The subsequent impacts of independent and combined drought and heat stress around flowering on maize grain filling: Field and laboratory investigation

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Abstract

ABSTRACT Drought along with high temperature around flowering is becoming more frequent to strongly influence maize production. However, the impacts of independent and combined drought and heat stress during this stage on subsequent grain filling have received limited attention. Here we investigated the response of grain development to three stresses (drought, heat and combined drought and heat stresses (DS, HS, DHS)) around flowering in ponds covered with a rain shelter in field. In additional, some grains were incubated in laboratory after pollination. Compared with control treatment, the decreased rate of grain weight was in the order of HS[?]DS[?]DHS in both experiments. In field, grain weight was decreased by 5-11% in stresses. The leaf senescence was accelerated together with decreased photosynthesis rate. Grain weight was still reduced largely in stresses with sufficient source supply in laboratory, which implicated a subsequent sink (grain) limit by early stresses. This mainly resulted from the disturbed carbohydrate metabolism and starch synthesis such as the activities of surcose invertase, sucrose synthase, ADP Glc pyrophosphorylase and starch synthase at early grain filling stage. This study provided information on how to promote drought and heat tolerance hybrids and mitigated management strategy.

1 | INTRODUCTION

As the first cereal crop in the world, maize production is important to ensure food security (FAO, 2020). Because of the increasing population and feed demand due to diet shift in many areas, the continuous improvement of maize yield is a great challenge in the new era. Maize is sensitive to abiotic stresses such as drought and heat, which are major environmental factors that limit crop growth and productivity (Bonfante et al., 2015; Lizaso et al., 2018; Rossini, Maddonni, & Otegui 2016; Shen et al., 2018; Waha, Müller, & Rolinski, 2013). Drought and heat stress has been becoming more sever in time and over space around the world since 1980 (Dai, Trenberth, & Qian, 2004; Sheffield, Wood, & Roderick, 2012; Wang, Marija, Dong, Susanne, & Bernd, 2014; Harrison, Tardieu, Dong, Messina, & Hammer, 2014). Heat stress is frequently associated with reduced availability of water (Lesk, Rowhani, & Ramankutty, 2016; Wang et al., 2018), and the occurrence of drought accompanied by heat stress is predicted to increase in future (IPCC, 2014). Therefore, the understanding of independent and combined drought and heat stress impacts on maize during the whole growth duration and the underlying mechanism is pivotal to promote breeding and targeted management measures.

Across the global maize production area, the combined drought and heat stress mostly occurred around flowering (Lesk, Rowhani, & Ramankutty, 2016; Wang et al., 2018). During this stage, the adverse impacts of the stresses on the male and female maize organs for the seed-set and seed formation have been frequently reported (Bassetti and Westgate, 1993; Edreira, Carpici, Sammarro, & Otegui, 2011; Marceau, Saint-Jean,

Loubet, Foueillassar, & Huber, 2012; Sánchez, Rasmussen, & Porter, 2014; Wang et al., 2018). The previous studies indicated that maize kernel number was significantly reduced in the stresses because of low pollen number and pollen viability, slow silk-growth and ovary-growth rate, and weak silk receptivity (Alam et al., 2017; Dresselhaus, & Franklin-Tong, 2013; Wilhelm, Mullen, Keeling, & Singletary, 1999; Oury, Tardieu, & Turc, 2016; Oury et al., 2016). For example, the drought stress from tassel emergence to 6 days after silking decreased the kernel numbers by 42-77% (Oury et al., 2016). The heat stress in 15 days from start of silking onwards reduced the kernel numbers by 75% (Edreira, Carpici, Sammarro, & Otegui, 2011). Most of current studies has analyzed the impacts on maize kernel number by the heat and drought stresses, but the persistent impact of the stresses around flowering on the subsequent maize kernel development has received limited attention. Furthermore, limited information is available on the individual and combined impacts of heat and drought stress during the early flowering stage on the qualitative aspects in maize grain weight.

For wheat, some studies indicated that the early (around flowering) drought and heat stress shortened leaf area duration (Cristina, Daniel, Calderini, & Gustavo, 2007), decreased chlorophyll index (Hlaváováet al., 2018) and reduced net photosynthetic rate (Wang, Marija, Dong, Susanne, & Bernd, 2014). The subsequent impact of early heat stress on grain weight in field was also observed (Abeledo, Savin, & Slafer, 1999; Calderini, Savin, Abeledo, Reynolds, & Slafer, 2001; Cristina, Daniel, Calderini, & Gustavo, 2007). For barley, the heat exposed to booting-anthesis stage decreased grain weight by 13.4% (Cristina, Daniel, Calderini, & Gustavo, 2007). For rice, grain yield was reduced by 24% because of the combined drought and heat stress at anthesis (Amjikarai, Chenniappan, & Dhashnamurthi, 2018). These observations showed grain development was limited by the source (canopy structure and function), which was affected by the early stress. Whether the sink (grain) development was also limited by the early stress around flowering if source supply was not limited since grain filling remained unclear. Meanwhile, the underlying physiological mechanism for the response of subsequent grain development with both limited and non-limited source supplies to the early heat stress should be further addressed.

It has been generally accepted that the grain weight was determined during the period since grains were actually set (Egli, 2004). Therefore, most studies for grain weight focused on the post-anthesis stage for grain filling (Royo, Abaza, Blanco, García, & Luis F, 2000). In this study, we hypothesized that the independent and combined drought and heat stress around flowering stage still has significant effect on the subsequent grain filling process even with adequate source supply although the stress was relieved since the beginning of grain filling. The purpose of this study was to (i) detect and compared the impacts of the drought stress, heat stress, and combined drought and heat stress around flowering on the subsequent kernel weight, (ii) compare the response of subsequent grain development to the early stresses with limited source supply in field and adequate source supply in laboratory incubation, (iii) investigate the underlying mechanism of the above responses. This study included three experiments (Figure 1). In Experiment 1 (Exp. 1), maize plants were subjected to drought stress, heat stress and combined drought and heat stress during the short period from tassel emergence to ovaries fertilization in ponds covered with a rain shelter, and the fertilized ovaries continued to grow in field. In Experiment 2 (Exp. 2), the fertilized ovaries in Exp. 1 were vitro cultured in the chamber with favorable growth conditions in laboratory incubation. Sufficient source supply was provided for kernel growth in vitro cultured, which was proved to be an useful method for investigating the grain development (Burle, Gengenbach, Robert, 1994; Zhang et al., 2017). In Experiment 3 (Exp. 3), maize plants were subjected to similar stress with Exp. 1 around flowering in pots, the fertilized ovaries were also vitro cultured for the measurement of carbohydrate metabolism and starch synthesis in grain. Our study reports for the first time about the subsequent impacts of independent and combined drought and heat stress around flowering on maize grain filling and thus necessities the need for development of maize genotypes resilient to these stresses, especially to combined stress environments.

2 | MATERIALS AND METHODS

2.1 Experimental site and management

The Exp. 1 and Exp. 2 was conducted under a half-electric rain shelter at the Shangzhuang Experimental Station of China Agricultural University, Beijing, China, (40° 08'N, 116° 10'E) in 2019. The half-electric rain

shelter contained 9 big ponds (2 m long, 4 m wide, and 1.8 m deep) and 9 small ponds (2 m long, 2 m wide, and 1.8 m deep) (Figure 1). Each pond was cemented on the four sides and the bottom, and each pond was filled with soil. The soil was classified as loam. The chemical properties of top 20 cm soil layer sampled prior to planting was total nitrogen 0.81 g kg^{-1}, organic matter 18.88 g kg^{-1}, rapid available phosphorus 30.31 mg kg^{-1}, rapid available potassium 100.47 mg kg^{-1}, soil bulk density 1.35 g cm^{-3}.

Three seeds (Zhengdan 958) per hole were sowed on May 17, 2019 in each pond and the seedlings were thinned to 7.5 plants m^{-2}at the 3-leaf stage with a row spacing of 60 cm and plant spacing of 20 cm. Prior to sowing, 120 kg N ha^{-1}, 50 kg P_2O_5 ha^{-1}, and 150 kg K_2O ha^{-1} was applied evenly to the soil surface. The additional 120 kg N ha^{-1} was applied by furrowing at the 12-leaf stage. Weeds, pests, and diseases were well controlled during the two seasons.

As a pot experiment, Exp. 3 was also conducted at the Shangzhuang Experimental Station in 2016. The same hybrid (Zhengdan 958) was grown in a 120 pots (diameter was 30 cm at the top and 25 cm at the bottom, 29 cm deep). In each pot, 20 kg naturally air-dried soil that was 1 cm sieved per pot was filled. The bottoms and inside of pots were perforated with small holes which allow free drainage. The soil was classified as loam. The chemical properties were total nitrogen 0.99 g kg^{-1}, organic matter 20.95 g kg^{-1}, rapid available phosphorus 24.03 mg kg^{-1}, rapid available potassium 87.33 mg kg^{-1}, soil bulk density 1.28 g·cm^{-3}. All the pots were buried in the 25 cm ditch avoiding high soil temperature. At the commencement of the experiment, fertilizer was applied prior to grown with the amount of 3.9 g N pot^{-1}, 12.5 g $P_2O_5pot^{-1}$, and 5.8 g K_2O pot^{-1}, and then 2.6 g pot^{-1} was top-dressed at V12 stage. Four seeds were sown in the central of each plot, at the V3 stage, the five maize seedlings were thinned to one plant per pot.

FIGURE 1

2.2 Imposition of the drought and heat stress

The Exp. 1 was arranged in a completely randomized block design with four replications. The ponds were kept from tasseling to ovaries fertilization under different stress treatments as follows: (I) Well-irrigated in field (CK), the soil potential was -0.12 MPa (II) Drought stress (DS), introducing a water deficit by no irrigation about 7 d before tasseling, the soil water potential was -0.51MPa. (III) Heat stress (HS), which was according to the method of Savin, Stone, & Nicolas (1996). The polyethylene film (100 μm thickness) was mounted on steel structures of 3.5 m height (as showed in Figure 1a), but leaving the bottom 50 cm of the four sides of each structure open and the top 30 cm open, in order to facilitate free gas exchange from top to bottom. The magnitude of the elevated canopy air temperature (about 3-5 higher than field) was the consequence of the greenhouse effect of the polyethylene enclosure (Cicchino, Edreira, & Otegui, 2010). (IV) Combined drought and heat stress (DHS) were realized by the both methods of DS and HS. The soil water potential (depth 40 cm) and air temperature (ear position) were recorded by Em50 (Decagon, America) and GSP-6 (Elitech, China), respectively. These sensors of Em50 were buried 40 cm underground of plot. The effects of stresses in experiment were obvious (Figure 2).

In Exp. 2, the fertilized ears were sampled. According to the methods of Burle, Gengenbach, Robert (1994) and Zhang et al. (2017), husk tissues and silks were gently removed with a fine scalpel blade, taking care to avoid breaking the ovary. Then the superior kernels (basal 18-20^{th} line) and inferior kernels (top 3 cm) of the ear were sectioned into blocks of 4×4 (16 grains) and seated into culture media (adequate source supply) in 25×100 mm Petri dishes such that the media contacted only the cob tissue (Figure. 1e). Kernels were incubated in thermostatic incubator (YIHENG TECH-NICAL.CO., LTD) with 25, relative humidity 75%. To sterilize for 20 minutes with ultraviolet lamp and wipe the inner wall with 75% ethanol to protect the culture materials from contamination before using thermostatic incubator.

In Exp. 3, the soil moisture regime of 120 pots was 60+-5% (well-irrigated) of soil water holding capacity before stress treatment. Then the pots were kept from tasseling to ovaries fertilization under different stress treatments as follows: (I) Well-irrigated in field (CK). (II) Drought stress (DS), introducing a water deficit (30+-5%) by no irrigation about 7 d before tasseling. (III) Heat stress (HS), the 30 pots were 60+-5% (well-water) of soil water holding capacity also transferred into greenhouse covered with polyethylene film. The

elevated canopy air temperature (about 3-5 higher than field) was also the consequence of the greenhouse effect of the polyethylene enclosure. (IV) Combined drought and heat stress (DHS) were realized by the both methods of DS and HS. The soil moisture and temperature were recorded by HD2 (IMKO Micromodultechnic GmbH, Germany) and GSP-6 (Elitech, China) during the stress treatment (Figure 2). When the ears were fertilized, the grain was cultured in vitro by the methods of Exp. 2.

FIGURE 2

2.3 Sampling and measurements

2.3.1 Measurement and sampling in Exp. 1

Four maize plants were selected in each treatment at the tasseling stage. The maximum length (L, cm) and real width (W, cm) of each leaf were measured using the formula (LxWx0.75) to calculate the total plant leaf area. The plant tagged were measured 9 times on DAS (Days after sowing) 61, 66, 75, 82, 89, 99, 108, 118, 123. The SPAD-502 chlorophyll meter was used to monitor chorophyll concentrations (a.u., i.e., SPAD reading) in ear leaf of selected plant. The middle section of the ear leaf was measured 5 times, avoiding the main leaf vein on DAS 59, 64, 69, 75, 82, 89, 99, 108, 118, 123. The net photosynthetic rate of the ear leaf was measured at 10:00-11:00am using Li-CorLI-6400XT Portable Photosynthesis System (Licoln, NE, USA) at DAS 64, 69, 75, 82, 89, 99,108, 123.

Plants growing well were tagged and hand-pollinated by enclosing ears in bags from silk emergence until the last emerged silks became senescent. 200 superior grains (middle grains of spike) and 200 inferior grains (upper grains of spike) from above plants were obtained to determine the grain dry weight at DAS 78, 88, 98, 108, 118, 123. All grains were dried at 70 to constant weight and weighed. The processes of grain filling were fitted by Logistic growth equation (Figure 3) (Gao et al., 2017):

$W=K/(1+ae^{-bt})$

Where W is the kernel weight (mg); K is the final kernel weight (mg); t is the time after pollination; a, b are coefficients determined by regression. The onset time of grain filling peak stage (d): t1=(lna-1.316) b^{-1}; The end time of grain filling peak stage (d): t2=(lna+1.316) b^{-1}; The end of grain filling (d): t3=(lna+4.595) b^{-1}; The slow-increase period (d): T1=t1; The fast-increase period (d): T2=t2-t1; The slight-increase period (d): T3=t3-t2; The mean grain filling rate (mg $grain^{-1}$ d^{-1}): V=K $t3^{-1}$; Mean grain filling rate of slow-increase period (mg $grain^{-1}$ d^{-1}): V1=W_{t1} $T1^{-1}$; Mean grain filling rate of fast-increase period (mg $grain^{-1}d^{-1}$): V2=(W_{t2}-W_{t1}) $T2^{-1}$; Mean grain filling rate of slight-increase period (mg $grain^{-1}$ d^{-1}): V3=(W_{t3}-W_{t2}) $T3^{-1}$.

FIGURE 3

2.3.2 Sampling in Exp. 2

Five superior and inferior grains from each treatment were sampled at DAS 78, 88, 98, 108 d after vitro culture in Exp. 2. All grains were dried at 70 to constant weight and weighed. The kernel weight and grain filling parameters were obtained by methods of Exp. 1.

2.3.3Measurements of soluble sugar and starch content in Exp. 3

The five grains sampled at DAP (Days after pollination) 10, 30, 40 after vitro culture for the measurement of soluble sugar and starch content with the anthrone-sulfuric acid method as follows. The grain pulverized to pass a 1 mm screen, 1 mg samples were extracted with of 80% ethanol. Extraction was done three times, followed by centrifugation at 4500 r/min for 10 min, supernatant was incubated at 100 degC for 20 min. The 0.2 mL supernatant was mixed with 1.25 mL of Anthron reagent (100 mg Anthron in 50 mL sulfuric acid). The mixture was heated in a boiling water for 10 min and cooled in water. OD was measured at 630 nm by using 1510 Microplate Reader (ThermoFisher, Shanghai, China).

The residue of the soluble sugar was mixed with 7 mL 3 mol L^{-1} of HCL, then heated in a boiling water for 10 min and cooled in water, followed by centrifugation at 4500 r /min for 10 min. The supernatant was mixed with 7 mL 3 of mol L^{-1}NaOH in 50 mL volumetric flask, and dilute with water to 50 mL. The starch

content were quantified by using 1510 Microplate Reader (ThermoFisher, Shanghai, China) at a wavelength of 625 nm after reaction with 0.5 mL solution from volumetric flask and 2.0 mL Anthron reagent at 45oC for 10-15 min.

2.3.4 Enzyme activity measurement in Exp. 3

The methods for enzyme activity of starch synthesis were slightly modified from those described by Nakamura,Yuki, Park, & Ohya, 1989 and Zinselmeier et al. 1995. Four grains from each treatment were sampled at DAP 10, 25 d after vitro culture in Exp. 3, then frozen in liquid nitrogen for 2 min and stored at -80 for enzyme activity measurements. The extract contained the following: 50 mM Hepes; 2 mM $MgCl_2$*$6H_2O$; 3% (V/V) $(CH_2OH)_2$; 1 mM DTT; 1 mM $EDTA_{-2}$ Na; 1 N NaCl.

ADP Glc pyrophosphorylase (EC 2.7.7.27) reaction system: 50 mM Hepes-NaOH (pH 7.2) 100 μL; 10 mM $MgCl_2{\cdot}6H_2O$ 50 μL; 15 mM DTT 100 μL; 1.5 mM ADPG 100 μL; 20 mM PPi 100 μL; Extract 200 μL.

Starch synthase (EC 2.4.1.21) reaction system: 50 mM Hepes-NaOH (pH7.2) 50 μL; 15 mM DTT 50 μL; 1.5 mM ADPG 50 μL; 1 mg Amylopectin; Extract 200 μL.

Sucrose invertase (EC 3.2.1.26) reaction system: CH_3COOH-CH_3COONa buffer (pH 4.8) 200 μL; 100 mM Sucrose 200 μL; Extract 100 μL.

Sucrose synthase (EC 2.4.1.13) reaction system: 50 mM Hepes-NaOH (pH7.2) 100 μL; 10 mM UDP 100 μL; 10 mM $MgCl_2{\cdot}6H_2O$ 50 μL; 100 mM Sucrose 150 μL; Extract 100 μL.

2.3.4 Hormone content measurement in Exp. 3

Four grains from each treatment in Exp. 3 were sampled at DAP 16, 32 d after vitro culture, then frozen in liquid nitrogen for 2 min and stored at -80 for GA_3, ZR, IAA, ABA measurements. The methods for hormones measurements from those described by Zhang et al. 2009, four frozen grains removed embryos were ground in an ice-cold mortar in 5 mL 80% (V/V) methanol extraction medium containing 1mM BHT as an antioxidant. The extract was incubated at 4 for 4 h and centrifuged at 5000 g for 15 min at the same temperature. The supernatants were passed through Chromosep C^{18} columns (C^{18}Sep-Pak Cartridge, Waters Corp, Millford, MA, USA), pre-washed with 10 mL 100% (V/V) and 5 mL 80% (V/V) methanol, respectively. About 5 mL purified fraction was collected and dried under N_2, and was dissolved in 1.5-3.0 mL phosphate buffer saline containing for analysis by an enzyme-linked immunosorbent assay (ELISA), which was provided by China Agricultural University.

2.4 Statistical analysis

All measurements described were comprised of at least 4 biological replicates. Data were separately analyzed for variance using SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA). Means were tested by least significant difference at the P 0.05 level (LSD 0.05).

3 | RESULTS

Compared with CK, kernel numbers (KNS) in DS, HS and DHS treatments was all decreased significantly (Figure 4a). The decrease rate of KNS among treatments was in the order of HS<DS<DHS. The KNS was decreased by 27%, 13%, 42% in DS, HS and DHS treatment, respectively. Furthermore, the subsequent impacts on grain filling of stress episodes around flowering was also observed in both field experiment in Exp. 1 and laboratory incubation experiment through grain vitro culture in Exp. 2 (Figure 4b, c).

3.1 The response of grain weight to early stress

episodes

For both Exp. 1 in field and Exp. 2 in laboratory, grain weight at physiology maturity in DS, HS and DHS treatments was all decreased significantly compared with CK (Figure 4b, c). Furthermore, the decreased grain weight by the early stresses was observed in both superior and inferior grains. For Exp. 1, the decreased rate of grain weight among treatments was in the order of HS<DS[?]DHS for superior grain and

DS[?]HS<DHS for inferior grain. Grain weight of superior grain was decreased by 7% in DS treatment, 5% in HS treatment and 8% in DHS treatment. For inferior grain, it was decreased by 6% in both DS and HS treatments and 11% in DHS treatment. For Exp. 2, the decreased rate of grain weight among treatments was in the order of DS[?]HS[?]DHS for superior grain and HS<DS[?]DHS for inferior grain. Compared with CK, grain weight of superior grain in DS, HS and DHS treatment was substantially decreased by 34%, 35% and 38%, respectively. Grain weight of inferior grain was decreased by 31% in DS treatment, 18% in HS treatment and 32% in DHS treatment.

FIGURE 4

3.2 The dynamic of grain filling process

The dynamic of grain dry matter accumulation was further investigated in both Exp. 1 and Exp. 2 (Figure 5). At the beginning of grain filling (78 DAS), no significant difference for grain weight (1000-grains weight) was observed among treatments. However, the subsequent grain filling process was significantly impacted by the early stresses for both superior and inferior grains.

FIGURE 5

In Exp. 1, the decreased grain filling rate and duration mostly occurred in phases I for superior grain (Figure 3, Table 1). As the most important grain filling period (Phase II, fast-increase period for grain dry matter) (Figure 3), the duration of grain filling in stress treatment was decreased (Table 1). Compared with CK, the growth duration was decreased by 4.7 days in DS treatment, 4.6 days in HS and 2.2 days in DHS treatment. For inferior grains, the response of grain filling was observed in the early stage (Phase I). For Exp. 2, the decreased grain weight in stress treatments was mainly because of the low grain filling rate while the growth duration was even extended for superior grains. During the Phase II, grain filling rate was decreased by 31.1% in DS treatment, 43.4% in HS treatment and 42.3% in DHS treatment. For inferior grains, the decreased grain weight in stress treatments also resulted from the lower grain filling rate compared with CK.

TABLE 1

3.3 The leaf area, chlorophyll content,and

net photosynthesis in stresses

To identify the source limit to grain weight by the early stresses, the leaf area, relative chlorophyll content (SPAD) and net photosynthesis rate of ear leaves was investigated in field in Exp. 1 (Figure 6). Because of the stresses since 63 DAS, leaf area decreased on 66 DAS in both DS and DHS treatments (Figure 6a). When the stresses were relieved, leaf area recovered for DS treatment on 75 DAS. However, leaf area was consistently lower in stress treatments compared with CK since 89 DAS. The large decrease for leaf area in stress treatments was observed in the middle-late grain filling stage. On 123 DAS (near physiology maturity), the leaf area was decreased by 33% in DS, 33% in HS and 36% in DHS.

The SPAD value was also decreased by the early stress at the middle-late growth stage (from 99 DAS to 123 DAS) (Figure 6b). On 123 DAS, the SPAD value was decreased by 30% in DS, 38 % in HS and 40% in DHS. The net photosynthesis rate was significantly reduced when stresses occurred on 69 DAS, but it recovered on 75 DAS when stress was eliminated in both DS and HS treatments (Figure 6c). The net photosynthesis rate continued to be lower in DHS treatment than CK after stress episodes. On 123 DAS, net photosynthesis rate was decreased by 16% in DS, 13% in HS and 40% in DHS. Furthermore, we found the reduction in leaf area, SPAD value, and net photosynthesis rate on 123 DAS compared with CK was in line with the decrease in 1000-kernel weight at physiological maturity (Figure 6d, e, f).

FIGURE 6

3.4 The soluble sugar content, starch content, andenzymes activities in grains

In the laboratory incubation experiment of Exp. 3, the soluble sugar content in both superior and inferior grains reduced with time and remained stable from the 30^{th} day after pollination (DAP) to grain physiology

maturity (Figure 7a). Similar soluble sugar content was observed among treatments at different stage in both superior and inferior grains with an exception of lower soluble sugar content in DHS treatment on the 10^{th} DAP. For starch content, it showed an increasing trend in both superior grain and inferior grains with time. In stress treatments, starch content in grain was significantly lower compared with CK. On the 40^{th}DAP, it was reduced by 12% in DS, 12% in HS, and 9% in DHS for superior grains.

FIGURE 7

The activities of the four enzymes in relation to starch synthesis in grain exhibited variable responses to stresses (Figure 8). Compared with CK, sucrose invertase activity was decreased by 30% in DS, 41% in HS and 30% in DHS on the 10^{th} DAP for superior grain. For inferior grain, it was reduced by 57% in DS, 40% in HS and 51% in DHS. Similar or even higher sucrose invertase activity was observed on the 25^{th} DAP in stress treatments (Figure 8a). For sucrose synthase activity, it was decreased by 30-45% on the 10^{th} DAP and by 28-39% on the 25^{th}DAP for superior grain in stress treatments. For inferior grain, it was decreased by 19-21% on the 10^{th} DAP in stress treatments (Figure 8b). For ADP Glc pyrophosphorylase activity, it was decreased by 21-67% on the 10^{th} DAP and by 16-50 % on the 25^{th} DAP for superior grain in the stress treatments compared with CK. For inferior grain, lower ADP Glc pyrophosphorylase activity was observed in both DS and DHS treatments (Figure 8c). For starch synthase activity, it was decreased by 22-34% on the 10^{th} DAP in stress treatment for superior grain (Figure 8d).

FIGURE 8

3.5 The hormone content in grains

In Exp. 3, the hormone contents related to grain filling changed with time in different treatments (Figure 9). Correlation analysis indicated that the average of grain filling rate was negatively correlated with the ZR content in both superior and inferior grains (Table 2). Compared with CK, ZR content was increased by 11% in DHS treatment on the 16^{th} DAP for superior grain (Figure 9a). It was increased by 19% in DS treatment, 20% in HS treatment and 16% in DHS treatment on the 16^{th} DAP for inferior grain. The mean grain filling rate was positively correlated with the IAA content in superior grains on the 32^{th} DAP. For IAA content, it was reduced by 21% in DS treatment, 26% in HS treatment and 20% in DHS treatment on the 32^{th} DAP for superior grain (Figure 9b).

FIGURE 9

TABLE 2

4 | DISCUSSION

We found the negative effects of DS, HS and DHS around flowering on maize grain filling. The early stress around flowering reduced kernel weight by shortened grain filling duration, accelerated leaf senescence, and decreased net photosynthesis in field condition. When the source supply was sufficient by vitro cultured in laboratory incubation, the early stress still significantly decreased kernel weight. These results clearly showed that the independent and combined drought and heat stress around flowering stage had significant impact on the subsequent grain filling even with sufficient source supply (Figure 10).

FIGURE 10

4.1 The reduced grain weight in field

Drought and heat stress are two main factors adversely affecting crop productivity. In the present study, the grain weight was decreased by 6-7% in DS treatment, 5-6% in HS treatment and 8-11% in DHS treatment under field condition (Figure 4), which was consistent with the previous reports (Jones, Roessler, & Ouattar, 1985; Westgate et al., 1994).

Multiple underlying mechanisms may contribute to the reduced grain weight in stresses in field. From a source (leaf) perspective, the drought and heat stress accelerated leaf senescence and decreased photosynthetic capacity (Hlaváová et al., 2018; Zhou, Zhou, He, Zhou, & Zhou, 2019). In this study, we found the leaf area

and SPAD were decreased by the early stress at the middle-late growth stage (from 99 DAS to 123 DAS) (Figure 6a, b). The net photosynthesis rate was decreased by 16% in DS treatment, 13% in HT treatment and 40% in DHS treatment on DAS 123 (Figure 6c). The inhibited leaf photosynthesis mainly resulted from the decrease of maximum photosynthetic electron transport rate and carboxylation efficiency (Wang et al., 2015). Furthermore, grain weight was reduced because the grain filling period was short in stresses (Chen et al., 2013; Mayer, Rattalino, & Maddonni, 2014; Plaut, Butow, Blumenthal, & Wrigley, 2004). Grain filling duration of superior grains was decreased by 4.7 days in DS treatment, 4.6 days in HS treatment and 2.2 days in DHS treatment at fast-increase period (Table 1). The results above showed that DS, HS and DHS treatment around flowering could shorten grain filling duration in superior grain, without sufficient increases in grain filling rate to compensate, resulted in grain weight reduction, which was inconsistent with previous researches (Jones, Roessler, & Ouattar, 1985; Yang et al., 2006). Some studies also showed that the drought and heat stress shortened the grain filling period, but the grain weight could be compensated by faster grain filling rate, enhanced pre-stored carbohydrate remobilization, and accelerated endosperm cell division (Jones, Roessler, & Ouattar, 1985; Yang & Zhang, 2006).

4.2 Grain development in laboratory incubation

The grain weight in vitro cultured incubation was still decreased largely by 18-38% in the stress treatments (Figure 4). This result showed the impacts of stresses exposed around flowering persisted when the sufficient source supply was provided. Through the dynamic of grain weight, we found the early stress did not shorten grain filling period, but the mean grain filling rate was reduced by 13-40% (Table 1).

Maize kernel development is mainly a process of carbohydrate metabolism and starch synthesis (Singletary, Banisadr, & Keeling, 1997). Some studies demonstrated that lack of assimilates within the embryo or endosperm limited maize kernel development in drought during grain filling stage (Westgate et al., 1994). In this study, the soluble sugar used to starch synthesis in the stress was relatively high (Figure 7a), suggested that the carbohydrate metabolism was not the main factor affecting final kernel weight. This was agreement with previous research in maize under water deficit (Oury, Tardieu, & Turc, 2016; Oury et al., 2016). However, the starch content of grain was significantly decreased in the DS, HS and DHS treatment (Figure 7b), indicating the sugar-to-starch synthesis was interfered in stresses during maize grain development (Jones et al., 1985; Singletary, Banisadr, & Keeling, 1994; Zinselmeier, & C, 1999).

Grain weight was reduced because the starch biosynthesis in endosperm cell was interfered in stress (Jones, Roessler, & Ouattar, 1985). Meanwhile, the rate of endosperm cell division was decreased with reduced starch granules in stress during maize seed development (Singletary, Banisadr, & Keeling, 1994). The enzymes activities for starch biosynthesis (surcose invertase, sucrose synthase, ADP Glc pyrophosphorylase and starch synthase) played a key role in this process (Figure 10). First, previous reports showed the sucrose invertase produced adequate glucose and fructose to allow grain set to proceed (Ruan, Jin, Yang, Li, & Boyer, 2010). In this study, sucrose invertase activity in grain was decreased by 30-57% in stress treatments on the early grain filling stage on 10^{th} DAP for superior grain (Figure 8a). Second, the sucrose synthase catalysed the cleavage of sucrose to form UDPG and fructose, and its activity was linked to sink strength in development grain (Kato, 1995; Yang, Zhang, Wang, Xu, & Zhu, 2004). The activity of sucrose synthase was decreased by 30-45% on the 10^{th} DAP for superior grain in stress treatments (Figure 8b) .Third, the ADP Glc pyrophosphorylase produced ADPG, which was the primer of the starch chain regarded as the rate-limiting enzyme in starch biosynthesis (Preiss, 1988; Smith and Denyer, 1992; Yang, Zhang, Wang, Xu, & Zhu, 2004). The activity of ADP Glc pyrophosphorylase was decreased by 21-67% on the 10^{th} DAP and by 16-50 % on the 25^{th}DAP for superior grain in the stress treatments compared with CK (Figure 8c). Finally, the previous found the starch synthase activity is positively correlated with the rate of starch synthesis (Keeling, Bacon, & Holt, 1993; Nakamura and Yuki, 1992). We also found starch synthase activity was decreased by 22-34% on the 10^{th} DAP in stress treatments for superior grain (Figure 8d)

The starch synthesis is not only affected by enzymes activity but also regulated by hormone content. It was reported that the content of Z+ZR and IAA in rice grains was related to the endosperm cell division and starch biosynthesis (Huang et al., 2016; Zhang et al., 2009). The DS, HS and DHS around flowering

increased ZR content by 11-20% in superior and inferior grains on 16^{th} and 32^{th} DAP (Figure 9a). The correlation analysis showed the mean grain filling rate was negatively correlated with the ZR content in both superior and inferior grains (Table 2). For IAA content, it was reduced by 20-26% in stress treatments on the 32^{th} DAP for superior grain (Figure 9b). The IAA content was positively correlated with grain filling rate in superior grains at 32^{th} DAP (Table 2). The IAA content mainly increased grain development in mid-grain filling, which was consistent with previous study (Yang, Zhang, Wang, Zhu, & Wang, 2001). In this paper, the ABA and GA_3 contents were also changed by DS, HS and DHS treatment around flowering (Figure 9c, d), however, there was no significant correlation with the mean grain filling rate (Table 2). The previous researches reported that the grain filling rate was closely associated with an enhanced ABA level in water-stressed grains (Yang, Zhang, Wang, Zhu, & Wang, 2001; Yang, Zhang, Wang, Xu, & Zhu, 2004). In addition, the GAs content was associated with the development of embryo at early grain filling stage (Schussler, Brenner, & Brun, 1991).

4.3 The implication for maize breeding

In this study, we found the subsequent impacts on grain filling of stresses episodes around flowering mainly resulted from the disturbing starch synthesis. Therefore, maize hybrids that have a high ability for starch synthesis in kernel are likely more tolerant to DS, HS and DHS. This should be received more attention in future breeding program. For example, the drought-tolerant lentil showed stronger ability of starch synthesis in seeds (Sehgal et al., 2018). Meanwhile, we found the grain weight was also reduced by shortened grain filling duration, accelerated leaf senescence, and decreased photosynthesis in field. This also implicated the importance of delaying senescence and improving photosynthetic capacity for maize breeding. For example, the drought-tolerant hybrid maintained greater photosynthetic rates (+8-10%) compared with the conventional hybrid (Lindsey, Barker, Metzger, Mullen, & Thomison, 2018). Meanwhile, the heat-tolerant germplasm emphasized the importance of sufficient CO_2 exchange rate (Neiff, Trachsel, Valentinuz, Balbi, & Andrade, 2016).

5 | CONCLUSION

The study showed that the negative effects of the stress around flowering persisted in the kernel. In field, the accelerated leaf senescence and decreased net photosynthesis rate was observed. Although the supply of source was sufficient in vitro cultured in the chamber, the effect of stresses imposed around flowering on kernel weight was inevitable. The kernel weight reduction mainly resulted from decreasing enzyme activities related to starch synthesis, which further reduced grain filling rate. It is necessary to further investigate the effect of the early stresses on kernel through the view of key proteins and genes. This information is important for breeding and selecting stress tolerant maize hybrids.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

The work was envisioned by Qingfeng Meng, Shoubing Huang and Pu Wang jointly led the research. Xiwei Liu wrote the manuscript with primary support from Qingfeng Meng. Guanying Chen was responsible for collecting data from Exp. 3. Chenchen Xu, Yonghong Yu and Jia Gao participated in the acquisition of experimental data. Xingya Wang contribution to analysis and interpretation of data. All co-authors commented on the manuscript.

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Figure legends

FIGURE 1 (a) Partial view of Exp. 1 and Exp. 2 with the drought and heat stress, with a detail on how the system looked like from inside the enclosures. (b) Partial view of Exp.3 with the drought and heat stress, with a detail on how the system looked like from inside the enclosures. (c) The stage exposed to drought, heat and combined drought and heat stress for each treatment. (d) The growth conditions in Exp.1. (e) Processing of maize grains cultivation in vitro in Exp. 2 and Exp. 3. CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 2 (a) Time courses of average soil water potential and temperature in Exp. 1 and Exp. 2. (b) Time courses of soil water content and temperature in Exp. 3. CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 3 Schematic diagram of logistic curve (black color), where W is the kernel weight (mg), K is the final kernel weight (mg), t is the time after pollination. a, b are coefficients determined by regression. Phase I: The slow-increase period (d); Phase II: The fast-increase period (d); Phase III: The slight-increase period (d).

FIGURE 4 Effects of drought, heat and combined drought and heat stress around flowering on kernel numbers and 1000-kernels weight at physiological maturity. (a) The kernel numbers in Exp. 1. (b) The 1000-kernels weight in Exp.1. (c) The 1000-kernels weight in Exp. 2. Values with the same letter are not significantly different at$P$ <0.05 within the same parameter of each hybrid. Bars denote the standard error of the mean (n=4). CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 5 Effect of drought, heat and combined drought and heat stress around flowering on grain dry matter accumulation. (a) The changes of 1000-kernels weight in superior and inferior grains in Exp. 1. (b) The 1000-kernels weight in superior and inferior grains in Exp. 2. Values with the same letter are not significantly different at$P$ <0.05 within the same parameter of each hybrid. Bars denote the standard error of the mean (n=4). CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 6 Effect of drought, heat and combined drought and heat stress around flowering on leaf area, SPAD and net photosynthesis (Pn; μmol CO_2 m^{-2} s^{-1}). (a) The changes of leaf area in Exp.1. (b) The changes of SPAD in Exp.1. (c) The changes of net photosynthesis in Exp.1. (d) The relationship between 1000-kernel weight and leaf area reduction at maturity. (e) The relationship between 1000-kernel weight and SPAD reduction at maturity. (f) The relationship between 1000-kernel weight and net photosynthesis reduction at maturity. Values with the same letter are not significantly different at P<0.05 within the same

parameter of each hybrid. Bars denote the standard error of the mean (n=4). CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 7 The soluble sugar content and starch content in superior and inferior grains under drought, heat and combined drought and heat stress around flowering. (a) The changes of soluble sugar in Exp. 3. (b) The changes of starch content in Exp. 3. Values with the same letter are not significantly different at P<0.05 within the same parameter of each hybrid. Bars denote the standard error of the mean (n=4). CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 8 Enzymes activity related to starch synthesis in superior grains and inferior grains under drought, heat and combined drought and heat stress around flowering in Exp. 3. (a) The sucrose invertase activity in superior and inferior grains. (b) The sucrose synthase activity in superior and inferior grains. (c) The ADP Glc pyrophosphorylase activity in superior and inferior grains. (d) The starch synthase activity in superior and inferior grains. Values with the same letter are not significantly different at P <0.05 within the same parameter of each hybrid. Bars denote the standard error of the mean (n=4). CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 9 Hormone content in superior grains and inferior grains under drought, heat and combined drought and heat stress around flowering in Exp. 3. (a) The ZR content in superior and inferior grains. (b) The IAA content in superior and inferior grains. (c) The GA_3 content in superior and inferior grains. (d) The ABA content in superior and inferior grains. Values with the same letter are not significantly different at P<0.05 within the same parameter of each hybrid. Bars denote the standard error of the mean (n=4). CK, control treatment. DS, drought stress treatment. HS, heat stress treatment. DHS, combined drought and heat stress treatment.

FIGURE 10 A schematic diagram of how drought, heat and combined drought and heat stress around flowering reduced kernel weigh. CK: control treatment; DS: drought stress treatment; HS: heat stress treatment; DHS: combined drought and heat stress treatment. Solid line indicated superior grain. Dotted line represented the inferior grain. The three lines from left to right represent DS, HS and DHS stress. The line thickness represents the influence degree of stresses.

Table legends

TABLE 1 Grain filling parameters under the condition of drought, heat and combined drought and heat stress around flowering in Exp. 1 and Exp. 2.

TABLE 2 Correlations of hormonal content in superior and inferior grains with mean grain filling rate at 86 and 102 days after pollination.

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FIGHRE 1

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FIGURE 2

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FIGURE 3

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FIGURE 4

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FIGURE 5

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FIGURE 6

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FIGURE 7

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FIGURE 8

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FIGURE 9

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FIGURE 10

TABLE 1 Grain filling parameters under the condition of drought, heat and combined drought and heat stress around flow

Parameters	
Exp. 1	
Phase I (days)	
Phase II (days)	
Phase III (days)	
Total Phase (days)	
V1 (mg grain^{-1} d^{-1})	
V2 (mg grain^{-1} d^{-1})	
V3 (mg grain^{-1} d^{-1})	
V (mg grain^{-1} d^{-1})	
Exp. 2	
Phase I (days)	
Phase II (days)	
Phase III (days)	
Total Phase (days)	
V1 (mg grain^{-1} d^{-1})	
V2 (mg grain^{-1} d^{-1})	
V3 (mg grain^{-1} d^{-1})	
V (mg grain^{-1} d^{-1})	

Note: Phase I: Slow-increase period; Phase II: Fast-increase period; Phase III: Slight-increase period; Total Phase: Filling d

TABLE 2 Correlations of hormonal content in superior and inferior grains with mean grain filling rate at 86 and 102 days

Superior	
Inferior	

, ** Indicate correlation significance at the 0.05 and 0.01 probability levels, respectively (n=12)