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Research article

Growth and photosynthetic responses in *Brassica napus* differ during stress and recovery periods when exposed to combined heat, drought and elevated CO_2

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ABSTRACT

This study was intended to investigate how an agronomically important crop *Brassica napus* will be able to cope with the combined impact of a heatwave (21/14 °C *vs.* 33/26 °C day/night) and drought under ambient or elevated CO_2 (800 *vs.* 400 μmol mol^{-1}) and to what degree their recovery will be ensured after the stress, when additional CO_2 is also removed. The obtained results revealed that, in the presence of an adequate water supply, *B. napus* performed well under heatwave conditions. However, drought fully negated all the advantages gained from hotter climate and led to a slower and incomplete recovery of gas exchange and retarded growth after the stress, regardless mitigating the effect of elevated CO_2 during the stress. The mechanism by which the elevated CO_2 diminished the adverse effect of a combined heat and drought stress on photosynthetic rate at saturating light (A_{sat}) was attributed to the improved plant water relations. However, it had little effect on the recovery of A_{sat}. In contrast, the mechanism by which photosynthesis was more impaired under the combination of heatwave and drought, compared to single drought treatment, was attributed mainly to the faster soil drying as well as faster and sharper decrease in stomatal conductance and subsequent in C_i/C_a. Keeping in mind that photosynthesis can acclimatize by downregulation to higher CO_2, the results of this study, showing a weak memory of mitigating the effect of elevated CO_2, highlight a potential risk of more intense and frequent heatwaves and droughts on *B. napus*.

1. Introduction

Together with a steady rise in atmospheric CO_2 concentration, extreme climatic conditions with extended drought periods and heatwaves, broadly defined as a period of consecutive days where conditions are hotter than normal (Perkins and Alexander, 2013), are predicted to increase in frequency and severity in many regions of the world (Mittal et al., 2014). Europe's climate has already become more extreme than previously could be foreseen (Toreti et al., 2013) with a doubling in the length of European heatwaves between of 1880–2005 (Della Marta et al., 2007). Extreme precipitation deficits can occur in any season, but those that lead to a significant soil water deficit are most likely in summer, when relative air humidity is low while air temperature is high, and the leaf area is the most extensive, causing high evapotranspiration (De Boeck et al., 2010) associated with increased leaf-to-air vapor pressure deficit (VPD). Recent studies have shown that VPD increases non-linearly with a sharp increase in air temperature (Duarte et al., 2016; Ruehr et al., 2016), suggesting that heatwaves accelerate soil water depletion. The combination of high leaf temperature and reduced water availability increases the severity of damage to leaf physiological functions and growth (De Boeck et al., 2011; Bauweraerts et al., 2013; Ruehr et al., 2016; Duan et al., 2017). Therefore, heatwaves together with the extended drought periods occurring during the summer may have the most dramatic impact on plant productivity in comparison with such extreme events occurring in spring or autumn (De Boeck et al., 2011).

The predicted reduction in food production and quality, according to the IPCC 2014 report, mainly resulting from heat and drought induced stress, is considered a significant future risk in many areas of the world (IPCC, 2014; Zandalinas et al., 2018). Several studies have demonstrated that increased temperature and drought can reduce crop yields by up to 50% (Lamaoui et al., 2018). Aside from this, the

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response of plants to the combinations of two or more abiotic stressors is different from that of a single stressor and cannot be directly extrapolated (Zandalinas et al., 2018). The combination of drought and heat stress could also evoke opposite responses of plants. For example, in some plants, under heatwave conditions, stomatal conductance did not change or increased (e.g. Ameye et al., 2012; Dreesen et al., 2012; Duan et al., 2017; Correia et al., 2018; Elferjani and Soolanayakanahally, 2018) as the plant attempts to cool its leaves by transpiration to avoid overheat and prevent deleterious damage while, under drought stress, plants tend to close their stomata to prevent water loss (Dreesen et al., 2012; Duan et al., 2017; Correia et al., 2018; Elferjani and Soolanayakanahally, 2018). Thus, stomatal responses to drought and heat combination represent a challenging situation in which plants must balance between preventing water loss and protecting from overheating (Zandalinas et al., 2018). Several reports have shown that plants subjected to this stress combination preferred to reduce their transpiration to avoid unnecessary water loss rather than leaf cooling (Dreesen et al., 2012; Duan et al., 2017; Correia et al., 2018; Elferjani and Soolanayakanahally, 2018). However, such a “conservational” strategy driven by water economy via reduced stomatal conductance at the cost of evaporative cooling could lead to excessively increased leaf temperature and the damage on photosynthesis (Teskey et al., 2015), which has been recognised as one of the most thermally sensitive metabolic processes (Wang et al., 2008). It has been shown that plants affected by combined heat and drought exhibited a higher leaf temperature than plants subjected to only heat stress, probably because of inability to increase transpiration due to closed stomata (Rizhsky et al., 2002, 2004; Duan et al., 2017). Nevertheless, the response to combined drought and heat stress may vary among different genotypes or different plant species (Aprile et al., 2013; Zandalinas et al., 2016; Dikšaitytė et al., 2018).

Carbon dioxide (CO_2) concentration has dramatically increased since pre-industrial period from 280 up to 412 μmol mol^{-1} (NOAA, 2019). The projections for the end of the 21st century based on RCP8.5 scenario show that CO_2 concentration may reach up to 936 ppm leading to elevation in air temperature by 2.6–4.8 °C relative to 1986–2005 (IPCC, 2013). The most important response of plants to elevated CO_2 is an increase in net photosynthetic rate, leading to improved growth and productivity (Leakey et al., 2009; Taub and Wang, 2013). It has been demonstrated that elevated concentration of atmospheric CO_2 diminished the adverse effects of stress induced by heatwaves on photosynthetic performance of *Pinus taeda* (Ameye et al., 2012) and *Quercus rubra* (Ameye et al., 2012; Bauweraerts et al., 2013) and enhanced photosynthetic thermotolerance in a group of C_3 plants (Wang et al., 2008) to acute heat temperatures.

The mechanisms by which elevated CO_2 can alleviate the adverse effect of abiotic stressors encompass enhanced photosynthesis (Biswas et al., 2013; Xu et al., 2014; Zinta et al., 2014), increased water use efficiency (WUE) due to reduced stomatal conductance (Leakey et al., 2009), increased levels of antioxidants and their activities, and other processes, such as reduced photorespiration, leading to a decline in the level of reactive oxygen species (ROS) (Mishra and Agrawal, 2014; Zinta et al., 2014; AbdElgawad et al., 2016). Nevertheless, studies on the interactive effects of heatwaves and drought in conjunction with elevated CO_2 on the agronomically important performance of plants are still very rare. To what extent crop performance would be affected by the combined heatwave and drought and whether elevated concentration of CO_2 would be able to diminish adverse effects of these stressors are the crucial questions of the current research. Furthermore, to get a more holistic view of stress effects on plants, plant recovery needs to be considered, as it could be dependent on species, stress severity and the legacy effects on plant (Ameye et al., 2012; Anderegg et al., 2015; Ruehr et al., 2016; Duan et al., 2017). It has been shown that after the extreme heat and drought events, the plant growth and physiological activities may partly recommence at high CO_2 concentrations, and the oxidative stress can be greatly alleviated, although they cannot reach the control plants level (Xu et al., 2010; Xu and Zhou, 2011; Zinta et al., 2014). In addition, stress exposure alters gene expression beyond the duration of the stress phase, forming a molecular “memory” (Crisp et al., 2016; Georgii et al., 2019). The key mechanism responsible for the acquired “stress memory” include sustained/accumulated levels of key signaling metabolites, plant hormones and proteins involved in their synthesis, or transcription factors and the kinases/phosphatases regulating their activity, as well as chromatin remodeling through histone modifications (Avramova, 2015; Lämke et al., 2016; Lämke and Bäurle, 2017). Even in the absence of a further stress challenge, plant performance can have signs of a stress-related memory after a successful stress recovery, significantly differing from untreated control plants (Xu et al., 2010; Hagedorn et al., 2016; Georgii et al., 2019). However, investigations on the recovery of crop physiological processes from the combination of heatwave and drought remain very scarce, and hardly anything is known about the role of CO_2 enrichment during the recovery in case of its elimination together with stress. This has encouraged us to examine the impact of combined heatwave and drought with and without elevated CO_2 treatment on the photosynthetic performance and growth of spring oilseed rape (*Brassica napus* L.) during the period of stress as well as the recovery following treatments under non-stressed ambient climate conditions without CO_2 enrichment.

The main goals of our study were (i) to determine whether there is a difference between the rate of gas exchange parameter changes during the exposure phase and recovery phase, and (ii) whether CO_2 fertilization has a memory effect on the recovery of gas exchange after its removal together with stress. We hypothesized that (i) during the stress, the elevated concentration of atmospheric CO_2 would alleviate the adverse effect of combined heat and drought by improving plant water status but, during the recovery, its role would be weak; (ii) the predominance of gas exchange reduction over its recovery under the combined conditions of heatwave and drought would reflect into slower and incomplete reversal of growth, regardless the mitigating effect of elevated CO_2 during the stress.

2. Materials and methods

2.1. Plant material and growth conditions

The experiments were conducted in three growth chambers (LESLAT, Lithuania) with a volume of 10 m^3 of each (2.0 × 2.0 × 2.5 m width, length, height) in the precisely controlled environment at Vytautas Magnus University, Lithuania. Spring oilseed rape seeds (*Brassica napus* L., var. ‘Fenja’) were sown and grown in plastic pots (64 cm diameter, 50 cm height) filled with a mixture of field top-soil (taken from Aleksandras Stulginskis University training area, Kaunas district), perlite, and fine sand (5:3:2, by volume).

Until the treatments, plants were grown under the ambient climate (AC) conditions of vegetation period. An ambient day/night air temperature averaged 21.1 ± 0.02 °C/14.1 ± 0.02 °C corresponding to the long-term average for Lithuania, with 14 h/10 h day/night photoperiod. A daytime CO_2 concentration averaged 406 ± 1.2 μmol mol^{-1} and the day/night relative air humidity (RH) averaged 56 ± 0.1%/73 ± 0.3%. A light level of about 270 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) was provided by a combination of natural daylight luminescent lamps (Philips, Waterproof OPK Natural Daylight LF80 Wattage 2 × 58 W/TL-D 58 W) and high-pressure sodium lamps (Philips MASTER GreenPower CG T 600 W). The plants were fertilized twice with the complex nutrient (NPK 12-11-18 + microelements) solution to the final N level of 160 kg ha^{-1}. Volumetric soil water content (SWC), measured with a Theta Probe ML2x sensor combined with a handset HH_2 moisture meter with a depth of 6 cm (Delta-T Devices Ltd., Cambridge, UK), for the well-watered plants was kept at 30% on average. Throughout the experiment, control plants were kept under the conditions mentioned above.

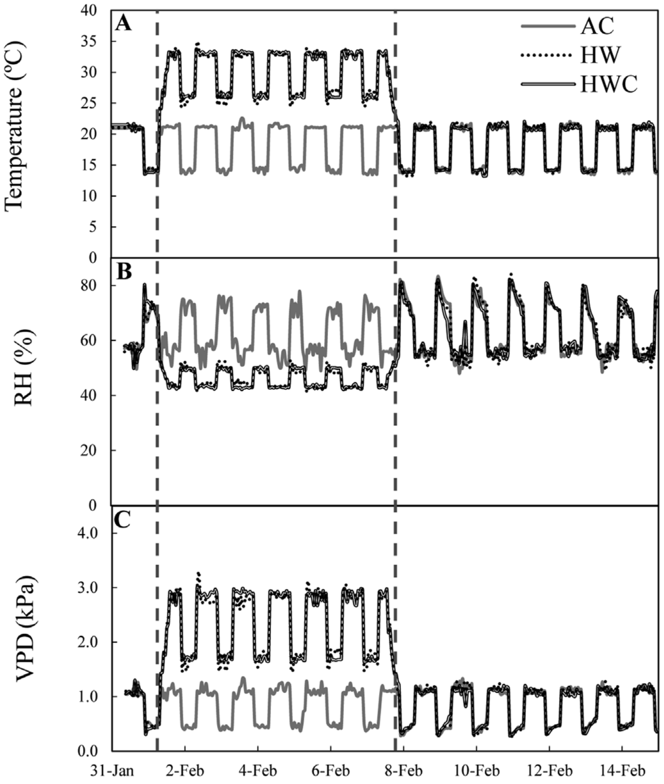

Fig. 1. Environmental conditions: (A) air temperature, (B) relative air humidity (RH) and (C) vapor pressure deficit (VPD) recorded in plant growth chambers throughout the experiment. Treatments: AC – ambient climate conditions, HW – +12 °C heatwave in ambient CO_2 concentration, HWC – +12 °C heatwave in elevated CO_2 concentration. The vertical dashed lines indicate a 7-day period during which HW or HWC were applied.

2.2. Experimental design and treatments

Heatwave (HW) and heatwave with elevated to 800 μmol mol^{-1} CO_2 (HWC) treatments were initiated at the three-leaf growth stage, i.e. BBCH 13, according to the BBCH (biologische bundesanstalt, bundessortenamt and chemical industry) scale (Meier, 2001) and lasted 7 days until the 7-day period of recovery began. Prior to treatments, plants were randomly assigned to one of the three groups: AC (ambient climate, i.e. 21/14 °C day/night temperature, CO_2 of 400 μmol mol^{-1}), HW – heatwave under ambient CO_2 level (33/26 °C, CO_2 of 400 μmol mol^{-1}), and HWC – heatwave under elevated CO_2 level (33/26 °C, CO_2 of 800 μmol mol^{-1}). The experiment was performed with a total of 36 pots (16 plants per pot), 18 pots for treatments and 18 pots for recovery (3 replications for each treatment).

On day 1 of treatment, the temperature in HW and HWC regimes was gradually increased from 21 °C to 33 °C between 8:00 h and 14:00 h in 4 steps: 24 °C at 8:00 h, 27 °C at 10:00 h, 30 °C at 12:00 h and 33 °C at 14:00 h. Then it was held at 33 °C/26 °C day/night cycle for 5 days before it was gradually decreased to 21 °C/14 °C day/night cycle on day 7 of treatment in 4 steps between 14:00 h and 20:00 h: 30 °C at 14:00 h, 27 °C at 16:00 h, 24 °C at 18:00 h and 21 °C at 20:00 h (Fig. 1A). The drought stress was applied to half of the pots (six) in all climate treatments (AC, HW and HWC) by withholding water for the same period of 7 days. After the heatwave was relieved (i.e. the temperature returned to the ambient day/night air temperature cycle of 21/14 °C), the drought-stressed plants were re-watered to the target SWC level of 30%, which was further maintained during the 7-day recovery period, according to the indication of the moisture meter (Fig. 2).

Plants in different treatments are identified in the following manner: A-W for well-watered plants grown under AC conditions, A-D – drought-stressed plants grown under AC conditions, HW-W for well-watered plants subjected to HW treatment, HW-D – drought-stressed plants subjected to HW treatment, HWC-W for well-watered plants subjected to HWC treatment, and HWC-D – drought-stressed plants subjected to HWC treatment.

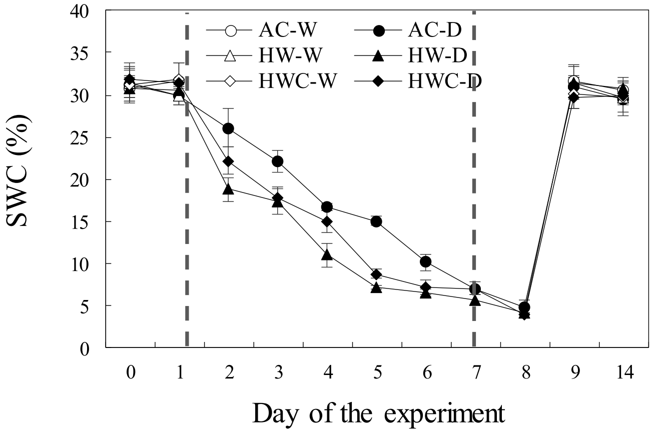

Fig. 2. Measured volumetric soil water content (SWC) at 12 cm depth throughout the experiment. Values are means ± SE ($n = 3$). Well-watered and drought-stressed plants grown under ambient climate conditions (AC-W and AC-D) are indicated by open and filled circles, respectively. Well-watered and drought-stressed plants subjected to +12 °C heatwave in ambient CO_2 concentration (HW–W and HW-D) are indicated by open and filled triangles, respectively. Well-watered and drought-stressed plants subjected to +12 °C heatwave in elevated CO_2 concentration (HWC–W and HWC-D) are indicated by open and filled diamonds, respectively. The vertical dashed lines indicate a 7-day period during which HW or HWC treatments were applied.

The preset values of the air temperature in the growth chambers were controlled manually at each of the growth chambers operating cupboards (Emerson Network Power S.r.l., Italy, model No. S06UC021V300020FX051260). The concentration of CO_2 was manipulated automatically by controlling the amount of injected CO_2 gas and chamber conditioner. The climate program was controlled by the IGSS 9–13175 software. Air temperature, relative humidity and CO_2 concentration were measured by an automated sensor (CO2HRT-D, Regin, Källered, Sweden), mounted in each chamber. With the exception of the 7-day heatwave episode in the HW and HWC chambers, the differences in air temperature, and RH between the chambers before the treatments and during the recovery period were negligible ($p > 0.05$) (Fig. 1A and B). The pots within the same growth chamber were rotated every day to minimize any potential chamber effects on plant performance.

2.3. Leaf gas exchange measurements

Light-saturated gas exchange measurements were conducted with a portable photosynthesis system (LI-6400XT, LiCor Biosciences, Lincoln, NE, USA), equipped with a 6 cm^2 leaf cuvette and LED light source (Li-6400-02B LED). Photosynthesis under saturating light conditions (A_{sat}; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}), leaf transpiration (E; mmol H_2O m^{-2} s^{-1}) and the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) were simultaneously measured or calculated by the LI-6400XT software before the treatments and every second day during the 7-day heatwave (HW and HWC) and/or drought treatments and the 7-day recovery period under unstressed ambient climate conditions between 10:00 a.m. and 3:00 p.m. At the start of the measurements, the CO_2 level in the leaf cuvette was set at the same CO_2 level as the plants were growing at, either 400 or 800 μmol CO_2 mol^{-1} for ambient (AC and HW) and elevated (HWC) CO_2 atmospheres, respectively. The temperature of the leaf cuvette was set to match the temperature in each treatment chamber, either 21 °C for AC or 33 °C for

HW and HWC treatments. The air flow rate through the assimilation chamber was maintained at 500 μmol s^{-1}. During all light-saturated measurements, PAR was set at 1500 μmol $m^{-2} s^{-1}$. The air humidity (RH) and VPD inside the leaf cuvette was allowed to vary with chamber conditions. Water use efficiency (WUE; μmol CO_2 $mmol^{-1}$ H_2O) was calculated as the light-saturated CO_2 assimilation rate divided by transpiration rate (A_{sat}/E). All measurements were conducted on the most recent fully developed lit leaves (randomly chosen three plants per pot were measured).

2.4. Growth measurements

Half of the pots (18) was harvested after 7-day treatments, and another half of the pots was harvested after the 7-day recovery period. For the evaluation of leaf area, all leaves per plant were removed and scanned using CanoScan 4400F (Canon, USA) and then the leaf area was evaluated using GIMP 2.8 software. Foliage, stem and root biomass were dried at 60 °C until constant dry weight was obtained.

2.5. Statistical analysis

The effects of drought, heatwaves and their combination on different parameters were analyzed using two-way ANOVA. Fisher's least significant difference (LSD) tests were used to evaluate statistically significant differences between the means of the investigated parameters. Student's *t*-tests were used to compare the difference between the two groups' means. The rate of leaf gas exchange parameters during the HW treatment and recovery was determined by linear regression, and the slope of the curve (b) was used as a prediction of the rate (change of x parameter per day). The significance of the difference between the linear regression slopes for different treatments was assessed using Z-test (Clogg et al., 1995):

$$Z = \frac{b_1 - b_2}{\sqrt{SEB_1^2 + SEB_2^2}}, \tag{1}$$

where b_1 and b_2 are the slopes of the linear regression for the different treatments, SEb – standard error of b coefficient. The differences between the slopes were considered to be statistically significant ($p < 0.05$) when the Z-test exceeded the critical value of 1.96.

All statistical analyses were performed by STATISTICA 8 software. The results were considered significant if $p \leq 0.05$.

3. Results

3.1. Growth responses during stress period

At the end of the treatments, the leaf area (LA), shoot dry weight (DW) and root to shoot ratio (R/S) were all affected by heatwaves and drought × heatwaves interaction, while root DW was only affected by heatwaves (Table 1). Drought as a single stressor did not reduce ($p > 0.05$) the growth under ambient climate (AC) conditions, while in combination with heatwave (HW) it induced the substantial reductions in LA, shoot and root DW by 48%, 35% and 41% respectively compared to control (well-watered plants grown under AC conditions) and by 41%, 32% and 34%, respectively, compared to single drought stress. LA and shoot DW of drought-stressed plants grown under heatwave at elevated CO_2 (HWC) treatment did not change significantly from controls and single drought-stressed ones, however root DW decreased by 34% and 26%, respectively. Consequently, R/S of drought-stressed plants grown under HW did not change from controls and drought-stressed ones grown under AC, while those under HWC decreased significantly by 25% and 21%, respectively, because the compensatory effect of elevated CO_2 was more pronounced for shoot than for root growth (Fig. 3A–D).

Under both heatwave treatments (HW and HWC), the above-ground growth of well-watered plants was modulated contrariwise to single drought effect. Both LA and shoot DW were enhanced by 54% and 33% respectively, under HW, and additionally reinforced by elevated CO_2 under HWC treatment up to 91% and 73% respectively compared to controls. As root DW of well-watered plants grown under HW and HWC treatments did not change significantly, the considerably increased shoot DW led to the reduction of R/S by 39% and 38%, respectively (Fig. 3A–D).

3.2. Post-stress growth responses

After stress, all the growth attributes were still affected by heatwaves and drought × heatwaves interaction (Table 1). After the relief of stress, single drought-stressed plants apparently possessed a lower growth as differences of LA, shoot and root DW from controls were bigger than those at the end of the treatment (Fig. 3E–G). LA, shoot and root DW of plants exposed to combined drought and HW did not regenerate at all and were by 55%, 44% and 39% respectively lower than that of control plants and by 41%, 34% and 27% lower compared to single drought-stressed plants. After the withdrawal of additional CO_2 together with the cessation of stress, drought-stressed plants grown under HWC treatment also possessed a significantly lower above-ground growth compared to control plants. LA and shoot DW of these plants were significantly lower by 38% and 34% respectively compared to controls, and by 19% and 22% ($p > 0.05$) respectively compared to drought-stressed plants grown under AC conditions (Fig. 3E–G). Nevertheless, drought affected plants grown under HWC conditions had a better regeneration of root DW compared to HW-treated ones. They recovered up to 79% of control level ($p > 0.05$) and almost did not differ (reduction of 5%) from single drought-stressed plants. That, in turn, translated into a significantly higher (by 21%) R/S ratio compared to control.

In contrast to drought effect, the well-watered plants fully recovered their biomass production and showed higher growth compared to

Table 1

Analysis of variance showing the level of significance for growth (leaf area (LA), shoot and root dry weight (DW) and root to shoot ratio (R/S)) and leaf gas exchange (photosynthetic rate at saturating light (A_{sat}), stomatal conductance (g_s), transpiration rate (E), intercellular to ambient CO_2 concentration (C_i/C_a), and water use efficiency (WUE)) parameters as affected by drought and heatwaves (HW and HWC) during the stress and after recovery. ‘*‘, ‘**‘, ‘***’ indicate significance levels at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively, ns is non-significant.

Stage	Treatment	Growth parameters				Gas exchange parameters				
		LA	Shoot DW	Root DW	R/S	A_{sat}	g_s	E	C_i/C_a	WUE
Stress	Drought	ns	ns	ns	ns	***	***	***	***	***
	Heatwaves	***	***	ns	***	***	**	***	ns	***
	Drought × heatwaves	***	***	***	**	***	***	***	**	**
Recovery	Drought	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Heatwaves	**	**	**	*	ns	ns	ns	ns	ns
	Drought × heatwaves	***	***	**	*	*	**	*	*	ns

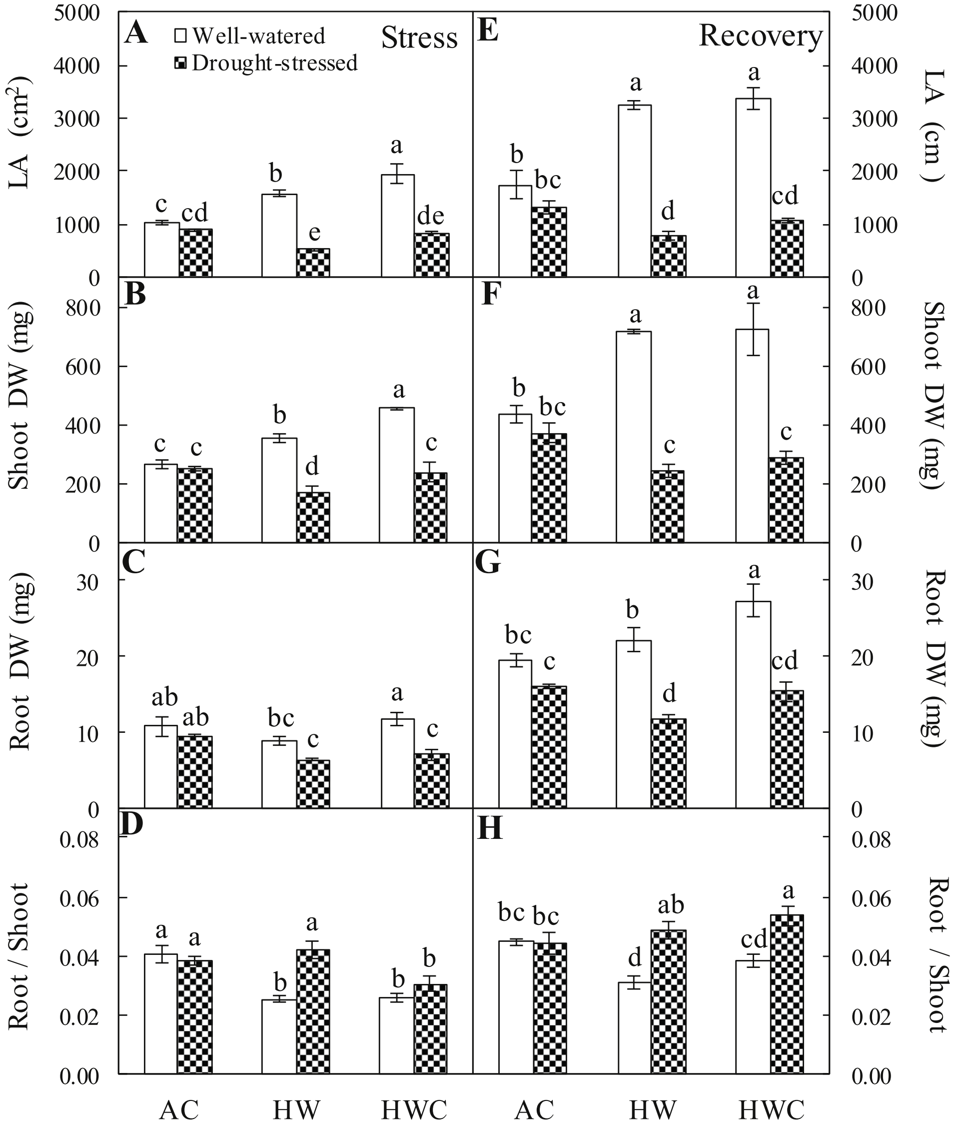

Fig. 3. Leaf area (LA), shoot and root dry weight (DW) and root to shoot ratio (root/shoot) of well-watered and drought-stressed plants during the stress (left panels, A-D) and after recovery (right panels, E-H). Values are means ± SE ($n = 3$). Treatments: AC–ambient climate conditions; HW–heatwave (+12 °C) in ambient CO_2 concentration; HWC–heatwave (+12 °C) in elevated CO_2 concentration. Different lowercase letters above the columns indicate significant difference ($p < 0.05$) among the treatments (Fisher's LSD).

control plants. LA and shoot DW of well-watered plants after the recovery period were up to 95% and 66% respectively higher than that of control plants and no significant difference was found between HW and HWC treatments ($p > 0.05$). Root growth also recovered. Root DW of both HW and HWC pre-exposed plants was higher than that of control plants, although a significant difference from controls (by 40%) was only found for HWC treated plants. While under both heatwave treatments R/S ratio was lower compared to controls, but significantly by 31% only under HW treatment.

3.3. Gas exchange responses during stress period

All gas exchange parameters except C_i/C_a were significantly affected by drought and heatwaves and their combination (Table 1).

Photosynthesis under saturating light conditions (A_{sat}) was 15.3 μmol CO_2 $m^{-2} s^{-1}$, on average, prior to the experimental treatments and did not differ significantly in control plants (AC-W) throughout the experiment (Table A1). The exposure to heatwave treatment of well-watered plants under both ambient and elevated CO_2 level, resulted in 29% and 69% ($p < 0.05$) higher A_{sat} values respectively compared to that of control plants (Figs. 4A and 5A and Table A1). In contrast, photosynthetic carbon fixation decreased sharply in plants exposed to single drought and combined drought and heatwave. Single drought led to a reduction of A_{sat} by 34%, while the decline in A_{sat} in combined drought and heatwave treatments was more pronounced (81% and 64% in HW-D and HWC-D treatments, respectively) (Fig. 5A). At the same time, SWC from the initial value of 31.3%, on average, had decreased to 7.0% in AC-D and HWC-D treatments, and to 5.6% in HW-D treatment (t-test, $p < 0.05$) (Fig. 2A). The data showed that HW and HWC had exacerbated the negative drought effect on A_{sat} that was by 72% and 46% respectively lower ($p < 0.05$) in HW-D and HWC-D treatments than under single drought stress (Fig. 5A).

Stomatal conductance (g_s) of well-watered plants treated with HW under ambient CO_2 level was significantly higher during the entire 7-day heatwave period, reaching its peak on the fifth day. The g_s of HWC-W treated ones tended to be slightly and insignificantly lower than that of control plants, during the entire experimental period (Fig. 4B and Table A1). A significant decline (−71% compared to control) in g_s of plants exposed to single drought was only recorded on the fifth day of treatment, when SWC dropped from 31.3% to 15.0% (t-test, $p < 0.05$)

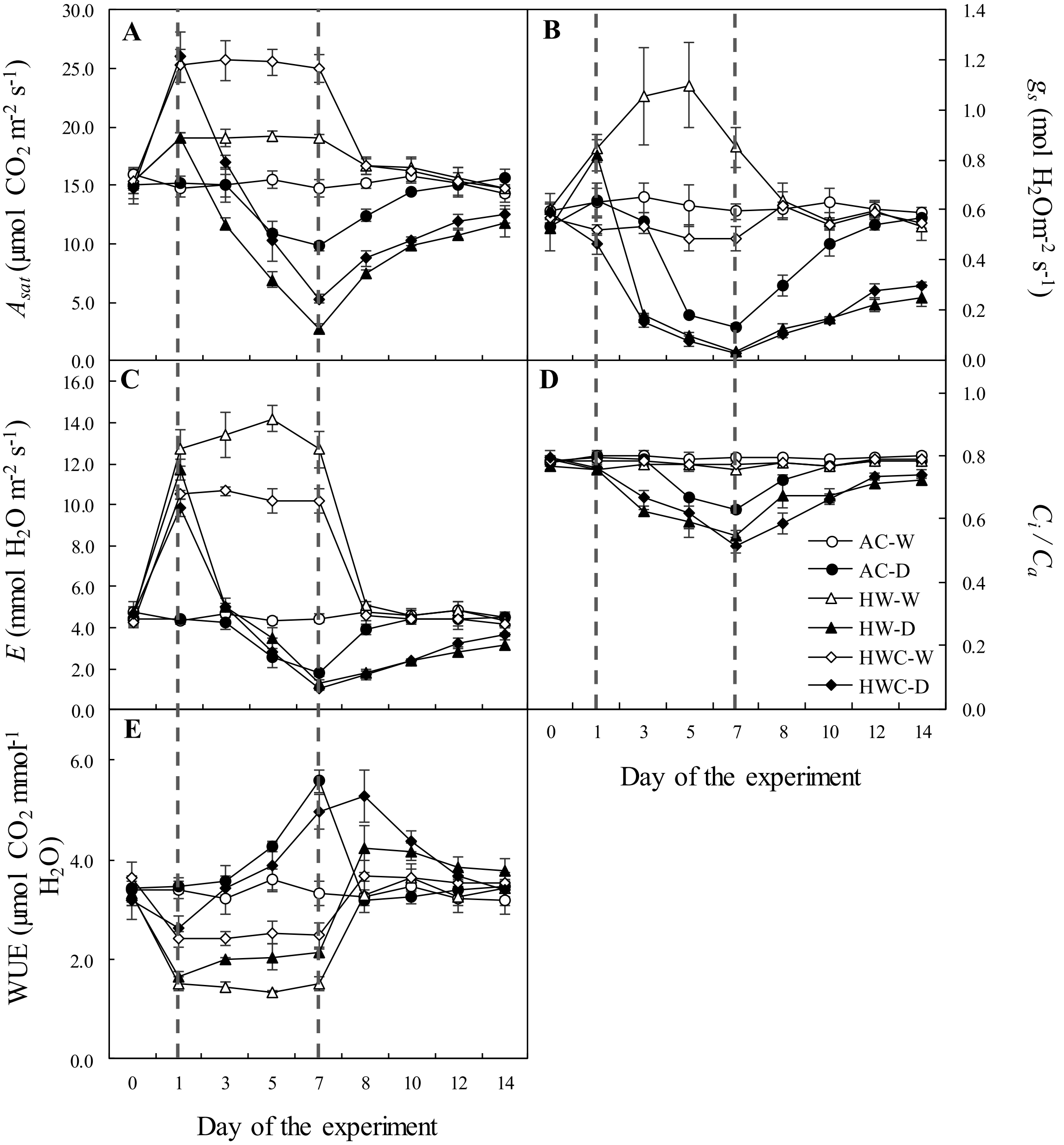

Fig. 4. Dynamics of gas exchange parameters: (A) leaf photosynthesis under saturating light conditions (A_{sat}), (B) stomatal conductance (g_s), (C) transpiration (E), (D) the rate of intercellular to ambient CO$_2$ concentration (C_i/C_a), and (E) WUE throughout the experiment. Values are means ± SE ($n = 9$). Well-watered and drought-stressed plants grown under ambient climate conditions (AC-W and AC-D) are indicated by open and filled circles, respectively. Well-watered and drought-stressed plants subjected to +12 °C heatwave in ambient CO$_2$ concentration (HW–W and HW-D) are indicated by open and filled triangles, respectively. Well-watered and drought-stressed plants subjected to +12 °C heatwave in elevated CO$_2$ concentration (HWC–W and HWC-D) are indicated by open and filled diamonds, respectively. The vertical dashed lines indicate a 7-day period during which HW or HWC treatments were applied.

(Figs. 2A and 4B and Table A1). While a significant decline in g_s of drought-stressed plants treated with heatwave under ambient and elevated CO$_2$ levels was observed earlier, i.e. on the third day of treatments, and decreased constantly till the last day of treatments. The lowest values of g_s of all drought-stressed plants, i.e. those grown under AC-D, HW-D and HWC-D treatments, were recorded on the last day of treatments when they dropped by 78%, 95% and 96% lower levels respectively compared to controls ($p < 0.05$). However, contrarily to A_{sat}, heatwaves under drought conditions (HW-D and HWC-D) did not add any significant effect on g_s, when compared to single drought treatment (AC-D) (Fig. 5B and Table A1).

The time course responses of transpiration (E) were similar to those of g_s in all treatments, although E in well-watered plants grown under HWC conditions during the 7-day period of treatment was significantly higher compared to controls (Fig. 4C and Table A1). At the end of the treatments, the E in well-watered plants grown under HW and HWC conditions was up to 84% and 29% higher respectively compared to control (Fig. 5C). The transpiration of plants exposed to single drought was only 40% of control value, and additional heat stress, especially under elevated CO$_2$ level, slightly reinforced the negative drought impact (Fig. 5C).

The ratio of intercellular to ambient CO$_2$ concentration (C_i/C_a) did not differ significantly throughout the experiment between the well-watered plants, except a small but still significant reduction of C_i/C_a in plants affected by heatwave on the first day of the treatment (Fig. 4D and Table A1). During all the treatment period, the drought-stressed plants exhibited the same pattern of changes in C_i/C_a as were the changes of A_{sat} and g_s. The reductions of C_i/C_a in drought-stressed

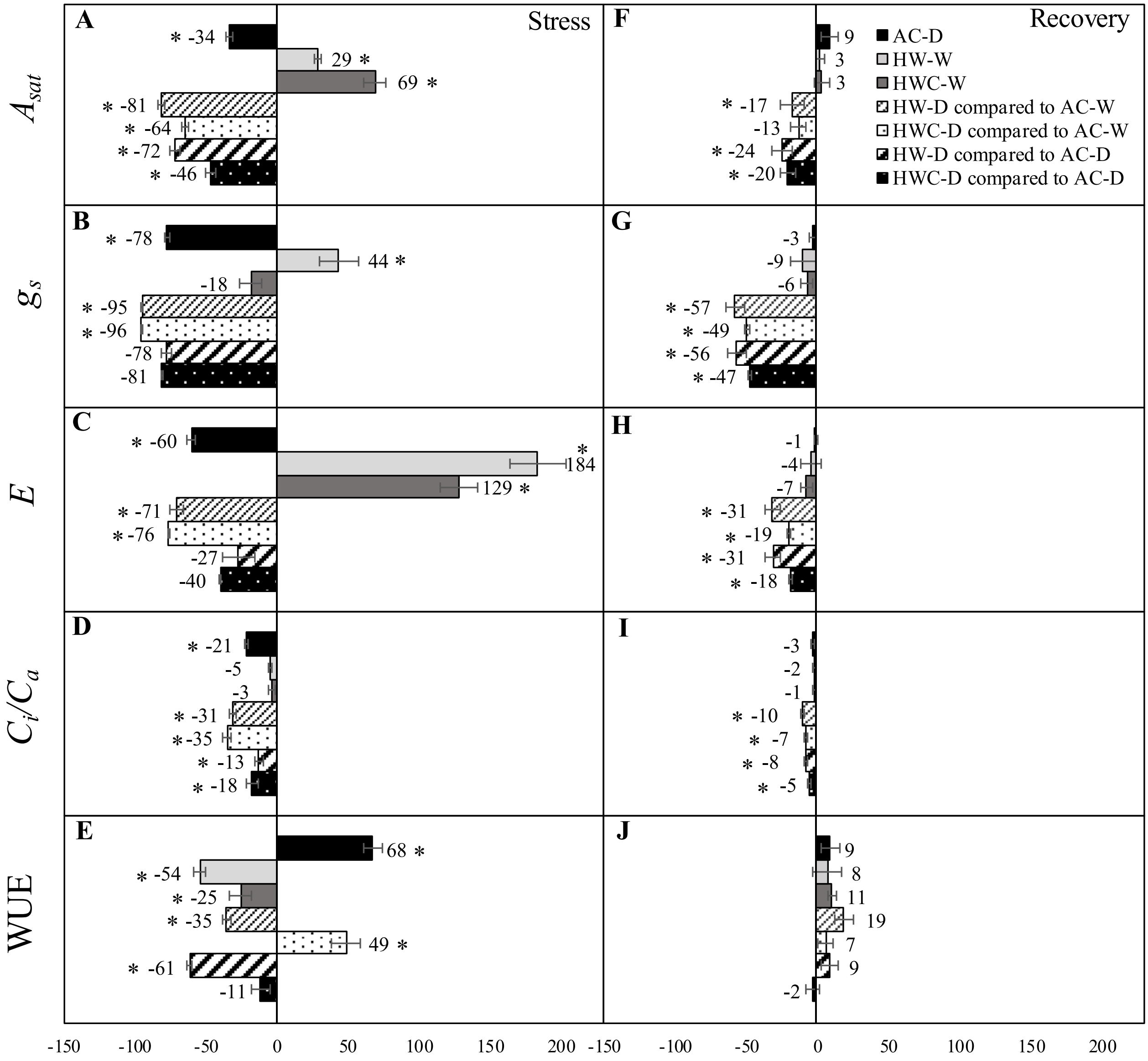

Fig. 5. The percentage changes in leaf gas exchange parameters (photosynthetic rate at saturating light (A_{sat}), stomatal conductance (g_s), transpiration rate (E), intercellular to ambient CO_2 concentration (C_i/C_a), and water use efficiency (WUE)) for drought-stressed plants grown in ambient climate conditions (AC-D), well-watered and drought-stressed plants subjected to heatwave in ambient CO_2 concentration (HW–W and HW-D, respectively), well-watered and drought-stressed plants subjected to heatwave in elevated CO_2 concentration (HWC-W and HWC-D, respectively) compared to control (well-watered plants grown under AC conditions, AC-W), and for the plants subjected to the treatments of combined drought and heatwave in ambient CO_2 concentration (HW-D) and combined drought and heatwave in elevated CO_2 concentration (HWC-D), compared to drought-stressed plants grown in ambient climate conditions (AC-D) on the last day (7th day) of treatments (left panels, A-E) and after 7-day recovery period (14th day) (right panels, F-J). '*' indicates a significant difference from control (AC-W or AC-D) at $p \le 0.05$ (Fisher's LSD).

plants grown under AC conditions were lower, while those treated with heatwave under ambient and elevated CO_2 levels demonstrated a sharper decrease in C_i/C_a (Fig. 4D and Table A1). At the end of the treatments, C_i/C_a in the AC-D, HW-D and HWC-D treatments decreased by 21%, 31% and 35% ($p < 0.05$) respectively compared to controls (Fig. 5I,D). In comparison to AC-D treatment, the reductions of C_i/C_a in the HW-D and HWC-D treatments were significantly lower (Fig. 5D and Table A1).

During the 7-day treatment period, WUE in well-watered plants treated with heatwave under ambient and elevated CO_2 levels substantially decreased compared to controls with a significantly greater reduction in HW-W treatment (Fig. 4E and Table A1). In HW-D, during the entire exposure period, it was also substantially lower than that in controls. However, in the drought-stressed plants grown under AC and HWC conditions, starting from the third day of treatments, WUE was higher compared to controls, and at the end of the treatments was up to 68% and 49% higher ($p < 0.05$) than that of control (Figs. 4E and 5E and Table A1). Comparing single drought and combined drought and heatwave impact on WUE, it could be seen that additional heat leads to lowered WUE of drought-stress plants, and under elevated CO_2 concentration the effect of heatwave was less pronounced (Fig. 5E).

As leaf gas exchange parameters were consistent with SWC changes during the simultaneous heatwave and drought treatment, we examined the relationship between leaf gas exchange and SWC. A_{sat}, g_s, C_i/C_a, and E of drought-stressed plants exhibited logarithmic functions with SWC (Fig. 6). The g_s (subsequently C_i/C_a as well) and A_{sat} exhibited the highest sensitivity to SWC changes (g_s $R^2 = 0.72$, A_{sat} $R^2 = 0.66$, $p < 0.001$, Fig. 6B,A). At a given SWC, simultaneous heatwave and drought resulted in lower g_s than under single drought and the response of g_s to SWC was not significantly affected by CO_2 level. While the

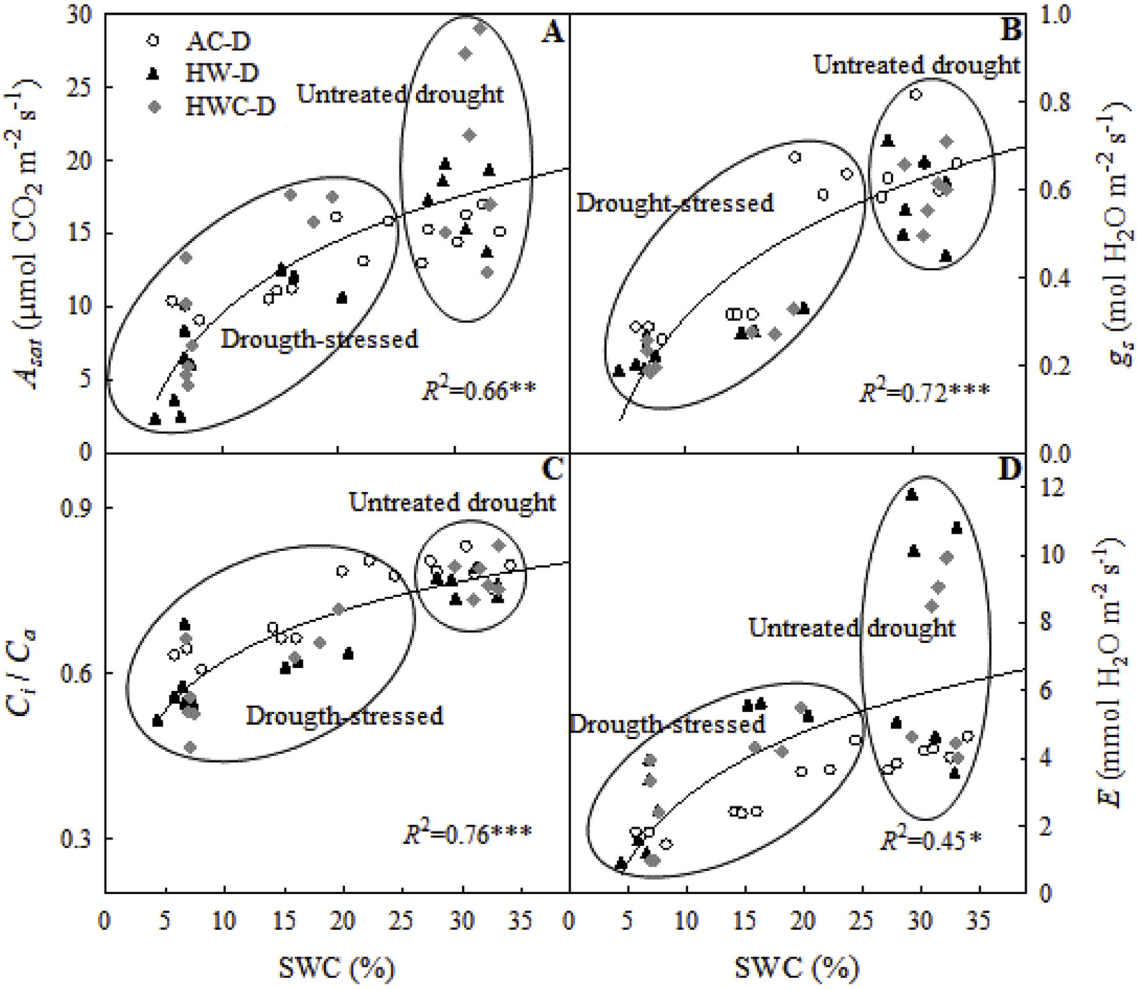

Fig. 6. (A) Leaf photosynthesis under saturating light conditions (A_{sat}), (B) stomatal conductance (g_s), (C) the ratio of intercellular to ambient CO_2 concentration (C_i/C_a), and (D) transpiration (E) of *Brassica napus* grown under different climate conditions during drought stress as a function of SWC. Points are the raw data of the measured variables. Data are fitted with logarithmic functions: y = a + b × log(x). The determination coefficients (R^2) are also given. Open circles represent drought-stressed plants grown under ambient climate conditions (AC-D); filled and black triangles represent drought-stressed plants subjected to +12 °C heatwave in ambient CO_2 concentration (HW-D); filled and gray diamonds represent drought-stressed plants subjected to +12 °C heatwave in elevated CO_2 concentration (HWC-D). '*', '**', '***' indicate significance levels at $p \le 0.05$, $p \le 0.01$ and $p \le 0.001$ respectively.

response of A_{sat} was altered by the CO_2 level. Under ambient CO_2, the A_{sat} began to decrease at higher soil SWC (less intense drought) than under elevated CO_2 level. Average to strong relationships (estimated by logarithmic functions) were also detected between A_{sat} and C_i/C_a ($R^2 = 0.52$, $p < 0.001$), A_{sat} and g_s ($R^2 = 0.62$, $p < 0.001$) as well as between C_i/C_a and g_s ($R^2 = 0.88$, $p < 0.001$) and E and g_s ($R^2 = 0.46$, $p < 0.001$) (data are not shown), indicating that stomatal conductance was the essential factor limiting carbon assimilation under drought stress. A weaker but significant effect of SWC was also determined on transpiration ($R^2 = 0.45$, $p < 0.05$, Fig. 6D), however at different SWC levels, the different pattern of E response among our treatments was observed. At medium SWC (15–20%), additional heat stress increased transpiration in comparison to single drought treatment, and elevated CO_2 diminished this increase. At low SWC (5–10%), additional heat stress decreased transpiration in comparison to single drought treatment, although elevated CO_2 led to higher transpiration compared to single drought and simultaneous drought and heat treatment.

3.4. Post-stress gas exchange responses

After stress, A_{sat}, g_s, E, and C_i/C_a were only affected by drought × heatwaves interaction, while WUE was not affected by any of these treatments or their combination (Table 1). After the relief of heatwave and additional CO_2, on the first day of recovery, A_{sat} of all the drought-stressed plants was still significantly lower compared to control plants, and it remained such in both heatwave treatments until the fifth day of recovery (Fig. 4A and Table A1). The reductions of A_{sat} by 17% ($p < 0.05$) and 13% ($p > 0.05$), respectively, were still apparent in drought-stressed plants grown under HW and HWC conditions after the 7-day recovery period, while a full recovery of A_{sat} was observed in the AC-D treatment (Fig. 5F). A_{sat} in the HW-D and HWC-D treatments, compared to AC-D, significantly decreased by 24% and 20%, respectively (Fig. 5F).

The recovery pattern of the g_s and E matched the A_{sat} exhibiting a steep recovery of g_s and E in AC-D treatment during the 7–10 days and full recovery at the end of the experiment, while the recovery of g_s and E in the HW-D and HWC-D treatments was slower and incomplete (Fig. 4B and C, 5G,H and Table A1). After the 7-day recovery period, g_s of drought-stressed plants grown under HW and HWC conditions was only 43% and 51% ($p < 0.05$) respectively compared to that in controls (Fig. 5G). Similarly, E remained lower by 31% and 19% ($p < 0.05$) respectively compared to control (Fig. 5H). Additional heat stress, under both ambient and elevated CO_2 levels, significantly impaired the recovery of both g_s and E in drought-stress plants, however the plants exposed to combined drought and HW under elevated CO_2 showed a slightly better recovery than those treated with drought and

HW without additional CO_2 (Fig. 5G and H).

The g_s of well-watered plants grown under HW conditions dropped to the control level after the heatwave was removed and did not differ significantly from the control during the 7-day recovery period. Likewise, the E of well-watered plants grown under HW and HWC conditions dropped to the control plants level after the cessation of heatwave and did not differ significantly during the entire recovery period (Fig. 4B and C, 5G,H and Table A1).

The recovery of C_i/C_a in the plants exposed to single drought stress (AC-D) was also faster and complete while in the HW-D and HWC-D treatments it was slower and incomplete (Figs. 4D and 5I and Table A1). After the 7-day recovery, C_i/C_a of drought-stressed plants treated with heatwave under ambient and elevated CO_2 levels was still significantly lower by 10% and 7% respectively compared to controls (Fig. 5I). Additional heat stress, under both ambient and elevated CO_2 levels, significantly impaired the recovery of C_i/C_a (Fig. 5I). WUE showed no statistically significant difference between the treatment after stress (Fig. 5E,J).

3.5. Rates of gas exchange parameter changes during stress and recovery

As some differences in the temporal pattern of gas exchange parameters of well-watered and drought-stressed plants exposed to HW treatments under ambient and elevated CO_2 concentrations were observed, the rate of gas exchange parameter changes was calculated (Table 2). A_{sat} and g_s of well-watered plants changed at a very low rate during both HW and HWC treatments, and no difference from the control was found (Z-test). However, in drought-stressed plants, A_{sat} decreased very fast, and the fastest decrease was observed in HWC-D treatment, where A_{sat} decreased 3.4-fold faster than in AC-D treatment, implying that heat accelerates the decline in A_{sat}. During the recovery period, A_{sat} of HW-D and HWC-D treated plants increased at a lower rate than during the treatments, which indicates their slower recovery compared to the response to stress. No significant differences were detected between the A_{sat} recovery rates of plants exposed to single drought and combined drought and heat (Table 2). During the heatwaves, plants closed their stomata faster when they were subjected to the simultaneous drought and heatwaves and opened them significantly slower after the cessation of stress. Both transpiration and C_i/C_a rates showed a similar pattern as g_s. The plants exposed to combined drought and heat lowered their transpiration more rapidly than those exposed to single drought. The fastest change in WUE was recorded in plants exposed to single drought and additional heat stress, regardless the fact that the level of CO_2 slowed the regulation of water use efficiency. Drought and heatwave led to an increase of WUE, and plants exposed to these stressors simultaneously increased their WUE at a higher rate compared with that of well-watered plants. However, WUE recovery rates did not differ from the WUE change rates during the imposed treatments, indicating that there was no difference between the response and recovery.

4. Discussion

4.1. Effect of single stressors on growth and physiology

Drought stress takes place when soil and atmospheric humidity are low, and the ambient air temperature is high. This condition is the result of an imbalance between the evapotranspiration flux and water intake from the soil (Lamaoui et al., 2018). The primary response of almost all the plants to moisture stress is a rapid closure of stomata to avoid further water loss through transpiration that enables to maintain hydraulic function but restricts CO_2 intake, which leads to the reduced assimilation (Fahad et al., 2017; Zandalinas et al., 2018). In this study, all these above - mentioned physiological adjustments providing an escape to the water stress also occurred with spring oilseed rapes grown under the imposed drought stress for 7 days (Fig. 4A–D). However, despite the significant reduction of A_{sat} during the last three days of exposure (5–7 days) (Fig. 4A and Table A1), the growth parameters of drought-stressed plants grown under ambient climate conditions were not significantly affected at the end of the treatment (Fig. 3A–D and Table 1).

The heatwave acted as a stimulant factor for the photosynthetic performance of well-watered plants significantly increasing the A_{sat} from the first day of treatment (Fig. 4A and Table A1) that translated into increased above-ground growth at the end of the treatment (Fig. 3A and B). A slight increase in air temperature appeared to have a positive effect on crop growth and productivity (Sage and Kubien, 2007; Hudson and Henry, 2009; Way and Oren, 2010). While extreme temperatures associated with heatwaves usually have an adverse effect on plants' photosynthetic performance and growth (Ameye et al., 2012; Bauweraerts et al., 2013; Duarte et al., 2016; Ruehr et al., 2016; Duan et al., 2017). In general, the tendency for elevated temperature to act as a positive environmental factor depends on whether it is within or beyond plants' optional growth and photosynthetic temperature range (Way and Sage, 2008; Feng et al., 2014). Therefore, the susceptibility to abiotic stresses may differ considerably among species or varieties of a crop (Chen et al., 2012; Wishart et al., 2014). For example, while a number of studies show quite strongly influenced photosynthesis by heatwaves in different woody plant (Bauweraerts et al., 2013; Duarte et al., 2016; Ruehr et al., 2016) and crop (Duan et al., 2017) species, in the study of Dreesen et al. (2012), the photosynthetic rate was not diminished in an experimental group of annual and biannual plants grown under well-watered conditions. Similarly, De Boeck et al. (2011) concluded that heatwaves as a single factor did not generate a significant response, which can be attributed to the fact that heat stress does not develop when plants have enough water at their disposal. The latter authors considered that the absence of marked and lasting effects of a summer heatwave could be attributed to the fact that leaf temperatures did not increase to damaging levels (causing, for example, chlorophyll degradation) as the abundance of soil water allowed plants to continuously cool their leaves via transpiration. In an agreement with that assumption, the results of our study also showed that E of well-watered plants substantially increased during the heatwave

Table 2
Rate of gas exchange parameters' changes during the stress (Str) and recovery (Rec) periods.

Treatments	A_{sat} (μmol CO^{-2} m^{-2} s^{-1} day^{-1})		g_s (mol H_2O m^{-2} s^{-1} day^{-1})		E (mmol H_2O m^{-2} s^{-1} day^{-1})		C_i/C_a		WUE (μmol CO^{-2} $mmol^{-1}$ H_2O day^{-1})	
	Str	Rec	Str	Rec	Str	Rec	Str	Rec	Str	Rec
AC-W	0.03	−0.16	−0.01	−0.00	0.00	−0.02	−0.02	0.00	0.01	−0.03
AC-D	−1.01*	0.51*	−0.10*	0.04*	−0.48*	0.09	−0.03*	0.01*	3.50*	0.05
HW-W	0.00	−0.33	0.00	−0.01	0.03	−0.09	0.00	0.00	−0.00	0.00
HW-D	−2.69*	0.71*	−0.12*	0.02*	−1.68*	0.22*	−0.03*	0.01	0.08	−0.08
HWC-W	0.05	−0.32	−0.01	−0.01	−0.07	−0.06	−0.00	0.00	0.02	−0.03
HWC-D	−3.45*	0.65*	−0.07*	0.04*	−1.43*	0.34*	−0.04*	0.03*	0.37*	−0.32*

Abbreviations of treatments are the same as in Fig. 4. '*', indicates a significant difference from control (A-W) (Z-test).

treatment (Figs. 4C and 5C). Furthermore, corresponding to higher transpiration, the stomatal conductance of well-watered plants grown under heatwave conditions (HW–W treatment) also significantly increased compared to controls (AC-W treatment), allowing the plants to keep their C_i/C_a ratio at the control one level (Fig. 4B,D and 5B,D). Thus, according to these results, it seems that in the presence of an adequate water supply, spring oilseed rape cultivar 'Fenja' has a rather high upper limit to temperature increase that allows it to perform well under heatwave conditions (+12 °C), at the early vegetative stage. However, it should be noted that different growth stages have different susceptibility to heat stress. For example, Angadi et al. (2000) determined that, during reproductive growth, dry matter of three *Brassica* species (*B. juncea*, *B. napus*, and *B. rapa*) was unaffected by moderate temperature stress (28/15 °C for 7 days *vs.* 20/15 °C, day/night), but diminished under high-temperature stress (35/15 °C *vs.* 20/15 °C, day/night) that was injurious to reproductive organs at different developmental stages. High temperature at flowering affected yield formation more than high temperature at pod development. Similarly, in another recent study with *B. napus*, it was found that heat had a significant adverse effect on the photosynthetic-related variables (photosynthetic rate, carboxylation efficiency and electron transport rates, and mesophyll conductance), as well as on growth, yield, and oil quality, when imposed during the flowering and silique-filling stages (Elferjani and Soolanayakanahally, 2018). In addition, the study of Angadi et al. (2000) demonstrated that investigated *Brassica* species differed in optimum temperatures from *B. juncea* and *B. rapa* having higher optimum temperature than *B. napus*, which suggests that the induced high temperature stress can also be cultivar dependent.

4.2. Effect of combined treatments on growth and physiology

Elevated concentration of atmospheric CO_2 additionally reinforced the stimulations of photosynthesis and above-ground growth of well-watered plants grown under heatwave conditions (Figs. 5A and 3A,B). While root growth changed significantly from control neither under HW nor under HWC treatment, that, in turn, led to the significant reduction of R/S (Fig. 3C and D). Similarly, the root to shoot ratio of *Poa pratensis* remained constant from 15 to 30 °C and then decreased at 35 °C under both ambient and elevated (800 *vs.* 400 μmol mol^{-1}) CO_2 conditions (Song et al., 2014). In our other recent study, it was also revealed that future climate conditions (+4 °C day/night air temperature and 800 μmol mol^{-1} of CO_2) considerably stimulated spring oilseed rapes' growth and to a lesser extent stimulated photosynthetic carbon assimilation (Kacienė et al., 2019). The positive effect of the increase in temperature within the optimum rage together with elevated CO_2 (Yoon et al., 2009; Juknys et al., 2011; Kacienė et al., 2017) or a less negative effect of extremely high temperatures under elevated CO_2 (Ameye et al., 2012; Yu et al., 2012) can be explained by the increased optimal temperature for plant growth (Long and Drake, 1991; McMurtrie and Wang, 1993) and net photosynthesis (Bernacchi et al., 2006; Alonso et al., 2009). The biochemical model of leaf photosynthesis proposed by Farquhar et al. (1980) shows that a relative increase in leaf CO_2 uptake with an increase in atmospheric CO_2 is greater at a high than at a low leaf temperature. This phenomenon could be explained by the fact that rising temperature favors oxygenation, as the solubility of CO_2 in water relative to that of O_2 declines with the increases in temperature and the activation energy requirement of the oxygenation reaction is greater than that of carboxylation, while CO_2 competitively inhibits oxygenation. As a result, the net increase in CO_2 uptake resulting from the suppression of photorespiration rises with temperatures (Long et al., 2004). It is estimated that the optimal temperature for many C_3 plants may increase by approximately 5 °C, as CO_2 increases by 300 mmol mol^{-1} (Long and Drake, 1991). Furthermore, because of higher CO_2 in the atmosphere, C_i/C_a did not reduce in HWC-W treatment either, although g_s slightly decreased (Fig. 4D,B and 5D,B). It has been observed that abscisic acid (ABA) shifts the heat-induced stomatal opening toward a higher temperature (Feller, 2006). Moreover, the CO_2 diffusion through stomata tends to be faster at higher temperatures.

Recent studies have revealed that heatwaves exacerbated the severity of drought-induced damage on plants' growth and physiology when applied simultaneously (Ruehr et al., 2016; Duan et al., 2017; Correia et al., 2018; Elferjani and Soolanayakanahally, 2018). Moreover, induced adverse effects were mostly larger than the sum of each of the stressors applied individually (De Boeck et al., 2011; Ruehr et al., 2016). In our study, heatwave and drought acted in a different manner. However, drought-induced stress on photosynthesis was also highly exacerbated under heatwave and heatwave with elevated CO_2 treatments, although under HWC treatment to a considerably lesser extent (Fig. 4A and Table A1). The data showed that A_{sat} in HW-D and HWC-D treatments was by 72% and 46% respectively lower than under single drought stress, suggesting an additive heat effect on A_{sat} (Fig. 5F). On the above-ground growth, however, drought-induced stress was only significantly exacerbated under HW treatment, showing also a potential additive manner of response of LA and shoot DW (Fig. 3A and B). Thus, in the agreement with other reports (Zinta et al., 2014; AbdElgawad et al., 2015), the results of this study also showed that elevated CO_2 ameliorated the adverse effect of combined drought and heatwave treatment on photosynthesis and above-ground growth during the stress. The mechanisms underlying the stress mitigating effect still have to be researched to understand them better. It has been found that, among the other aspects of the mitigating effect of CO_2 enrichment that include relatively increased growth (Xu et al., 2014), enhanced photosynthesis (Biswas et al., 2013; Xu et al., 2014; Zinta et al., 2014), and increased WUE and NUE (Leakey et al., 2009; Palmroth et al., 2013), a very important role is likely to be played by up-regulated antioxidant defenses metabolism as well as reduced ROS production (e.g. photorespiration) resulting in a decreased oxidative pressure (Zinta et al., 2014; AbdElgawad et al., 2016).

Opposing to the well-watered conditions, imposed drought stress under heatwave (HW and HWC) treatments significantly decreased stomatal conductance, resulting in considerable reductions of E and C_i/C_a under both HW-D and HWC-D treatments and increased WUE under HWC-D treatment (Fig. 4B–E and 5B-E). Thus, similarly to the effect under the single drought stress, these observations also suggest the direct stomatal limitations on photosynthesis because of restricted diffusion of CO_2 from the environment into the leaf. Moreover, even a close relationship between A_{sat}, g_s, C_i/C_a, and E of all drought-stressed plants with volumetric SWC was found (Fig. 6), as well as between A_{sat} and C_i/C_a, A_{sat} and g_s, C_i/C_a and g_s, and E and g_s (data are not shown), indicating that stomatal conductance was the primary factor responding to drought stress under both single and combined treatments. The highest g_s sensitivity to SWC compared to photosynthesis and transpiration was also recorded in naked oat (*Avena nuda*) (Wang et al., 2017). However, the values of g_s, C_i/C_a and A_{sat} in the HW-D and HWC-D treatments in responses to SWC during the stress period (days 3–7) were mainly continually lower compared to those in AC-D treatment (Fig. 6A–C and Table A1). In accordance to this, in the study with *Eucalyptus radiata*, it was found that elevated temperature treatments reduced A_{sat} and g_s at higher volumetric SWC than ambient temperature, indicating that sensitivities of A_{sat} and g_s to declining soil moisture increased in elevated temperature treatments (Duan et al., 2014). This is also consistent with the study of Zhao et al. (2013), in which a large g_s of *Thuja occidentalis* was found in the high-temperature treatment as long as the water supply was sufficient, followed by a sharp decline in soil water depletion. The reason for this is that high VPD associated with high temperature often accelerates evapotranspiration, thereby aggravating soil water depletion and further lowering g_s (Ohsumi et al., 2008; McDowell et al., 2011). Apparently, in the present study, more impaired photosynthesis and growth under the combined conditions of drought and heatwaves (HW and HWC), comparing with drought stress alone, were also partly attributed to the faster soil drying induced by higher evapotranspiration (through higher VPD) and as a result of this faster and sharper decrease in g_s and subsequent in C_i/C_a (Figs. 1C, 2 and 4B,D). Nevertheless, the values of A_{sat} in the HWC-D treatment in responses to SWC during the stress period (days 3–7) tended to be between AC-D and HW-D treatments (Fig. 6A and Table A1), which suggests that CO_2 enrichment could have alleviated drought stress, perhaps via improved WUE (Fig. 5E).

Reductions in photosynthetic rate are directly linked to WUE, which is one of the most critical parameters in crop response to osmotic imbalances. Numerous reports have shown that decreases in net photosynthetic rate, in response to drought stress, are associated with increased WUE as a result of stomatal closure (Lamaoui et al., 2018). While such responses were determined in AC-D and HWC-D treatments, the behavior of WUE in HW-D treatment was in an opposite manner (Figs. 4E and 5E), because of a higher decrease in A_{sat} than in E (Fig. 5A,C and Table A1). As it was found that the reductions in C_i/C_a ratio in both HW-D and HWC-D treatments during the stress period were similar (Figs. 4D and 5D), while A_{sat} and SWC in HW-D treatment were continually lower (Figs. 4A, 5A and 2), we presumed that a better photosynthetic performance in HWC-D treatment must be connected to an improved plant water relation. For C_3 plants, at high CO_2 concentrations, the WUE is increased through the direct increase in the metabolic substrate of carbon fixation and the decreased transpiration rate (Xu et al., 2013). We examined the relationship between leaf gas exchange and SWC, and the data of A_{sat} plotted against SWC showed that at a given SWC, the response of A_{sat} to the simultaneous heatwave and drought treatment was altered by CO_2 level. Under ambient CO_2, the A_{sat} began to decrease at higher soil SWC than under elevated CO_2 level (Fig. 6A). Improvements in SWC and photosynthetic assimilation under elevated CO_2, when soil dried out, were also found in the study of LeCain et al. (2003) with C_3 and C_4 grasses in a semi-arid ecosystem. Although the reduction in E in the HW-D and HWC-D treatments did not differ significantly during the stress period (Figs. 4C and 5C and Table A1), a weaker but significant effect of SWC was also determined on transpiration (Fig. 6D). It was found that at medium SWC (15–20%), additional heat stress increased transpiration in comparison with single drought treatment, while elevated CO_2 diminished this increase. Hence, by improving WUE, elevated CO_2 could have diminished the adverse effect of drought on A_{sat} (Figs. 4A and 5A) and shoot growth (Fig. 3A,C) to a significant level under heatwave conditions. However, it was not enough in order to fully negate the induced damage in simultaneously heatwave and drought affected plants' leaf physiology and growth. Moreover, the data of E plotted against SWC showed that at low SWC (5–10%), elevated CO_2 led to even higher transpiration compared to single drought and simultaneous drought and heat treatment (Fig. 6D). So, in accordance to other reports (Urban, 2003), these results show that a higher CO_2 concentration in the atmosphere also has the potential to exacerbate heat stress by reducing foliar cooling via transpiration. Also, consistently with previous studies (Norby and Luo, 2004; Dermody et al., 2007; Albert et al., 2011), our observations confirm that a combination of high temperature and drought may eliminate the improvement in water status under elevated CO_2 in the long term.

Another reason for a worse photosynthetic performance under HW-D and to a lesser extent under HWC-D treatments could be an increased leaf temperature due to lower cooling by reduced transpiration (Figs. 4C and 5C). It is known that the combination of drought and heat stress might require antagonistic responses (Mittler and Blumwald, 2010), and that was the case in our study regarding E. While during HW and HWC treatments, well-watered plants increased their transpiration to reduce leaf temperature, drought-stressed ones grown under HW treatments had markedly reduced E (Figs. 4C and 5C), so their leaf temperature remained higher. It was shown that if the heat stress occurred simultaneously with drought, plants are not able to open their stomata and their leaf temperature can be up to 2–5 °C higher (Rizhsky et al., 2002, 2004), which may be largely above the temperature optimum for photosynthesis. In the present study, the leaf temperature of drought-stressed plants grown under HW and HWC conditions was ~2 °C higher, compared to HW and HWC treated ones grown under well-watered conditions (data are not shown) that could exceed the upper temperature limits for photosynthesis. Furthermore, once the thermal threshold is exceeded, non-stomatal limitations, such as structural alterations in chloroplast protein complexes, increased photorespiration, increased mitochondrial respiration, inactivation of Rubisco attributed to reduced activity of Rubisco activase, decreased activity of photosystem II (PSII) and many other damages could occur, further reducing carbon assimilation (Teskey et al., 2015; Sita et al., 2017; Zandalinas et al., 2018).

In summary, the results of our study demonstrate that probably the final higher response of A_{sat} to HW-D and HWC-D treatments, compared to drought stress alone (AC-D), is a combination of faster soil drying and increased leaf temperature under combined conditions of drought stress and high temperature (HW or HWC). However better performance of HWC-D treated plants, compared to those grown under HW-D conditions, might be related to the alleviation of drought stress by CO_2 enrichment due to improved plant water status via enhanced WUE.

4.3. Single and combined stress responses during recovery

The leaf gas exchange parameters of well-watered plants affected by heatwaves (HW, HWC) reversed after re-establishment of the control growing condition (Fig. 4A–E and 5F-J). The above-ground growth parameters increased even more during post-stress period, however, when the additional CO_2 was removed, no differences were observed between HW and HWC treatments (Fig. 3E and F), suggesting no memory effect of CO_2 enrichment after its removal. However, the root growth of plants grown under HWC conditions markedly increased, and their R/S ratio did not differ significantly from control, while those grown under HW conditions still had lower R/S (Fig. 3G and H). Thus, at the whole plant level, a marginal fertilization effect of elevated CO_2 might still be apparent even after its cessation. In contrast, plants affected by drought during the post-stress period possessed a lower growth than during the stress. After 7-day recovery, their LA, shoot and root DW compared to controls had decreased even more than at the end of the treatment (Fig. 3E–G), despite quite a fast and full recovery of gas exchange (Fig. 4A–E and 5F-J). Since the A_{sat}, g_s, and C_i/C_a of drought-stressed plants grown under AC conditions, towards the end of the exposure, all were significantly lower compared to controls (Fig. 4A–D, 5A-D and Table A1), these results indicate that compared to gas exchange, the effect of drought was lagged on the growth.

Both LA and shoot DW of HW-D and HWC-D treated plants as well as root DW of HW-D treated plants were significantly lower compared to controls (AC-W) after the 7-day recovery period. LA and root DW of drought-stressed plants grown under HW conditions were also significantly lower compared to drought-stressed ones grown under AC conditions (AC-D) (Fig. 3E–G). In accordance with this, A_{sat}, g_s, E, and C_i/C_a of HW-D and HWC-D treated plants, during the recovery period, all reversed at a slower and considerably lower strength, compared to AC-D treatment, with insignificant trend of a better recovery of HWC compared to HW treatment (Fig. 4A–D and Table A1). After 7-day recovery, A_{sat}, g_s, E, and C_i/C_a of both HW-D and HWC-D treated plants remain significantly lower compared to controls and AC-D treated ones (Fig. 5F–I). These findings are in line with other recent studies, showing the most restrictive effect of these stressors' combination compared to single effect of each stress conditions, leading to incomplete recovery (Ruehr et al., 2016; Duan et al., 2017). However, what has also been shown besides a complete reconstitution of transpiration and photosynthetic capacity along with a relaxation of water potentials after one week of recovery is also an increased rate of carbon gain compared to non-stressed controls for gray poplar trees that had experienced three weeks of drought-heat stress, both a periodic and a chronic (Georgii et al., 2019). Although in the latter study of Georgii et al., both drought-heat stress scenarios were imposed under elevated to 500 μmol mol^{-1} CO_2 level that was also during the recovery period, and no significant difference in net CO_2 assimilation rate, after recovery, was found between stress affected trees and non-stressed trees grown under elevated CO_2 level, suggesting that even a moderately increased CO_2 during the recovery may support photosynthesis. However, our study demonstrated that CO_2 enrichment had little effect on the recovery of A_{sat} and other gas exchange parameters, when additional CO_2 was also removed after the cessation of stress (Fig. 4A and Tables 2, A1). Similarly, in the study with spring barley (*Hordeum vulgare*), Žaltauskaitė et al. (2019) also found that elevated CO_2 had the potential to mitigate the adverse effect induced by heatwave and drought combination on barley's leaf gas exchange parameters during the stress but had no effect during the recovery. Furthermore, the results of the present study show that, in both HW-D and HWC-D treatments, A_{sat} decreased at a faster rate while, during the recovery period, it increased at a lower rate than during the treatments, indicating a slower recovery compared to the response to stress, and so did other gas exchange parameters, except WUE (Table 2). Thus, it could be stated that, regardless mitigating the effect of elevated CO_2 during the stress period, the rate of recovery of gas exchange parameters of plants experiencing combined heat and drought stress was slower than its rate of reduction during the period of treatments that led to a slower and an incomplete recovery of growth.

5. Conclusions

In conclusion, the results of the present study clearly showed that, at the early vegetative stage, up to 12 °C higher than ambient air temperature improved the photosynthetic performance and the growth of *B. napus* under well-watered conditions. CO_2 enrichment additionally reinforced those stimulations. Gas exchange of drought-stressed plants was modulated contrariwise to the effect of heatwave, but the down-regulation of photosynthesis by single drought stress was legged on the growth during the stress and became more apparent after the stress. Despite the opposing responses of gas exchange and growth to heatwaves and drought, drought-induced stress was highly exacerbated under heatwave conditions. A more impaired photosynthesis and growth under the combination of heatwaves and drought was attributed to the faster soil drying induced by higher evapotranspiration (through higher VPD) and, as a result, a faster and sharper decrease in g_s and subsequent in C_i/C_a. In addition, increased leaf temperature due to lower cooling by reduced transpiration could also play a significant role in down-regulation of A_{sat}. The elevated concentration of CO_2 diminished the adverse effect of a combined heat and drought stress on A_{sat} and shoot growth to a considerable extent. This was most likely the result of improved plant water relations. However, the rates of recovery of a combined drought and heat stress-affected gas exchange parameters were slower than its rates of reduction during the period of treatments that reflected into slower and incomplete recovery of growth, regardless mitigating the effect of elevated CO_2 during the stress. Hence, despite that the heatwave and drought acted in a different manner, these results highlight that drought might fully negate all the advantages gained from hotter climate and lead to a slower and incomplete recovery of gas exchange and retarded growth after the stress, confirming that water availability plays a predominant role during the heatwave conditions.

Contributions

Austra Dikšaitytė: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Akvilė Viršilė: Resources.

Jūratė Žaltauskaitė: Writing – review & editing, Supervision.

Irena Januškaitienė: Data curation.

Gintarė Juozapaitienė: Data curation.

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Appendix A. Supplementary data

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