingly, the V+A+Z pigments and Lut are involved in the regulation of thermal energy dissipation (Li et al. 2009), indicating that there is greater photoprotective demand under this scenario of water scarcity. Interestingly, alongside its high V+A+Z, HYD1 also showed a higher PRI index under drought (Fig. 5), which is additional evidence of an enhanced photoprotective pool. Lastly, Fig. 5 is a spider plot that includes the main variables and traits measured for each of the varieties. It summarises the strategy for each of the varieties under drought, indicating that the high yielding varieties, HYD1 and HYD2, tolerate drought.

The strategy for the high yield capacity of HY1 variety

Our studies have shown that drought negatively affected grain yield in all the genotypes except HY1 (NE3001; Table 1). This genotype has been described as an elite material selected for its high-yielding performance in yield potential conditions (Graef et al. 2009). Our data also highlights the fact that HY1 can be identified as tolerant to water shortages under greenhouse conditions. Indeed, HY1 showed the highest photosynthesis rates under both water availability conditions, explaining in part the reasons for its higher yields. However, if HY1 does not

increase its stomatal opening to fix more carbon, how can we explain its higher photosynthetic rates reflected by its higher A_N and the same Vc_{max} as the HDY varieties? One explanation could be that the high-yielding varieties under well-watered conditions did not show an increase in LMA, and showed lower values under these conditions than the varieties that produce more under drought (Table 1). This aspect is worth noting because lower LMA has been related to higher mesophyll conductance in several crops (Flexas et al. 2008, Galmés et al. 2011, Niinemets et al. 2009). If HY1 had higher mesophyll conductance, because its g_s is the same as the HYD, its mesophyll conductance/g_s ratio would be increased. An increased mesophyll conductance/g_s, has been demonstrated to increase transpiration efficiency under drought without a negative impact on carboxylation (Barbour et al. 2010, Galmés et al. 2011b); therefore, this could explain the higher photosynthetic rates observed in HY1 under drought.

Growth parameters (Table 1) also reveal that part of the high yield capacity of HY1 under drought is due to its high HI. Harvest index (HI), defined as the weight of seed divided by the total weight of above ground biomass, is an indicator of the amount of biomass that is derived from the reproductive biomass relative to the total biomass. Similar to observations in other crops, the fact that HY1 had a high HI is an indicator of this variety having favouring conditions to tolerate drought, due to its capacity to accumulate biomass in the vegetative period, and later under drought, to remobilise this biomass for seed formation (Beebe et al. 2013, Polania et al. 2016, Polania et al. 2016). Interestingly, we found an increase in leaves of the manganese superoxide dismutases (MnSOD) for the variety HY1 (Fig. 5; with no changes for the rest of SODs), indicating no oxidative stress in any of the SOD´s locations; Alscher et al. 2002). MnSOD is a constitutive antioxidant enzyme in mitochondria, and it can vary between species and varieties, but in general, it is quite stable under environmental stresses (Asensio et al. 2012). MnSOD was found to increase in senescent roots in soybean plants (Asensio et al. 2012), indicating that an increase in MnSOD could be an aging symptom. This is in accordance with the data obtained for the variety HY1, which may initiate drought-induced senescence.

Conclusion

The objective of this study was to determine the effects of drought on five NAM parent cultivars of soybean in order to determine traits for selecting cultivars with greater drought tolerance. Overall, we found that the effects of drought on the yield, photosynthetic parameters, and biochemical traits varied greatly depending on the variety and treatment. In general, the data demonstrated that high-yielding varieties were able to cope with drought via a number of plant defence mechanisms (larger xanthophyll and antioxidant pools) and investing in growth (LMA) and productivity, all associated with a higher intrinsic WUE. Besides, the HY1 variety (N3001) was found to be more tolerant to drought than was previously thought, showing high yield and WUE under drought conditions. Its tolerance strategy involves transitioning to reproductive

stages faster (shorter life and flowering cycle) to avoid the drought period. Indeed, the increase in seed yield (Table 1) and the higher activity of MnSOD (Fig. 5) suggest that this variety has better allocation and partitioning of assimilates to developing seeds, a response well documented in crop plants such as cereals (Bruce et al. 2002), and it may initiate drought-induced senescence (Asensio et al. 2012). Field experiments will be needed to confirm this data.

Author contributions

J.B., A.S.S., I.A., and R.E. conceived the experiments; A.S.S. selected the soybean seed cultivars; R.E., J.B., and I.A. performed the experiments; R.E. also supervised the whole project and wrote the manuscript; I.A. gave experimental advice and conceived and supervised the whole project. All the authors interpreted the data and contributed to drafting the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Definition of terms and formulae of the OJIP-test parameters used for analysis of the chlorophyll *a* fluorescence transients in Table 2 and Fig. 5 following the formulas of Strasser et al. (2000, 2004)

Figures

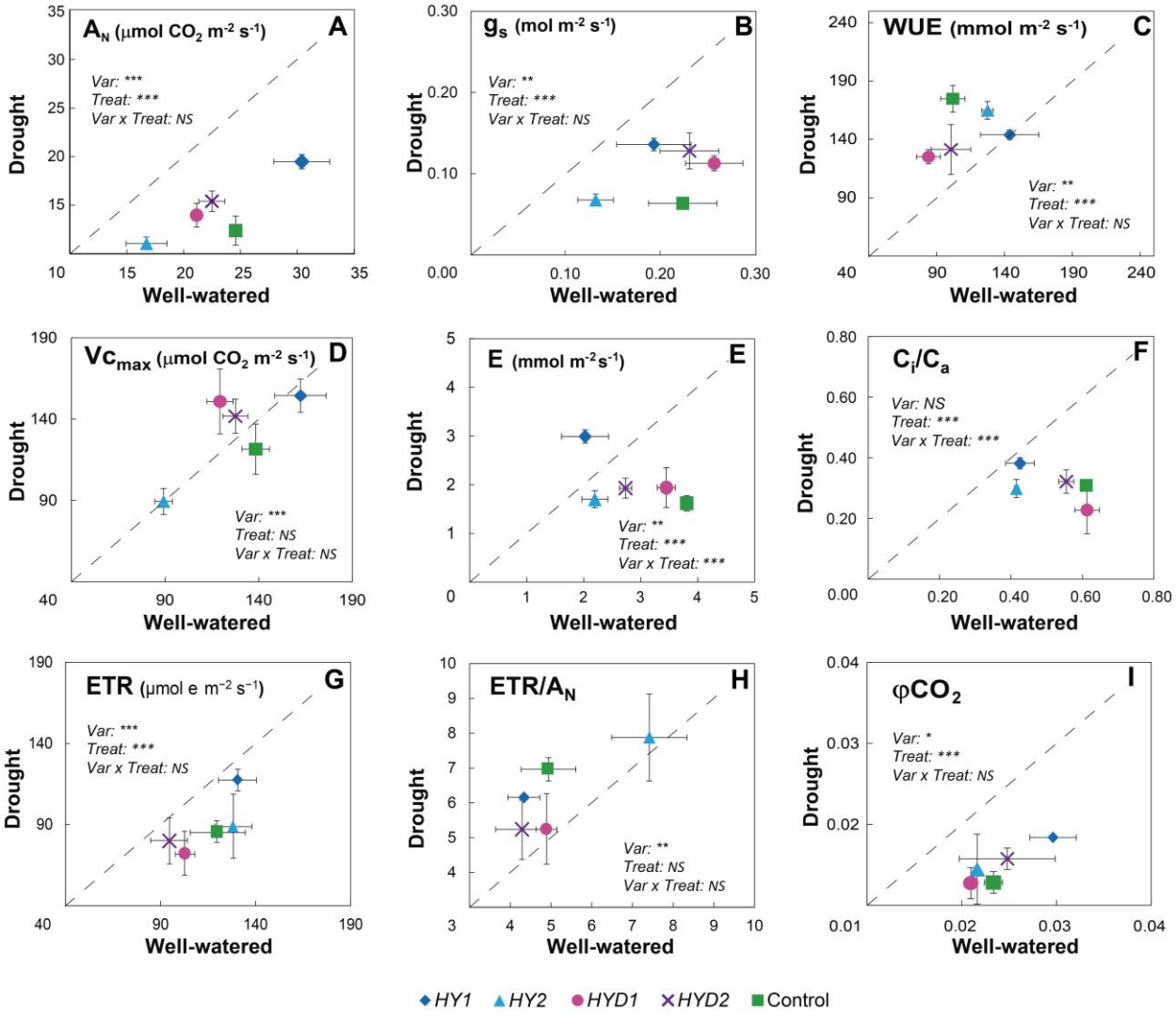

Fig. 1. Bisector plots representing the relationship of gas exchange parameters under drought and well-watered conditions in soybean cultivars. (A) net CO_2 assimilation (A_N, µmol CO_2 $m^{-2}s^{-1}$), (B) stomatal conductance (g_s mol $m^{-2}s^{-1}$), (C) intrinsic water use efficiency (WUE, mmol m^{-2} s^{-1}), (D) the maximum rate of rubisco carboxylase activity ($Vc_{max,}$ µmol CO_2 $m^{-2}s^{-1}$), (E) transpiration (E, mmol m^{-2} s^{-1}), (F) the intercellular to atmospheric ratio of the CO_2 mole fraction ($C_i/C_{a,}$), (G) electron transport rate (ETR; µmol e $m^{-2}s^{-1}$), (H) ETR/An, (I) quantum efficiency of CO_2 uptake (φCO2). Data are presented as the mean for each of the varieties ± SE (n=4). Dotted lines represent the regression with slope 1 (y=x). Data points above the line indicate that the parameter was more affected by drought compared to well-watered conditions. Inside panels indicate the analysis of variance of the effects of treatment and variety. In order to standardise variances, one datum was replaced by the mean of the group and 1 degree of freedom was subtracted from the residual (Winer BJ, Brown DR, 1991).

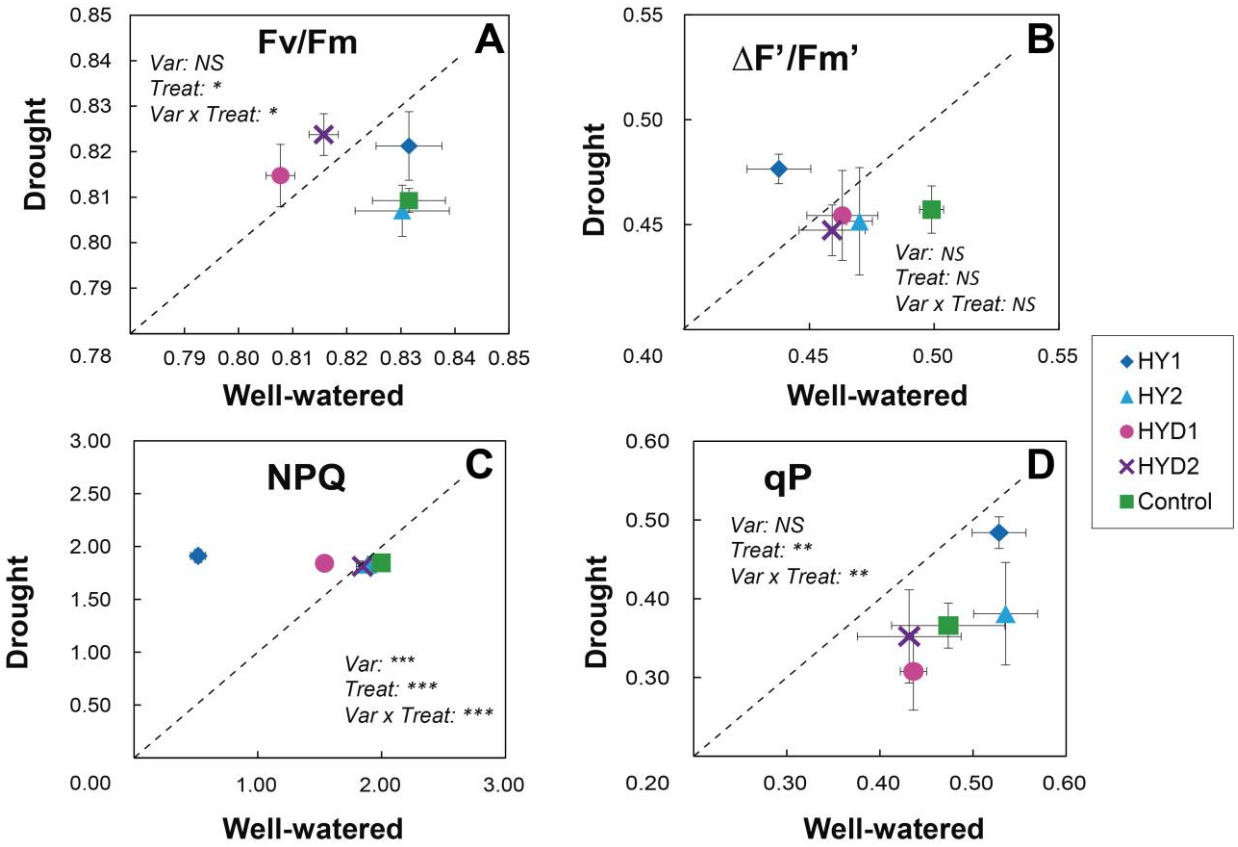

Fig. 2. Bisector plots representing the relationship of fluorescence parameters under drought and well-watered conditions in soybean cultivars. (A) maximal quantum efficiency (Fv/Fm), (B) fluorescence-based photochemical yield of photosystem II (ΔF′/Fm'), (C) non-photochemical quenching (NPQ) and (D) chemical quenching (qP). Dotted lines represent the regression with slope 1 (y=x). Data are presented as the mean for each of the varieties ± SE (n=4-6). Data points above the line indicate that the parameter was more affected by drought compared to well-watered conditions. Inside panels indicate the analysis of variance of the effects of treatment and variety. NPQ errors are smaller than the symbols.

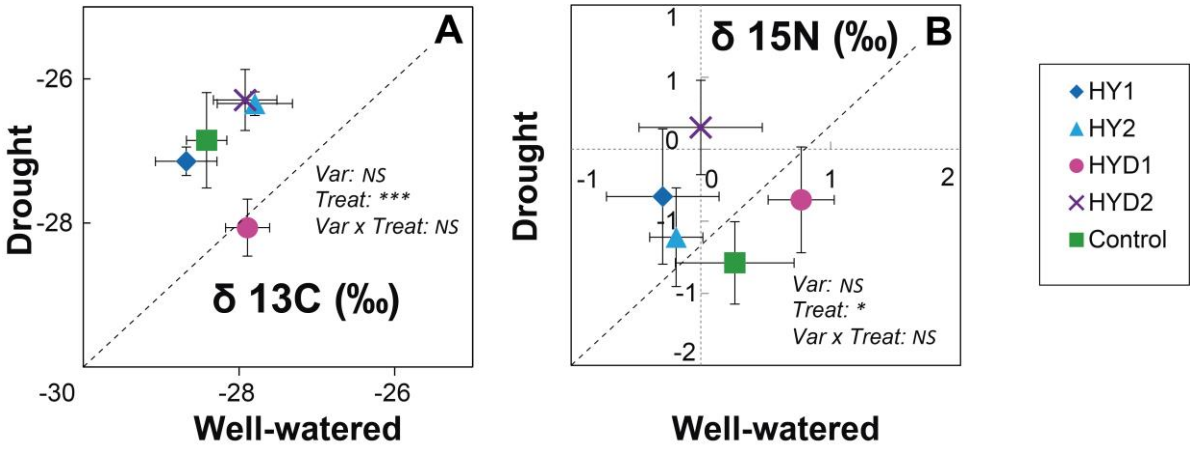

Fig. 3. Bisector plots representing the relationship of isotopic signature under drought and well-watered conditions in soybean cultivars. (A) carbon isotope composition ($\delta^{13}C$, ‰) and (B) nitrogen isotope composition ($\delta^{15}N$, ‰). Dotted lines represent the regression with slope 1 (y=x). Data are presented as the mean for each of the varieties ± SE (n=4). Data points above the line indicate that the parameter was more affected by drought compared to well-watered conditions. Inside panels indicate the analysis of variance of the effects of treatment and variety.

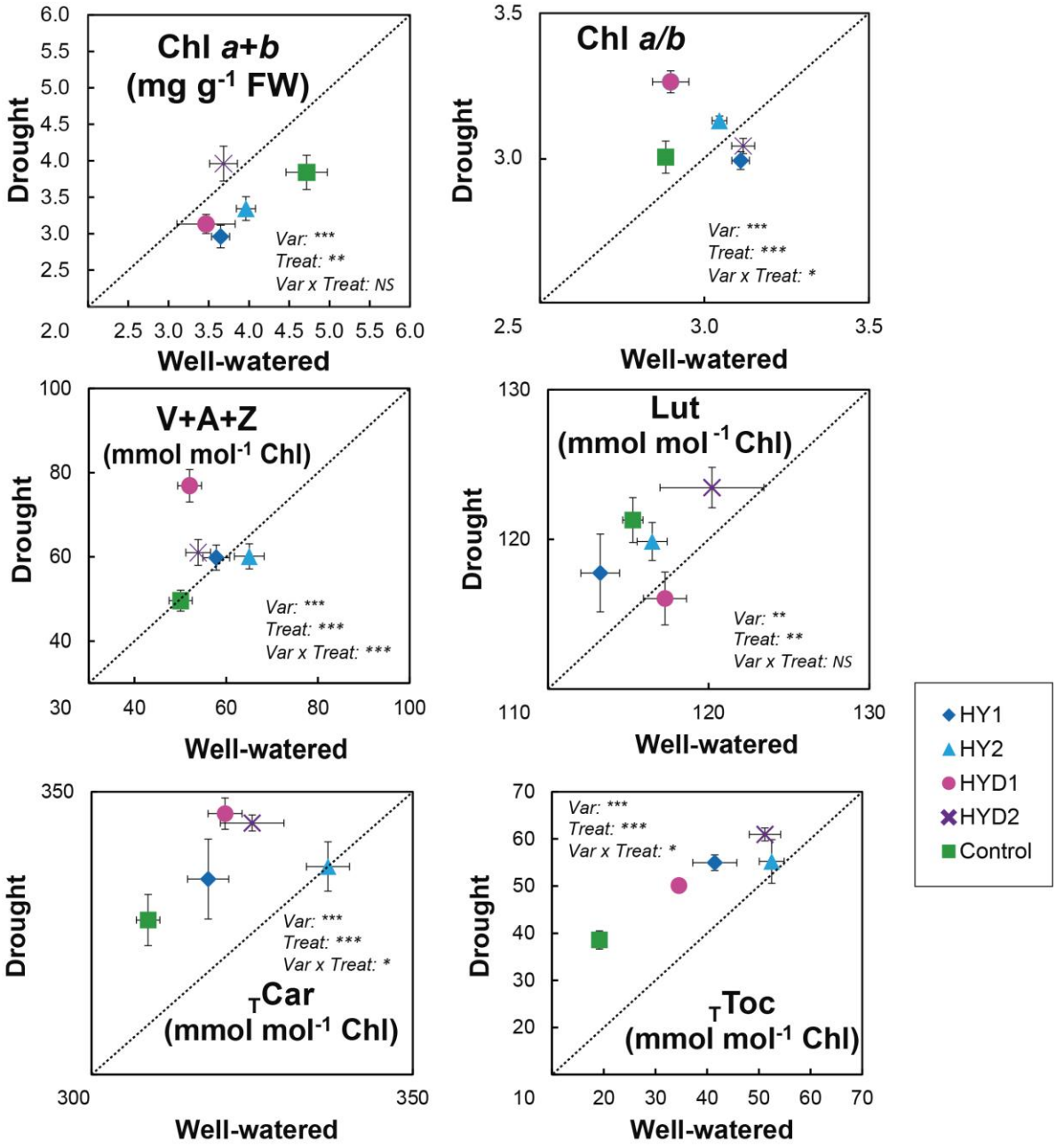

Fig. 4. Bisector plots representing the relationship of pigments and tocopherols under drought and well-watered conditions in soybean cultivars. (A) total chlorophylls (Chl *a*+*b*, mg g^{-1} FW), (B) the ratio of chlorophyll a to b (Chl *a/b*), (C) total xanthophyll pigments (V+A+Z, mmol mol^{-1} Chl), (D) lutein content (L, mmol mol^{-1} Chl), (E) total carotenoids ($_T$Car, mmol mol^{-1} Chl) and (F) total tocopherols ($_T$Car, mmol mol^{-1} Chl). Dotted lines represent the regression with slope 1 (y=x). Data are presented as the mean for each of the varieties ± SE (n=5). Inside panels indicate the analysis of variance of the effects of treatment and variety.

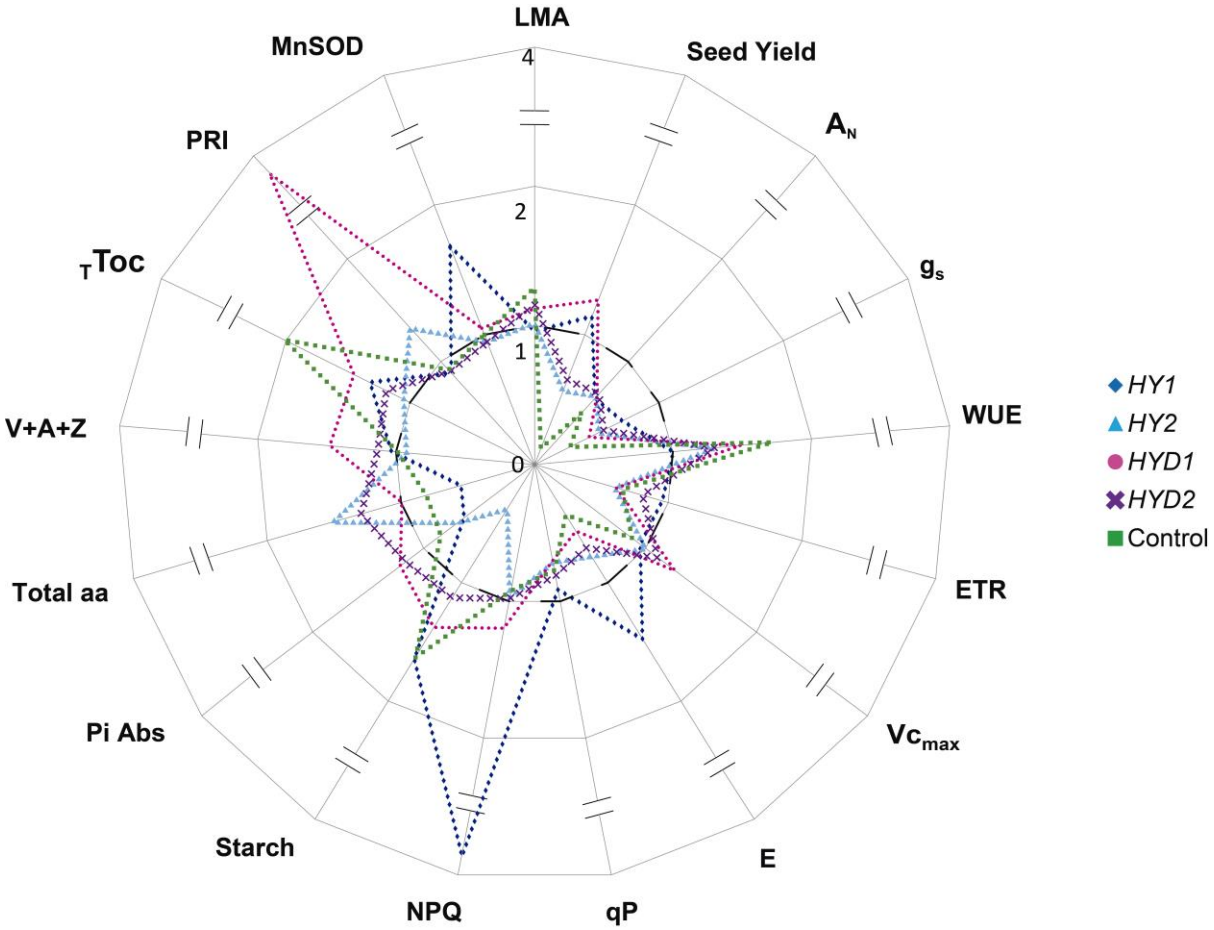

Fig. 5. Spiderplot showing the effect on the main parameters measured: net CO_2 assimilation (A_N), stomatal conductance (g_s), water use efficiency (WUE), electro transport rate (ETR), the maximum rate of rubisco carboxylase activity (VC_{max}), transpiration rate (E), photochemical quenching (qP), non photochemical quenching (NPQ), starch, performance index (PiAbs), total xanthophyll pigments (V+A+Z), total carotenoids ($_T$Car), the photochemical reflectance index (PRI) and mitochondrial antioxidant manganese superoxide dismutase (MnSOD). All the data were normalised to their respective controls under well-watered conditions. Thus, value 1 indicates the control values, while the deviation from 1 indicates the impact of drought on the parameters analysed. SE is not shown for clarity but was <10% of the means in all cases.

Table 1. Seed yield, biomass, harvest index (HI), leaf mass area (LMA) in the five varieties (HY1, HY2, HYD1, HYD2, Control) under well-watered and drought. Data are means ± SE (n=4-10) (A). Two-way analysis of variance (ANOVA) for the effects of variety (Var.) and water treatment (Treat.), with their interaction factor (Var.×Treat.) on production traits, with between- and within-subject factors carried out to assess statistical significance of mean differences is shown. **P*<0.05; ***P*<0.001; ****P*<0.0001; n.s., not significant (B). Different letters denote statistically significant differences at α=0.05 after Student–Newman–Keul test.

A Var./ Trait.	**Seed yield** (g FW plant^{-1})		**Biomass** (g DW plant^{-1})		**HI**		**LMA** (g^1 DW cm^{-2})	
	WW	**D**	**WW**	**D**	**WW**	**D**	**WW**	**D**
HY1	17.1±2.2^{ab}	21.9±3.7^{a}	40.1±2.2^{bc}	27.0±3.6^{c}	0.42±0.04^{b}	0.73±0.10^{a}	305.1±11.4^{ab}	292.3±14.6^{ab}
HY2	15.5±1.4^{ab}	8.8±1.9^{b}	58.5±6.3^{ab}	29.1±4.3^{c}	0.27±0.05^{bc}	0.30±0.03^{bc}	252.2±11.9^{bc}	256.9±8.8^{abc}
HYD1	11.7±1.8^{b}	12.9±1.9^{ab}	40.9±4.8^{bc}	40.1±3.4^{bc}	0.29±0.01^{bc}	0.31±0.03^{b}	280.7±10.8^{abc}	314.5±22.5^{ab}
HYD2	17.1±2.0^{a}	10.1±1.8^{b}	60.8±8.0^{a}	32.2±1.6^{c}	0.33±0.04^{b}	0.29±0.05^{bc}	276.2±7.4^{abc}	317.8±27.0^{a}
Control	15.4±1.4^{ab}	1.4±0.5^{c}	53.6±0.8^{ab}	20.7±3.1^{c}	0.30±0.03^{bc}	0.09±0.05^{c}	224.7±6.0^{c}	285.9±6.3^{ab}

B	**d.f.**	**$F_{seed\ yield}$**	**Sig. seed yield**	**F Biomass**	**Sig. Biomass**	**F HI**	**Sig. HI**	**F LMA**	**Sig. LMA**
Var	4	**4.15**	*	**2.71**	*	**17.6**	***	**5.37**	**
Treat	1	**6.20**	*	**51.08**	**	0.54	ns	**7.98**	*
Var*Treat	4	**3.88**	*	**4.27**	**	**7.48**	***	2.14	n.s.

Table 2. Numerical values for fluorescence parameters derived from the chlorophyll *a* fast florescence transient in leaves of soybean varieties: (i) normalized data as V_j and V_i, (ii) quantum yields and flux ratios as φ_{Po}, Ψ_o, φ_{Eo}, (iii) performance index ($Pi_{\_Abs}$) and (iv) specific energy fluxes per Q_A^-reducing photosystem II centers as ABS/RC, TR_0/RC, ET_0/RC, DI_0/RC. Definitions and formulae are given in materials and methods and in the Appendix SI. Values are means ± SE from independent measurements (n=4). Two-way analysis of variance (ANOVA) for the effects of variety (Var.) and water treatment (Treat.), with their interaction factor (Var.×Treat.) on production traits, with between- and within-subject factors carried out to assess statistical significance of mean differences is shown. **P*<0.05; ***P*<0.001; ****P*<0.0001; n.s., not significant. Different letters denote statistically significant differences at α=0.05 after Student–Newman–Keul test. No letters indicate no significant differences.

		HY1		HY2		HYD1		HYD2		Control		ANOVA		
		WW	D	WW	D	WW	D	WW	D	WW	D	Var. F./Sig	Treat. F./Sig	Var*Treat F./Sig
Normalized data	V_j	0.49±0.02^{bc}	0.51±0.03^{bc}	0.46±0.02^{c}	0.54±0.02^{bc}	0.55±0.02^{b}	0.54±0.01^{bc}	0.50±0.02^{bc}	0.49±0.01^{bc}	0.50±0.01^{bc}	0.53±0.01^{bc}	**2.93***	2.98$^{n.s.}$	2.15$^{n.s.}$
	V_i	0.79±0.03	0.73±0.02	0.78±0.01	0.77±0.02	0.80±0.03	0.80±0.02	0.80±0.02	0.74±0.01	0.77±0.02	0.76±0.02	1.39$^{n.s}$.	**5.30***	1.02$^{n.s.}$
Quantum yields and flux ratios	φ_{Po}	0.83±0.01	0.82±0.01	0.83±0.01	0.81±0.01	0.81±0.01	0.81±0.01	0.82±0.00	0.82±0.00	0.83±0.00	0.81±0.01	0.99$^{n.s..}$	5.51*	3.053*
	Ψ_o	0.51±0.02^{bc}	0.49±0.03^{bc}	0.54±0.02^{b}	0.46±0.02^{bc}	0.45±0.02^{c}	0.46±0.01^{bc}	0.50±0.02^{bc}	0.51±0.01^{bc}	0.50±0.01^{bc}	0.47±0.01^{bc}	**2.93***	2.98$^{n.s}$	2.14$^{\mathbf{n.s.}}$
	φ_{Eo}	0.42±0.02^{bc}	0.40±0.03^{bc}	0.45±0.02^{b}	0.37±0.02^{bc}	0.36±0.02^{c}	0.37±0.01^{bc}	0.41±0.02^{bc}	0.42±0.01^{bc}	0.41±0.01^{bc}	0.38±0.01^{bc}	**2.75***	3.73$^{n.s}$.	2.39$^{n.s.}$
	φ_{Do}	0.16±0.01^{c}	0.18±0.01^{bc}	0.17±0.01^{bc}	0.19±0.01^{b}	0.19±0.01^{b}	0.19±0.00^{bc}	0.18±0.00^{bc}	0.18±0.00^{bc}	0.17±0.00^{bc}	0.19±0.01^{b}	0.99$^{n.s.}$	**5.51***	**3.05***
Specific energy fluxes	ABS/RC	2.29±0.07	2.48±0.05	2.42±0.10	2.54±0.09	2.37±0.10	2.33±0.07	2.57±0.08	2.37±0.06	2.35±0.09	2.59±0.11	1.23$^{n.s.}$	0.54$^{n.s.}$	2.59$^{n.s.}$
	TR_o/RC	1.90±0.05	2.02±0.02	2.01±0.07	2.05±0.05	1.91±0.07	1.90±0.06	2.09±0.06	1.95±0.04	1.96±0.07	2.09±0.07	1.62$^{n.s}$.	0.56$^{n.s.}$	1.84$^{n.s..}$
	ET_o/RC	0.96±0.03^{abc}	1.00±0.05^{abc}	1.09±0.06^{a}	0.94±0.03^{abc}	0.85±0.03^{c}	0.88±0.05^{bc}	1.05±0.05^{ab}	0.99±0.02^{abc}	0.97±0.02^{abc}	0.99±0.02^{abc}	**5.21****	1.08$^{n.s.}$	1.76$^{n.s.}$
	DI_o/RC	0.39±0.03	0.46±0.03	0.41±0.03	0.49±0.04	0.46±0.03	0.43±0.01	0.47±0.03	0.42±0.02	0.40±0.02	0.49±0.04	0.30$^{n.s.}$	3.42$^{n.s.}$	2.83*
	Pi_{ABS}	2.44±0.16^{c}	2.02±0.30^{abc}	2.24±0.18^{bc}	1.64±0.06^{ab}	1.62±0.15^{ab}	1.62±0.09^{ab}	1.67±0.12^{ab}	2.13±0.05^{bc}	2.21±0.15^{bc}	1.39±0.09^{a}	**4.48****	**8.51** **	**5.73****

Table 3. Aminoacids (μmol g^{-1} FW) in the five varieties (*HY1. HY2. HYD1. HYD2.* Control) under well-watered and drought. Data are means ± E.S. (n=4). Two-way analysis of variance (ANOVA) for the effects of variety (Var.) and water treatment (Treat.) with their interaction factor (Var.×Treat.) on amminoacids with between-and within - subject factors carried out to assess statistical significance of mean differences is shown. *P<0.05; **P<0.001; ***P<0.0001. n.s. not significant. Different letters denote statistically significant differences at p_0.05 after Student–Newman–Keul test. No letters indicate no significant differences.

	HY1		HY2		HYD1		HYD2		Control		ANOVA		
	WW	D	WW	D	WW	D	WW	D	WW	D	Var. F./Sig	Treat. F./Sig	Var*Treat F./Sig
Asp	0.7±0	0.7±0.1	0.7±0.1	0.6±0	0.7±0	0.6±0	0.7±0.1	0.6±0	0.8±0.2	0.8±0.1	1.10 $^{n.s}$	1.75$^{n.s}$	0.08 $^{n.s}$
Asn	1.1±0.5	2.4±0.6	0.7±0.4	2.0±0.6	0.8±0.2	2.2±1.2	0.2±0.4	1.0±0.5	1.8±1	0.2±0.3	0.77 $^{n.s}$	2.04 $^{n.s}$	1.36 $^{n.s}$
Glu	1.7±0.2	1.7±0.2	1.3±0.2	1.2±0.2	1.6±0.1	1.3±0.1	1.5±0.2	1.3±0.1	1.9±0.4	1.4±0.3	3.15 $^{n.s}$	2.00 $^{n.s}$	0.38 $^{n.s}$
Gln	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.0	3.02 $^{n.s}$	0.35 $^{n.s}$	0.93 $^{n.s}$
Gly	0.5±0.0^{a}	0.4±0.1^{a}	0.3±0.0^{b}	0.4±0.1^{a}	0.3±0.0^{b}	0.3±0.0^{b}	0.3±0.0^{b}	0.3±0.0^{b}	0.3±0.0^{b}	0.3±0.0^{b}	**6.17** ***	1.02 $^{n.s}$	**4.46** **
Ser	0.8±0.1	0.6±0.0	0.7±0.2	0.7±0.1	0.6±0.1	0.6±0.1	0.5±0.0	0.6±0.1	0.6±0.1	0.6±0.1	1.60 $^{n.s}$	0.03 $^{n.s}$	0.73 $^{n.s}$
Phe	0.6±0.1^{b}	0.6±0.1^{b}	0.4±0.0^{b}	1.1±0.3^{b}	0.4±0.0^{b}	0.5±0.1^{b}	0.4±0.0^{a}	0.4±0.1^{b}	0.4±0.0^{b}	0.4±0.0^{b}	**2.76** *	**5.80** *	**2.63** *
Tyr	0.5±0.1^{ab}	0.4±0.0^{b}	0.4±0.0^{b}	0.7±0.1^{a}	0.4±0.0^{b}	0.5±0.0^{b}	0.4±0.0^{b}	0.4±0.0^{b}	0.4±0.0^{b}	0.4±0.0^{b}	2.44 $^{n.s}$	1.878 $^{n.s}$	**3.54** *
Val	0.4±0.1^{b}	0.4±0.1^{b}	0.2±0.0^{b}	0.8±0.3^{a}	0.2±0.0^{b}	0.3±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.3±0.1^{b}	**2.97** *	**4.79** *	**2.83** *
Leu	0.7±0.1^{ab}	0.5±0.1^{ab}	0.3±0.0^{b}	0.8±0.2^{ab}	0.3±0.0^{b}	0.4±0.0^{b}	0.3±0.0^{b}	0.4±0.1^{b}	0.3±0.0^{b}	0.4±0.1^{b}	**3.19** *	2.62 $^{n.s}$	**3.14** *
Ile	0.5±0.1^{b}	0.5±0.1^{b}	0.3±0^{b}	0.9±0.2^{a}	0.3±0.0^{b}	0.4±0.0^{b}	0.3±0.0^{b}	0.4±0.1^{b}	0.3±0.0^{b}	0.4±0.0^{b}	**3.18** *	**5.83** *	**2.88** *
Ala	10.6±1.1^{b}	5.5±0.5^{a}	7.7±1.3^{a}	6.4±0.7^{a}	7.3±1.5^{a}	5.5±0.6^{a}	4.7±0.5^{a}	7±0.6^{a}	6.6±1.2^{a}	4.3±1.0^{a}	2.27 $^{n.s}$	**7.45** ***	**3.86** ***
Thr	0.2±0.2	0.1±0.1	0.2±0.2	0.1±0.1	0.5±0.1	0.5±0.0	0.5±0.0	0.3±0.2	0.7±0.3	0.4±0.1	2.85 *	1.21 $^{n.s}$	0.48 $^{n.s}$
Met	0.3±0.1	0.2±0.0	0.±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.60 $^{n.s}$	0.68 $^{n.s}$	2.51 $^{n.s}$
Lys	0.2±0.0^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	**10.06** ***	**10.74** **	**7.92** ***
His	0.2±0.1^{b}	0.0±0.0^{ab}	0.0±0.0^{a}	0.2±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.1±0.1^{ab}	0.1±0.1^{ab}	0.1±0.1^{ab}	0.1±0.1^{ab}	**3.62** *	0.00 $^{n.s}$	**7.19** ***
Pro	0.8±0.1^{b}	1.9±0.8^{b}	0.4±0.1^{b}	5.4±2.2^{a}	0.4±0.1^{b}	0.7±0.2^{b}	0.3±0^{b}	1.2±0.5^{b}	0.4±0.1^{b}	1.4±0.7^{b}	2.37 $^{n.s}$	**8.02** *	2.32 $^{n.s}$
Arg	0.8±0.1	0.5±0.1	0.7±0.1	0.6±0.1	0.7±0.1	0.5±0.1	0.4±0.0	0.5±0.0	0.5±0.1	0.5±0.1	1.43 $^{n.s}$	2.98 $^{n.s}$	1.71 $^{n.s}$
GABA	0.9±0.2^{b}	0.6±0.0^{a}	0.6±0.0^{a}	0.6±0.1^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.3±0.1^{a}	0.4±0.1^{a}	0.5±0.0^{a}	**7.93** ***	1.49 $^{n.s}$	**3.15** *