Heat waves imposed during early pod development in soybean (*Glycine max*) cause significant yield loss despite a rapid recovery from oxidative stress.

Running head: Impacts of heat waves on soybean physiology.

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Abstract

Heat waves already have a large impact on crops and are predicted to become more intense and more frequent in the future. In this study heat waves were imposed on soybean using infrared heating technology in a fully open-air field experiment. Five separate heat waves were applied to field-grown soybean (*Glycine max*) in central Illinois, three in 2010 and two in 2011. Thirty years of historical weather data from Illinois were analyzed to determine the length and intensity of a regionally realistic heat wave resulting in experimental heat wave treatments during which day and night canopy temperatures were elevated 6 °C above ambient three days. Heat waves were applied during early or late reproductive stages to determine if and when heat waves had an impact on carbon metabolism and seed yield. By the third day of each heat wave, net photosynthesis (*A*), specific leaf weight (SLW) and leaf total non-structural carbohydrate concentration (TNC) were decreased while leaf oxidative stress was increased. However, *A*, SLW, TNC and measures of oxidative stress were no different than the control ca. 12 hours after the heat waves ended, indicating rapid physiological recovery from the high temperature stress. That end of season seed yield was reduced (~10%) only when heat waves were applied during early pod development stages indicates the yield loss had more to do with direct impacts of the heat waves on reproductive process than on photosynthesis. Soybean was unable to mitigate yield loss after heat waves given during late reproductive stages. This study shows that short high temperature stress events that reduce photosynthesis and increase oxidative stress resulted in significant losses to soybean production in the Midwest, U.S. The study also suggests that to mitigate heat wave-induced yield loss, soybean needs improved reproductive and photosynthetic tolerance to high but increasingly common temperatures.

Introduction

Heat waves can dramatically impact crop and forest productivity (Filewod and Thomas, 2014; Bauweraerts et al., 2013; Ciais et al., 2005). In 2003, a heat wave in Europe caused an estimated 13 billion dollars in agricultural loss (Parry et al., 2007). During a heat wave that covered most of eastern Russia in 2010, wildfires emitted 10 Tg of CO_2 (Konovalov et al., 2011). Heat waves are predicted to increase in frequency and intensity with global climate change (Seneviratne et al., 2012; Meehl and Tebaldi, 2004). However, few experimental efforts have been made to investigate how heat waves impact crop physiology and quantify their impact on yield.

Soybean (*Glycine max*) is the fourth most important commodity crop globally and is grown on over 67 million acres of the United States (National Agricultural Statistics Service, 2011). Soybean is sensitive to periodic temperature increases and in the Midwestern United States may currently be growing close to or even above its average optimum seasonal temperature (Ferris et al., 1999; Lobell and Asner, 2003; Kucharik and Serbin, 2008). Additionally, soybean reproductive development typically has a lower temperature optimum (ca. 26 °C) than vegetative growth (ca. 30 °C; Hatfield et al., 2011). Thus, heat waves both current and future can be expected to have large physiological and economic effects on soybean.

There are a number of ways that heat wave temperatures might affect plant productivity. In soybean, two of the processes that are the most sensitive to temperature and central to yield and productivity are net photosynthesis (*A*) and pod set (Zinn et al., 2010). Photosynthesis can be affected by temperature in direct or indirect ways. Indirectly, high temperature events increase the vapor pressure deficit and evaporative demand (DeBoeck et al., 2010), which more rapidly depletes soil moisture causing stomatal closure, and leading to decreased rates of *A*. The largest direct high temperature effect on C3 plants such as soybean, is the decrease in net *A* due to the increase in the rate of photorespiration at elevated temperature. Higher temperatures alter the enzymatic properties of rubisco and decrease the solubility of CO_2 relative to O_2. Both of those factors favor increase oxygenation of ribulose-1,5-bisphospate (RuBP) by rubisco at elevated temperature (Badger and Andrews, 1974). Rubisco activase can also be directly affected by elevated temperature and has been linked to declines in photosynthesis at high temperatures (Salvucci and Crafts-Brander, 2004). The permeability of the thylakoid membrane increases with temperature, which compromises the membrane's ability to maintain transmembrane ion gradients necessary for photosynthesis (Schrader et al., 2004). The potential for damage to the

water evolving complex and photosystem II (PSII) is also greater as temperatures increase beyond optimum, which can be detected as a decrease in the operating efficiency of photosystem II (φPSII) using fluorescence techniques (Baker, 2008). The hardiness of PSII and rubisco activase to resisting temperature stress is species dependent, but generally the operating efficiency of PSII begins to decline above 35 °C in nearly all species (Gamon and Pearcy, 1990).

The transition from a fertile flower to a developing pod is known as pod set. As in all legumes, pod set in soybean is highly sensitive to temperature and photo-assimilate availability (Boote et al., 2005). The rates of pod set and seed growth in soybean are relatively consistent from 22 to 30 °C but decrease sharply at higher temperatures (Egli and Wardlaw, 1980).

Many temperate plants have an extensive capacity to mitigate damage during and recover from high temperate events. High temperatures have been observed to stimulate the development of flowers and pods on secondary and tertiary inflorescences in soybean and thus moderate yield reduction (Koti et al., 2005; Zheng et al., 2002). Furthermore, soybean has an extended flowering period producing flowers for 20 to 30 days (Castro et al., 2009; Smith et al., 1988), which implies a potential for yield recovery following a heat wave by increased pod set. Photosynthetic and respiratory acclimation can also occur in response to high temperatures (Rosenthal et al., 2014). This acclimation can include but is not limited to: decreased daytime respiration, increased electron transport capacity and the synthesis of a more heat stable rubisco activase (Way and Yamori, 2014; Sage and Kubien, 2007). Increased oxidative stress, which is frequently associated with high temperature events, is expected to slow the repair of the photosynthetic

machinery (Nishiyama et al., 2001). Although recovery is not instantaneous, damage to PSII usually recovers after heat stress.

We hypothesized that if leaf temperatures within the heat wave treatments consistently exceed optimal temperatures then (1) *photosynthesis will be decreased in the short term due to stomatal closure and increasing the proportion of photorespiration relative to carbon assimilation,* and (2) *damage, which compromises ϕPSII, will cause reductions in carbon assimilation that will persist for a period after the end of the heat wave and affect season long yield.* Also, given the length of the heat wave treatments relative to the length of the developmental periods over which they were applied, (3*) any damage done to reproductive structures or processes during the heat waves will have negligible impact on yield due to the long flowering period for soybean and its consequent ability to compensate for yield loss*. To investigate these predictions we elevated plant canopy temperatures in field-grown soybean by 6 °C for 72 h periods at discrete crop developmental stages over two growing seasons in order to determine the effects of heat waves in central Illinois on soybean physiology and productivity.

Methods

Site and heating infrastructure description

Field-grown soybean (*Glycine max cv.* Pioneer 93B15) was exposed to three heat waves during 2010 and two heat waves during 2011 using arrays of infra-red lamps at the SoyFACE research facility located on the University of Illinois research farm, Savoy, IL, USA. The 32 ha field site is planted half in corn and half in soybean, and these crops are rotated yearly. The site is tile drained and employs standard agronomic practices for the region. Soybeans were planted in 0.75

m row spacing at ~350,000 plants per hectare in 2010 and 0.4 m row spacing and ~350,000 plants per hectare in 2011. Within a linear, 1 m stretch of a 0.75 m spaced row in 2010 there was an average of 27 plants. In 2011, there was an average of 17 soybean plants in a linear, 1 m stretch of 0.4 m spaced rows.

The IR heating method in this experiment was modified from Kimball (2005) and fully described in Ruiz-Vera et al. (2013). The circular heated area of each heat wave subplot was 7 m^2. In 2010 the heat wave treatments covered a width of 5-6 rows of plants (0.75 m row spacing) and 11-13 rows in 2011 (0.4 m row spacing). Heaters (Salamander Ceramic Infrared Heaters with Aluminum Extrusion Reflector Assembly Housings; Mor Electric Heating Association Inc., Comstock Park, MI, USA) were hung in a hexagonal arrangement 1.2 m above the canopy and mounted at a 45° angle facing into the plot. Each heater contained four ceramic heating elements (Mor-FTE 1000W heaters; Mor Electric Heating Association Inc.). Two infrared radiometers (IRR) (SI-121; Apogee Instruments, Inc., Logan, UT, USA) were positioned in each block at the height of the heaters, and monitored the canopy surface temperature in the heat wave plot and the control plot. The IRR canopy temperature data were relayed to a central computer. The electrical output to the heaters was continuously modulated to maintain surface canopy temperatures in the heat wave plots at the target temperature difference using a PID controlled dimmer system (Model LCED-2484, 240V, 35A; Kalglo Electronics Co., Inc., Bethlehem, PA USA).

The experiment was a randomized complete block design (n=3 in 2010, n=4 in 2011). In 2010, when heat waves were applied at three different crop developmental stages, each block contained four subplots: the control subplot and three treatment subplots for (one for each of the three heat wave treatments). The subplots were arranged side by side. The order of the subplots was randomized within each block. An additional 4.6 m wide row of soybean at the same row

spacing was planted around each rectangular block as a buffer zone. In 2010 this consisted of 6

buffer rows of plants at 0.75 m row spacing and 12 buffer rows of plants at 0.4 m row spacing in

2011. Blocks were designed the same way in 2011 but two instead of three heat wave treatments

were conducted.

The heating infrastructures were relocated during the growing season to the different heat

wave plots, such that each set of subplots was heated only a single time but at different

developmental stages. Heaters and IRRs were installed one week prior to each heat wave and

removed three days after the heat wave ended. Table 1 provides the timing of each heat wave for

both years. Heat wave one in 2010 is referred to as Wv10.1, the second as Wv10.2 and the third

as Wv10.3. The two heat waves in 2011 are designated as Wv11.1 and Wv11.2.

Establishing the characteristics of a historically representative heat wave

To define a heat wave in central Illinois, 30-years of historical weather data from June, July and

August were analyzed to identify a target temperature and duration that qualified as a realistic

heat wave for central Illinois. We determined the frequency of days that were 2 to 8 $^\circ$C above the

30-year mean for that month (MRCC Applied Climate System;

http://mrcc.isws.illinois.edu/MACS/). Temperature aberrations that were 7 or 8 $^\circ$C above the 30-

year mean were too infrequent to be considered historically representative. Over the 30-year

period from 1970 to 2000 less than 1% of days in June, July or August experienced a day 7 or 8

$^\circ$C above the 30-year mean. Events where the temperature was 5 degrees above the 30-year mean

were deemed too common: ca. 10% of days in June, July and August from 1970 to 2000 had

mean daily temperatures 5 $^\circ$C above the monthly mean. Focusing on events where the

temperature was 6 °C above the monthly mean, two-day long heat waves were deemed too frequent. There were 18 occasions between 1970 to 2000 when there was a two day long 6 °C heat wave, meaning there was a better than 50% chance each year of experiencing such an event. Three day-long 6 °C heat wave events occurred approximately once in every five years and were thus qualified as a representative heat wave for central Illinois.

During each heat wave treatment, infrared heaters were turned on at 4:30 a.m. The target temperature for heated subplots was 6 °C above ambient temperatures except under two circumstances. First, if the canopy temperature dropped below the Fourier-transformed 30-year mean at any time during a heat wave treatment, the canopy temperature was elevated 6 °C above the 30-year mean. This ensured credible heat wave treatments even on days that were cooler than average, which happened on day two of the second heat wave in 2010 (Wv10.2). Second, to ensure that leaves or plants were not killed, heat wave subplots were not heated above 40 °C. This upper limit temperature threshold was not necessary to invoke during any heat wave reported in this study.

Development

In 2010 the developmental stage of five plants in each plot was measured two days before and two days after each heat wave. In 2011 five plants were tagged in each plot, and their development was tracked over the course of the growing season. Observations were made every 2-3 days. The vegetative and reproductive stage classifications followed Fehr and Caviness (1977).

Pod painting

In 2011 to track temporal pod development throughout the canopy, ten neighboring plants within the area designated for final harvest in the control and Wv11.2 subplots were flagged for pod painting. Beginning at R2 each flagged plant was checked for newly developed pods every 2-3 days. Pods were marked during early development (pod length = 10mm) and dotted with water-based acrylic paint on the proximal and distal tips (Egli and Bruening, 2006). The two colors used to paint each pod represented the number of days the pod entered early development after the beginning of full flowering (R2). For example, white was used to represent the number zero and dark blue was used for the number nine. At final harvest, a pod bearing a white acrylic mark on its proximal tip and a dark blue mark on its distal end reached 10mm and had been marked nine days after the beginning of R2. Additionally, the node placement and height was recorded for each painted pod.

Gas exchange and chlorophyll fluorescence

Midday and diurnal gas exchange and fluorescence measurements were taken *in situ* on the youngest most fully expanded leaf using open path infra-red gas analyzers (LI-COR-6400, LiCOR, Lincoln, NE, USA) equipped with fluorescence heads. Midday gas exchange and accompanying chlorophyll fluorescence measurements were taken between 11:30 a.m. and 1 p.m. on the day preceding each heat wave (day 0), the first day of the heat wave (day 1), the final day of each heat wave (day 3), and the day after the heat wave (day 4/recovery). Midday gas exchange data for these time points were extracted from diurnal gas exchange data taken on days 3 and 4. Diurnal gas exchange and leaf fluorescence measurements were done on days 3 and 4 to

assess recovery. Due to rain, the Wv11.2 recovery diurnal was done two days after the end of the treatment. Photosynthetic photon flux density (PPFD) in the leaf chamber was adjusted to match ambient light intensity, which was measured at an on-site weather station described previously (Leakey et al., 2004). Block temperatures were adjusted before each time point so that leaf temperatures within the gas exchange chambers matched average values measured by the IRRs in each plot. The measurement chamber relative humidity ranged between approximately 50% and 70%.

In 2011, photosynthesis vs. intercellular [CO_2] (A/C_i) curves were measured using the same instrumentation described for midday and diurnal gas exchange measurements. The youngest most fully expanded leaves from 2 plants per plot were harvested pre-dawn and then taken back to the lab, where the petioles were recut under water and stored in low light with the re-cut petiole in water until measured. This protocol was favored over in field measurements on attached leaves because of the greater precision of the laboratory measurements and to ensure that any photoinhibition or down regulation of photosynthesis due to photoprotection was fully relaxed. Leaves were allowed to acclimate (1700 µmol PPFD for 10 minutes) before beginning A/C_i measurements. The light level for all measurements was 1700 µmol PPFD and the block temperature of the gas exchange chamber was set to 25 ^{o}C. Plots of A vs. C_i were used to solve for V_{cmax} and J_{max} using the equations of Farquhar et al. (1980). CO_2 concentrations in the leaf chamber were adjusted in the following order: 400, 300, 200, 100, 50, 400, 600, 800, 1000, 1200, 1500 ppm. When necessary, measurements were corrected to leaf temperatures of 25 °C using the temperature responses of Bernacchi et al. (2001). ϕPSII was measured and calculated as described by Baker (2008).

Respiration

In 2011, the dark-adapted rate of leaf respiration was measured as CO_2 efflux *in situ* from the youngest most fully expanded leaf on the second night of Wv11.1 (DOY 195) and Wv11.2 (DOY 233). Measurements started two hours after sunset and were completed by 2 am. Two subsamples were measured per plot. Whole leaf respiration rates were measured with LiCor 6400 instruments using a customized leaf chamber that enclosed an entire trifoliate (Gillespie et al., 2012). Leaf temperatures inside the chamber were kept at the temperature measured by the IRR for the control or heat wave plots. After leaf respiration reached a uniform rate, approximately 10 minutes after clamping onto a leaf, the values were logged and the measured leaf was tagged, removed from the plant and stored in a sealed plastic bag for leaf area determination. Immediately after the gas exchange measurements, trifoliate area was determined (LiCOR-3100 leaf area meter (LI-COR, Inc.) and then used to normalize respiration rates to leaf area.

Leaf water potential

Leaf water potential (WP) was measured on three 0.5 cm^2 leaf discs collected from the youngest fully expanded leaf. Samples were taken at the same time as midday gas exchange measurements. Leaf disks were sealed into steel chambers containing thermocouple psychrometers (C-30, Wescor Inc., Logan, UT) and taken back to the lab where they equilibrated at 25 ^{o}C (Leakey et al., 2006). After thermal equilibration, WP was measured using an integrated dew point micro-voltmeter (HR-33T; Wescor) after which the chambers were submerged in liquid nitrogen for 60 seconds and the thermal equilibration and measurement were repeated to determine osmotic potential (OP). The turgor potential (TP) was calculated as WP–OP. The

thermocouples were calibrated independently each year using sucrose solutions ranging in concentration from 0 to 1.60 M; this curve was used to correct raw data from the thermocouple psychrometers.

Soil moisture

Soil volumetric water content was measured in 2010 and 2011 using a time domain reflectometry soil moisture meter with 20 cm rods (Field Scout TDR 300, Spectrum Technologies Inc., Aurora, IL, USA). Soil moisture was measured the day preceding, the first day, the third day and the day after each heat wave. On each of these days, six soil moisture readings were taken per subplot (three between row and three within row) between noon and 2 pm.

Tissue biochemical analysis

Specific leaf weight (SLW; g m^{-2}) was determined on three leaf disks sampled at midday, dried in an oven at 50 °C for one week and weighed. Leaf discs were always sampled from the middle leaflet, avoiding the mid-vein. Leaf total non-structural carbohydrates (TNC) was determined using ~30 mg of tissue from single leaflets of five plants per plot that were combined and ground in liquid nitrogen.

Glucose, fructose and sucrose were extracted in ethanol and measured as glucose equivalents with a continuous enzymatic substrate assay (Jones et al., 1977). Starch content in the remaining pellets of the ethanol extracts was converted to glucose by incubation with exo- and endoamylases (Geigenberger et al., 1996). Starch was subsequently measured as glucose equivalents. For analysis of ascorbate content, one leaf disk (1.34 cm^2) was sampled from 3

plants per plot and immediately frozen in the field in liquid nitrogen and then stored at -80 °C. Leaf ascorbate content, including reduced, oxidized and total ascorbate, was determined using the methods of Gillespie and Ainsworth (2007).

Final harvest

In 2010 and 2011, soybean plants were harvested from rectangular, two row sections of each subplot (0.6 m^2) that were not touched by destructive sampling during the season. There were two buffer rows of plants between edge of the treatment and the area that was sampled for final harvest. On each plant the number of pods were counted and the stems and pods were separated. The tissues were dried in an oven at 65 °C for a week, weighed and then the pods were mechanically threshed to determine seed yield. Two hundred randomly selected seeds were counted out from each subplot and weighed to determine average individual seed weight. In addition, to examine yield components by height in 2011, ten extra plants from Wv11.2 and the control plots were harvested. The pods in each 10 cm increment were counted, dried, and the seeds were counted and weighed.

Statistics

Analyses were performed on subplot means (n=3, 2010; *n*=4, 2011) in SAS using the MIXED procedure. Heat waves were analyzed independently as a mixed model ANOVA. In all cases, block was considered a random effect and the effect of a heat wave was fixed. For development and midday gas exchange data, DOY was included in the model as a repeated measure. Also, for

the temperature profile and yield component by height data, measurement height was included in the model as a repeated measure. *In situ* gas exchange data and temperature profile data were analyzed separately for each DOY. T-tests were performed on SLW, TNC and yield measurements.

Results

Background meteorological conditions were variable and canopy heating treatments were successful, affecting leaf water potential only when soil moisture was low.

During each heat wave, the infrared heating system successfully elevated canopy temperatures. Wv10.1, Wv10.3, Wv11.1 and Wv11.2 were all within 15% (i.e., +/- 0.9 °C) of the target temperature difference 85% of the time. Wv10.2 was not controlled as well as the other heat waves. It was within 15% of the target 47% of the time. However, Wv10.2 was still within 2 ^{o}C of the target temperature ~70% of the time. Nighttime temperatures were lower than the Fourier-transformed 30-year mean (see Methods) during Wv10.1 and Wv10.2, leading to nighttime temperature differentials of 10 °C during Wv10.1 and 8 °C during Wv10.2 (Table 2). The larger canopy volume during Wv10.2 relative to Wv10.1 and the lower than average night time temperatures contributed to the less successful application of the Wv10.2 heat wave treatment, as the IR heaters had difficulty maintaining greater canopy temperature differentials.

Soil moisture was only affected by Wv10.1, when the heaters at 1.2 m above the canopy were the closest to the ground and the row spacing was wider resulting in more soil infrared absorption that any of the other heat waves. The decreased soil moisture during Wv10.1 did not lead to changes in leaf water potential (Table 3). Midday leaf water potentials were affected in two of the five heat waves, both of which coincided with the driest periods of the growing

seasons (Table 3). Compared to the control, leaf water potential was significantly reduced by 0.29 MPa on the day 3 of Wv10.3 (p = 0.0082) and 0.16 MPa on day 3 of Wv11.2 (p = 0.02).

Heat waves reduced primary metabolism but the reductions were transient.

Midday gas exchange measurements in 2010 and 2011 were similar in that there were consistent reductions in A and g_s during the heat wave treatment later in the season when background conditions were hotter and drier (Fig. 1, Table 3). However, even when the heat wave treatments caused reductions in midday A, there was typically full recovery of photosynthesis on the day following the heat wave. During Wv10.3, there was a significant day × treatment effect on A, g_s and ΦPSII (Fig. 1j, k, l). These parameters all decreased during Wv10.3, but then recovered completely by the day following the heat wave treatment.

The heat wave caused reductions in ΦPSII only during Wv10.3 and Wv11.2, which coincided with the hottest and driest portions of the growing seasons. The ΦPSII reduction was significant during Wv10.3 and Wv11.2, and multiple statistical comparisons of the least squared means showed that there were no differences between the heated and control plots at midday on the day following the heat waves (data not shown). Furthermore, any differences in ΦPSII at midday were transient and did not persist on day four, the day after the heat wave (Fig. 1d, h, l, p, t).

Reductions in A are typically associated with decreases in SLW (Oren et al., 1986) and that was the case in our study. SLW was significantly decreased (p < 0.1) on the first and third day of each heat wave but was never significantly different from the control on the day after the heat wave (Fig. 2c, f, i, l, o). This included an initial average decrease of 13% on the first day of

the treatment relative to control. By the last day of the heat wave, the decreases in SLW were less pronounced but still significant. Therefore, it was unlikely that permanent structural changes in the leaf were altered by the heat wave. To investigate the mechanism underlying the decrease in SLW, we monitored changes in leaf carbohydrate content throughout the heat waves. Decreased amounts of leaf total non-structural carbohydrate (TNC) content were observed in all five of the heat waves (Fig. 2a, d, g, j, m). Similar to the changes for SLW, the decrease in TNC content was evident only during the heat wave treatment and again returned to control levels by the day following the conclusion of the heat wave.

Rapid change in oxidative stress and primary metabolism were followed by a quick recovery.

To assess the impact of the heat waves on the oxidative stress status of leaves, we quantified the concentration of reduced and oxidized ascorbate in the leaf. Although ascorbate levels are not a comprehensive monitor of all aspects of plant oxidative stress, ascorbate is a major antioxidant that plants use to detoxify harmful reactive oxygen species (ROS) generated by oxidative stress. The percent of reduced ascorbate was significantly decreased during every heat wave except Wv10.2 (Fig. 2b, e, h, k, n), indicating that for these heat waves the treatment was sufficient to induce strong oxidative stress. In addition, total ascorbate levels (i.e., reduced plus oxidized) were decreased by day three in every heat wave except Wv10.2 (data not shown). Similar to SLW and TNC, reduced ascorbate in the heat wave treatments returned to control levels during the day following the heat wave.

Leaf photosynthetic physiology was altered but there were no differences in whole leaf respiration.

To better understand the mechanisms that drove the decrease in A, SLW and TNC seen during the heat waves, A vs. C_i curves were measured on day two of Wv11.1 and Wv11.2; and night-time respirations rates were measured on night two of Wv11.1 and Wv11.2 (Fig. 3a, b). During Wv11.1 there was a significant increase in V_{cmax} and no change to J_{max} (Fig. 3a). Wv11.2 saw a reduction in both V_{cmax} and J_{max} relative to the control. Nighttime respiration rates were no different than the control in either Wv11.1 or Wv11.2 (Fig 3b).

Heat waves did not speed or slow development but caused reductions in yield when applied during pod set.

The heat waves in 2010 or 2011 did not alter plant development, neither detectably speeding nor slowing reproductive or vegetative growth. Although the heat waves were applied during a small window of season-long growth, they could span significant portions of a given reproductive stage; for instance, although Wv11.2 spanned only 5% of all reproductive growth (R1-R5), ~30% of R4 occurred during Wv11.2 .

There was a consistent ca. 10% reduction in yield for heat waves applied during reproductive phases that coincided with early pod set: 10% during Wv10.2 (p = 0.07) and 17% in Wv11.2 (p = 0.06) (Table 4). Though the decrease in yield in Wv10.3 was similar in magnitude to Wv10.2, it was not statistically significant (p = 0.58) due to increased variance in yield in the Wv10.3 subplots. The decrease in yield for Wv11.2 and Wv10.2 can be attributed to a statistically significant decrease in the number of pods per m^2 of land area (Table 4). Within each

year individual seed weight and the number of seeds per pod were unaffected by the heat wave treatment (Table 4; Fig. 4a, b).

From the ten subsampled plants in the pod painting analysis, there were 1234 pods in the control plots and 1098 pods in the Wv11.2 plots where 90% of those pods had distinguishable marks. There were 1122 marked pods from the control and 998 marked pods in the Wv11.2 plot. The application of acrylic paint used to mark pods had a varied effect on pod development. Compared to the plants used in the yield component by height analysis, which were not marked with acrylic paint, there were 6.3 fewer pods per plant in the control of the pod painting experiment ($p = 0.05$). However, there was no difference in the number of pods per plant between painted vs. unpainted plants from the heat wave treatment ($p = 0.24$). Plants began setting pods on the lower regions first, closest to the ground and set the most pods 8-12 days after R2 (Fig. 5a-f). During the Wv11.2 heat wave, 18-21 days after the onset of R2, there was a reduction ($p = 0.05$) in the number of pods produced relative to the control (Fig. 5e). The significant reduction was driven by a 54% decrease in the number of pods 30-50 cm from the ground. No other yield component was significantly affected by Wv11.2 (Table 4; Fig. 4a, b).

Discussion

This is the first field experiment to use IR heaters to study the effects of a regionally defined heat wave on any crop. A +6 °C, 3-day heat wave applied to soybean strongly impacted leaf physiology (e.g., A, g_s, ϕPSII, TNC, and oxidative stress) especially when background soil moisture levels were low (Wv11.2). In most cases, there was rapid recovery for all these physiological parameters within 24 h of the end of the heat wave. V_{cmax} was increased during

Wv11.1 but both V_{cmax} and J_{max} decreased during Wv11.2. Heat waves did not alter the rate of reproductive or vegetative development. However, there were significant reductions in yield when heat waves were applied during reproductive stages that coincided with early pod development. The fact that yield was only reduced during early pod set, despite large leaf physiological and photosynthetic responses during every heat wave, suggests that a significant portion of the yield reduction was due to effects of the high temperature directly on reproductive processes.

The first hypothesis that photosynthesis would be decreased in the short term in response to heat waves due to reduced stomatal conductance and increasing the proportion of photorespiration relative to carbon assimilation was supported. Except for Wv11.1 midday A and g_s were significantly reduced on all days during every heat wave. The greater stimulation of A by elevated [CO_2] observed for the heat wave plants in A vs. C_i analysis taken during Wv11.1 and 11.2 (data not shown) confirmed the expected increase in the proportion of rubisco catalyzed oxygenation relative to carboxylation at higher temperature. The second hypothesis that damage, which compromises ϕPSII, will cause reductions in carbon assimilation that will persist for a period after the end of the heat wave and affect season long yield was partially rejected. Oxidative stress and damage to the photosynthetic machinery did not cause a long-term decrease in photosynthesis. Observed reductions to ϕPSII did not result in any lingering reductions in photosynthesis after the heat waves. The rapid recovery of ϕPSII after the heat wave treatment was surprising since drought and an increased amount of oxidative stress are expected to slow the repair of the photosynthetic machinery (Souza et al., 2004; Nishiyama et al., 2001) possibly signifying changes in ϕPSII during heat waves were driven more by photoprotection than by damage to PSII. Overall the data indicated that reduced ϕPSII was only an ancillary component

of the impacts of effects heat waves on soybean. Carbohydrate deprivation experiments in soybean suggest that it takes prolonged reductions (7 to 14 days) in *A* to cause pod abortion (Egli and Breuning, 2006). The significant reduction in seed yield during relatively short heat waves suggests that yield loss was due primarily to direct impacts on reproductive processes.

The third hypothesis that any direct damage done to reproductive structures or processes will have negligible impact on yield due to the long flowering period for soybean and its consequent capacity to compensate for yield loss was partially supported. Reproductive development a spanned long enough period to tolerate heat waves that occurred during early flowering stages (R1) without significant yield loss. However, heat waves had a significant effect on end of season yield when applied during early pod development: Wv10.2 was applied during R3 and Wv11.2 was applied during R4. This observation is consistent with historical soybean yield data showing that yield is negatively correlated to temperature increases above the 30-year mean in July and August (Tannura et al., 2008). The simplest explanation for the decrease in seed yield during R3 (Wv10.2) and R4 (Wv11.2) is small pod availability; pod abortion is most sensitive while pods are in early development and less than their maximum size (Egli and Breuning, 2006). Assuming that the pattern of pod development was the same in 2010 and 2011, early heat waves (Wv10.1 and Wv11.1) did not cause yield loss because they were applied during R1, before the development of small pods (e.g., Fig. 5a). Furthermore, the latest heat wave (Wv10.3) did not cause yield reductions during R5 because plants were past the point where they were producing small pods and the treatment duration was not sufficiently long to decrease long-term *A* and assimilate availability (e.g., Fig. 5f). These results suggest that short heat waves will have the largest impact on seed yield when they occur during the developmental

periods that coincide with the greatest rates of small pod development (e.g., Fig. 5c). Another possible explanation for the decrease in yield during R3 and R4 is that early soybean flowers (R1) abort at a much lower rate than flowers that develop later (R3) (Heitholt et al., 1986). Heat wave treatments during early reproductive stages, Wv11.1 and Wv10.1, had no effect on yield. It is possible that plants in early flowering were able to moderate yield loss because they were still setting pods from early flowers, even after the heat wave. However, due to the high abortion rate of later developing flowers, few pods would be able to set after heat waves applied during later reproductive stages.

Soybeans have the ability to compensate for the loss of pods by increasing the seed size (Munier-Jolain et al., 1998), measured in this study as individual seed weight (Table 4, Fig. 5b). In experiments where soybean plants were manually depodded, although there was a decrease in the total number of seeds per plant at final harvest, the individual seed weight of the depodded plants was increased relative to the control (Munier-Jolain et al., 1998). Since the heat wave treatments were relatively short and reduced pods without affecting A long term, it is somewhat surprising that there was no compensatory increase in seed weight. However, the extent to which soybean can adjust to pod loss is limited and seed weight does not always increase after depodding. For example, after 90% of soybean pods were experimental removed 6 days after R2 there was no change in final, individual seed dry weight despite the fact that the sucrose concentrations within the depodded seeds were greater than the control (Egli and Bruening, 2001).

Differential acclimation of photosynthesis may also explain why there was no reduction in yield caused by early reproductive heat waves. Thermal acclimation has been observed within two days of exposure to elevated temperatures (Sage and Kubien, 2007). During Wv11.1 V_{cmax}

was increased compared to the control. However, acclimation of V_{cmax} during Wv11.1 did not lead to persistently higher rates of A or increased biomass. It is likely and expected that acclimation occurred only in leaves that experienced the treatment and not in leaves that formed after the heat wave. In 2010 the effect of each heat wave on A was tracked throughout the season on later cohorts of leaves that did not directly experience the heat wave treatment. During Wv10.3 there was no difference in A between the control and plants that experienced Wv10.1 (data not shown). Later in the season, when the acclimated leaves became shaded, photosynthesis would be limited by electron transport (i.e., light-limited), negating the impact any persistent increase in V_{cmax} would have on A. Wv11.1 was applied during V7 (seven fully developed trifoliate leaves) whereas at the end of the season plants had ca.18 trifoliates on average. V_{cmax} may have decreased during Wv11.2 due to the remobilization of nitrogen in rubisco for seed fill (Schiltz et al., 2004).

Although long term A was not decreased in this experiment, multiple heat waves within a season or longer heat waves have the potential to reduce yield by reducing carbon uptake. The heat waves in this experiment were short compared to the European heat wave of 2003 and the Russian heat wave of 2010 (Fink et al., 2004, Barriopedro et al., 2011). During those events, month long temperatures were 6°C higher than average.

Respiration measurements were not made during each heat wave but it is clear that elevated nighttime respiration during the heat wave plots could also contribute to loss in carbon gain. A number of variables including light and water availability can alter leaf respiration rates but plant mitochondrial respiration responds most consistently to temperature (Atkin et al.,

2005). Nighttime respiration is also correlated to daily photosynthesis and soluble sugar and starch concentration (Whitehead et al., 2004). Wv11.1 and Wv11.2 night-time CO_2 fluxes were the same in the heated and control plots (Fig. 3b). It is likely that the expected suppression of night-time respiration rates due to decreases in midday A, SLW and TNC in Wv11.1 and Wv11.2, was compensated for by the stimulation due to the higher temperature resulting in no difference in night-time respiration rates between the control and heat wave plants. Prolonged decreases in A coupled with enhanced rates of respiration could be one mechanism by which heat waves decrease productivity and yield. Furthermore, when considering future responses to heat waves, plant respiration is increased under elevated CO_2 (Leakey et al., 2009), but it is uncertain how respiration in plants under elevated CO_2 will respond to heat waves.

This study illustrates the important impact that short-term high temperature events can have on plant productivity. Despite rapid recoveries of A, ϕPSII, TNC and SLW within 24 hours of the end of a heat wave, heat waves that coincided with early pod developmental (R3, R4) stages led to a 10% reduction of yield. As climate models predict an increase in the frequency and intensity of heat waves (Meehl and Tebaldi, 2004), it is important to identify the process in plants that need to be adapted to tolerate climate extremes under a variety of field situations. Further research is needed to explain how heat waves affect yield under varying degrees of drought stress as well as interactions with increasing atmospheric $[CO_2]$. High temperature tolerant pod set and development in soybean should also be considered a target for introgression to improve capability of salvaging yield after extreme events.

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Year	Start Date/(DOY)	Vegetative stage	Reproductive stage	Name
2010	July 3 (184)	V6	10% of plants in R1	Wv10.1
2010	July 27 (208)	V14	R3 (Beginning pod)	Wv10.2
2010	Aug 13 (225)	V18	R5 (Seed fill)	Wv10.3
2011	July 14 (195)	V7	10% of plants in R1	Wv11.1
2011	Aug 19 (231)	V15	R4 (Full pod)	Wv11.2

Table 1. The reproductive timing of each heat wave and its abbreviation used in the text.

Wv	Control		Heat wave		Tdiff
	day	night	day	night	
10.1	23.6 ± 0.8	13.3 ± 0.3	31.3 ± 0.4	23.6 ± 0.1	8.7 ± 0.4
10.2	25.6 ± 0.1	19.1 ± 0.1	32.6 ± 0.02	27.1 ± 0.1	7.4 ± 0.1
10.3	27.8 ± 0.1	21.2 ± 0.1	33.8 ± 0.1	27.4 ± 0.1	6.1 ± 0.2
11.1	27.8 ± 0.4	18.1 ± 0.3	33.7 ± 0.4	25.4 ± 0.1	6.3 ± 0.2
11.2	25.3 ± 0.2	17.6 ± 0.9	31.5 ± 0.3	27.2 ± 1.7	7.0 ± 0.2

Table 2. Nighttime and daytime Infrared Radiometer (IRR) canopy temperatures during each heat wave. Temperatures are reported in degrees Celsius. "Tdiff" is the average temperature difference between the heated and control plots.

Wv	day	Soil Moisture (%)				Leaf Water Potential (MPa)			
		control	heat wave	s.e.	ANOVA	control	heat wave	s.e.	ANOVA
10.1	day 0	41.4	40.7	± 2.7	day	-0.71	-0.87	± 0.09	-
	day 3	34.8	32.8		day*trt	-0.8	-0.85		
10.2	day 0	31.4	29	± 1.5	day	-0.61	-0.66	± 0.05	-
	day 3	39.4	36.1			-0.69	-0.74		
10.3	day 0	23.3	22	± 2.3	day	-0.81	-0.76	± 0.08	trt
	day 3	24.1	16.5			-0.77	-1.07		
11.1	day 0	30.2	31	± 4.2	day	-0.6	-0.61	± 0.03	-
	day 3	30.3	32.3			-0.52	-0.59		
11.2	day 0	19.3	.	± 1.1	.	-0.69	-0.7	± 0.06	trt
	day 3	23.2	.			-0.75	-0.86		

Table 3. Soil moisture concentration and leaf water potential during each heat wave. This table shows the mean soil volumetric water content by percentage (left columns) and the leaf water potential (WP, right columns) in megapacals (MPa) on the day before each heat wave started (day 0) and the last day of each heat wave (day 3) in the control and heat wave subplots as well as the standard error (s.e.). The ANOVA column tests for statistical differences between the heat wave and the control plot; "day" indicates a significant effect of day, "trt" is a significant effect of heat wave and "day*trt" is a significant day by heat wave interaction ($p < 0.1$).

Wv	Seed yield (gm^{-2})		Pods per m^{-2} (#)		Seeds per pod (#)		Seed weight (g)	
10c	341.2 ± 7.3		1065.9 ± 12		2.76 ± 0.05		0.139 ± 0.003	
10.1	337.7 ± 35	(0.93)	1038.7 ± 48	(0.62)	2.76 ± 0.04	(0.97)	0.14 ± 0.009	(0.86)
10.2	306 ± 9.7	(0.07)	934.4 ± 48	(0.06)	2.76 ± 0.02	(0.4)	0.141 ± 0.007	(0.51)
10.3	313.2 ± 46	(0.36)	1043 ± 81.8	(0.79)	2.64 ± 0.06	(0.18)	0.137 ± 0.018	(0.9)
11c	340.3 ± 20		1499.6 ± 40.2		2.11 ± 0.03		0.11 ± 0.004	
11.1	345.4 ± 45	(0.92)	1499 ± 154	(1)	2.34 ± 0.22	(0.39)	0.11 ± 0.004	(0.88)
11.2	280.8 ± 7	(0.06)	1245 ± 49.9	(0.02)	1.95 ± 0.14	(0.29)	0.109 ± 0.00.	(0.84)

Table 4. Seed yield and yield component data from each heat wave and its control in 2010 and 2011. The parentheses beside the treatment mean contain the p-value of the statistical comparison made against the control for that year.
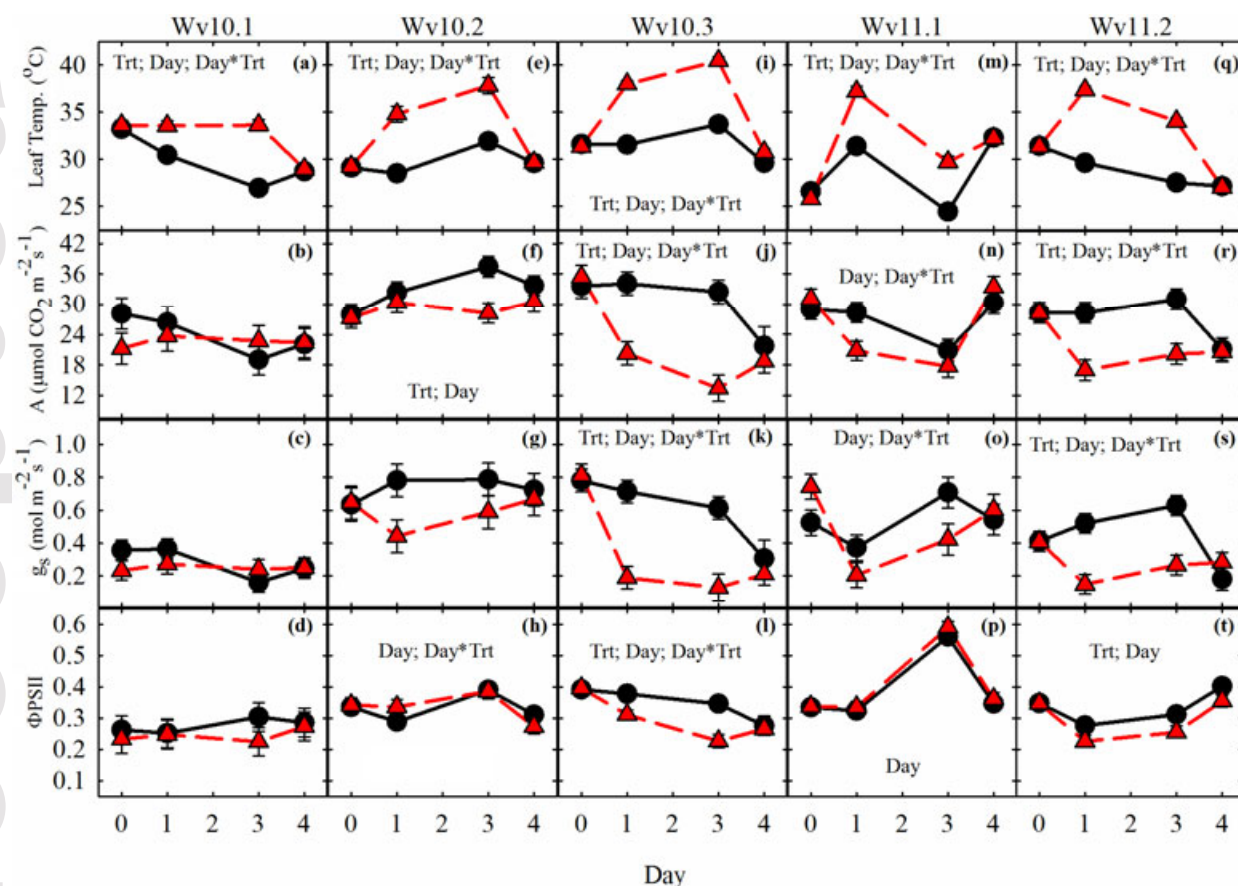

Figure 1. Midday leaf physiological data. Including: leaf temperature (Leaf Temp.; a, e, i, m, q), photosynthetic rate (A; b, f, j, n, r), stomatal conductance (g_s; c, g, k, o, s) and the operating

efficiency of photosystem II (ϕPSII; d, h, l, p, t). The columns from left to right are each of the five heat waves in chronological order across the two years of the experiment from the beginning of the 2010 to the end of 2011 growing season. Day 0 is the day before the heat wave. Days 1-3 are during the heat wave, and day four is the day after the heat treatment was turned off. The red triangles and the dashed lines are measurements from the heat wave plots. The black circles and solid lines are the control. Text within each graph indicates significant repeated measures terms ($p < 0.1$). "Trt" indicates a significant heat wave effect. "Day" is a significant effect of day and "Day*Trt" notes significant treatment by day interactions.
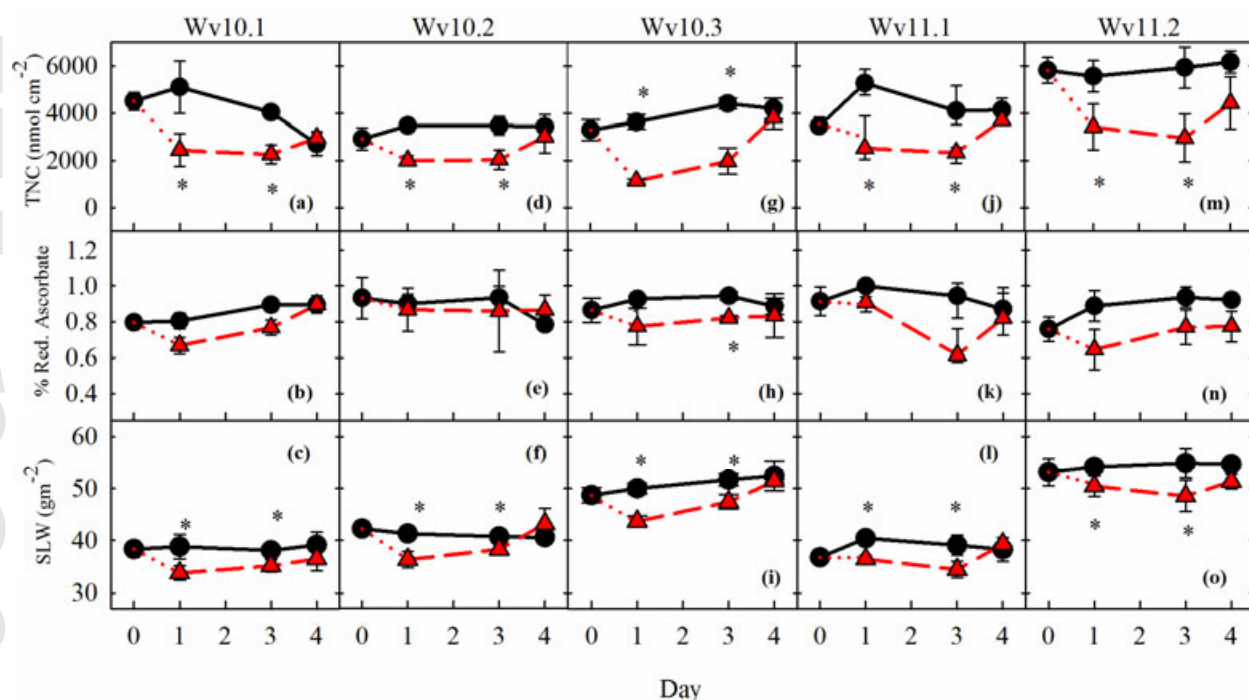

Figure 2. Heat waves induced changes in total leaf nonstructural carbohydrate (TNC) concentration, the percent of reduced ascorbate and specific leaf weight (SLW) at midday over the course of the heat wave. The X-axis is the day of the heat wave. The black circles are measurements made in the control plots and the red triangles are from the heat wave plots. Asterisks indicated significant differences ($p < 0.1$) between the heat wave and the control treatment within a day.
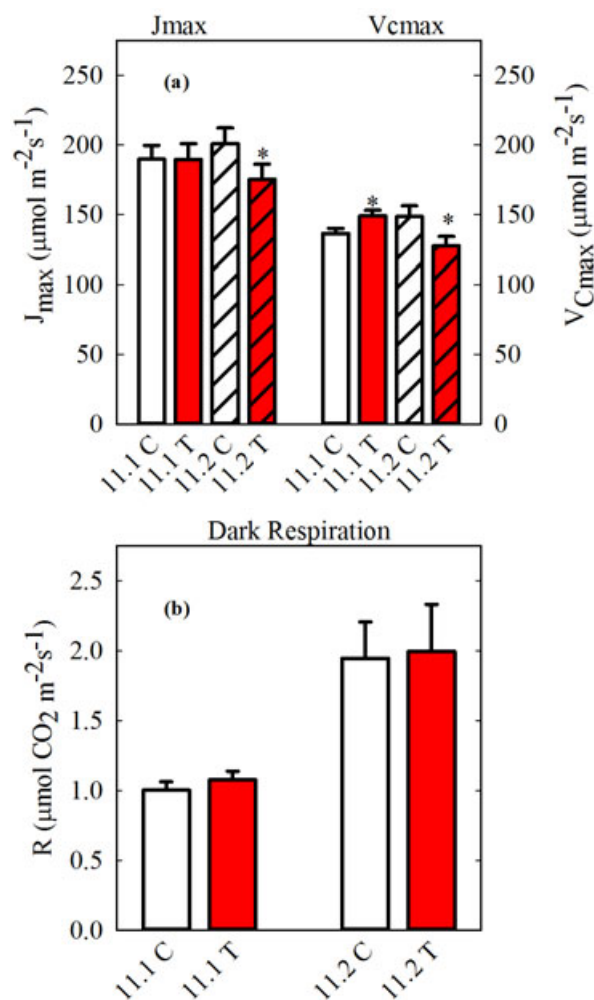
Figure 3. The effect of 2011 heat waves on V_{cmax} and J_{max} and nighttime leaf respiration (R). Figure 3a shows $V_{c,max}$ and J_{max} values from *A vs. C_i* curves taken during Wv11.1 and 11.2. An asterisk denotes a significant difference from the control ($p<0.1$). Figure 3b shows the rates of nighttime respiration in the control (C) and heat wave plots (T).
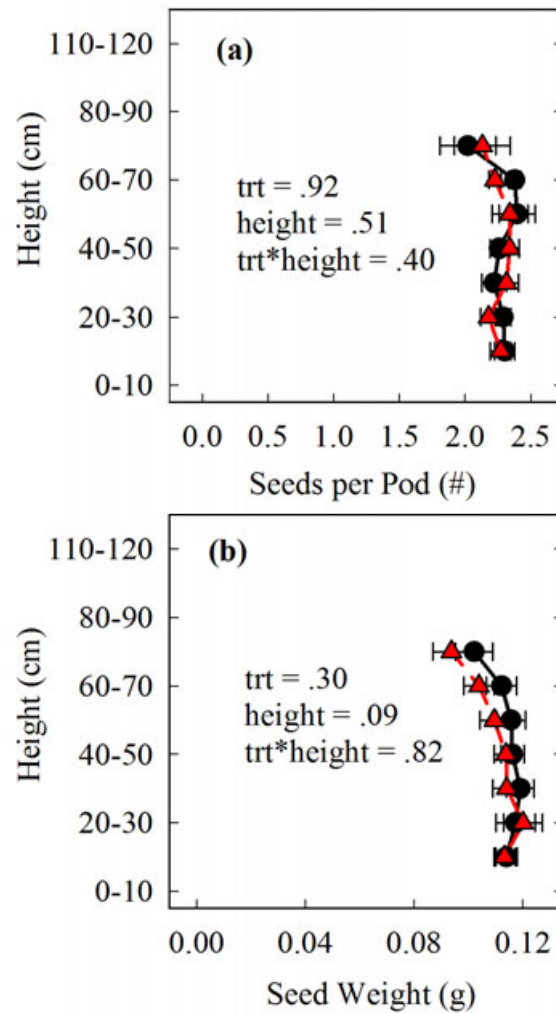
Figure 4. Analysis of Wv11.2 yield components at varying height classes. The text within each graph shows the results of a repeated measures statistical analysis. The black circles depict measurements from the control and the red triangles are from the heat wave plots. An asterisk represents a significant difference between the heat wave and the control treatment within a height class (p < 0.01).
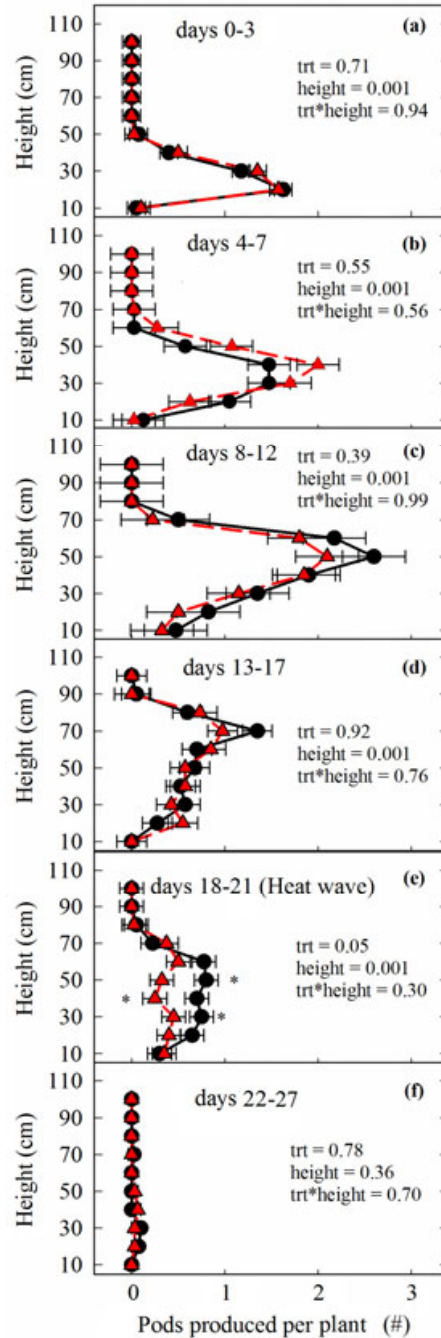
Figure 5. Spatial and temporal changes in pod set during Wv11.2. Panels a-f are from the beginning of R2 (day 0) to the beginning of R5 (day 27). The heat wave was applied on days 18-21. Statistical tests are the same as in Figure 4.