



Additive effects of heatwave and water stresses on soybean seed yield is caused by impaired carbon assimilation at pod formation but not at flowering

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ABSTRACT

Heatwave (HW) combined with water stress (WS) are critical environmental factors negatively affecting crop development. This study aimed to quantify the individual and combined effects of HW and WS during early reproductive stages on leaf and nodule functioning and their relation with final soybean seed yield (SY). For this purpose, during flowering (R2) and pod formation (R4) soybean (*Glycine max* L. Merr.) plants were exposed to different temperature (ambient[25°C] versus HW[40°C]) and water availability (full capacity versus WS[20% field capacity]). HW, WS and their combined impact on yield depended on the phenological stage at which stress was applied being more affected at R4. For gas exchange, WS severely impaired photosynthetic machinery, especially when combined with HS. Impaired photoassimilate supply at flowering caused flower abortion and a significant reduction in final SY due to interacting stresses and WS. On the other hand, at pod formation (R4), decreased leaf performance caused additive effect on SY by decreasing pod setting and seed size with combined stresses. At the nodule level, WS (alone or in combination with HW) caused nodule impairment, which was reflected by lower leaf N. Such response was linked with a poor malate supply to bacteroids and feed-back inhibition caused by nitrogenous compounds accumulation. In summary, our study noted that soybean sensitivity to interacting heat and water stresses was highly conditioned by the phenological stage at which it occurs with, R4 stage being the critical moment. To our knowledge this is the first soybean work integrating combined stresses at early reproductive stages.

1. Introduction

Soybean (*Glycine max* (L.) Merr.) is the most cultivated legume with over 124.9 million hectares worldwide and the fourth most important crop in terms of seed production (i.e., 353 million tons produced in 2020) (FAOSTAT, ProdStat. Core Production Data Base, Electronic resource, 2022. (<http://faostat.fao.org/>) (accessed March 4, 2022)). This legume is a rich source of high quality proteins and oil for livestock and human consumption (Ainsworth et al., 2012; Medic et al., 2014). However, soybean production is severely limited by several abiotic factors (Raza et al., 2020; Ostmeyer et al., 2020). Among them, exposure to periods of extreme heat (or heatwaves) is recurrent in primary soybean production areas (Deryng et al., 2014; Perkins, 2015; Herritt and Frittschi, 2020). Additionally, since much of the cultivated area is rain-fed, lack of precipitation is the other limiting factor affecting soybean

production (Liu et al., 2004).

Indeed, high-temperature and water stress are the most influential climate variables that negatively affect plant growth and development, causing significant yield losses on a global scale (Matiu et al., 2017). In legumes, the effects of heat (Nahar et al., 2016) and water (Farooq et al., 2009) stresses physiology and metabolism have been studied widely in isolation, but their potential combined impact is less clearly defined (Awasthi et al., 2014). Plant responses to heat and water stress have much in common and mainly depend on growth stage and the duration and intensity of stress (Farooq et al., 2017). Overall, at the leaf level, both stresses decrease photosynthesis rate, due not only to an increase in internal resistance to CO₂ diffusion caused by stomatal closure, but also due to an increase in photodamage of photosystem II (PSII) and leaf temperature, thereby favoring ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) oxygenase activity (Prasad et al., 2008; Ergo

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et al., 2018). At the legume nodule level, water stress has been widely reported to decrease nitrogenase activity by reducing carbohydrate and O₂ availability to the bacteroid and by increasing accumulation of N-compound and oxidative stress (Aranjuelo et al., 2008; Larrainzar et al., 2009; Soba et al., 2019). However nodule function under heat (especially under combined stresses) has not been characterized in detail, highlighting the necessity to conduct further studies considering these factors (Aranjuelo et al., 2014).

In terms of productivity, both stresses impair legume grain yield at all growth stages. However, damage during reproductive stages (flowering and pod formation and filling) is the major reason for yield loss (Pushpavalli et al., 2015; Sehgal et al., 2017). Yield losses during reproductive stages have been directly linked to a decrease in photosynthetic performance, resulting in a poor synthesis/translocation of assimilates to flowers and pods that causes weak pollination and higher rates of flower abortion and, during pod and grain filling, poor cell division (during pod and grain filling) that resulted in fewer and smaller seeds with both high temperature (Djanaguiraman, 2010; Puteh et al., 2013; Bhandari et al., 2017) and water stress (Farooq et al., 2017; Du et al., 2020). Additionally, number composition and viability of pollen grains is highly sensitive to high temperature (Djanaguiraman and Prasad, 2013) and water stress (Al-Ghazawi et al., 2009), which lead to decreased pod set percentage. As a final consequence, both stresses negatively affect seed yield (SY) and their components such as number of pods per plant (PN), number of seeds (SN) or thousand seed weight (TSW) (Puteh et al., 2013; Egli et al., 2005).

As shown, the influence of heat and water stress (as single-stress factors) on soybean physiological and metabolic processes are well documented; however, studies focused on effects of combined stresses (especially short periods of extreme heat or heatwaves) are scarce. Nonetheless, these two abiotic stresses often occur together and are expected to increase in the near future (Schär et al., 2004; Vautard et al., 2007; IPCC, Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, 2014.). Previous work has shown that pathways regulating plant responses to heat and water stress are largely distinct (Rizhsky et al., 2004) and that we cannot extrapolate plant responses to stress combinations simply by adding the two single stress responses (Mittler, 2006; Zandalinas et al., 2018). In general, heat and water stress in combination has been shown to more severely impact physiology and yield compared to heat or water stress alone (Balfagón et al., 2020). This emphasizes the need to study stress combinations to identify traits that confer tolerance to heat and water stress for developing more tolerant varieties (Rizhsky et al., 2004; Ahmed et al., 2013; Cohen et al., 2021). In legumes, the combined effects of heat and water stress on physiological and yield parameters have been studied in some species, e.g., peanut (*Arachis hypogaea* L.) (Hamidou et al., 2013), chickpea (*Cicer arietinum* L.) (Awasthi et al., 2014; Canci and Toker, 2009), lentil (*Lens culinaris* Medikus) (Sehgal et al., 2017) and soybean (Ergo et al., 2018; Jumrani and Bhatia, 2018). However, the specific effect of heatwave alone or in combination with water deficit has not yet been addressed. Additionally, most combination stress studies have focused on the grain filling period (R5) with a lack of studies during earlier key reproductive stages (from the beginning flowering to full pod formation, i.e. R1-R4). Finally, legume nodule performance under combined stresses and their relation with flowering and seed formation are clear gaps demanding more attention.

Therefore, understanding the effect of heatwave, water stress, and their interactions at flowering and full pod stages on developmental and biochemical processes at leaf and nodule levels is valuable for improving grain yield under future climatic conditions. With this in mind, the present study aimed to (i) analyze the effects of heatwave and water stress (alone or in combination) on soybean leaf and nodule performance during flowering and pod formation; and (ii) link these effects on leaf and nodule functioning at these specific stages with final soybean seed yield.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was conducted with Sumatra soybean cultivar (*Glycine max* (L.) Merr.). Sumatra is the maturity group I cultivar and is the most cultivated in Spain cultivar for human consumption. Seeds were germinated in Petri dishes and, germinated plants were transplanted to 3 L black pots (one plant per pot) containing a substrate filled with 2:1 (v/v) perlite/vermiculite. The experimental design was as follows; two factors: treatment (Control, heatwave (HW), water stress (WS) and heatwave x water stress combination (HW x WS)) and phenological stage in which the treatments were applied (full flowering (R2)) and full pod stage (R4) (Fehr et al., 1971). Ten replications for each treatment combination of the two factors (80 pots in total) were used. Five replications for biomass, biochemical and metabolic measurements just after the imposition of treatments and five replications for yield and yield parameters at maturity. Randomized block design was used in order to avoid spatial effects in greenhouse.

During the first and second week, plants were inoculated twice with commercial *Bradyrhizobium japonicum*. To ensure that the sole N source was N₂ fixed by nodules, the plants were watered with an N-free nutrient solution exactly as described by Soba et al. (Soba et al., 2019) and microelements as recommended by Hoagland and Arnon (Hoagland and Arnon, 1950) twice weekly.

Pots were placed in a controlled greenhouse at 25/18 °C (day/night) under natural daylight. The greenhouse was located at the Institute of Agrobiotechnology (IdAB) (42° 47'N, 1° 37'W; Pamplona, Spain). Soybean plants growth under these conditions until the imposition of the treatments at R2 and R4 in each case. At full flowering (R2) and full pod stage (R4) the different stresses were applied in two independent set of plants.

For water stressed plants, suppression of irrigation was maintained for 7 days until 20% of full substrate water-holding capacity was reached, while plants in well irrigated groups were maintained at full substrate water-holding capacity.

Heatwave treatments were conducted in a growth chamber (Fitoclima, 1200 PLH, Aralab Bio, Portugal). Before heat wave imposition, plants were grown under greenhouse similar conditions for three additional days for proper chamber acclimation. Growth chamber conditions were set to 14 h of light (1000 μmol m⁻² s⁻¹), 25 °C and 50% relative humidity during the light hours (6 am to 8 pm) and 18 °C and 70% relative humidity during darkness. Temperatures were increased from 18° to 25°C, 1 h after lights were turned on and returned to 18 from 25 °C, one h after lights were turned off. After these three days, of maintaining photoperiod and humidity, the temperature treatment was initiated, and was imposed as follows: the temperature was increased gradually from 30 °C at 6:00 am to reach 40°C by 9:00 am and held constant until 5:00 pm. The temperature was then reduced from 40° to 30°C from 5:00 pm to 8:00 pm and maintained at 30 °C until 6:00 am. Since elevated temperatures occurring in the field during the growing season often come in the form of heatwaves that generally last a few days, temperature treatments were imposed over the course of 4 days.

Just at the end of the different treatments, half of the plants of each set were harvested; gas exchange measurements were conducted before plants were separated into leaves, shoot, roots and nodules; samples were immediately frozen in liquid N and stored at - 80 °C for further analyses. Subsamples of each organ were oven dried for 48 h at 60 °C prior to dry weight (DW) determinations. The remaining plants from each treatment were maintained until maturity harvest under controlled greenhouse conditions. At maturity harvest (R8), aboveground biomass, yield and yield components such as PN, SN and TSW were recorded.

The same four treatments and set of measures were carried out during full pod stage (R4) with the other set of the plants.

2.2. Gas exchange and chlorophyll fluorescence determinations

Gas exchange and chlorophyll fluorescence were measured at the end of the different treatments (imposed at R2 and R4 developmental stages). The former were conducted with a Li-Cor 6400 portable gas exchange photosynthesis system (LI-COR, Lincoln, NE, USA) on fully expanded apical leaves. Measurements were carried out at the end of the treatments under conditions similar to growth conditions (1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and 400 $\mu\text{mol mol}^{-1} \text{CO}_2$) and block temperature depending of the group (control and WS; 25°C and HW and HW x WS; 40°C). Photosynthetic CO_2 assimilation (A_n) was determined using equations developed by von Caemmerer and Farquhar (von Caemmerer and Farquhar, 1981). Stomatal conductance (g_s) was determined as described by Harley et al. (Harley et al., 1992). Plants were dark-adapted for 50 min prior to dark respiration (R_D) measurements (Nogués et al., 2004).

Fluorescence parameters were measured with a fluorescence chamber (LFC 6400–40; LI-COR) coupled to the Li-Cor 6400. Light-adapted variables included electron transport rate (ETR), quantum yield of photosystem II photochemistry (Φ_{PSII}), photochemical quenching (qP), and non-photochemical quenching (qN). Leaves were then dark-adapted for 20 min and maximum quantum yield of PSII photochemistry (F_v/F_m) were measured.

2.3. Leaf and grain nitrogen content

The nitrogen (N) content in leaf samples were determined based on sample dynamic combustion, using an elemental analyzer (FlashEA1112, ThermoFinnigan, Waltham, Massachusetts, USA) equipped with a MAS200R autosampler. Samples were weighed in a tin capsule (MX5 microbalance, Mettler-Toledo, Columbus, Ohio, USA) and introduced into a quartz reactor filled with WO_3 and copper before being heated at 1020 °C. The combustion gas mixture was carried by a helium flow to a WO_3 layer to achieve a complete quantitative oxidation, followed by a reduction step in a copper layer to reduce nitrogen oxides and SO_3 to N_2 and SO_2 . The resulting components, N_2 , CO_2 , H_2O , and SO_2 were separated in a chromatographic column (Porapak 2 m, Santa Clara, California, USA) and detected with a thermal conductivity detector.

2.4. Chlorophyll and anthocyanins content

Chlorophyll (Chl) and anthocyanins (Anth) content was estimated using a portable non-destructive a DUALEX sensor (Dualex Scientific, Force A, France). Chlorophyll was measured in $\mu\text{g}/\text{cm}^2$ in the range of 5–80 $\mu\text{g}/\text{cm}^2$. Anthocyanins content were measured using relative absorbance units from 0 to 1.5.

2.5. Organic acids determinations

Organic acids were determined in frozen tissue collected just at the end of the treatments at both phenological stages (R2 and R4). For oxalic, oxaloacetic, malic, citric and succinic acid extractions, leaf and nodule fresh weight frozen material was ground to a fine powder in liquid nitrogen with a pestle and mortar and 50 mg was weighted and resuspended in 500 μL of HClO_4 1 M, left at 4 °C for 30 min and centrifuged for 15 min at 14,000 g at 4°C. Organic acids contents from supernatants were then determined by High Performance Liquid Chromatography (HPLC). Chromatographic separation was performed using a Waters Alliance HPLC system (Waters, Milford, MA, USA) and a Synergi Hydro-RP 80 Å column (250 × 4.6 mm i.d., particle size = 4 μm ; Phenomenex) with a flow of 0.8 mL/min mobile phase (20 mM KH_2PO_4 , adjusted to pH 2.9 with concentrated H_3PO_4) and 40 °C column temperature. The total run time per sample was 20 min. Organic acids were monitored at 210 nm and quantified based on standard calibration curves generated with authentic oxalic, oxaloacetic, malic, citric and succinic acids (Sigma-Aldrich). Before statistical analysis quantification

was normalized to DW in order to avoid any discrepancy due to changes in tissue water content.

2.6. Free amino acid and sugar determinations

Free amino acid and soluble sugars determinations were conducted on leaf and nodule samples collected just at the end of the treatments at both phenological stages (R2 and R4). For amino acids, frozen plant tissues were ground to a fine powder in liquid nitrogen and a sub-sample was lyophilised. Lyophilised plant tissue (20 mg) was homogenized in 400 μL of 80% ethanol using a vortex, incubated at 80 °C for 1 h, and centrifuged at 14,000 g and 4 °C for 10 min until the pellet was completely dehydrated. The pellet was re-suspended in 100 μL of milli-Q water and centrifuged at 14,000 g and 4 °C for 10 min before supernatant collection. The amino acid content in the supernatant was determined by HPLC (Waters Corporation, Barcelona, Spain) after derivatization with a ACCQ-Fluor™ Reagent kit (Waters, Milford Massachusetts, USA) based in borate buffer, acetonitrile and, AQC derivatizing reagent (6-aminoquinolyl-n-hydroxysuccinimidyl carbamate) as previously described (Cohen and Michaud, 1993).

For sucrose (Suc), glucose (Glu) and fructose (Fru) determinations, lyophilised plant tissue (25 mg) was first homogenized in 0.5 mL of 100% ethanol before another 0.5 mL of 80% ethanol was added and mixed using a vortex. Samples were incubated at 70 °C for 90 min, centrifuged at 14,000 g for 10 min prior to supernatant collection. The supernatant was used to determine Suc, Glu and Fru content with an ionic chromatographer (ICS-3000, Thermo Scientific™, Waltham, Massachusetts, USA). Starch was determined in leaf pellets after another ethanol extraction, using a amyloglucosidase test kit (R-BiopharmAG, Darmstadt, Germany).

2.7. Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 20.0. (IBM Corp. Armonk, NY, USA). Statistical differences between measurements of different treatments and phenological stages were evaluated independently with one way Analyses of Variance (ANOVA). Interactions between these two factors were evaluated with two way ANOVA. Tukey' post hoc tests were used to determine statistical differences between treatments at R2 and R4 independently. All data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test). The resulting *P*-values were considered to be statistically significant at $p < 0.05$. Asterisks indicate significant differences: * $p < 0.05$, ** $p < 0.01$.

3. Results

3.1. Biomass and yield parameters

With the exception of root DW, soybean biomass parameters at the end of R2 and R4 stress period were significantly affected by treatments (Fig. 1 and Table S1). In addition, the influence of phenological stage on biomass was always significant, but no interactions were significant for any biomass parameter. Overall, at flowering, drought had significant effect on biomass in opposition to heatwave; however, at pod formation only combination of stresses had a significant effect, except for nodule biomass (Fig. 1 and Table S1). For example, total biomass was reduced by 45.0% and 39.1% at R2 and R4 respectively when both stresses were applied in combination. Leaf and nodule biomass were parameters exhibiting the greatest reduction under the studied stresses. Finally, root/shoot ratio showed significant increases under combined stresses (Table S1).

Phenological stage had no effect on SY or its components with only thousand seed weight (TSW) and seed n° per pod showing significant differences between stages (Fig. 2). Seed yield, SN and PN exhibited

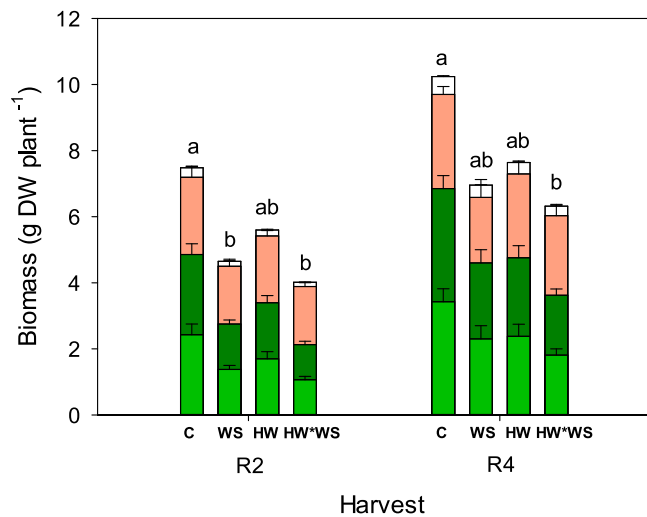


Fig. 1. Leaf, stem, root and nodule biomass (g DW plant⁻¹) of soybean plants grown under four conditions (control, HW, WS and HW x WS) and harvested at two different stages (R2 and R4). Bars correspond to mean ± SE of n = 5 for each tissue type. Results of statistics for total biomass (the sum of leaf, stem root, and nodule) are shown (two-way ANOVA, *p* < 0.05). Letters indicate significant differences independently for each harvest (R2 and R4) (Tukey posthoc test *p* < 0.05).

significant differences among treatments. The interaction between phenology and treatments was significant for SY and SN. When episodes of heat and water stress occurred concomitantly, SY was decreased at both the R2 and R4 stages (33.2% and 54.3%, respectively) (*p* < 0.05) in comparison to the control. Similarly, WS plants at both stage and HW plants at R4 also exhibited reduced SY (Fig. 2 and Table S1). Similarly to SY, PN values decreased (at both phenological sta) when the stresses were applied at the same time. In comparison, for SN and TGW the effect

of HWxWS was significant when the stresses were applied at R4. Water stress only reduced PN at R2. On the other hand, HW reduced PN and SN when the stress was applied at R4.

3.2. Gas exchange and chlorophyll fluorescence

Photosynthetic rate and *g_s* were found to be significantly lower under water stress and combined heat and water stress (Table 1). This decrease was found when stresses were applied both at R2 and R4. However, no significant differences were found when heat wave occurred alone. Transpiration (*T_r*) values under HW were significantly higher than the control and values for WS and HWxWS were significantly lower; therefore, higher water use efficiency (WUE) values were found in water stress treatments. Higher vapor pressure deficit (VPDL) and leaf temperature (*T_{leaf}*) values (*p* < 0.05) were noted for plants under heat-wave (and their combination with water stress) at both growth stages. On the other hand, the highest *R_p* values were found in plants subjected to heatwave alone followed by plants under combined stresses (Table 1).

Among chlorophyll fluorescence parameters, phenological stage had no significant effect on any parameter. Parameters for light acclimation (i.e., *F_v/F_m*, *φPSII*, *ETR*, *qP* and *qN*), were significantly reduced under WS (alone or combined with HW). The dark-adapted parameter *F_v/F_m* did not exhibit differences for stages or treatments (Table 1).

Finally, Chl and Anth pigments showed antagonistic trends. For example, leaves of HW plants had higher Chl content whereas Anth was lowest in these plants.

3.3. Carbohydrates

The three most important carbohydrates implied in glycolysis (Sucrose, glucose and fructose) were assessed in leaves and nodules. Additionally, starch, the most abundant storage carbohydrate in plants, was analyzed in leaves (Table 2). Leaf carbohydrate concentration generally decreased under water stress particularly at flowering and

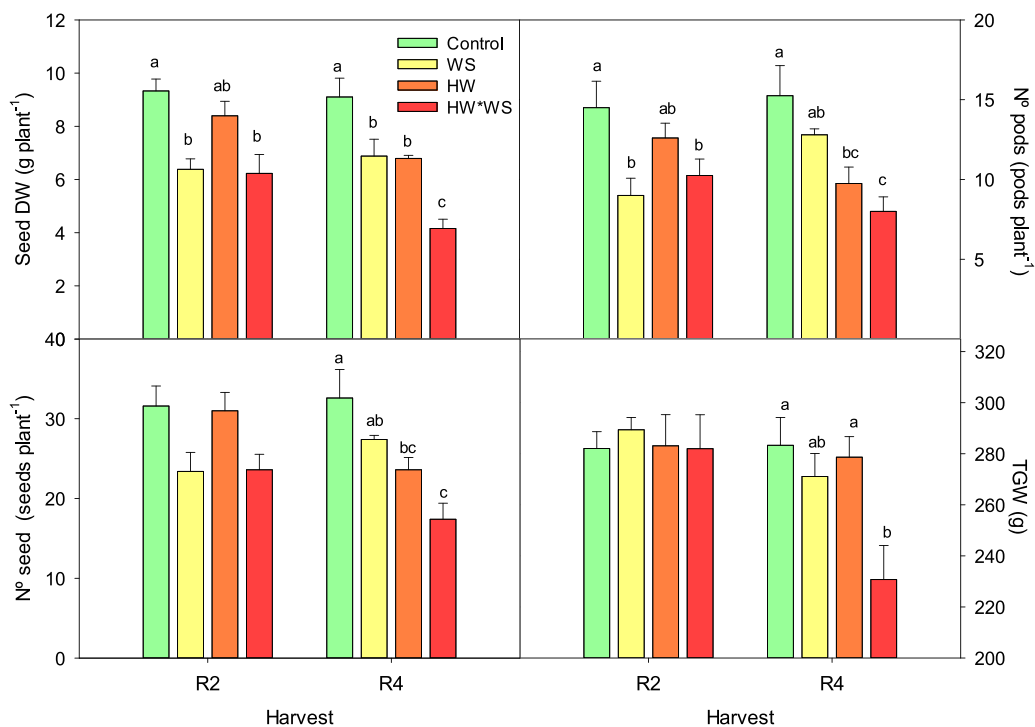


Fig. 2. Seed DW, N° pods, N° seed, and thousand seed weight (TSW) for soybean plants grown under four water and temperature treatments: control (non-heat-stressed and non-water-stressed), water-stressed (WS), heatwave (HW), and heatwave × water-stressed (HW × WS) plants at two developmental stages (R2 and R4 harvest). Bars show mean and standard errors (n = 5). Letters indicate significant differences independently for each harvest (R2 and R4) (Tukey posthoc test *p* < 0.05).

Table 1

Mean values ($n = 5$) of leaf gas exchange, chlorophyll fluorescence parameters and Chl and Anth content for soybean plants just after the four treatments [control (C), water stress (WS), heatwave (HW) and their combination (HWxWS)] were applied at two phenological stages [flowering (R2) and pod formation (R4)]. P – phenology; T-treatments.

	R2				R4				ANOVA		
	C	WS	HW	HWxWS	C	WS	HW	HWxWS	P	T	Interac.
A_n	22.9 a	4.6 b	19.2 a	3.6 b	23.4 a	7.6 b	18.7 a	3.1 b	n.s.	**	n.s.
g_s	0.36 a	0.02 b	0.27 a	0.01 b	0.29 a	0.04 b	0.23 a	0.02 b	n.s.	**	n.s.
C_i	274.8	141.6	278.8	175.6	245 a	88c	224 ab	141 BCE	n.s.	*	n.s.
T_r	4.70 b	0.37c	7.37 a	0.40c	4.02 b	0.72c	6.83 a	0.89c	n.s.	**	n.s.
VPDL	1.30 b	1.63 b	2.54 a	2.77 a	1.34c	1.83c	2.88 b	3.90 a	n.s.	**	n.s.
WUE	64c	211 b	65c	376 a	82 b	185 a	92 b	149 a	n.s.	**	n.s.
$T^{\circ}\text{leaf}$	26.1c	28.3 b	32.8 a	35.5 a	25.7 b	27.3 b	32.2 a	34.2 a	n.s.	**	n.s.
RD	-2.33 ab	-1.82 b	-3.66 a	-3.36 ab	-2.73 b	-2.84 b	-3.67 a	-3.01 ab	n.s.	*	n.s.
F_v/F_m	0.79	0.80	0.82	0.81	0.81	0.82	0.82	0.80	n.s.	n.s.	n.s.
F_v'/F_m'	0.56 a	0.39 b	0.52 a	0.32 b	0.58 a	0.50 b	0.49 b	0.43c	n.s.	**	n.s.
ΦPSII	0.30 a	0.10c	0.24 b	0.09c	0.28 a	0.18 BCE	0.25 ab	0.12c	n.s.	**	n.s.
ETR	157 a	53 b	135 a	49 b	156 a	102 BCE	137 ab	65c	n.s.	**	n.s.
qP	0.51 a	0.24 b	0.47 a	0.27 b	0.49 a	0.36 b	0.51 a	0.27 b	n.s.	**	n.s.
qN	2.32 a	1.66 b	2.10 a	1.50 b	2.43 a	2.01 ab	1.96 ab	1.77 b	n.s.	**	n.s.
Leaf N	3.61 ab	3.19 b	4.65 a	3.60 ab	3.56 ab	3.36 b	4.28 a	3.84 ab	n.s.	**	n.s.
Chl	36.0 a	33.7 b	38.3 a	32.0 b	41.1 a	37.1 b	40.2a	34.6 b	**	**	*
Anth	0.09 a	0.09 a	0.07 b	0.09 a	0.08 ab	0.08 ab	0.07 b	0.09 a	n.s.	**	n.s.

An: leaf net CO₂ assimilation ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); g_s : stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$); C_i : internal CO₂ concentration (ppm); T_r : transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$); VPDL: vapor pressure deficit (kPa); WUE: water use efficiency ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$); $T^{\circ}\text{leaf}$: foliar temperature ($^{\circ}\text{C}$); RD: respiratory rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); F_v/F_m : maximum photochemical quantum yield of PSII; ΦPSII : effective photochemical quantum yield of photosystem II; ETR: maximum rate of electron transport ($\mu\text{mol e}^{-} \text{ m}^{-2} \text{ s}^{-1}$); qP : photochemical quenching; qN : non-photochemical quenching; Leaf N: Leaf N concentration (%); Chl: chlorophyll content (Dualex units); Anth: anthocyanins (Dualex units). Letters indicate significant differences (Tukey posthoc test; $p < 0.05$) independently for each developmental stage. Significance levels: * ($p < 0.05$), ** ($p < 0.01$), n.s. (differences not significant; $p < 0.05$).

when water and heat stress were combined (Table 2). Sucrose (the main sugar in nodules), was significantly higher at both growth stages when stresses were applied alone or in combination. Leaf starch content showed similar trends at both developmental stages, with significant decreases under heat stress (particularly under water stress and their combination with heat).

3.4. Organic acids

Organic acids are important metabolites involved in redox regulation and energy balance in higher plants. In the present study, five target organic acids (oxaloacetate, malate, citrate, succinate and oxalate) were analyzed in leaves and nodules. In leaves, HW alone has not significant effect on leaf organic acids, except for the case of oxalate which was increased under this stress in both phenological stages. On the other hand, WS alone only reduced significantly, compared to control plants, the levels of citrate and malate during flowering (Fig. 3). However, when combined with HW, reductions in these two dicarboxylic acids were significant at both stages; additionally succinate was also reduced at R2 stage when both stresses were applied concomitantly. When compared to WS, HW produced a significant increase on malate and oxalate levels.

In nodules, water stress reduced citrate, malate and succinate contents at flowering in comparison to the control; however, when the stress was applied at R4 only malate content was reduced (Fig. 3). Significant lower levels of malate were observed in plant suffering HW at both stages. When combined with HW, the reduction was generally occurred at both phenological stages with the exception of oxaloacetate (OAA). Levels of oxalate were higher in nodules of plants under HWxWS.

3.5. Amino acids

At the two phenological stages, 18 amino acids were quantified in leaf and nodule tissue across the four different water and heat treatments. Quantitations were normalized to sample DW to avoid discrepancies due to changes in water content under WS conditions. Significant differences in amino acids between treatments and phenological stage are shown in Table 3.

In leaves, when the treatments were applied at flowering, a significant accumulation in all amino acids (except for glutamine (Glu) and arginine (Arg)) were found under heatwave and interacting stresses. In contrast, at R4, the trend was not clear; while plants under drought accumulated higher contents of proline (Pro), branched-chain amino acids (isoleucine (Ile), leucine (Leu) and valine (Val)) were found to be accumulated under the combination of stresses. Alanine (Ala), the main amino acid found in leaves, showed a significant decrease at both phenological stages in plants under drought stress when compared to heatwave (Table 3).

For nodules, significant differences were mainly seen in amino acids involved in long-distance transport of N [aspartate (Asp) and asparagine (Asn)], osmoregulatory functions [γ -aminobutyric acid (GABA)] and branched-chain amino acid [isoleucine (Ile), leucine (Leu) and valine (Val)] (Table 3). In this tissue, Asn was the most abundant amino acid found. Further, amino acid content showed a significant increase at both developmental stages in nodules of plants under WS and HWxWS when compared to nodules of plants subjected only to HW. Aspartate and Glu were also important amino acids in terms of concentration in nodules. Levels of Asp were always higher in nodules subjected to drought in comparison with the rest of treatments. On the contrary, highest levels of Glu were always found in HW nodules. GABA levels were higher in nodules of plants under the combination of stresses. Similarly to Asp, glycine (Gly) levels were higher in nodules of plants under drought.

4. Discussion

Physiological and metabolic mechanisms underlying SY responsiveness of soybean plants subjected to high temperature or water stress have been extensively studied; however, much less information is available on combined heatwave and water stresses that often occur in combination under field conditions (Schär et al., 2004; Vautard et al., 2007). Additionally, scant information currently exists regarding effects of interacting stresses on nodule performance and their implication for overall plant fitness and seed yield. In the current study, consequences (physiological, biochemical and metabolic) of short periods of extreme heat [heatwave (HW), water stress (WS) and their combination (HW x WS)] during reproductive period [flowering (R2) and full pod (R4)]

Table 2
Mean values (n = 5) and standard error for carbohydrates content (umoles/g) of leaves and nodules determined at flowering (R2) and pod formation (R4) for soybean grown under control (C), water stress (WS), heatwave (HW) and their combination (HWxWS) conditions.

	LEAF															
	R2 HARVEST				R4 HARVEST				R2 HARVEST				R4 HARVEST			
	Control	WS	HW	HWxWS	Control	WS	HW	HWxWS	Control	WS	HW	HWxWS	Control	WS	HW	HWxWS
Sucrose	25.93 ^a	7.57 ^{bc}	15.37 ^b	3.31 ^c	7.57	9.77	11.5	5.39	16.19 ^b	31.47 ^a	37.20 ^a	40.37 ^a	12.27 ^b	21.85 ^a	26.81 ^a	31.75 ^a
Glucose	11.08 ^{ab}	0.63 ^b	20.57 ^a	1.01 ^b	43.39 ^a	8.08 ^{bc}	17.31 ^b	2.3 ^c	2.7 ^a	1.46 ^{bc}	1.59 ^b	0.80 ^c	7.36 ^a	2.64 ^b	3.87 ^{ab}	1.64 ^b
Fructose	8.37 ^b	4.67 ^{bc}	15.06 ^a	2.47 ^c	24.23	12.21	20.92	8.28	n.d.	2.77	n.d.	2.50	n.d.	4.51 ^{ab}	3.13 ^b	5.22 ^a
Starch	554.8 ^a	72.2 ^c	262.7 ^b	23.0 ^d	613.5 ^a	34.6 ^c	380.0 ^b	20.5 ^c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Letters indicate significant differences (Tukey posthoc test; $p < 0.05$) independently for each developmental stage

were studied in leaves and nodules of soybean plants and their effect on SY was analyzed.

4.1. Leaf function

4.1.1. Photosynthetic performance

Leaves have a primary role in the production and transport of photoassimilates to seeds, thus, leaf function is closely related to final SY (Zandalinas et al., 2018; Aluko et al., 2021). In our experiment, leaf biomass was the plant tissue most affected by the stresses (Fig. 1 and Table S1). As observed in SY, leaf biomass was significantly decreased under WS (alone or in combination with HW) during flowering; however, leaf reduction was not significant under HW. These results are indicating that, when the treatments were applied at R2, drought effect on leaf biomass were predominant over heatwave and when combined the effects were not significantly more detrimental than the effect of drought. These results at flowering are in opposition with previous works in arabidopsis (Vile et al., 2012), soybean (Jumrani and Bhatia, 2018), chickpea (Awasthi et al., 2014), and wheat (Bakhshandeh et al., 2019) that found an additive effect of combined stresses on biomass. One possible explanation could be due to the phenological stage of the plants (most of the previous works were focused in vegetative and grain filling stages) and/or the short period of heat applied in the present study. On the other hand, when stresses occurred during full pod formation, only the combination of stresses significantly reduced both, leaf and total biomass (51.6% and 39.1%, respectively).

Photosynthetic function in leaves was inhibited when WS (alone or in combination with HW) was imposed at both R2 and R4 stages, as reflected by sharp reductions in A_n , g_s and Chl concentration (Table 1 and Fig. 4). However, photosynthetic parameters of HW plants were not affected at any developmental stage, which is in contrast to previous soybean studies (Vu et al., 1997; Jumrani and Bhatia, 2019). This, in part, could be due to the short duration of the heatwave conducted in the present work compared with other studies (with lower temperatures but longer periods of time). In plants under combined stresses, stomatal response to WS prevailed over that of HW at both key developmental stages. Therefore, in the balance between preventing water loss and protecting from over-heating it seems that the prevention of water loss via stomatal closure prevails over evaporative heat loss for cooling the leaf surface. Similar results in soybean have been shown recently by Katam et al. (Katam et al., 2020). However, Jumrani and Bhatia (Jumrani and Bhatia, 2019) found negative effects on photosynthesis under both temperature and water stresses. Our data suggest that plants under water scarcity suffer a sharp decrease in g_s in order to reduce water loss through transpiration (Table 1), which lead to a reduction in CO_2 uptake for photosynthesis and a subsequent reduction in plant productivity (Awasthi et al., 2014; Lawson and Blatt, 2014). In plants under combined HW x WS, high vapor pressure deficit (VPDL) caused a supplementary source for stomatal closure (Lobell et al., 2014). An additional effect of lower g_s is the observed increase in WUE (Table 1), mainly due to decreased water loss by transpiration, as widely observed in crops (Gorthi et al., 2019).

Maintenance of Chl in leaves is considered to be an important factor for maintaining higher A_n and, hence, better yields under environmental stresses such as heat and water deficit (Sharma et al., 2020). The observed greater loss of Chl under WS or HS x WS plants compared to HW alone has previously been reported for bentgrass (*Agrostis stolonifera* L.) (McCann and Huang, 2007) and legumes such as chickpea (Awasthi et al., 2014) and soybean (Jumrani and Bhatia, 2019). Recently, loss of Chl in soybean leaves has been associated with N-deficiency due to an impaired nodule functioning (Soba et al., 2021). This, along with a possible increase of reactive oxygen species (ROS) production, as suggested by chlorophyll fluorescence measures (Lim et al., 2007), are likely behind the lower Chl content in water stressed soybeans.

Soybean plants under WS (combined or not with heatwave), reduced ETR and $\Phi PSII$, compared to control conditions, especially at flowering

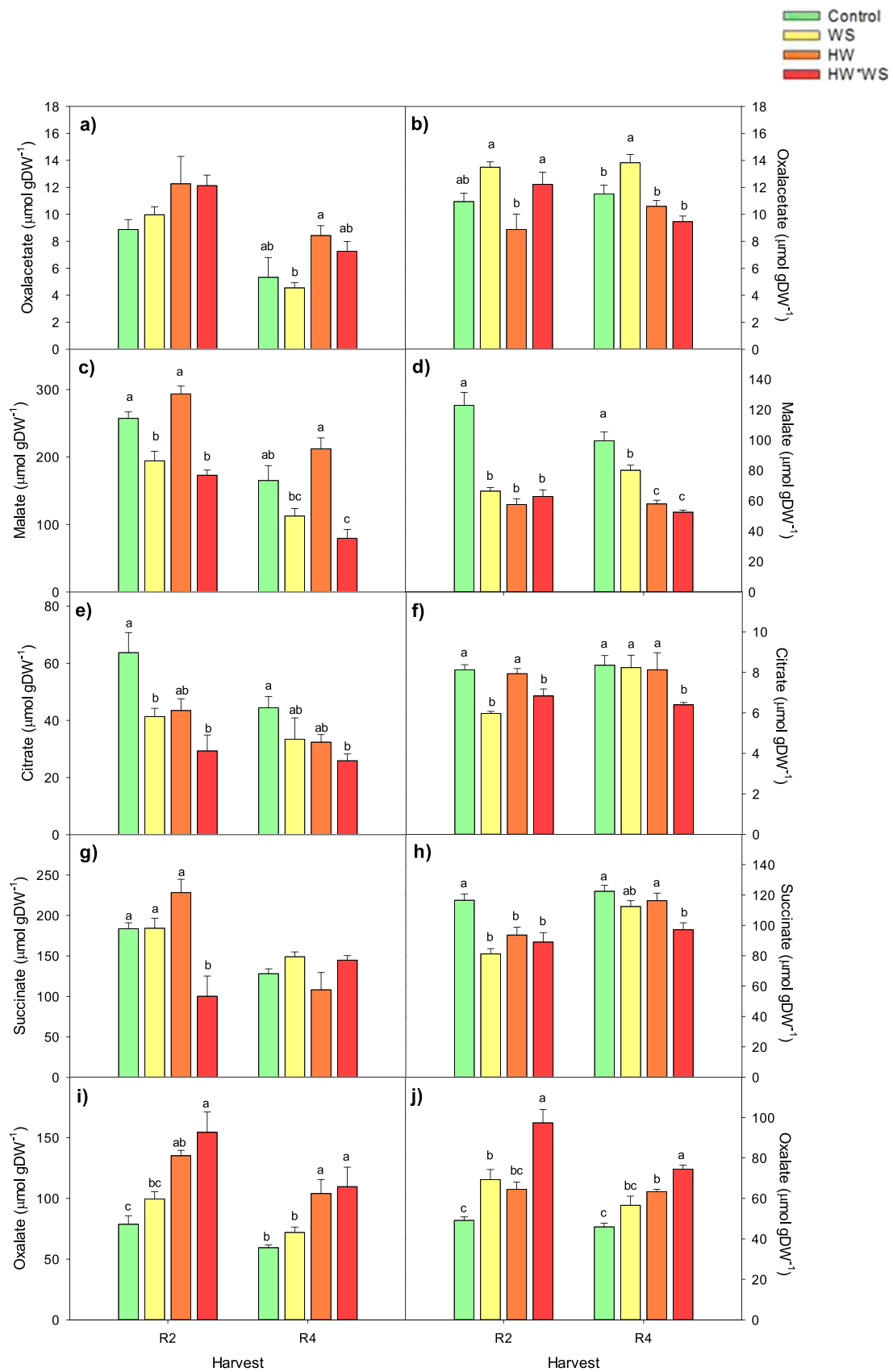


Fig. 3. Organic acids contents in leaves (a, c, e, g, i) and nodules (b, d, f, h, j) of soybean plants grown under four water and temperature treatments: control (non-heat-stressed and non-water-stressed), water-stressed (WS), heatwave (HW), and heatwave \times water-stressed (HW \times WS) plants at two developmental stages (R2 and R4 harvest). Bars show mean and standard errors (n = 5). Letters indicate significant differences independently for each harvest (R2 and R4) (Tukey posthoc test p < 0.05).

Table 3
Mean values (n = 5) of amino acid composition (nmol/g DW) of leaves and nodules determined at flowering (R2) and pod formation (R4), for soybean grown under control (C), water stress (WS), heatwave (HW), and their combination (HWxWS) conditions.

	LEAF															
	R2 SAMPLING				R4 SAMPLING				R2 SAMPLING				R4 SAMPLING			
	Control	WS	HW	HWxWS	Control	WS	HW	HWxWS	Control	WS	HW	HWxWS	Control	WS	HW	HWxWS
Asp	0.41 ^{ab}	0.35 ^b	0.45 ^a	0.39 ^{ab}	0.41 ^b	0.47 ^{ab}	0.55 ^a	0.45 ^{ab}	0.38 ^b	0.80 ^a	0.39 ^b	0.38 ^b	0.45 ^b	0.86 ^a	0.37 ^b	0.42 ^b
Glu	0.54	0.42	0.55	0.44	0.55	0.55	0.49	0.43	0.59 ^{ab}	0.39 ^b	0.81 ^a	0.58 ^{ab}	0.39 ^{ab}	0.35 ^b	0.44 ^a	0.42 ^{ab}
Ser	0.26 ^{ab}	0.25 ^b	0.34 ^a	0.28 ^{ab}	0.28 ^c	0.31 ^{cb}	0.46 ^a	0.38 ^{ab}	0.3	0.25	0.25	0.26	0.32 ^a	0.24 ^b	0.22 ^b	0.23 ^b
Asn	-	-	-	-	-	-	-	-	1.92 ^b	3.96 ^{ab}	1.41 ^b	6.12 ^a	1.04 ^{bc}	2.3 ^{ab}	0.35 ^c	2.53 ^a
Gly	0.15 ^b	0.16 ^c	0.20 ^a	0.21 ^a	0.18 ^b	0.19 ^{ab}	0.21 ^a	0.19 ^{ab}	0.24 ^{ab}	0.29 ^a	0.20 ^b	0.23 ^{ab}	0.23 ^{ab}	0.27 ^a	0.19 ^b	0.19 ^b
Gln	0.25 ^b	0.28 ^b	0.34 ^a	0.33 ^a	0.28	0.29	0.29	0.33 ^a	0.26	0.28	0.29	0.28	0.28	0.27	0.29	0.27
His	0.16 ^b	0.19 ^b	0.23 ^a	0.24 ^a	0.19	0.2	0.2	0.21	0.17	0.21	0.21	0.21	0.19	0.17	0.19	0.18
Thr	0.25 ^b	0.27 ^b	0.35 ^a	0.35 ^a	0.29	0.3	0.29	0.29	0.28	0.32	0.29	0.3	0.28	0.29	0.28	0.28
Ala	2.5 ^{ab}	1.88 ^a	2.83 ^a	2.24 ^{ab}	3.04 ^a	1.93 ^b	3.47 ^a	1.62 ^b	3.82	3.54	3.97	4.25	3.07	2.97	2.35	3.03
Arg	0.26	0.51	0.3	0.47	0.23	0.24	0.27	0.29	0.22	0.24	0.24	0.23	0.23	0.23	0.24	0.22
GABA	0.29 ^b	0.29 ^b	0.45 ^a	0.21 ^b	0.28 ^b	0.28 ^b	0.35 ^a	0.27 ^b	0.29 ^b	0.49 ^{ab}	0.44 ^{ab}	0.55 ^a	0.27 ^b	0.35 ^b	0.41 ^b	0.63 ^a
Pro	0.12 ^b	0.14 ^b	0.16 ^{ab}	0.21 ^a	0.11 ^b	0.21 ^a	0.15 ^{ab}	0.18 ^{ab}	0.66	0.51	0.52	0.49	0.36	0.42	0.28	0.37
Tyr	0.27 ^c	0.3 ^{cb}	0.37 ^{ab}	0.43 ^a	0.3	0.31	0.31	0.32	0.29 ^b	0.35 ^{ab}	0.37 ^a	0.37 ^a	0.30 ^b	0.31 ^{ab}	0.34 ^a	0.32 ^{ab}
Val	0.1 ^b	0.11 ^b	0.14 ^a	0.15 ^a	0.11 ^b	0.13 ^{ab}	0.12 ^{ab}	0.14 ^a	0.12 ^b	0.19 ^{ab}	0.17 ^{ab}	0.25 ^a	0.13 ^b	0.14 ^b	0.15 ^b	0.20 ^a
Met	0.18 ^b	0.19 ^b	0.23 ^a	0.23 ^a	0.19	0.2	0.2	0.2	0.25 ^a	0.24 ^{ab}	0.22 ^{ab}	0.21 ^b	0.23 ^a	0.22 ^a	0.21 ^{ab}	0.19 ^b
Ile	0.21 ^b	0.21 ^b	0.27 ^{ab}	0.30 ^a	0.21 ^b	0.22 ^b	0.23 ^{ab}	0.25 ^a	0.20 ^b	0.26 ^a	0.24 ^{ab}	0.26 ^a	0.21 ^b	0.22	0.23	0.23
Leu	0.19 ^b	0.21 ^b	0.26 ^a	0.25 ^a	0.21 ^b	0.21 ^{ab}	0.22 ^{ab}	0.24 ^a	0.20 ^b	0.24 ^a	0.23 ^{ab}	0.23 ^{ab}	0.26	0.22	0.23	0.23
Phe	0.26 ^c	0.29 ^{bc}	0.33 ^{ab}	0.36 ^a	0.26 ^b	0.27 ^b	0.29 ^{ab}	0.33 ^a	0.26	0.28	0.28	0.28	0.37	0.27	0.29	0.28

Letters indicate significant differences (Tukey posthoc test; p < 0.05) independently for each developmental stage

stage. Nevertheless, increasing temperatures alone did not influenced ETR and ΦPSII values, which was in accordance with the higher A_n observed in this treatment. The present study would indicate that drought has a more pronounced effect on PSII function than heatwave. Similar results have been shown recently in tomato plants (Zhou et al., 2019) and olive trees (Haworth et al., 2018). On the other hand, Jumrani and Bhatia (Jumrani and Bhatia, 2019) found significant decline in soybean ETR and ΦPSII with both high temperature and water stress. Additionally, previous observations on the impact of high temperatures combined with water deficit indicated that PSII inhibition was more sensitive to heat stress than to water stress (Jumrani and Bhatia, 2019; Havaux and Strasser, 1992; Martinazzo et al., 2012; Killi et al., 2020), which was in opposition to our observations. One possible explanation for this contrasting response could be the intrinsic nature of heatwaves (i.e., short period of extreme high temperature (Haworth et al., 2018)) in opposition to reported high temperatures maintained during entire phases of plant growth in previous experiments (Jumrani and Bhatia, 2019; Killi et al., 2020). Non-photochemical quenching (q_N) decreased at flowering under WS alone and in combination with HW; however, at R4, the decrease was only significant in HW x WS plants. This indicates that soybean under such conditions may not be able to thermally dissipate portions of absorbed light energy in the PSII antennae to avoid photo-oxidative damage (Aranjuelo et al., 2005; Méndez-Espinoza et al., 2019) (Fig. 4). In addition to chemical protection, Anth are plant pigments known for their photoprotective role under stress (Kovinich et al., 2015). In our study, a significant increase in Anth could be seen under HW alone when compared to HW x WS. Many studies have confirmed that plants accumulate Anth for various photoprotective functions under different stresses (Hughes et al., 2005; Moustaka et al., 2020). This possible photoprotective role of Anth is in accordance with the photo-inhibition of the PSII (ETR and ΦPSII) observed in leaves of plants under combined stresses when compared to heatwave (Table 1).

Taking into account leaf biomass, gas exchange and chlorophyll fluorescence data, WS had a dominant effect over HW, especially when applied at flowering. The biochemical response of photosynthesis was similar for plants under both WS and HW x WS. This inhibition was caused by stomatal and non-stomatal limitations of photosynthesis. On the other hand, plants under HW were capable of maintaining leaf function without significant inhibitions compared to control plants.

4.1.2. Carbon and nitrogen metabolism

According to our results, the described decrease in photosynthetic performance under water stress is translated into a lower synthesis of non-structural carbohydrates such as soluble sugars (Suc, Glu and Fru) and starch, as previously described (Balfagón et al., 2020). Decreases in leaf soluble sugars content under drought (alone or combined with heatwave) are especially relevant at flowering, in particular Suc content. These results are in accordance with a recent study (El Habti et al., 2020) that found a significant reduction in soluble sugars under combined drought and heat stress in wheat grains. Leaf Suc content is determined by several factors others than A_n , such as Suc export to flowers/pods or nodules (Ruan, 2014). Since PN at physiological maturity seem to be reduced in plants under interacting stresses imposed at flowering (Fig. 2) but not the supply of Suc to nodules (Table 2), we suggest that at this stage export of Suc to reproductive sink may be compromised by water scarcity at this stage, which results in significant flower abortion (Al-Ghazawi et al., 2009). During R2 and R4, low starch concentration in leaves were found under WS (alone or in combination with HW) when compared to control or HW plants. This indicates poor starch synthesis or/and high starch degradation required to maintain Suc supply when A_n are low (Ruan, 2014). Lower starch synthesis under combined stresses has been also related with lower content of Glu and Fru, the first substrates in the starch biosynthesis pathway (El Habti et al., 2020).

Protection of photosynthesis from photoinhibition through photo-respiration is well known to dissipate excess light energy and reduce formation of ROS (Kozaki and Takeba, 1996) but also likely results in

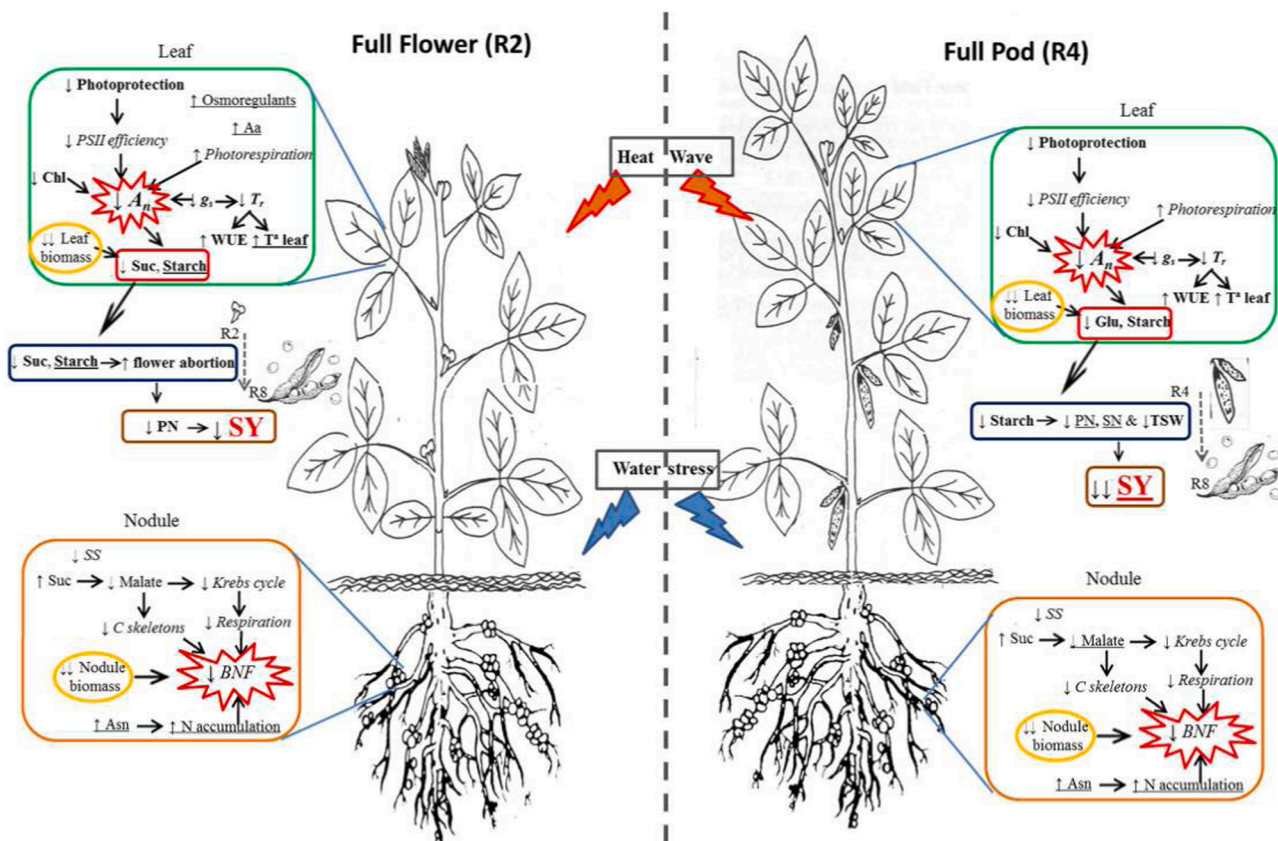


Fig. 4. Effects of combined heatwave and water stress applied at full flower and full pod on soybean physiology and metabolism. All physiological, biochemical and metabolic parameters that appear in the image were significantly affected under combined stresses in comparison to control plants. Additionally, parameters in bold were differently affected ($p < 0.05$) under combined stresses in comparison with heatwave alone and parameters underlined were differently affected ($p < 0.05$) under combined stresses in comparison with water stress alone. Italic indicates processes not directly measured in the present study but likely affected (from related parameters) under combined stresses.

loss of C previously fixed by photosynthesis (Farquhar et al., 1980). The observed high levels of Gly and Ser in soybean plants under HW (alone or combined with WS) has been related to increased photorespiratory activity under stress conditions (Soba et al., 2021; Novitskaya et al., 2002; Fahnenstich et al., 2008). Additionally, the most likely means of oxalate accumulation is by glyoxylate oxidation (Yu et al., 2010; Igamberdiev and Kleczkowski, 2018) that can occur during photorespiration. These two observations indicate higher levels of photorespiration during heatwave treatments as suggested in previous works (Prasad et al., 2008; Peñuelas and Llusià, 2002; Rollins et al., 2013). This is probably due to higher leaf temperature (Table 1) that increased oxygen solubility and affinity by Rubisco (Brooks and Farquhar, 1985).

Leaf N content at R2 and R4 was significantly higher under heatwave than under water stress (Table 1). In accordance, leaf nitrogenous amino acids (Asp, Alanine (Ala)) were decreased by water limitation compared to heatwave (Table 3). Since nodules were the sole N source for plants, lower leaf N and nitrogenous metabolites content may indicate impaired nodule performance under water stress (Soba et al., 2019). Another explanation for lower Asp and Ala accumulation in WS and HW x WS leaves is lower transpiration rate (Table 1). Lower transpiration has been noted to possibly involve alterations in long-distance transport of N compounds between underground and aerial plant tissues, provoking an accumulation of N compounds in nodules of plants under water stress (Serraj et al., 2001). The lower leaf N concentration could be involved in the observed lower Chl concentration in WS and combined stresses treatments, and may be another reason for impaired photosynthetic performance in both treatments (Evans, 1989; Xue et al., 2004; Sanz-Saez et al., 2019).

4.2. Nodule performance

Along with leaves, nodule was the tissue more severely affected (in terms of biomass) by occurrence of stresses (Fig. 1 and Table S1). Nodule biomass reduction was significant for the two stresses and their combination during R2 and R4, with reductions ranging from $\approx 40\%$ when stresses were applied alone to more than 50% when applied in combination.

Biological nitrogen fixation (BNF) in legume nodules is fueled by C fixed through photosynthesis (Aranjuelo et al., 2014). Although photosynthesis is severely inhibited by WS (and their interaction with HW), limited supply of photosynthates to nodules does not appear to be directly involved in nodule performance, given that all treatments promoted an accumulation of Suc in nodules (Table 2). Increased nodule Suc under abiotic stress has been previously reported in legumes under water stress (González et al., 1995; Ramos et al., 1999; Gálvez et al., 2005) and has been mainly attributed to inhibition of sucrose synthase (SS) activity (González et al., 1995; Gordon et al., 1999). The limited number of studies on high temperature and nodule performance also showed that decreases in nodule function was not due to limited carbohydrate supply (Aranjuelo et al., 2014).

In our experiment, lower contents of malate and succinate in nodules of plants under HW, WS, or combined stresses were observed. These two dicarboxylic acids are the main respiratory substrates for bacteroids and are produced by the cleavage of Suc by SS and subsequent glycolytic activities, which probably indicates that phosphoenolpyruvate carboxylase (PEPC) was also negatively affected (Lodwig and Poole, 2003). Malate and succinate contents have been shown to be limited in nodules of pea (Gálvez et al., 2005) and soybean (Ladrera et al., 2007) under

water stress, probably due to this SS and/or PEPC down-regulation (González et al., 1995). Therefore, these observations reinforce the idea of an adequate supply of photoassimilates from leaves to nodules but with a SS down-regulation provoking low provision of dicarboxylic acids that supply the energy and C skeletons required by bacteroids to fix N_2 by nitrogenase (Aranjuelo et al., 2014).

Additionally, the lower leaf N demand under abiotic stresses has been described to cause amino acid accumulation in nodules; in this regard, several compounds such as glutamine (Gln), Asn, Asp and ureides have been suggested to be involved in a N feedback mechanism (Larrainzar et al., 2009; Soba et al., 2019; Aranjuelo et al., 2014; Serraj et al., 2001; King and Purcell, 2005; Sulieman and Schulze, 2010). In our case, WS (alone or in combination with HW) caused an increase in Asn and Asp (Table 3), which are two of the most abundant amino acids in soybean nodules. Lower transpiration rates observed in both treatments (Table 1) may be involved in this specific amino acid accumulation through alterations in long-distance transport of N compounds between underground and aerial plant tissues (Serraj et al., 2001). Additionally, oxaloacetate could mostly be used to accumulate Asp/Asn, at the expense of malate production.

Nodule biomass was severely affected by HW, WS and their combined effect, similarly, nodule performance was also affected. On one hand, nodule function under combination of HW and WS seem to be affected similar to WS alone but with more severe consequences. Under these conditions, nodule performance seems to be limited by (i) poor respiratory activity to bacteroids (due to poor SS/PEPC activity rather than reduced transport of photoassimilates from leaves to nodules) and (ii) accumulation of nitrogenous compound (Asp and Asn) likely due to reduced transpiration (Fig. 4). Both traits have been associated with decreased nitrogenase activity (Serraj et al., 1999). On the other hand, HW nodules seem to be affected by poor cleavage of Suc into malate (the main supply of energy and C skeletons required by the bacteroid), but high transpiration rates avoid N-feedback due to amino acids accumulation. As a consequence, aboveground N availability in the WS treatment (leaf N concentration; Table 1) was negatively affected in comparison to HW. However, in plants under combined heat and water stresses, leaf N content was statistically similar to HW plants, probably due to a concentration effect caused by lower leaf biomass and N demand.

4.3. Linking plant physiology and soybean seed yield

Grain yield under HW, WS, and their combination is the final manifestation of physiological effects occurring during and after imposed stresses. In this regard, biomass measurements at flowering and pod formation were similar to final seed yield (Figs. 1 and 2). For example, analysis of total biomass (R2 and R4) and final SY, revealed a similar reduction in water stressed plants (alone or in combination with HW) at R2; however, a greater effect was observed with R4 plants under combined stresses. The simultaneous occurrence of HW and WS has been documented to potentially cause an additive impact on SY that is considerably larger than single stress effects. This has been observed in cereals such as barley (Rollins et al., 2013), wheat (Matiu et al., 2017; Zampieri et al., 2017) and legumes like soybean (Jumrani and Bhatia, 2018; Matiu et al., 2017).

In our study, a significant decrease of 54.3% compared to the control was only observed when the stresses were applied during grain filling. This decrease was greater than those observed for HW (-25.4%; $p < 0.05$) and WS (-24.4%; $p < 0.05$). On the other hand, similar to observations during flowering, (Ergo et al., 2018) did not observe additive or multiplicative effects on soybean grain yield due to interaction of both stresses. For instance, WS (alone or in combination with HW) applied at flowering resulted in similar significant decreases (31.6% and 33.2%; respectively), while plants under HW exhibited a slight non-significant decrease (-9.9%). Similar results have been reported for tomato (Nankishore and Farrell, 2016), groundnut (Awasthi et al., 2014;

Hamidou et al., 2013), and lentil (Sehgal et al., 2017). Furthermore, SY is primarily a function of pod numbers (PN), seed number (SN), and thousand seed weight (TSW) (Jumrani and Bhatia, 2018). In our study, reductions in SY was mainly due to PN and SN diminutions, which closely followed the same trend as SY (Fig. 2). However, changes in TSW only explained seed yield variation in plant under combined stresses during pod formation (R4). Similar results have been shown in heat and water stressed field-grown soybean (Ergo et al., 2018). Maintenance of TSW can be seen as a compensatory effect by HW plants due to reduced SN (Andriani et al., 1991). Nevertheless, plants under WS during pod formation displayed a slight (non-significant) decrease in TSW while a greater decrease ($p < 0.001$) was seen in HWxWS. In this regard, there is ample evidence supporting that heat stress episodes and/or water stress during grain filling decrease soybean SY mainly by decreases in TSW and to a lesser extent in SN (Ergo et al., 2018; Brevedan and Egli, 2003; Rotundo and Westgate, 2010; Dornbos and Mullen, 1992). Seed weight can be modified until late seed filling which extends the capacity of soybean seeds to modify their weight in response to abiotic stresses (Borrás et al., 2004), such as the additive effects of HW x WS. However, we observed significant reductions in PN and SN in plants subjected to heatwave. This result highlights that important pod and seed number diminutions can be expected at this developmental stage due to a large overlapping of reproductive phases; this was also accentuated by indeterminate growth of the genotype used in our study. Earlier studies showed that the end of the critical period for pod and seed number determination is close to the grain filling stage (Board and Tan, 1995; Egli, 1997).

Our findings suggest that water stress applied at flowering decreased SY due to flower abortion caused by insufficient Suc supply from leaves (Farooq et al., 2017; Du et al., 2020). However, good photosynthetic performance in plants under HW allows SY maintenance similar to control plants. The TGW yield parameter was not affected under any treatment since the photosynthetic machinery was probably able to recover from stress during grain filling and photoassimilates supply was not affected. Therefore, since photosynthetic effects and reduction in sugar supply were similar in water stressed plants and plants under combined effects (Tables 1 and 2), final SY were similarly reduced and no additive effect was observed (Fig. 2). On the other hand, reductions in SY under combined stress applied at late pod formation were significantly higher compared to other treatments. This SY reduction was due to diminutions in PN, SN, and TSW, which suggests poor pod setting due to low photoassimilates supply and low TSW of formed seeds. This may indicate that photosynthetic capacity of these plants may not have fully recovered when stresses were terminated. Collectively, reduced PN (and SN) and lower TSW observed in plants under combined stresses can help explain the additive effects of heatwave and water stress on SY during late pod formation (Puteh et al., 2013; Egli et al., 2005). Additionally, we suggest that reductions of PN is most critical for SY determination during full pod formation since later PN reduction would not be expected (pods are already formed) and only TSW should be modified (Ergo et al., 2018; Brevedan and Egli, 2003; Rotundo and Westgate, 2010; Dornbos and Mullen, 1992), as opposed to R4 where both PN and TSW can be altered by abiotic stresses.

5. Conclusions

Our study indicates that water stress had a higher impact than heatwave on physiological and biochemical parameters and combined effects were more similar to water stress than heatwave especially when applied at flowering. Water stress (alone or combined with heatwave) negatively affected photosynthetic performance caused by closed stomata and photodamage, which impaired Suc and starch metabolism in leaves and reproductive organs during flowering and pod formation. Nodule functions were affected by water stress and combined stresses due to poor carbon supply to bacteroids and N-compound accumulation that negatively affected leaf N content in water stressed plants. These

whole-plant effects caused a decrease in seed yield at maturity. At flowering, fewer flower sets reduced seed yield equally in water stress and combined stress treatments, but not in the heatwave treatment. However, the seed yield effect from heatwave and water stress at pod formation was exacerbated when both stresses were applied concomitantly due to both less pod setting and smaller seeds. Our study highlights that, in current and near-future environmental conditions, it is crucial to increase our knowledge of combined stress effects for a better agronomic practices and breeding programs. Within this context, it is crucial to identify the agrophysiological factors and cultivars that contribute to sustain a more resource efficient crop development under changing environmental conditions. In this context, such findings suggest that pod formation is the critical moment for soybean production under these combined abiotic stresses. However, more research needs to be done with other genotypes and crop species to test if the findings described in this study are a general trend.

CRedit authorship contribution statement

David Soba: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft. **Cesar Arrese-Igor:** Conceptualization, Investigation, Writing – review & editing. **Iker Aranjuelo:** Conceptualization, Investigation, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.plantsci.2022.111320](https://doi.org/10.1016/j.plantsci.2022.111320).

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