

ABSTRACT

CARRILO RAMOS, SELVIN. Genetic Variation in Heat Stress Response of Soybeans. (Under the direction of Dr. Kent O. Burkey).

Climate warming has become an escalating research topic for its looming impacts on crop production systems and therefore exacerbation of global and national food security. Considering the projected global increase in temperatures, a full season heat stress study was conducted in 2021 to assess the responses of elite breeding lines to elevated air temperature (2-5 °C), a range aligned with the expectation in temperature increase in the coming decades. Various chlorophyll fluorescence (Chl) and agronomic traits were measured to explore whether these increases in temperature caused adverse responses in water stress-free conditions and to identify promising heat tolerant genotypes. From the thirteen breeding lines evaluated, SW_AABB, N16-7526, FW_aabb, RIL#1360, and S14-1625 were the best performing genotypes in leaf-level and yield related parameters. Genotypes SW_AABB and S14-1625 had the highest quantum yield of photosystem II (Φ_{PSII}) and lowered their leaf temperature the most (-5 °C), whereas N16-7526 showed the least leaf temperature differential (LTD) (-3 °C). SW_AABB showed greater seed yield, seed number, pod number, stem biomass, plant height, branch number, and short crop cycle (planting to R-8 stage). In contrast, N16-7526 showed high seed weight, stem biomass, and HI, but longer crop cycle, shorter plant height, and low branch number. Interestingly, S14-1625 exhibited the shortest crop cycle and low branch number but did not show seed yield reductions in response to elevated temperature mainly attributed to increased main stem pod number, branch pod number, and stem biomass. These differential genotypic responses to full season heat stress indicate that the screened germplasm exhibited contrasting physiological and agronomic traits and that Chl parameter Φ_{PSII} and LTD seemed an efficient screening technique in this study. Irrespective of not statistically significant main effects of warming, yield losses ranged from -3%

to -39% and days to maturity were delayed 7 days on average. The genetic sources identified in this preliminary screening should pave the way for more detailed study in field experiments and biochemical analysis to determine their potential inclusion in heat-stress breeding programs for the development of heat-tolerant varieties.

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Genetic Variation in Heat Stress Response of Soybeans

by
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DEDICATION

Dedicated to my family.

BIOGRAPHY

Selvin Carrillo was born in a rural community in Huehuetenango, Guatemala growing staple foods, and surrounded by smallholder farmers and enchanting mountains. He earned both a bachelor's degree in Agronomy and a specialization in agricultural research at the University of San Carlos in Guatemala. Before starting his program at NC State, he worked on a multicultural project for the Inter-American Institute for Cooperation on Agriculture (IICA) to identify resilient strategies and livelihoods to mitigate climate change variability in highly vulnerable communities, did an internship through the Worldwide Farmers Exchange Program in Hawaii', United States in the area of vegetable production, worked at the Instituto de Ciencia y Tecnología Agrícolas (ICTA) in maize research, and implemented high-value horticulture and rural development projects in areas with high levels of malnutrition and migration rates in his country through the Farmer-to-Farmer Program/Partners of the Americas. His experience working locally and abroad, plus growing up on an agricultural farm, gave him a broad perspective of the challenges to overcome food insecurity and the limitations farmers face to increase productivity. These experiences ignited his desire to study abroad and pursue a master's degree as a Fulbright fellow at North Carolina State University. While at NC State, he was awarded membership to the Honor Society of Phi Kappa Phi and was selected as a Future Leader Fellow for the Association for International Agriculture and Rural Development (AIARD). Under the direction of Dr. Kent O. Burkey, his master's project focused on the physiological and agronomic heat stress responses of soybeans.

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Chapter 1. Literature review

The climate warming crisis for soybeans: exploring heat stress effects on soybeans for various physiological and agronomic responses, including some technological challenges.

ABSTRACT

Heat stress (HS) —linked to global warming— threatens productivity of economically important crops. Studies report that heat stress causes soybean [*Glycine max* (L.) Merr] yield reduction over 50% in brief and season-long elevated temperatures driven in part by a reduction in photosynthesis, pod set, and harvest index mainly at reproductive stages. The effects will likely worsen if no urgent action is taken to develop appropriate screening technologies, establish standard phenotyping techniques, assess tolerance of modern and wild germplasm, and study the interaction with other environmental stresses (e.g., water, CO₂, air pollution, light); which can aid in the identification of climate-smart varieties. Irreversible scenarios, at least for the next three decades, prompt the need to conduct basic and applied research to understand heat stress responses of soybeans and other crops and to implement mitigation strategies. This review draws from pertinent literature on heat stress (e.g., heat shock, heat waves, warming) to illustrate the multitude of ways in which soybeans may be at risk during global warming. A description of impacts on yield, physiological and agronomic traits, interaction with other environmental stresses, mitigation strategies, and future research are highlighted.

Keywords: warming, heat stress, *Glycine max*, soybean, screening, environmental stress, abiotic stress.

INTRODUCTION

Temperature, which influences the internal biological reactions that control growth and development, influences photosynthesis and respiration, sets the cultivation boundaries for crops (Geange et al., 2021), and prevails as a concerning climate change factor (e.g., water stress, salinity, ozone, and CO₂) that will affect crop yields (Jin et al., 2017; Piao et al., 2017).

Temperature increase projections by the end of the 21st century are 2.1 °C to 5.7 °C globally (IPCC, 2021) and 2 °C to 3.8 °C for the Midwest U.S. (Jin et al., 2017). The increase in temperature variability is a concern because of most cultivated species have lower heat tolerance limits (Geange et al., 2021). Thus, crops need to be bred to perform well at higher temperatures.

Plant heat stress (HS) is the exposure of species to air temperatures beyond its optimum physiological range, for a period of minutes to months, to sustain maximum productivity, causing acute and chronic impacts on overall plant performance. It is not uncommon that HS often refers to heat shock, heat waves, and climate warming. Heat shock may occur once at any growth stage, lasting from minutes to hours, often observed in molecular studies; heat waves are intermittent stresses from a few hours to days usually imposed in experiments on critical growth stages (e.g., soybeans) (Siebers et al., 2015); and warming is a general temperature rise, on average, usually over a month (S. Jagadish et al., 2021). The effect on plants depends on stress duration, frequency, and intensity of the specific environment, which has become unpredictable and unavoidable recently. For most species, temperatures >45 °C or 10-15 °C above optimum may cause irreversible damage (Panthee & Gotame, 2020; Taiz et al., 2015). HS not only threatens crop systems by exacerbating food production but also posits additional pressure on irrigation-dependent zones (Parker et al., 2020a). These effects thus may ultimately drive food insecurity and social unrest in regions that rely on monoculture and staple foods.

HS impacts on soybeans may cause yield reductions over 50% (Table 1.1). These losses have been predicted by crop simulations, statistical models, and field experiments at the global, regional, national, and local scales (Ahumada & Cornejo, 2021; Burkey et al., 2020; Jin et al., 2017; Parker et al., 2020b; Piao et al., 2017; X. Wang et al., 2020; L. Zhang et al., 2016). Among these methods, field experiments, particularly artificial warming, are a more direct and realistic measure; models, however, cover a wide range of time and natural variability (Piao et al., 2017). HS is difficult to determine by visual inspection, but as the heating magnitude increases the impairment of photosynthetic and reproductive tissues may lead to disruption in plant development, yield reduction, and even death. How the plant senses heat and responds to HS has been studied in model plants, but it is still unclear in crops. Some cell HS sensing mechanisms were reviewed recently, implying that this stress is sensed in all cellular and intracellular compartments and responsible for protein denaturation (Mittler et al., 2012; H. Zhang et al., 2022).

HS in soybeans has distinct effects and requires specialized approaches to measurement, assessment, and mitigation. This review focuses on the latest research on HS in soybeans and intends to: (i) report contemporary literature on soybean heat stress (ii) highlight warming effects on photosynthetic and agronomic traits (iii) explore technology challenges peculiar to warming experiments and (iv) address some mitigation strategies and future perspectives posed by the climate crisis. This is a broad review that focuses on soybeans, a staple crop with moderate sensitivity to heat stress.

SOYBEANS

Soybean is a warm-season legume and the fourth most produced crop worldwide; with Brazil, United States, and Argentina leading production. In 2021, the United States was the

second-largest producer worldwide with 112.5 million metric tons (www.soystats.com). In 2022 the expected production area is 37 million ha (91 million acres) (USDA NASS <https://www.nass.usda.gov>). Besides economic and food security implications (e.g., meat restrictions, culture), the seeds have high protein content, and soybean is able to form a symbiotic relationship with nitrogen-fixing bacteria in the nodules (Mousavi-Derazmahalleh et al., 2018).

Soybeans achieve maximum plant performance and yield growing within optimum temperature ranges and when plants collect maximum light energy to use in photosynthesis, without any other stress. The optimum crop mean growing temperature reported across literature is 28.3 °C, with a minimum and maximum of 11.4 °C and 39.4 °C, respectively (Nicole D Jackson, 2021). These thresholds vary by region and genotype; for example, preferred season temperatures for two cultivars were 22-26 °C in South Korea (Choi et al., 2016), which was similar to models and regional trials conducted in the United States (Hatfi et al., 2011). Soybean plants begin to experience heat stress when air temperature exceeds 30 °C, with a damaging threshold of 40 °C at the reproductive stage (Prasad et al., 2017). At temperatures > 30 °C during the night, the plant is prone to photosynthetic aberrations due to its inability to efficiently synthesize carbohydrates; particularly the breakdown of stored starch in the chloroplast that was synthesized during the day. Temperature response may vary within species, in soybeans, for example, the optimum intraspecific temperature for photosynthesis differs between genotypes (Bunce, 2019).

The Southeast U.S. is suitable for growing diverse soybean maturity groups (MG), typically 4 to 8. This was observed by Morris et al. (2021) who tested MGs 2-8 across North Carolina (NC) and found that soybean plants emerged faster in June-July than on earlier planting

dates, probably due to warmer temperatures. The study also emphasized that MGs 4-8 had similar yields when planted later in the season, but yield was high when soybeans were planted before June. Ideally, growers and extension specialists prefer flexibility to choose from a wide range of cultivars and MGs based on the potential yield and production system—double crop, rotation, planting date preference, and the environment—highlighting the need for a diverse portfolio of cultivars to choose from in a changing climate (Santachiara et al., 2017). Regardless of planting date or double crop (wheat before soybeans), the exposure of soybeans to high temperatures during grain filling is latent, for example. Santachiara et al. (2017) investigated contrasting MGs at reproduction (R1-R5) under elevated temperature and observed no significance in seed number, biomass, and yield, despite differences in growth cycle length. Longer crop cycles may be explained by poor light use efficiency of the canopy to absorb solar radiation for optimum growth and development, heat stress effects, and nutrient deficiencies.

Genetic variability for heat stress tolerance

Modern germplasm is essential to understand heat stress responses because of the need to find genetic sources for specific geographic zones (Sadok et al., 2020). Djanaguiraman et al. (2018) described diverse genotypic variations in physiological and agronomic traits on soybean germplasm at the R4-R6 stages under high day temperatures. It is unclear, however, if these variations are unique to high day temperatures or interactions with other abiotic stresses. This finding suggests that potential exists to find tolerant soybean genotypes and it is the genetic composition of cultivars that often determines the amount of intercepted light and yield benefit. Soybean MGs with improved efficiency to capture light energy would increase yield (Morris et al., 2021). The genotypic responses under warming may provide better parameter estimates even when differences are not significant. Thus, by determining genotypic acclimation potential, we

could predict impacts, and even consider interactions with more environmental factors (photoperiod, water, light intensity, and CO₂).

Species with narrow temperature limits (resilience) and genetic diversity are at more risk of failure than those with ample adaptation thresholds, thus it is critical to understand the plasticity of species (Geange et al., 2021). Boehm et al. (2019) evaluated 93 soybean cultivars from MGs 5 to 7 and concluded that the yield threshold potential has not been reached, but the protein content for MGs 2, 4, and 6 showed reductions. These protein concentration decreases may be greater under abiotic stress, as noted recently where not all genotypes will experience equal effects even if the heat stress is the same (Ortiz et al., 2021).

Warming impacts on soybeans

Warming studies focus primarily on trees, particularly forests (S. Jagadish et al., 2021). Also, heat effects have been investigated in California for high-value crops, but far less on row crops (Parker et al., 2020b). Piao et al. (2017) reported that ~ 25 experimental sites studied warming (>2 months exposure) in major crops worldwide, for instance, and ~3 assessed soybean yield responses. Climate warming experiments target temperatures up to 5-7 °C (Choi et al., 2016; Jagadish et al., 2021; Kimm et al., 2021), which may produce less plant morphological and developmental damage than heat waves or heat shocks. Constraints to studying long-term responses —planting to maturity— might result from the lack of appropriate exposure technology to evaluate large germplasm collections; limited research on key molecular, physiological, developmental, and agronomic mechanisms in crops; few economic resources for infrastructure maintenance and labor; and lack of standardized phenotyping tools; thus, reasons that hinder the screening process and impeded resilient germplasm identification.

Soybean growing areas began to dry and showed abnormal warming since the 1980s (He et al., 2022), implying that temperature increases may shift soybean-growing regions northward as of the 2060s (Hoffman et al., 2020). To sustain food production, it is critical to understand plant heat responses at all levels due to interannual, seasonal, intra, and interspecific variability. Soybeans yield reductions due to heat stress, demonstrated by models and field experiments, are quite significant: 3.1% globally and ~6.8% in the United States for every 1 °C increase; 11.6% yield reduction with a +4 °C scenario (Piao et al., 2017). This study also reported that soybean (among maize, wheat, and rice) was less sensitive to heat, probably explained by its relatively high-temperature limits (~40 °C) and canopy shade provided to flowers, avoiding direct sunlight (Prasad et al., 2017).

Models predicted that warming would reduce soybeans yield by ~10.6% per 1 °C globally (X. Wang et al., 2020). In Argentina, through time series models a study concluded that 10 days of high temperature (>30 °C) would decrease yield by 10% (Ahumada & Cornejo, 2021). Interestingly, Hatfi et al. (2011) found contrasting yield responses reported in the literature: 2.4% reduction for the South, but 1.7% increase in the Midwest, United States per ~ 1 °C rise. These contrasting results point out that specific environments may aggravate soybean loss, but regions with mean temperatures slightly below the optimum temperature may benefit from climate warming. The geographic zone will ultimately determine the warming severity and extreme heat events (Bitu et al., 2013). Elite lines crop yield responses to warmer climates remains uncertain, and a better understanding is critical for the development of mitigation strategies and breed varieties.

TECHNOLOGY TO ASSESS CROP RESPONSES

Evaluating heat stress responses

Since plants interact with the surrounding environment, the technology deployed to assess heat effects needs careful consideration. The use of labs, growth chambers, glasshouses, greenhouses, and open-air field systems is typical to address the question of how heat stress affects plants (S. Jagadish et al., 2021).

Various challenges emerge in high temperature treatment, in mimicking real-world stress conditions in experiments. The study of photosynthesis in the lab, for instance, precludes our ability to obtain a concrete idea of how it will respond in outdoor environments in which the plant will ultimately develop (Bita et al., 2013). Indeed, experiments that capture real-world dynamic environmental factors that drive photosynthesis such as light intensity are preferred (Cruz & Avenson, 2021). A constraint in warming research is the inability to evaluate large germplasm collections at once, and so is the lack of plant performance assessment under dynamic microclimate conditions and the temperature fluctuations throughout the crop cycle (S. Jagadish et al., 2021; Murchie et al., 2018). Geange et al. (2021) noted a few long-term experiments (over a month) in which only ~6% of the exposure systems used in temperature stress captured naturally occurring extremes such as heatwaves. This research also suggested that warming is a realistic approach for incremental temperature increases as observed in global trends, providing the plant an opportunity to acclimate, endure or survive future stress.

In soybeans just a few long-term heating technologies have been used in full crop cycles; for instance, infrared heating arrays (Kumagai & Sameshima, 2014) or ceramic heating arrays in the temperature free-air controlled enhancements (T-FACE) project developed to study high-temperature in the field (Kimm et al., 2021). These systems have successfully imposed

temperature stress; however, the exposure area is limited and may not apply to large screenings for breeding programs. Sinclair et al. (1995) initially proposed an exposure system model to study warming in conjunction with CO₂ in wheat, which may be adapted to a larger scale by taking advantage of new engineering advances.

Phenotyping tools and methods for heat tolerance

In field conditions, phenotyping tools can detect early plant changes that could predict environmental and biological stresses. Although phenotyping is a holistic process, the use of reliable nondestructive, high-throughput tools to identify photosynthetic traits and crop species performance under varying climate conditions is essential for improving stress tolerance (Fernández-Calleja et al., 2020; Araus et al., 2018; Araus & Cairns, 2014). Different machines and instruments ranging from satellites to unmanned aerial vehicles to portable devices are of current use, but they vary in cost, specificity, and size (Araus et al., 2018). Hand-held and affordable tools offer an advantage for *in situ* heat stress measurements and the ability to combine environmental and biological data of crops and forests, besides connecting and uploading the *data* to an open network in real-time (Kuhlgert et al., 2016).

The capacity of instruments to capture multiple parameters in a single measurement permits researchers to capture relationships, prevents diurnal or circadian changes in plant status between measurements, eliminates the burden of various equipment and specialized knowledge, and optimizes time. The study of Carrera et al. (2021), conversely, relied on multiple instruments to measure photosynthetic parameters. With the aid of remote and accurate portable devices deployed easily in the field, the timely prediction of plant heat stress and performance, and the identification of related genes through quantitative genetics will accelerate the discrimination of plant populations in the field (Cruz & Avenson, 2021).

Both the development of “high-throughput phenotyping” and long-term “envirotyping” (description of environmental factors) to screen large numbers of wild plants, breeding lines or cultivars will enhance the discovery of tolerant varieties (Bita et al., 2013; Sadok et al., 2020). Depending on the research objective and economic resources —low, medium, and high-throughput— phenotyping may be used to estimate photosynthetic efficiency, yield components, and related parameters. For a review of the uses, advantages, and limitations of phenotyping tools refer to (Hein et al., 2021). Modern controlled-environment screening technology combined with phenotypic tools may capture the complex target traits, such as physiological responses, in vast screenings (Murchie et al., 2018).

Traits to screen in heat stress

For soybeans, few studies have captured diverse MGs and wild germplasm in their research, suggesting that sources of heat tolerance remain to be discovered. Screening of wild or advanced soybean germplasm and determination of genetic variability for important physiological, morphological, and agronomic traits is a pathway to distinguish tolerant and susceptible genotypes.

Traits found in the literature to discriminate soybean genotypes based on heat stress responses are presented in Table 1.2, yet additional traits can be explored, as has been done in wheat (Ullah et al., 2021). Recently, Kimm et al. (2021) used a canopy chamber with above ambient temperatures of 1.5 °C to 6 °C and found that sun-induced fluorescence (SIF) yield was a sensitive indicator for elevated temperature stress due to its ability to capture physiological information, suggesting enhanced remote sensing optimization may be an improved tool for monitoring crop growth experiencing abiotic stress. The same study demonstrated SIF as a more

stable stress indicator than remote sensing for leaf area index (LAI) and harvest index (HI; more variable) when monitoring productivity and physiology.

Additional thermotolerance indicators such as seed or viability, leaf temperature, and stay-green, can be explored in soybeans. For example, in other legumes such as common bean, lower leaf temperature resulted in tolerant genotypes —the upper canopy seemed more sensitive to heat stress (Deva et al., 2020). These leaf temperature measurements were suggested at the reproductive stage (R-stage) rather than the vegetative stage (V-stage) (Kumar et al., 2017). Stay-green trait allows more sugars to be synthesized and greater biomass accumulation due to more photosynthesis, an attribute that may be underutilized.

DETERMINING HEAT STRESS

A challenge to assessing heat stress responses is that there are often no obvious whole plant signs, making it difficult to visually quantify the damage or utilize remote sensing techniques that rely on canopy changes such as wilting; therefore, visual damage assessment is not used to evaluate heat stress because of its confounding effects with water stress. Common methods to assess heat stress responses and indicators of thermal tolerance are chlorophyll fluorescence (Chl), reactive oxygen species (ROS), electrolyte leakage, heat shock proteins (HSP) (N. R. Baker, 2008; N. Baker & Rosenqvist, 2004; Geange et al., 2021; Maxwell & Johnson, 2000) and more recently ‘omics’ (e.g., Genome-wide association, epigenomics, and DNA modification), the latter more applied to breeding. The selection of technique(s) is based on the research objectives, knowledge, and economic reasons.

Chlorophyll fluorescence (Chl)

Chlorophyll fluorescence (Chl) is a widely used, non-destructive screening technique that approximates the plant photosynthesis status. It persists as one of the most useful techniques to

study heat stress through various parameters (e.g., F_v/F_m , Φ_{PSII}), described below. These parameters give rapid insights into how the plant genetics and photosynthetic apparatus respond to environmental stresses *in situ* in a non-invasive way (not to provide a full plant photosynthetic status) before any non-reversible visual damage is observed. Any study related to photosynthesis may incorporate Chl parameters for better informed decisions (Maxwell & Johnson, 2000). Examples of selection of heat-tolerant plants based on Chl were made in cotton (Wu et al., 2014), rice (Ferguson et al., 2020), fava beans (Benita et al., 2018), common bean (Traub et al., 2018), tomato (Poudyal et al., 2019), wheat (Posch et al., 2019) and forests (*Guarea Guidonia* and *Ocotea sintenisii*) (Carter et al., 2021). To measure Chl, pulse amplitude modulated (PAM) fluorimetry is an efficient, reliable, non-destructive technique to assess plant responses and derive more metrics (e.g., F_v/F_m) (Araus et al., 2018; Cruz & Avenson, 2021; Geange et al., 2021; Kuhlger et al., 2016).

Chl is the proportion of light energy absorbed by chlorophyll molecules that is re-emitted as light, rather than being passed through photosystem II (PSII) or being re-emitted as heat (Maxwell & Johnson, 2000), and it involves various photochemical and non-photochemical parameters (Table 1.3). Photosystem II quantum efficiency (Φ_{PSII}) —the proportion of light captured by photosystem II (PSII) used in photochemistry— serves as a monitor of ion rates and thus ATP and NADPH production (N. R. Baker, 1989, 2008). These two light-dependent molecules provide the chemical energy that the Calvin cycle needs to fix CO₂. A decline in Φ_{PSII} might indicate a reduced electron transport rate. In HS field conditions, tolerant genotypes had higher Φ_{PSII} values in cotton (Wu et al., 2014) and common bean (Traub et al., 2018), the latter with low nonphotochemical quenching (NPQ: energy dissipated as heat).

The maximal PSII efficiency (Fv/Fm) provides information on the maximum efficiency of PSII, so it is interrelated with Φ_{PSII} . Deviations (lower) from the usual values, ~ 0.83 , are expected in stressed plants (Maxwell & Johnson, 2000), which emphasizes that changes in this parameter reflect plant responses to environmental stress such as photoinhibition; however, it should be interpreted with caution due to confounding effects with other photosynthetic parameters (N. R. Baker, 2008). Recent findings suggest that heat-sensitive and tolerant cultivars can be identified with Fv/Fm: a study on wheat discriminated from 1274 entries to 41 tolerant genotypes (Kumari Sharma et al., 2012); higher concentrations of Fv/Fm and chlorophyll content were found in tolerant tomato varieties (Nankishore & Farrell, 2016); during a heat wave, Fv/Fm was a reliable approach to identify heat tolerant germplasm in the lab followed by field evaluations (Poudyal et al., 2019). However, Kalaji et al. (2011) found no significant effect of temperature stress on Fv/Fm in spring barley seedlings before 7 days of stress, implying the weak stress magnitude or premature photosynthetic machinery.

Quenching parameters determine the efficiency at which heat dissipation from excess light occurs. Non-photochemical quenching (NPQ) differences may point to leaf photoprotection or photoinhibition, which is related to plant genetic composition (Cruz & Avenson, 2021). In barley, NPQ was the most sensitive parameter and was used to discriminate genotypes under water stress conditions (Fernández-Calleja et al., 2020).

Literature validates that individual or combined Chl parameters may be used in heat tolerance screenings (Table 1.4). In soybeans under heat stress, for example, a decrease of Fv/Fm and relative chlorophyll content (SPAD), coupled with an increase of $\sim 33\%$ in Fo/Fm ratio (thylakoid membrane damage) was observed (Carrera et al., 2021; Djanaguiraman et al., 2011); inferring changes in overall leaf structure and ultrastructure (like increased leaf thickness). In

common bean, Φ_{PSII} , NPQ, and electrolyte leakage were used to discriminate tolerant genotypes (Traub et al., 2018). These and other Chl parameters (F_o , F_q'/F_m' or Φ_{PSII} , F_v'/F_m') were suggested for heat stress screenings (N. Baker & Rosenqvist, 2004), but F_v/F_m is the most widely used across species because tolerant genotypes that express higher F_v/F_m also produce higher biomass and yield (Kalaji et al., 2017).

Fluorescence and gas exchange, along with rapid sampling tools, offer a potential for environmental stress screening and provide a greater physiological performance understanding; fluorescence parameters, however, are highly variable to environmental conditions (N. R. Baker, 2008; Maxwell & Johnson, 2000). Sharma et al. (2014) warned that intact and detached leaves yielded contrasting chlorophyll fluorescence data under heat stress: the detached leaves were more affected. A comprehensive review of applications and common Chl questions is given by (Kalaji et al., 2014, 2017). Chl is the first screening step, then biochemical (ROS, HSP) and quantitative approaches (e.g., QTL, GWAS) may further discern tolerance mechanisms.

Reactive oxygen species (ROS)

ROS are unstable molecules due to unpaired oxygen atoms that can alter conventional cellular processes such as cell division, growth, and signaling. ROS is interrelated with antioxidants because cells constantly supply antioxidative enzymes to counteract the presence of oxidative molecules, but under extreme temperatures ROS may outperform antioxidants (ROS scavenging agents), inducing abnormalities in proteins and provoking cell damage or death (H. Zhang et al., 2022). HS increases ROS levels, but its concentration (e.g., hydrogen peroxide, superoxide, singlet oxygen), would be greater under short-term exposure than in warming conditions (Geange et al., 2021; S. Jagadish et al., 2021).

Another technique to assess heat effects is measuring electrolyte leakage. Even though more applicable to lab experiments due to less sensitivity than Chl, it is used to monitor the Mn and Ca ion concentrations changes in the cell and tissue injuries, and to derive extra parameters.

Heat shock proteins (HSP)

HSP are involved in HS responses by preventing stress damage and interacting with other proteins to avoid instability, maintain functionality, and prevent protein aggregations and denaturation (J. Zhang et al., 2022). HSP increase is associated with resilient species and possibly gene expression alterations that may result in tolerance mechanisms; thus, it is common to measure when investigating heat tolerance. Das et al., (2016) by studying two soybean varieties found that regulation of the higher concentration of Elongation Factor Tu Family (EF-Tu) proteins along with other HSP may activate heat tolerance. This finding was later confirmed by (H.-Y. Zhang et al., 2022), pointing out that protein GmEF8 (EF-Tu family) overexpression conferred heat stress and drought tolerance in soybeans. Through a proteome analysis of soybean seedlings, tissue-specific responses were found and HSP were involved in protecting chloroplast proteins from heat stress (Ahsan et al., 2010). Heat stress might be perceived in all cellular compartments due to alterations in protein stability, however, thermal tolerance is complex to discern at the molecular level (H. Zhang et al., 2022).

HSP related to growth and heat tolerance were recently reported. For instance, GmHsp90A2 (Huang et al., 2019) and GmDNJ1 (HSP40) (K. P. Li et al., 2021a) were related to increased chlorophyll content and provided heat tolerance when interacting with other proteins. A higher concentration of HSP (HSP70 and HSP17.6) was found in two genotypes exposed to HS (Krishnan et al., 2020) and Gm2-MMP was associated with heat and humidity stress in leaves (Liu et al., 2018). These studies concluded that HS reduced chlorophyll content and may

lead to plant death. Although HSP increases have been observed in short-term heat experiments (Ahsan et al., 2010), the HSP accumulation in long warming conditions remains unclear (S. Jagadish et al., 2021). Modern genomic editing approaches like *CRISPR/Cas9* may accelerate the discovery of HSP involved in thermotolerance.

Nowadays, the lack of standardized parameters, technology systems (growth chamber, heating arrays), techniques (Chl, ROS, HSP, membrane stability), experimental designs, and basic protocols to fully understand heat stress in crops prevail, with most of the work done in model plants. The costs involved to impose heat stress in field conditions may be the greatest constraint. These limitations prevent the replication and advancement in the identification of tolerant germplasm (Geange et al., 2021).

HEAT STRESS EFFECTS IN SOYBEANS

The looming effects of heat stress on metabolic and enzymatic functions alter plant growth and development, later reflected in soybean yield (Khan et al., 2020). Alterations are observed at the physiological, developmental, and reproductive levels.

Physiology

HS alters the photosynthetic machinery due to its effects on biochemical reactions, metabolic processes, and enzyme activity. For example, high leaf temperature minimizes Ribulose-1,5-bisphosphate carboxylase-oxygenase activity (Rubisco; CO₂ fixing enzyme and carboxylation catalyzer) by either inhibition or unstable Rubisco-activase and causes PSII aberrations by disruption of the oxygen evolving complex that affect protein synthesis due to electron transport processes (L. Zhu et al., 2018). To avoid HS leaves, close their stomata to reduce transpiration and to limit the movement of nutrients. Also, leaves could open their stomata to increase transpiration to avoid HS through evaporative cooling in water-stress free

conditions. Since stomata control CO₂ supply, it may lead to reduced photosynthesis and leaf overheating as temperature increases. Similarly, leaves may curl under high air temperature and low water potential, meaning less area to absorb light energy and maintain normal transpiration, so curled leaves without water absence point out to an indirect effect of heat stress (Taiz et al., 2015).

Photosynthesis rate reductions under HS have been documented in soybeans: reductions of 22% from gametogenesis to full bloom (Djanaguiraman et al., 2018), 6-10% at anthesis and seed filling (L. Zhang et al., 2016), 9-16% near flowering (Tacarindua et al., 2013), and 20% at R2 stage (Djanaguiraman et al., 2011), presumably due to chloroplast and photosynthetic machinery damage (e.g., PSII, NPQ, chlorophyll content) (Djanaguiraman et al., 2013) that can result in lower biomass accumulation —11 to 27% reduction in soybeans (Tacarindua et al., 2013). Soybeans may achieve maximum biomass at temperatures < 37 °C (Choi et al., 2016). For these reasons, plants are unable to accumulate enough carbon, leading to negative effects on productivity. HS also increased nighttime respiration (Ortiz et al., 2021). Combined effects of multiple genes result in alterations in the lipid, cytoplasmatic, nucleotides, and associated molecular components (Rustgi et al., 2021).

Additional reviews of the physiological, morphological, anatomical, phenological, and molecular responses under HS are addressed by (Allakhverdiev et al., 2008; Ul Hassan et al., 2021; Zhao et al., 2020), including HS and other abiotic stresses (Borsani et al., 2021); however, less focus has been on crop species and long-term HS (Zhao et al., 2020).

Vegetative and reproductive stages susceptibility

The threshold temperature for soybeans vegetative (V) and reproductive (R) stages are 30 °C and 26 °C, respectively (Choi et al., 2016; Kimm et al., 2021; Siebers et al., 2015),

hypothesizing that reproduction is more sensitive and a reason for greater research attention. In contrast, Hatfi et al. (2011) reported in a review optimum temperatures of 26 °C and 23 °C for V and R stages, stating that seed reduction is expected for means > 23 °C during reproduction; additionally, the study noted that seed growth rate, seed size and harvest index (HI) will be null at mean temperatures of 39 °C, which is in concordance with (Boote et al., 2005; Prasad et al., 2017).

During the vegetative period, the seedling stage is the most sensitive for various reasons: the low leaf number to cover the bare ground (reflected sunlight, thermal radiation), the photosynthetic machinery may be immature, the small root system is unable to fully mobilize water uptake, and the sunlight induces warmer soil surface or growing media that may kill the seedlings. Older and fully developed plants, conversely, may possess a canopy that covers the surface and cools the soil.

Uniform seed germination and good plant stand for any crop are vital for crop profitable production. Heat stress reduced seed germination and vigor of soybeans (Cohen et al., 2020), which is concomitant with (Prasad et al., 2015), where day/night temperatures of 35/30 °C produced low germination and seedling vigor. These reductions were observed in the lab and confirmed in the field (Egli et al., 2005), however, the two genotypes studied from MG4 and MG5 differed in the degree of sensitivity. Moreover, in a study with 64 soybean genotype seedlings, high temperature (40/32 °C) reduced root growth but increased shoot growth; the MG3 seemed more sensitive (Alsajri et al., 2019). Contrasting one conventional with an exotic soybean genotype, germination was faster and higher under heat stress for the landrace (Gillman et al., 2019), theorizing that landraces may exhibit higher tolerance; this was proposed earlier (Prasad et al., 2017). A slight increase in temperature at this stage may accelerate seed

germination if no other limitation occurs. In other legumes, the temperature was associated with node number development (Kibbou et al., 2021). In general, V stages offer greater tolerance over R stages (Boote et al., 2005).

The reproductive stage determines the crop yield and constitutes a highly sensitive period (S. Jagadish et al., 2021). Stages such as anthesis, pollen production, and pod set, are known to be critical for yield. Albeit soybeans abort flowers, the increased temperature can exacerbate that loss and reduce seed set, pod set, and seed number.

In soybeans, Sapra & Anaele (1991) reported flowering as a susceptible stage and following investigations revealed pollen sterility, and pod set and seed-set reduction (Djanaguiraman et al., 2013; Prasad et al., 2015). In an evaluation of soybeans with a 4 to 7 °C temperature increase, anthesis was earlier by ~ 4 days and the overall development by ~4.5 days (L. Zhang et al., 2016). Similar impacts are not uncommon across plant species, for instance, pollen viability and plant size were greatly reduced in tomato cultivars after exposure to heat waves (Poudyal et al., 2019). The variability at the flowering stage thus could be further explored to identify tolerant germplasm. Salem et al. (2007) screened 44 soybean genotypes from diverse MGs and used pollen germination as a basis for heat tolerance, but no specific pattern of tolerance was observed across MGs. Under short stress during flowering, soybeans may recover partially, but it is more difficult as the plant transitions to later stages. Warming, conversely, may impede the possibility of recovery at all.

Intraspecific variation occurs under heat stress, so it may occur at any stage of plant growth. Still, limited information exists on the extent to which elevated temperatures affect specific soybean development stages (Cohen et al., 2020), genotypic variation, and degree of tolerance (Burkey et al., 2020; Ortiz et al., 2021). Yet other phenological stages remain

unexplored (e.g., pistil, stamen, and floral meristem initiation) (Jagadish et al., 2021). In soybeans, Choi et al. (2016) estimated the optimum temperature for seed number at ~ 26 °C, but further analysis of two genotypes found that the optimum temperature during critical R stages (R5-R7) found that the optimum temperature was lower than 26 °C and different for both. This infers that genotypes within MG may indeed respond differently to HS.

Yield

Both yield and heat stress are polygenic, so yield as a complex trait could be a decisive argument to determine plant responses and the selection of tolerant genotypes (Bita et al., 2013). These selection criteria were employed as well on modern drought-tolerant lines (water-conservation traits) (Devi et al., 2014).

Heat disrupts cell division at meiosis, causing ovary and pollen sterility, which in turn reduces pod and seed number. Boote et al. (2005) suggested that temperatures > 26 °C begin to limit yield due to pollination inhibition and grain size reduction; above >37 °C HS greatly inhibits pod formation leading to a reduced pod number. For instance, high daytime temperatures of 36.5 °C to 38.6 °C from gametogenesis to full bloom reduced 11% pod-set, mainly attributed to sterile pollen (Djanaguiraman et al., 2018). At grain filling (R5) occurs the greatest effect on yield — the allocation of nutrients and energy is towards pod development and seed filling, making the plant susceptible to any stress. When optimum temperatures are between 21- 30 °C, each additional Celsius degree would cost a 4.2% yield decrease (Hoffman et al., 2020). Seeds per pod are likely the yield component less affected by temperature and other environmental stresses.

Harvest index (HI) is adversely affected by any reduction in its components —grain weight and biomass. For this parameter, soybeans have a higher temperature threshold (~39 °C)

compared with other crops and legumes (Prasad et al., 2017), which could drop to zero above 40 °C (Boote et al., 2005). HI reductions in soybeans under high temperatures are significant: 5 - 32% (Tacarindua et al., 2013) and 41% (Zhang et al., 2016). Both decreases in HI and leaf area index (LAI) were observed as well (Kimm et al., 2021). Effects on seed weight and yield are quite similar: 21% and 45% reduction for 100-seed weight and yield, respectively (L. Zhang et al., 2016); 25% yield loss for soybean cultivar “Jake” (under warming) (Burkey et al., 2020); and overall seed weight reduction in two genotypes (Cohen et al., 2020).

Moreover, heat stress reduced oil and affected seed protein content (Ortiz et al., 2021), produced seed wrinkling and discoloration plus negative effects on lipoxygenase, the β -subunit of β -conglycinin, sucrose binding protein, and Bowman-Birk protease inhibitor (Krishnan et al., 2020). Leaf soluble sugars and starch decreased ~ 26% and 20%, respectively (L. Zhang et al., 2016), with the same results for tocopherol and isoflavone, especially during seed filling (Chennupati et al., 2011). Overall, heat stress delays vegetative development and maturity, increases flower abortion and pod-set failure, and reduces grain weight; therefore, it is the primary driver of yield loss in soybeans.

REGULATION OF LEAF TEMPERATURE

Elevated temperature increases leaf transpiration —through stomata (guard cells open)—and residual transpiration —through the cuticle (closed stomata)— due to a disruption in the water movement, but only those plants able to minimize water loss through stomata or cuticle will not experience thermal damage (Bueno et al., 2019). Without any water limitation, heat-sensitive plants may raise their stomatal conductance to avoid heat (Nankishore & Farrell, 2016) or to protect against photosynthetic damage (e.g., PSII) as was inferred for soybeans where leaf

temperature decreased $\sim 5\text{ }^{\circ}\text{C}$ (Kumar et al., 2017), but plants could lower up to $12\text{ }^{\circ}\text{C}$ (Khera & Sandhu, 1986).

Among the traits controlling transpiration rates in the leaf, “latent cooling”, that could maintain or increase yield in climate change scenarios are membrane permeability, cuticle permeability, and water viscosity (Sadok et al., 2020). These leaf permeability changes are often observed in the $30\text{-}40\text{ }^{\circ}\text{C}$ range, inducing electrolyte leakage; however, ambiguity exists on whether high temperature causes membrane instability. Moreover, cuticular transpiration, not from stomata, seems to increase faster above $35\text{ }^{\circ}\text{C}$ due to melting of cuticular waxes, pointing out that waxes play a role in these changes (Bueno et al., 2019). The authors finally caution that is risky for genotypes with higher leaf cooling in water-limited conditions, particularly for long-term heat stress events.

Lower leaf temperature, as an indicator of transpiration rates, was associated with low chlorophyll (Prasad et al., 2015), but also low chlorophyll may avoid overheating by less absorbed light. The reduced transpiration trait was utilized to screen tolerance in young rice plants (Ferguson et al., 2020). Tissue temperature during V and R-stages deserves more attention; for instance, the evaluation of genotypes with wider leaves may be prone to greater stress due to excessive absorption of solar radiation or with large stomata that may facilitate high transpiration to provide heat tolerance but with the constraint of a rapid depletion of soil water.

HS caused delayed leaf senescence (green stem disorder) affecting the mobilization of resources to seed filling in soybeans (Tacarindua et al., 2013), possibly explained by increased leaf palisade and spongy layer thickness (Djanaguiraman et al., 2011). Under drought conditions, leaf cooling capacity and stay green traits were identified to select tolerant genotypes (Kumar et al., 2017). However, a recent study on maize involving vapor pressure deficit (VPD) presumes

that water conservation may depend on the root system rather than the leaves (Jafarikouhini et al., 2022); such research is lacking in heat stress. Compared with the canopy, the root system is more sensitive to HS due to a less variable microenvironment and lower temperatures.

Both estimates of tissue and air temperatures are preferred for comparisons across populations and experiments; the air temperatures will just determine the exposure system accuracy. Determining tissue temperature, however, becomes complicated and requires various environmental and plant factors (Jagadish et al., 2021), but portable infrared devices could measure this parameter in preliminary studies (Deva et al., 2020).

INTERACTION WITH OTHER ENVIRONMENTAL STRESSES

Plant heat stress may be coupled or confounded with additional stresses. In field evaluations, the control of soil compaction, moisture, fertility, and homogeneity; and in controlled environments, the control of light, air moisture, and VPD would be an extra challenge; thus, it is vital to reduce confounding errors (Kimm et al., 2021).

Interestingly, soybean studies reported that combined factors may yield additive effects only: drought, temperature, and biotic stress (Grinnan et al., 2013); temperature, CO₂, and light radiation (even though detrimental effects on petals, pollen, and pollen germination) (Koti et al., 2005); warming and elevated ozone (Burkey et al., 2020). Similar to tomatoes under well-watered and heat-stressed conditions (Nankishore & Farrell, 2016) —all reported no interactions. Conversely, in soybean exists evidence of additive and interactive effects of CO₂ and elevated air temperatures (J. T. Baker et al., 1989; Bryan Heinemann et al., 2006; Sicher, 2013; Thomey et al., 2019).

Discrepancies exist on how to approach abiotic effects studies on parameters such as yield. Proponents that each factor may be studied separately to account for specific effects and future

independent improvement strategies for adaptation or environmental manipulation when possible (Grinnan et al., 2013; Piao et al., 2017) contrast with the multifactor approach (Balfagón et al., 2020; Geange et al., 2021; Jin et al., 2017). In soybeans, Prasad et al. (2015) suggested that heat and drought stresses are associated and may be studied together, for instance, but recent research implies that physiological and other molecular traits for tolerance could be stress-specific (Taiz et al., 2015; H. Zhang et al., 2022).

Under high temperatures, plants may cool down through increased transpiration, but in drought conditions, they will close their stomata to save water. Even if water is available in the soil, stomatal closure can still happen when temperature exceeds the plants physiological capacity—it would reduce CO₂ uptake, which increases leaf temperature. Thus, while some traits may favor heat tolerance, they can be harmful to drought tolerance (Traub et al., 2018). Water supply may provide a buffer capacity for heat stress—plants would rather cool down by consuming extra water to prevent photosynthetic damage—, so heat causes less damage, which is critical to determine whether genotypes have water-saving or water-consuming traits (Sadok et al., 2020). Jagadish et al. (2021) also noted that even under well-watered conditions, the reduction in yield components is significant for various crops. If plants can acclimate their transpiration rates to high temperatures, this could be a trait for improvement (Posch et al., 2019). A minimal transpiration rate would benefit yields at critical stages of development.

Heat and drought stress have been studied vastly in soybean (Cohen et al., 2020; Das et al., 2016; Ergo et al., 2018; Jumrani et al., 2018), tomato (Nankishore & Farrell, 2016), faba (Kibbou et al., 2021), crops and trees (L. Zhu et al., 2021), and major global crops (He et al., 2022). In soybeans, both stresses induced disruption of various proteins including Rubisco, electron transport, Calvin cycle, and C fixation (Das et al., 2016). Regardless of water

availability, genotypes with contrasting transpiration responses to VPD may be evaluated for future climates. Recent reviews suggest that transpiration rates under warming conditions are genotype-dependent, even for non-cultivated plants (Sadok et al., 2020). A study also added that heat stress was more relevant in limiting root development than water and mechanical stress (soil compaction), and that soil temperatures $< 30\text{ }^{\circ}\text{C}$ favor soybean root growth and elongation (de Moraes & Gusmão, 2021).

Light energy drives photosynthesis and minimal light should be intercepted when measuring photosynthetic parameters, otherwise biases occur using artificial light or prolonged time lapses between measurements (Cruz & Avenson, 2021). Well-watered plants may perform well when $\text{PAR} > 250\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ (S. Jagadish et al., 2021) and avoid excess heat (Nankishore & Farrell, 2016), even in sensitive genotypes (Panthee & Gotame, 2020). High temperature and light effects have been studied in cowpea seedlings (Osei-Bonsu et al., 2021), but rarely in soybeans.

Increased vapor pressure deficit (VPD) — the difference between saturating moisture and relative humidity (air moisture) — is usually concomitant with HS. HS and VPD are important for local warming scenarios since stomate closure is driven in part by the increase in VPD to save water loss and conserve soil moisture (Jafarikouhini et al., 2022). The Impacts of VPD and high temperature on soybean yield are obvious (Hoffman et al., 2020; L. Zhang et al., 2016); for instance, each 1 hPa increase at grain filling reduced yield by 1.3% (Tacarindua et al., 2013).

High temperature and humidity worsened seed vigor in soybeans through reduced seed nutrient storage and negative effects on various pathways in the leaf, pod, cotyledon, and embryo (Wei et al., 2020) and grain weight and quality in rice (with decreased relative humidity) (Yan et

al., 2021). HS is more harmful with high humidity due to saturating water vapor that would limit transpiration (Wu, 2014). Recent reviews on HS and other environmental stresses are available: high temperature coupled with either water, salinity, or irradiation (Balfagón et al., 2020); drought, salinity, and extreme temperatures (H. Zhang et al., 2022); and temperature, CO₂ and ozone (Hatfi et al., 2011).

In summary, it is fundamental to address heat stress independently to disentangle its implications in plant development/biology and ameliorate such effects, then unravel interactions (Bitá et al., 2013; Prasad et al., 2017), but experimental challenges to impose heat independently or in conjunction with additional stresses exist to date. Heat and drought, for instance, may produce more unique responses when they occur in combination than when they occur individually. Therefore, high temperature in the presence of other stress (biotic or abiotic) has higher negative implications for plants.

MITIGATION STRATEGIES

Plants can tolerate or avoid heat stress through specific traits or aided with management practices and environmental manipulation (e.g., water, nutrients, light).

Unlike other crops, flowering is not directly exposed to sunlight in soybeans; this ameliorates the heat and serves as an avoidance mechanism, except in determinate varieties where the apical inflorescence may be exposed. Most soybean flowers, reproductive organs, and pods are shaded by leaves (Prasad et al., 2017). In common beans exposed to heat stress, leaf cooling resulted in an avoidance mechanism (Deva et al., 2020). Leaf cooling through increased transpiration is more typical of arid environments (Sadok et al., 2020).

Studies have observed that plants exposed to non-lethal and constant HS, either short or long-term, promote heat tolerance and improve responses to subsequent heat stresses, however, it

is unknown the extent a plant is genetically capable of achieving this adaptation. Genetic variation within species constitutes an adaptation mechanism to environmental changes (Mousavi-Derazmahalleh et al., 2018), and genomic or ‘omics’ technology can help identify such variation in breeding programs. The use of GWAS may help identify tolerant loci, but more work is needed to optimize costs of genomic characterization and QTL analysis to find heat-tolerant genes (L. Zhu et al., 2018). In wheat, Waheed Riaz et al. (2021) highlighted traits that can be used to select tolerant germplasm through conventional breeding. In soybean seedlings, adaptive and tolerance mechanisms may be tissue-specific (Ahsan et al., 2010).

Management strategies and agronomic approaches

Some mitigation strategies to offset the negative impacts of heat stress have been deployed (e.g., irrigation, cultural and site management, planting dates).

In soybeans, delayed planting was suggested to avoid high temperatures during the seed filling period and a strategy in cooler areas environments (Egli et al., 2005). Early planting (Early Soybean Production system (ESPS)) or shorter soybean cycles may be appropriate, but the unpredictability of heat waves may coincide with seed filling or maturation; for example, affecting seed germination the following season (Rustgi et al., 2021). In chickpeas, no clear conclusions were stated on the effects of sowing date and heat stress (Sadras et al., 2015). In modeling corn yield responses, Wang et al. (2021) found that increasing planting density of conventional varieties may not be feasible (e.g., more competition with light, water, nutrients, and other critical resources), but pointed out that genetically modified plants were less affected by HS. These results need to be tested in soybeans, however.

Biological approaches to mitigate HS have been used as well. Bacteria *B. cereus* SA1 was found to provide thermotolerance to a soybean variety by increasing Chl and other

metabolites (Khan et al., 2020). The use of arbuscular mycorrhizal fungi (AMF) enhanced photosynthesis in maize exposed to heat (Mathur et al., 2021). In the case of osmoprotectants, antioxidants, and phytohormones, these may drive a complicated chain of reactions (Borsani et al., 2021). Some bio-stimulants have also been reported to enhance thermotolerance (Khan et al., 2020).

Integrative solutions —planting dates, irrigation, bio stimulants, relative tolerant varieties—may buffer heat stress and reduce yield losses. Even though the ability to tolerate and recover is important in HS studies, under warming scenarios the recovery component may not be of great importance, instead is the plants genetic background that determines the overall tolerance and performance. Recovery could be observed in the yield components (S. Jagadish et al., 2021). It remains unclear whether the findings of short-term heat exposures and other abiotic stresses are also applied to climate warming, which is a more realistic approach to developing resilient crops for the predicted climate scenarios. A more holistic, systems perspective, is needed along with ‘omics’ approaches to better understand climate warming effects on crops and ecosystems (Bita et al., 2013).

FINAL COMMENTS

In general, ~24% fewer studies are on heat relative to cold experiments, of this proportion the focus is on intraspecific, single species, being cereals and fiber crops the most studied; for legumes, ~13% fewer focus on heat, with a wider gap for studies of wild species (Geange et al., 2021). These summaries point out concerns on heat tolerance studies on both cultivated and wild species given the increasing short and long-term temperature extremes and warming, highlighting the need for more HS research.

Although soybeans are the 4th commodity produced in the world, no public breeding program is focused on developing heat resilient varieties that may alleviate the projected losses, which could threaten future US production. A decade ago, the International Maize and Wheat Improvement Center (CIMMYT), for instance, initiated a standalone program to discover and breed for heat stress traits in maize, which is now in its final stage for the generation of heat-resilient inbred lines and hybrids (Prasanna et al., 2021). Research programs aimed to enhance or discover tolerant traits to face environmental stresses are a necessity because climate-smart plants will gradually have to replace antiquated varieties to meet food demands (Prasad et al., 2015).

If the canopy (leaf) temperature is not beyond the upper-temperature limit, at which denaturation and death seem unavoidable, species still have the potential to acclimate to gradual temperature changes (L. Zhu et al., 2018). Cultivated land may benefit from temperature increases if the season average fluctuates ~ 20 °C: an increase of 2-4 °C may maximize soybean yields, including wild species and other crops (Choi et al., 2016). For instance, Kumagai & Sameshima (2014) increased temperature to test cultivars adapted to suboptimal temperatures and observed an increase in yield for late MGs, presumably due to an increase in photosynthesis. Here, however, the negative ecological effects could outweigh the yield gains.

The advent of easy-to-install weather stations and wireless field monitoring systems will automate data collection of environmental and biological variables that are central to the quality of experiments and to contrast across crops and growing conditions. Experiments that automate the data collection in multifactor studies (e.g., heat with CO₂, light, or water deficits) can quickly give insights to make data-driven decisions. Remote sensing, especially thermal-sensing and imaging could detect early signs of heat stress. Therefore, a holistic approach across disciplines

in both cultivated and wild species will uncover tolerant traits that breeding programs can improve or pass on to existing cultivars.

Statistical tools

Statistical approaches used to identify resilient soybean genotypes under environmental stresses have been demonstrated. Based on historical data and machine learning to quantify yield responses across the US, yield starts to decrease at 30 °C (Hoffman et al., 2020). Evidence of soybean biochemical variability (V_{\max} and J_{\max}) and acclimation in-field full-season canopy warming (up to 6 °C increase) was partially achieved (~50%) by hyperspectral reflectance and machine learning methods (Kumagai et al., 2021).

Usual statistical models such as multiple regression, multivariate techniques (Principal Component Analysis, Factor Analysis, Discriminant Analysis), and additive main-effects and multiplicative interaction (AMMI) can disentangle the complex relationship between biological and environmental factors. The multivariate techniques provide better graphical displays and interpretation of genotypes interacting with the environment (Mohammadi et al., 2020; Yeater et al., 2015). Modeling combined environmental stressors will enlighten sustainable production in a changing climate (Burkey et al., 2020).

Ultimately, a careful selection of statistical models coupled with reliable screening tools for data collection (e.g., fluorescence, stomatal and mesophyll conductance, CO_2 , etc.) may shed light on discovering efficient photosynthesis and genetics for diverse environments (Cruz & Avenson, 2021). Similarly, more exploration is necessary of new photosynthetic models (Bambach et al., 2022).

CONCLUSION

This review highlights the urgency to study HS impacts on crop growth and development and yield to discover heat resilient physiological and agronomic traits. Experiments and crop modeling suggest that HS constitutes the greatest threat to world food security by its chronic effects on yield. HS tolerance, like yield, is a polygenic trait that requires molecular, physiological, and biochemical disciplines, and critical research to maintain and increase productivity. To this end, neither heat tolerant cultivars have been developed nor breeding programs have started to implement a portfolio that includes warming and cold temperatures — far less extreme temperature events, which are precursors of acclimation to changing climate (Geange et al., 2021). If a feasible protocol is developed to identify heat stress early on, significant progress can be made towards advancing cultivar selection, such that traits can be faster incorporated into improved populations. Albeit advances in other environmental stresses, limited information is available on the contribution of such improvements to HS heat tolerance. A starting point to develop field-ready varieties is to explore available crop germplasm (Bailey-Serres et al., 2019). A pressing question is: are we ever going to find multiple stress-tolerant crops that are as productive as they are today?

Large enough infrastructure to assess heat responses alone is often a limitation, but technological advances in exposure systems and phenotyping instruments can speed the identification of promising cultivars and untap diversity. Multifactor experimentation proponents of heat with additional stresses collide with those that believe in the single heat stress approach. The use of adequate statistical models, screening facilities, and phenotyping tools could aid in the efforts to address the climate warming crisis.

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Table 1.1. Target temperature increase, technology approach to evaluate heat stress, and impact in soybeans.

Heat treatment	Screening technology	Genotypes studied	Duration	Yield impact	Reference
+3.5/ +2.4 °C Day/night above ambient	Air Exclusion System (AES)	Cultivar “Jake”	Full-season field warming	25% seed yield loss	(Burkey et al., 2020)
+6 °C above ambient	Infrared heating	One commercial cultivar (Pioneer 93B15)	3-day periods at early pod development	~ 10% reduced seed yield	(Siebers et al., 2015)
30/22 °C (control); and 38/30 °C Day/night with (CO ₂ and radiation)	Controlled environment chambers (SPAR) (2 m long x 1.5 m wide)	6 soybean genotypes from contrasting MGs	Season-long heat stress		(Koti et al., 2005)
20/12 °C (low); 30/22 °C, 40/32 °C (high)	Controlled environment. Soil-plant-atmosphere-research (SPAR)	64 commercial soybean seedlings from MGs 3-5	20 days after sowing	Root, shoot, and physiological reductions for low and high temperature	(Alsajri et al., 2019)
+4.5 °C above ambient	Open-air field chambers	6 soybean genotypes	28 days during seed filling	Reduced oil and seed protein, but not yield (well-watered)	(Ortiz et al., 2021)
30/22 °C, 34/24 °C, 38/26 °C, and 42/28 °C Day/night	Greenhouse with mean temperatures 26 °C, 29 °C, 32 °C, and 35 °C.	Genotype EC 538828 in cement pots		13 to 52% per plant reduction	(Jumrani et al., 2018)
30/22 °C, 34/24 °C, 38/26 °C, and 42/28 °C Day/night	Greenhouses 6 x 3 m	Twelve genotypes		8-65% seed yield reduction	(Jumrani et al., 2017)

Table 1.1 (continued).

28/20°C (control); 38/28°C, and 45/28 °C Day/night	Growth chamber. Means (28 °C, 38 °C, and 45 °C)	Four soybean genotypes (pots with topsoil)	7- day heatwave	Reduced photosynthesis, Fo, and Fv/Fm	(Herritt et al., 2020)
10 °C above optimum (28 °C)	Greenhouse and growth chambers	One soybean genotype	14 days	20.2 % photosynthetic rate reduction; thylakoid damage	(Djanaguiram an et al., 2011)
+1.5 °C, +3 °C, and +5 °C above ambient	Four Greenhouses	Two soybeans	Full season		(Choi et al., 2016)
Increase of 4 °C to 7 °C	Infrared radiator (heaters)	One commercial soybean	Diurnal full season	Reduction of 45% seed yield, 21% 100-seed weight, and 41 % harvest index.	(L. Zhang et al., 2016)
High daytime temperature 36-38 °C	Natural field conditions	22 genotypes (breeding lines and PIs)	Gametogen esis to seed- set	NA	(Djanaguiram an et al., 2018)
Above 32 °C	Greenhouse simulation with no additional increases	Two commercial cultivars	6 h d ⁻¹ heating for 21 days at R4-R6 stage.		(Carrera et al., 2021)
+1 °C, +2 °C, and +3 °C above ambient	Temperature gradient chamber	One genotype	Field-like conditions	30-40% yield reduction	(Tacarindua et al., 2013)
30/22°C (control); 34/26°C, 38/30°C, and 42/34 °C Day/night	Polycarbonat e greenhouse	Cultivar Maverick (indetermin ate)	During summer, autumn, and spring at sowing, R1, and R5	Decrease internode length, but increased node number.	(Allen et al., 2018)

*Heat stress could be studied with other factors, but just additive heat effects are reported.

NA: study objectives were not to quantify yield impacts.

Table 1.2. Potential traits for screening soybean heat tolerance.

Trait	Reference
Membrane lipid unsaturation	(Rustgi et al., 2021)
SPAD /relative chlorophyll content	(Ergo et al., 2018)
Canopy temperature	(Ergo et al., 2018)
In vitro pollen germination	(Djanaguiraman et al., 2018)
Canopy reflectance at the visible spectrum	(Djanaguiraman et al., 2018)
Pollen germination	(Koti et al., 2005)
Seed quality	(Rustgi et al., 2021)

Table 1.3. Summary and description of common fluorescence parameters.

Abbreviation*	Description
Fo	Minimal fluorescence of dark-adapted leaves
Fo'	Minimal fluorescence of light-adapted leaves
Fm	Maximal fluorescence of dark-adapted leaves
Fm'	Maximal fluorescence of light-adapted leaves
$Fv/Fm = [(Fm-Fo)/Fm]$	Maximum quantum yield of PSII in dark-adapted leaves
$Fv'/Fm' = (Fm'-Fo')/Fm'$	Maximum quantum yield of PSII in light-adapted leaves
Fs	Steady-state fluorescence
Fv'	Variable to maximal fluorescence of light-adapted leaves
qP	Photochemical quenching
qN	Non-photochemical quenching.
ETR	Electron transport rate
$\Phi_{PSII} = [(Fm'-Fs)/Fm']$ or Fq'/Fm'	PSII quantum efficiency (Phi2 in MultispeQ)

*Refer to (N. R. Baker, 2008; Cruz & Avenson, 2021) for more detail and its mathematical formulas.

Table 1.4. Common fluorescence parameters used to assess photosynthetic performance in response to heat stress.

Parameter /trait	Crop	Technology/facility	Indication	Reference
Fv/Fm	Tomato (<i>Solanum lycopersicum</i> L.)	Chamber (screening from 28 to 2 tolerant). Phenotyping from lab to field.	Higher values showed heat tolerance and were related to leaf cooling.	(Damodar et al., 2020)
Fv/Fm	Soybean (<i>Glycine max</i> L. Merr.)	Greenhouse and growth chambers	Decreased under HS	(Djanaguiraman et al., 2011)
Fv/Fm	Faba bean (<i>Vicia faba</i> L.)	Greenhouse and growth chambers	Higher levels in tolerant genotypes	(Zhou et al., 2018)
Fv/Fm and Φ_{PSII}	Upland cotton (<i>Gossypium hirsutum</i> L.)	Single-leaf sample, growth chamber, and field screening for	1762 > 44 > 10 > 3 genotypes (higher values for vegetative heat tolerance)	(Wu et al., 2014)
Fo/Fm	Soybean (<i>Glycine max</i> L. Merr.)	Greenhouse and growth chambers	Increased under two weeks of HS. Damage to thylakoid membranes	(Djanaguiraman et al., 2011)
Fv/Fm	Wheat (<i>Triticum aestivum</i> L.)	Chambers	Selection of 41 cultivars from 1274 entries	(Kumari Sharma et al., 2012)
Φ_{PSII}	Arabidopsis	Chamber	Acute reduction under HS	(Gao et al., 2020)

Chapter 2. Exploration of heat stress effects on photosynthetic parameters of modern soybean breeding lines

ABSTRACT

High temperature stress prevails as a concerning climate change factor that may decrease soybean yields as the average temperature projections continue to increase by the end of the 21st century. The increase in temperature variability may exceed crop heat tolerance limits and threaten food production. To assess genotypic responses to increased temperature, thirteen elite soybean breeding lines with contrasting background profiles were grown in a Temperature Gradient Greenhouse (TGG) with season-long elevated air temperature of +2 °C and +4 °C above ambient, and with no water limitation. Photosynthetic responses through chlorophyll fluorescence (Chl), as well as leaf temperature depression (LTD) and relative chlorophyll content (SPAD), were measured during the 2021 growing season to investigate whether they show differential responses in stress tolerance and predict final yield performance. Although effects of season-long elevated temperature on these parameters were not significant, genotypes were different from each other on all parameters analyzed. Genotypes significantly differed in photosystem II (PSII) quantum efficiency (Φ_{PSII}), maximum efficiency of PSII (F_v'/F_m'), LTD, and SPAD. Genotype SW_AABB was able to direct a large proportion of light energy into photochemistry (Φ_{PSII}) and dissipated less heat (NPQ) among genotypes. Genotypes RIL#1360, FW_aabb, SW_AABB, S14-1625, and KS4520NS showed enhanced transpiration capacity, which could reduce heat stress via lower leaf temperatures. These differential genotypic responses to heat stress indicate that the screened germplasm has contrasting physiological mechanisms to regulate leaf temperature and that Chl could be an efficient selection strategy to

identify potential heat-stress tolerant genotypes. The genetic sources identified here could be used in subsequent field experiments and biochemical analysis to determine their potential inclusion in the development of heat-tolerant varieties in heat stress breeding programs. Thus, both TGG and Chl may aid to streamline the process of improving climate-smart varieties.

Keywords: heat stress, *Glycine max*, soybean, leaf temperature differential, chlorophyll fluorescence.

Variable abbreviations: photosystem II operating quantum efficiency (Φ_{PSII}), non-photochemical quenching (NPQ), fraction of open Photosystem II centers (qL), non-regulatory energy dissipation or other energy losses (NO), linear electron flow (LEF), maximum quantum efficiency in the light (F_v'/F_m'), maximum fluorescence yield (F_m), steady-state fluorescence (F_s), minimal fluorescence in the light (F_o'), proton motive force parameters (Electrochromic Shift: ECSt and ECSm; steady-state proton flux: v_{H^+} ; ATP synthase proton conductivity: g_{H^+}), leaf temperature differential in degrees Celsius (LTD), relative chlorophyll content (SPAD).

INTRODUCTION

Plant heat stress (HS) recently received greater attention for its potentially detrimental effects on crops and food security concerns over the next decades (Bita et al., 2013; Geange et al., 2021; Piao et al., 2017; X. Wang et al., 2020). On average, the projected temperature rise by the end of the century is between 2.1 °C and 5.7 °C globally (IPCC, 2021) and 2 °C to 3.8 °C for the Midwest, United States (Jin et al., 2017), but for certain tropical agricultural areas, these projections may be greater (Bailey-Serres et al., 2019). Most HS studies have focused on short-term exposure such as heat shock and heat waves in the lab, glasshouses, or field conditions, whereas warming has focused primarily on trees (Jagdish et al., 2021). Typically, a limitation to studying long-term HS (climate warming) is the lack of proper technology to expose crops to HS

throughout the season. For instance, recent studies noted that less proportion of experiments focus on HS over a month and that only ~6 % of the exposure systems used to assess temperature stress captured naturally occurring heat events such as heat waves (Geange et al., 2021). Piao et al. (2017) reported that ~ 25 experimental sites studied warming, >2 months exposure, in major crops worldwide, and ~3 assessed soybean yield responses.

Soybean [*Glicine max.* (L.) Merr.] optimum mean cardinal temperature reported across literature is 28.3 °C, with minimum and maximum temperatures of 11.4 °C and 39.4 °C, respectively (Nicole D Jackson, 2021). Independent of the frequency and intensity, soybean begins to experience HS above 30 °C, but for sensitive stages, this threshold could be lower (26 °C) (Choi et al., 2016; Kimm et al., 2021; Siebers et al., 2015). Soybean also constitutes the second largest crop produced in the United States and is a source of food, fuel, oil, and fiber. To date, it is not well known whether available modern soybean germplasm developed to cope with other environmental stresses (e.g., drought, ozone) possess heat tolerant traits, leaving a gap in their heat tolerance responses. Moreover, in previous studies, these genetic sources have been underexplored under full-season warming due to technology limitations in terms of size and number of genotypes evaluated (Choi et al., 2016; Kimm et al., 2021; Koti et al., 2005; L. Zhang et al., 2016). Research suggests that a starting point to develop tolerant field-ready varieties is to explore available germplasm (Bailey-Serres et al., 2019).

It is well known that HS becomes difficult to assess through visual symptoms due to its concurrence with drought. Recent advances to rapidly detect plant physiological status through fluorescence-based parameters permits researchers to identify desirable plant phenotypes. Techniques such as Chlorophyll fluorescence (Chl), which can be used to assess the proportion of light energy transformed into chemical bonds in the chloroplast (Maxwell & Johnson, 2000),

has promised a rapid non-invasive screening technique that serves as a physiological indicator of plant stress (N. Baker & Rosenqvist, 2004; Kalaji et al., 2014, 2017). Current portable, handheld devices using Pulse Amplitude Modulations (PAM) fluorometry facilitate these measurements and permit to derive additional parameters such as the maximal quantum efficiency of photosystem II (PSII) ($F_v/F_m = F_m - F_o/F_m$) and PSII quantum efficiency ($\Phi_{PSII} = F'_q/F'_m$) (Kuhlgert et al., 2016). For example, as an overall indicator of photosynthesis and its relationship with light efficiency, Φ_{PSII} was previously used on the final screening test in cotton (Wu et al., 2014).

Additional leaf-level parameters such as LTD and SPAD can be measured nondestructively for checking temperature regulation and plant health. However, it is unclear whether fluorescence or leaf-level parameters may be a proxy to predict final genotypic responses (e.g., seed yield) in the absence of water-limiting conditions. Detecting early heat stress responses may help to identify heat tolerant genetic sources in large plant populations (Kalaji et al., 2011). Thus, sensitive parameters to HS could help streamline the screening of tolerant germplasm in changing environmental conditions.

The objective of this study was to screen diverse soybean genetic sources under long-term heat stress to determine whether chlorophyll fluorescence or leaf-level traits predict crop performance that may facilitate the identification of tolerant germplasm. To achieve this, we utilized a TGG facility and a rapid non-invasive phenotyping tool through single leaf screenings. This study aimed to explore the overall performance of available germplasm under warming conditions and not to study in-depth physiological or biochemical aspects.

METHODS

Monitoring of environmental conditions

Air temperature and relative humidity sensors (HOBO U23 Pro v2, Onset, Bourne, MA, USA) were installed in each temperature treatment ($n=9$) at the top of the plant canopy, with approximately weekly adjustments as the plants were growing. Data from an in-house weather station was used to compare with TGG environmental parameters. Water supply was controlled through a digital irrigation timer (Rain Bird, Tucson, AZ, USA).

Plant material and crop husbandry

Thirteen soybean genotypes were selected for favorable yield and/or slow wilting traits belonging to Maturity Groups 4 to 7. These genotypes differed in origin, tolerance to environmental stresses (contrasting background profiles), and determinacy (Table 2.1). For instance, some common traits of soybean drought-tolerant lines were described previously (Devi et al., 2014). Four seeds per genotype, previously treated with Mefenoxam, were hand-sown at 2.5 cm depth on 7 or 21 June 2021 to account for MG determinacy, in 15-L sanitized black plastic pots. Pots were first sanitized with 10% chlorine, filled up to $\frac{3}{4}$ with commercial growing media (Sunshine Mix#8/Fafard-2, Sunagro), mixed with 60 g of slow-release fertilizer (Osmocote Plus 15-9-12), and finally complete filling. Growing media was soaked with tap water prior to planting. All seeds were treated with Mefenoxam (fungicide Apron max) before sowing. The pots were 0.29 m tall and 0.30/ 0.25 m (top/bottom) in diameter with bottom drain holes (~1.8 cm diameter) to drain excess water and covered/wrapped around with an insulation layer (Reflectix, Markleville, IN, USA) to maintain root zone temperature. Pots were arranged in 6 rows (8 pots per row) in each replicate with two additional border rows parallel to the panel wall that distributed the air and one perpendicular row on each side of the TGG vinyl wall. After

emergence seedlings were thinned to two and then to one plant per pot, then grown to maturity. All pots were irrigated two times a day (200 and 1000 h) using a digitally controlled drip system as described above. Daily water meters monitoring ensured that the dripper time and delivery rate provided complete saturation to the growing media. Constant irrigation and well-drained growing media avoided water and soil physical limitations to focus the experiment on heat responses only rather than confounding variables.

A bamboo stick was placed next to each plant followed by an antirust metal cage in each pot to prevent plants from bending later in the season. To prevent pest pressure, a surface insecticide (Imidacloprid) followed by a drench fungicide solution (Mefenoxam and Pentachloronitrobenzene) was applied at ~ 27 days after planting (DAP). There was only one pesticide application ~63 DAP to control spider mites. Some larvae and weeds were removed manually as needed.

Screening technology, research site, and experimental design

A Temperature Gradient Greenhouse (TGG) adapted from a base prototype to study heat stress (Sinclair et al., 1995) was built from north to south at the Lake Wheeler Farm of North Carolina State University, Raleigh, NC, USA (35 43'45'' N, 78 40'43'' W; elevation 120 m) for season-long day/night temperature treatments. TGG consisted of three zones, where each zone corresponded to one temperature treatment (whole plot), which was then subdivided into 3 subplots for replication (Figure 2.1).

In a split-plot design, unheated air was distributed to the first zone (T1= unheated ambient control), and heated air to zones two (T2= unheated ambient air + 2 °C) and three (T3= unheated ambient air + 4 °C). Germplasm was subset into MGs to account for determinacy and

then randomized within their corresponding MG in the subplots. The study consisted of 432 individual plants plus borders. The experiment layout is shown in Figure 2.1.

There were two reasons for assigning temperature levels to main plots: i) the low to high-temperature gradient as the air moved longitudinally through the TGG from north to south, and ii) to allocate precision in the experiment where large differences in stress responses are expected among genotypes.

Data collection

Chlorophyll fluorescence and leaf-level parameters

Non-destructive photosynthetic measurements were taken from the third uppermost recently fully developed leaf's middle leaflet (T-3). The top of the canopy was chosen due to direct exposure to sunlight and active photosynthesis; therefore, more production of photosynthates (e.g., sucrose and starch). One randomly selected plant (e.g., experimental unit) from each genotype in each replicate was measured to have three measurements for each genotype-treatment combination. Measurements were once a week on cloudless days for four consecutive weeks between 1000 - 1200 h, when leaves were photosynthetically active and light intensity was sufficient to support photosynthesis (400–700 nm). Measurements were timed to be completed each day by 1200 to prevent any human hazards since the temperature in T3 reached over 42 °C between 1200 and 1400 h.

All chlorophyll fluorescence parameters, LTD and SPAD were measured using the MultispeQ V 2.0 instrument linked to the PhotosynQ platform (www.photosynq.org) (Kuhlgert et al., 2016) utilizing the rapid information-dense experimental sequence “Photosynthesis RIDES” protocol. This protocol allows collection of relative humidity, vapor pressure deficit (VPD), and photosynthetic active radiation (PAR) parameters simultaneously. We deliberately

avoided measuring all selected plants from a particular treatment before moving to the next, instead, measurements were by replicate across treatments to account for light intensity and daytime changes during the 1000 - 1200 h measurement period. Readings showing any warning message were discarded and repeated immediately for data accuracy. All data can be accessed through the PhotosynQ platform.

Statistical analysis

All statistical analyses and plots were conducted in R software version 4.2.1. (R Core Team, 2022). The lme4 package was used to determine significance of factors (Bates et al., 2015) and the emmeans package for pairwise comparisons (Lenth, 2022). By default, emmeans uses the Kenward-Roger method to compute the degrees of freedom. The typical $\alpha = 0.05$ and Tukey method was used to determine differences.

To account for factors that may influence photosynthetic measurements, we transformed PAR to its square root and time of measurement into a numeric variable (0 to 24) to incorporate them into statistical models. These transformations make covariates effects on chlorophyll parameters linear. For chlorophyll fluorescence variables, treatment and genotype were fixed effects along with the corresponding interaction, transformed PAR and time of day were covariates, and replicate nested within treatment was treated as random. LTD and SPAD were analyzed as described above, except for covariates.

To further explore outcome fluorescence variables, we performed a Principal Component Analysis (PCA). By computing summary statistics, we verified that all variables were in the recommended ranges and that there were no missing data points. Data were fitted using the “prcomp” function from the MASS package (Venables & Ripley, 2002). Associations between traits and genotypes were also explored through PC biplots.

RESULTS

The experiment was carried out to determine the effects of season-long HS on the physiology and productivity of modern soybean breeding lines and commercial cultivars with contrasting abiotic tolerance traits using a TGG. Specifically, we aimed to determine if any fluorescence-based or leaf-level parameters predict or shed light on the potential heat stress tolerance of tested genotypes.

Environmental conditions during chlorophyll measurements

Mean air temperature difference recorded by MultispeQ was +1.3 °C and +2.5 °C with respect to T1 (34.8 °C) during photosynthetic measurements (Table 2.2). The minimum and maximum temperatures reflect that measurements were made across treatments to capture a wide range of light intensities. Mean difference recorded by sensors during the day for the four-week period when fluorescence measurements were taken was +2.4 °C and +3.2 °C relative to T1 (Table 2.3). Average relative humidity (57%) and atmospheric pressure (1006 mbar) changed little across treatments, suggesting that there were no additional limiting factors for photosynthesis.

Chlorophyll fluorescence

Significant main effects were found only for genotypes across all analyzed variables, except for NO where treatment main effects were significant. Although small differences, temperature and interaction effects were not significant. Linear mixed effect models were used to examine individual dependent variable contributions to main effects.

We found differences among genotypes in the maximum quantum efficiency of Photosystem II (PSII) in the light (F_v'/F_m') ($p = .001$), PSII quantum efficiency (Φ_{PSII}) ($p = .002$), non-photochemical quenching (NPQ) ($p = .013$) and minimal fluorescence yield (F_o') ($p =$

.001), while for NO both treatment ($p = .008$) and genotype ($p < .001$) main effects were observed (Table 2.4). The NO indicates energy directed somewhere else or unknown direction. The Φ_{PSII} and NPQ were affected by genotype. Based on marginal means, Φ_{PSII} was significantly higher in SW_AABB, Woodruff, and S14-1625, approximately 14% above Magellan, which had the lowest value. Temperature effects were not significant for Φ_{PSII} , but interestingly FW_aabb tended to increase (11%) and Holladay to decrease (-7%) from T1 to T3 (Figure 2.2). For NPQ, SW_AABB and Holladay were significantly lower, with approximately 17% lower NPQ than Magellan, which had the highest value.

Fv'/Fm' was relatively consistent across treatments, but genotypes showed differences. Holladay showed the greatest average (0.71) and was significantly different only from Magellan and N11-10295. Treatments were not significant ($p < .075$), but most genotypes showed a slight trend reduction in T2 and T3 (up to 6%), except for Holladay and S14-1625 in T2 where they increased 1% and 3%, respectively. Fo' was significantly lower in RIL#1360, AGS 738RR, SW_AABB, S14-1625 and FW_aabb, approximately 5-9% lower than Magellan. In general, Fo' seemed to decrease in all genotypes as HS increased, except in Magellan and Woodruff in T2.

Leaf temperature differential and relative chlorophyll content

Genotypes had significantly different leaf temperature differentials ($p < .001$), SPAD ($p < .001$), and leaf thickness ($p = .002$) (Table 2.6).

Contrasting temperature responses were observed for leaf temperature differential among genotypes. Mean canopy temperatures during Chl measurements were 30.5 °C, 31.8 °C, and 33 °C for T1, T2, and T3, respectively. N16-7526 cooled the least at elevated temperature (-3 °C) and was different from all genotypes, except N16-7558, Holladay, and N09-13890. Genotypes RIL#1360, FW_aabb, SW_AABB, S14-1625, and KS4520NS cooled off the most (< -4.4 °C),

except Magellan. RIL#1360 decreased its temperature the most (-5.4 °C). Although there was no interaction effect ($p = .0502$), FW_aabb and SW_AABB cooled below average temperature differential (-4.3 °C) but seemingly were less able to cool at higher temperature (T3); conversely, N16-7526, N16-7558, and Holladay were above the average and seemed to cool off as temperature increased (Figure 2.3).

Genotype significantly affected SPAD ($p < .001$). Overall, N16-7558, Holladay, KS4520NS, N16-7526, and RIL#1360 had significantly higher SPAD (>51) than the rest. Even though there was no interaction effect, FW_aabb decreased 10% from T1 to T3, whereas Holladay and RIL#1360 increased 4% and 8% from T1 to T3, respectively. S14-1625 remained somewhat consistent (~49 units) across treatments.

Leaf thickness was greatest in N16-7558 (1.35 μm) but was not statistically different from Holladay, N11-10295, FW_aabb, Woodruff, and RIL#1360.

Multivariate analysis

Principal components (PC) analysis showed that PC1 and PC2 explained 79% of the total variation for leaf-level trait data (Table 2.8). The first eigenvalue for PC1 is 2.7, which explains 42% of the total variability, with approximately four of the original variables contributing to the overall variation due to temperature levels and genotype. The eigenvector elements indicate that Φ_{PSII} , qL , and gH^+ (the steady-state rate of proton flux) exhibit jointly most of the variation (~42%) in PC1; NPQ, F_v'/F_m' , and F_m' explain an additional 37% of the variation in PC2; and leaf temperature differential and SPAD are the dominant weights explaining 11% of the variation in PC3 (Table 2.9). One cluster of genotypes was observed towards Φ_{PSII} . Φ_{PSII} was grouped with SW_AABB, S14-1625, RIL#1360, and FW_aabb (Figure 2.4); thus, these genotypes exhibited similar characteristics in their Photosystem II (PSII) operating efficiency.

DISCUSSION

We found that among the screened germplasm there is significant genetic variability with respect to photosynthetic machinery to utilize light radiation and leaf temperature regulation. The fluorescence parameter Φ_{PSII} appeared to be a valid screening method to discriminate genotypes. For instance, SW_AABB was able to direct a large proportion of light energy into photochemistry (Φ_{PSII}) and dissipate less heat (NPQ). Conversely, Magellan was less efficient to use light radiation, exhibited higher energy dissipation as heat (NPQ)—less energy available for photochemistry—, and suffered higher photosynthetic damage in the thylakoid membrane, which implies a lower efficiency of the light reactions. Genotypes that cooled the most such as RIL#1360, FW_aabb, SW_AABB, S14-1625, and KS4520NS exhibited “consumptive” water characteristics. The apparent similar transpiration cooling across treatments may indicate that genotypes were actively transpiring to avoid overheating; the water loss thus removed heat from the leaf. In the PC biplot with fluorescence-based parameters, genotypes were spread on different directions, revealing that genotypes have different background profiles to harvest light and may respond differently to stress conditions.

We speculate some temperature effects within this experiment context or with previous studies, regardless of p-values: “larger values do not imply lack of importance or effect” (Wasserstein & Lazar, 2016).

Chlorophyll fluorescence

Chl has been used in both biotic and abiotic stress tolerance studies, including heat tolerance on various species (Carter et al., 2021; Ferguson et al., 2020; Posch et al., 2019; Wu et al., 2014; Zhou et al., 2018) to screen germplasm collections. Within the complex photosynthetic apparatus, the Photosystem II (PSII) is a thermolabile component prone to the displacement of

light-harvesting complexes and thylakoid membrane damage under high temperatures. Since Φ_{PSII} is a heat-sensitive parameter, greater values imply higher tolerant operating quantum efficiency to heat stress, thus a more tolerant photosynthetic apparatus (Kalaji et al., 2017; Wu et al., 2014).

It was suggested previously that in the absence of water limiting conditions, the increased photorespiration in stressed C_3 reduces CO_2 assimilation rates even though linear electron transport remains similar to unstressed leaves, in which case Φ_{PSII} would not be a reliable indicator of CO_2 assimilation rates (N. Baker & Rosenqvist, 2004; Taiz et al., 2015). These reductions in carbon fixation are attributed mainly to the Rubisco capacity to catalyze both oxygenation and carboxylation. Even though in this study genotypes showed higher Φ_{PSII} estimates on average than a previous study (< 32) that evaluated two soybean genotypes on 21-day heat treatment in field conditions (Ergo et al., 2018), we found similar non-significant heat stress effects. This implies that water supply may offset heat effects and genotypes were able to maintain high photosynthetic rates or acclimate somewhat rapidly.

On average, Fv'/Fm' decreased -17% across genotypes relative to usual values (~0.83) of plants grown under stress-free conditions (Maxwell & Johnson, 2000). Previous studies reported declines of -5.4% on faba beans under 3-day heat stress (Zhou et al., 2018). In line with this study, Herritt et al. (2020) found that Fv/Fm in soybeans was relatively unchanged below 38 °C, but overall significant reductions of 5 to 10% were observed at 45 °C, except for one genotype that was lower but not different at 45 °C on a 7-day heat treatment. Additionally, in a cotton screening of 1762 accessions exposed at 25 °C and 55 °C h^{-1} , Fv/Fm showed minimal variation compared with the control (Wu et al., 2014). Either short- or long-term heat stress results point out that Fv/Fm is relatively stable at various heating intensities and stages of development in

soybeans, despite marked reductions in photosynthesis, damage to the oxygen-evolving complex, and damage to PSII reaction centers (Jumrani et al., 2017; P. Li et al., 2009; Ortiz et al., 2021). A possible explanation in this study is that at longer wavelengths (>700 nm) the PSI emits higher fluorescence, which may underestimate F_v'/F_m' (Kalaji et al., 2014).

Overall, our findings demonstrated that the relatively high leaf pigments (e.g., Chl) observed here compared with previous studies imply that genotypes performed well in in these stress conditions. However, the reductions observed for some genotypes in F_v'/F_m' , Φ_{PSII} , and F_o' suggest reduced light efficiency, thylakoid membrane disorganization, CO₂ assimilation inhibition, electron transport inhibition (N. Baker & Rosenqvist, 2004; Djanaguiraman et al., 2018) and heat-induced PSII photodamage (Wu et al., 2014); thus, declines in photosynthesis rates (not measured). PSII is a major ROS producer, which can lead to additional reactions with surrounding biomolecules such as proteins, pigments, and DNA (Moller 2007). These hypothetical changes in stomatal activity and ROS production will require further investigation. Recent studies in soybeans also point out that increased chlorophyll may be related to increased heat shock proteins (HSP) that confer heat tolerance (Huang et al., 2019; K. P. Li et al., 2021b).

It is important to remember that photosynthetic phenotype can be highly variable and difficult to interpret due to its high sensitivity to the surrounding environment and interaction with dependent and independent-light reactions, including non-photosynthetic pigments (e.g., anthocyanins, flavonoids) (N. Baker & Rosenqvist, 2004; Kalaji et al., 2014; Maxwell & Johnson, 2000). This dependence of photosynthetic processes on the environment, like temperature, is central because crop productivity —and crop yield— rely on photosynthetic rates.

Leaf temperature, SPAD and leaf thickness

Leaf temperature revealed the contrasting canopy cooling ability across genotypes. This latent cooling can help the plant to either avoid heat or to protect from photosynthetic damage (e.g., PSII) at temperatures above 35 °C and implies changes in leaf permeability and probably cuticular transpiration (Bueno et al., 2019; Sadok et al., 2020). Water is responsible for maintaining cell turgor and promoting cell elongation. The constant water supply in this study may have allowed normal cell growth and therefore leaf area to be affected little. Well-watered plants permit the leaf to buffer physiological changes (e.g., maintain photosynthesis, stomatal conductance, protein synthesis, wall synthesis and cell expansion; and less accumulation of abscisic acid and solutes) due to minimal effect of the water potential (Taiz et al., 2015). For example, in common beans, tolerant genotypes decreased their upper leaf temperature the most during daytime (-5.2 °C) to maintain physiological function and prevent biochemical damage (Deva et al., 2020). To some extent, when water is abundant during the day, the stomata can diffuse both water (transpiration) and CO₂ (uptake) in greater quantities simultaneously, which favors plant growth and reproduction.

The stay-green trait or SPAD reduction is a direct result of chlorophyll degradation and chloroplast reduction and damage; therefore, chlorotic leaves minimize photosynthetic efficiency (Djanaguiraman et al., 2011). Previous studies reported significant decreases in SPAD values among soybean genotypes that were mainly attributed to high temperature (Djanaguiraman et al., 2018; Jumrani et al., 2017). The dissimilar responses across genotypes in this study point out to different chloroplast capacity.

The genotype with the maximum leaf thickness in our study (N16-7558 = 1.35 µm) was below the minimum observed for soybean genotypes in previous studies (1.42 µm) (Jumrani et

al., 2017), however, that study found reductions of ~44% at 35 °C (121-127 μm), which is in line with the range in this study (1.08-1.35 μm). Conversely, Djanaguiraman et al. (2011) found that HS induced greater leaf thickness, and increased glucose and fructose by 83% (leaf reducing sugars) in soybeans. These contrasting results, increase or decrease, were observed among the screened genotypes here. Though no effect of temperature, RIL#1360 and Magellan tended to increase leaf thickness as temperature increased, whereas N11-10295 and SW_AABB were thinner at higher temperatures. Increases in leaf thickness may imply structural and ultrastructural leaf changes and aberrations in resource acquisition and use.

Limitations

Even though the TGG demonstrated to increase temperature +2.4 °C and +3.2 °C relative to unheated ambient air (Figure 3.1), there was no treatment effect on the studied variables. Some reasons could be the nature of the experimental design where we lose precision to detect whole plot factor effects in favor of the subplot factor. Another reason may be that the tested soybean genotypes are elite breeding lines, were grown without water limitation and soybeans have relatively high heat tolerance compared with other crops (Prasad et al., 2017). For instance, recent studies reported that soybean cultivars relative to plant introductions had higher optimum temperatures, higher photosynthetic rates, and less thylakoid damage (Djanaguiraman et al., 2018). Additionally, we did not establish a set of genotypes outside the TGG, which could have allowed us to make additional comparisons of treatment effects. Measurement of growing media temperatures was outside the scope of this research, but we suggest a useful parameter to account for.

The chlorophyll parameters analyzed seemed to vary little across treatments and genotypes. One reason could be that plants were measured at least a month after temperature

treatments were initiated, providing ample time for heat acclimation. Higher stress intensity (40 °C to 50 °C) and photosynthetic measurements throughout the day may provide insights not detected in this study. Previous studies in wheat detached leaves noted photosynthetic efficiency changes at 40 °C, but not at 35 °C where PSII stayed active (Mathur et al., 2014). Temperatures above 45 °C, however, may trigger protein denaturation and lipid oxidation (Taiz et al., 2015).

Based on previous research and our findings, we imply that higher heat stress intensities may yield more pronounced genotypic and treatment differences; for instance, Djanaguiraman et al. (2018) applied treatment differences of ~ 8 °C and observed maximum temperatures of 51 °C. However, we aimed to explore responses to the available germplasm within the expected warming projections and without water limitations. In field conditions, it is well known that both heat and drought stress coincide and have a greater impact during the warmer months. Similar not statistical significance has been observed previously, highlighting that the combined drought and heat stress may produce significant effects (Ergo et al., 2018; Nankishore & Farrell, 2016). It is unclear whether the identified genotypes will show the same trend under restricted water conditions due to the conflicting physiological responses (e.g., respiration and leaf temperature) to both stresses.

CONCLUSIONS

This study constitutes one of the first screening efforts to find heat-tolerant genetic sources among soybeans improved for other abiotic stresses in the Southeast, US. We identified promising genotypes with photosynthetic phenotypes that could potentially aid in the identification of heat-tolerant germplasm.

Genotypes SW_AABB, Woodruff, and S14-1625 had the highest Φ_{PSII} . The higher Φ_{PSII} values of SW_AABB and S14-1625 may be explained by the seemingly increasing, but not significant higher light use efficiency of PSII at higher temperatures (Figure 2.2). Both SW_AABB and S14-1625 were also among the genotypes that lowered their leaf temperature the most. Interestingly, N16-7526 showed the least temperature differential (-3 °C). We theorize that SW_AABB and S14-1625 may perform well under well-watered environments due to higher transpiration, a “consumptive” strategy, whereas N16-7526 may be better suited for water-saving environments due to lower transpiration, a “saving” strategy. Thus, these genotypes promise to be tolerant genetic sources that need additional heat stress field testing and molecular-biochemical analysis. This can streamline transferring tolerance traits into commercial varieties to improve climate resilient soybeans or diversify breeding programs' portfolio.

Although additional physiological (e.g., photosynthetic rate and stomatal conductance), biochemical (e.g., ROS, antioxidants) and field studies may confirm these findings in subsequent tests, Chl parameter Φ_{PSII} and leaf temperature differential seemed an efficient screening technique in this study. Future long-term studies are needed before endorsing these parameters, however. We identified F_v'/F_m' as a relatively invariable trait in our study, so it may not be a good screening indicator to detect heat responses in non-water-limiting conditions.

The study of crop productivity and yield responses due to photosynthetic activity is important because, unlike animals that define their cell functions during embryogenesis, plant cells respond to surrounding changing conditions, which may result in adaptations to new environments, especially due to the increasing levels of variation in temperature that may make prediction models ambiguous. The increased temperature variability suggests that heat stress should be considered as a stand-alone program for identifying advanced and exotic novel

germplasm. Understanding increased temperature effects on crops may unravel levels of stress tolerance, direct future research pathways, and protect the value chain from vulnerabilities.

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Table 2.1. Breeding lines and cultivars measured for fluorescence and leaf temperature parameters.

Breeding line/cultivar	Maturity Group	Origin/Provider	Original parental source(s) of drought tolerance
Magellan	4	Kansas State University	PI 595362
KS4520NS	4	Kansas State University	Unknown
FW_aabb	4	University of Missouri	Magellan and PI 567731
SW_AABB	4	University of Missouri	Magellan and PI 567731
S14-1625	4	University of Missouri	S05-11482 x S07-5451
RIL#1360	5	University of Missouri	Pana x PI567690
Holladay	5	USDA_ARS, North Carolina	PI 572239 N85-578
N16-7526	5	USDA_ARS, North Carolina	Holladay x Fiskeby III
N16-7558	5	USDA_ARS, North Carolina	Holladay x Fiskeby III
N09-13890	7	USDA_ARS, North Carolina	12.5% PI 416937, 25% PI 407948
N11-10295	7	USDA-ARS, North Carolina	12.5% PI 416937. Drought-Protein
Woodruff	7	University of Georgia	PI 416937
AGS-738RR	7	University of Georgia	Unknown
Jake*	5		

*Used as a border plant

Table 2.2. Summary of environmental parameters during chlorophyll fluorescence measurements.

	2021				
	Mean (°C)	Min (°C)	Max (°C)	RH (%)	PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
T₁	34.8	25.6	40.2	57.0	797
T₂	36.1	29.6	39.7	57.1	834
T₃	37.3	32.1	40.2	56.9	848

Mean, minimum and maximum temperatures; and mean of relative humidity and photosynthetically active radiation by treatment (T1, T2, T3). Chlorophyll fluorescence (Chl) measurements were between 1000 and 1200 h. Photosynthetically active radiation (PAR) and Relative Humidity (RH).

Table 2.3. Mean, 12 h mean day and night, and maximum temperatures during the four-week period when fluorescence measurements were taken.

	Temperature (°C)			
	Mean	Day	Night	Max
Ambient air	26.3	29.7	24.6	38.1
T₁	26.4	29.6	24.5	37.7
T₂	28.6	32.0	26.9	40.4
T₃	29.3	32.8	27.5	41.6

Temperatures correspond to the four-week period when fluorescence measurements were taken (From July 21st to August 12nd, 2021). Ambient air is the temperature outside the greenhouse. T1, T2, and T3 correspond to the three temperature treatments inside the TGG.

Table 2.4. p-values for chlorophyll fluorescence traits.

	Fv'/Fm'	Φ_{PSII}	NPQ	NO	Fo'
PAR ²	<0.001	<0.001	<0.001	0.022	0.807
Daytime	0.001	0.407	0.176	<0.001	<0.001
T	0.075	0.596	0.467	0.009	0.110
G	0.001	0.002	0.013	<0.001	<0.001
T x G	0.905	0.766	0.849	0.844	0.449

Covariates: square root of Photosynthetically Active Radiation (PAR²), time of day (Daytime).
Effects of treatment (T), genotype (G), and treatment by genotype (T x G).

Table 2.5. Estimated marginal means for chlorophyll fluorescence parameters.

	Fv'/Fm'	Φ_{PSII}	NPQ	NO	LEF	Fo'
Holladay	0.71 a	0.49ab	0.26b	0.25	175.65	293.33
AGS 738RR	0.69abc	0.49ab	0.28ab	0.23	174.24	284.26
FW_aabb	0.69abc	0.49ab	0.28ab	0.23	175.86	287.10
KS4520NS	0.68abc	0.47ab	0.29ab	0.24	168.87	294.93
Magellan	0.68bc	0.45b	0.31a	0.24	165.10	299.26
N09-13890	0.69abc	0.49ab	0.28ab	0.23	171.58	288.73
N11-10295	0.68c	0.49ab	0.29ab	0.22	171.72	287.59
N16-7526	0.70abc	0.48ab	0.27ab	0.25	171.29	293.97
N16-7558	0.70ab	0.48ab	0.27ab	0.25	172.21	292.27
RIL#1360	0.69abc	0.49ab	0.28ab	0.23	173.19	283.91
S14-1625	0.69abc	0.51a	0.26ab	0.23	181.84	285.59
SW_AABB	0.70abc	0.52a	0.25b	0.23	185.58	284.87
Woodruff	0.69abc	0.51a	0.27ab	0.22	179.99	287.89

Means ($n=9$) with different letters within a column were significantly different ($\alpha=0.05$; HSD).

Table 2.6. p-values of leaf temperature differential, leaf temperature, relative chlorophyll content (SPAD), and leaf thickness.

	Leaf temperature Differential (°C)	Leaf temperature (°C)	SPAD	Leaf thickness
T	0.9951	0.1007	0.8710	0.9841
G	<0.001	<0.001	<0.001	0.0021
T x G	0.0502	0.5238	0.5469	0.5716

Effects of treatment (T), genotype (G), and treatment by genotype (T x G).

Table 2.7. Estimated marginal means of leaf temperature differential, leaf temperature, relative chlorophyll content, and leaf thickness for thirteen soybean genotypes.

	Leaf temperature differential (°C)	Leaf temperature (°C)	SPAD	Leaf thickness
Holladay	-3.63 ab	31.87	53.61	1.23
AGS 738RR	-3.84 bc	32.46	49.57	1.08
FW_aabb	-5.27 d	31.19	47.28	1.16
KS4520NS	-4.89 d	31.25	52.60	1.09
Magellan	-4.64 cd	31.49	48.91	1.08
N09-13890	-3.71 ab	32.49	48.94	1.12
N11-10295	-3.86 bc	32.47	45.83	1.21
N16-7526	-3.00 a	32.48	51.18	1.11
N16-7558	-3.35 ab	32.06	54.22	1.35
RIL#1360	-5.40 d	30.83	51.11	1.16
S14-1625	-5.09 d	31.15	48.84	1.12
SW_AABB	-5.22 d	31.23	48.98	1.13
Woodruff	-3.88 bc	32.24	47.44	1.16

Means ($n=9$) with different letters within a column were significantly different ($\alpha=0.05$; HSD).

Table 2.8. Eigenvalues and proportion of variance for the first five principal components (PCs) of the PC analysis.

Principal component	Eigenvalues	Variance proportion	Cumulative variance
PC1	2.67	0.42	0.42
PC2	2.5	0.37	0.79
PC3	1.4	0.11	0.90
PC4	0.8	0.04	0.94
PC5	0.62	0.02	0.96

Only the first five PCS are listed because three explain 90% of the variability.

Table 2.9. The variables with the largest influence for each principal component (PC1–PC4).

Trait	PC1	PC2	PC3	PC4
Fv'/Fm'	-0.11	-0.39	0.17	-0.19
Φ_{PSII}	0.28	-0.27	-0.02	-0.26
NPQ	-0.10	0.40	-0.06	0.30
NO	-0.34	-0.16	0.13	-0.02
qL	0.37	-0.03	-0.13	-0.15
LEF	0.10	0.23	0.57	-0.19
F _s	-0.37	-0.07	-0.01	-0.09
F _o '	-0.33	-0.10	-0.24	-0.35
F _m	-0.23	-0.33	-0.08	-0.25
SPAD	-0.24	-0.15	0.50	0.31
ECSt	-0.19	0.35	-0.17	0.01
ECSm	-0.32	0.12	-0.31	0.14
vH+	0.05	0.39	-0.09	-0.56
gH+	0.35	-0.11	-0.02	0.15
LTD	-0.13	0.31	0.40	-0.31

PC1 to PC4 are eigenvector elements that define the linear combination of variables for the photosynthetic data variables, including relative chlorophyll content (SPAD) and leaf temperature differential (LTD)

Variable abbreviations: photosystem II operating quantum efficiency (Φ_{PSII}), non-photochemical quenching (NPQ), fraction of open Photosystem II centers (qL), non-regulatory energy dissipation or other energy losses (NO), linear electron flow (LEF), maximum quantum efficiency in the light (Fv'/Fm'), maximum fluorescence yield (Fm), steady-state fluorescence (F_s), minimal fluorescence (F_o'), proton motive force parameters (Electrochromic Shift: ECSt and ECSm; steady-state proton flux: vH+; ATP synthase proton conductivity: gH+), leaf temperature differential in degrees Celsius (LTD), relative chlorophyll content (SPAD units).

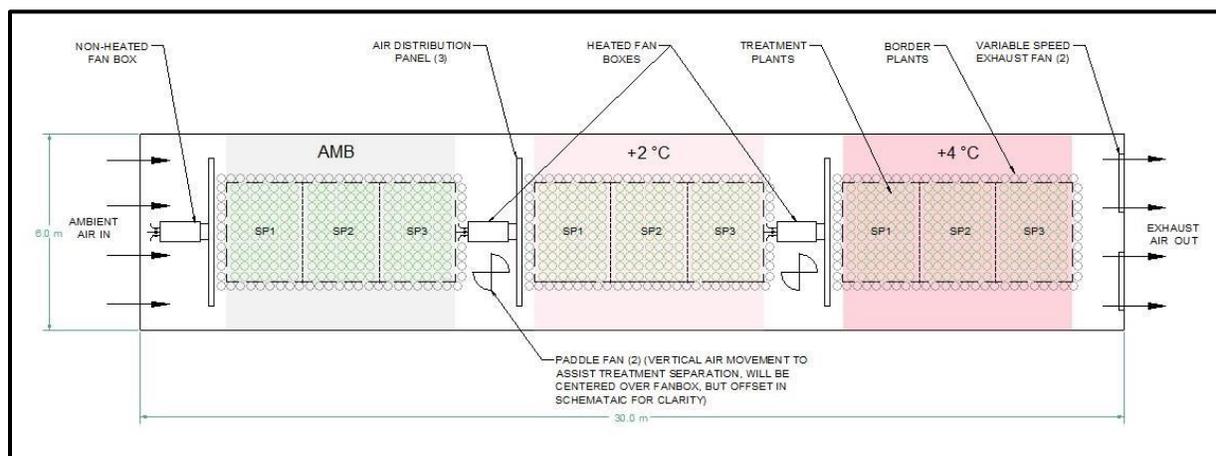


Figure 2.1. Schematic of the temperature gradient greenhouse.

Airflow is from left (north) to right (south) in the drawing with the air heated in fan boxes with electrical resistance heaters as it passes from the unheated ambient air (AMB) control section to the +2 °C section with additional heating as air passes into the +4 °C section. Within each treatment section there are three sub-plots (SP) for replication. Schematic courtesy of USDA-ARS air quality team, Raleigh, NC.

TGG of dimensions 30 m x 6 m (L x W) was divided into three main zones of approximately 10m x 6m each and then into subplots for replication. By-layer panels of 4.6 m x 0.20m x 1.25 m (L x W x H) were deployed just before the air enters each zone and perforated on the south side with ~297 equally spaced holes (2.7 cm diameter) to distribute the air evenly to the plant canopy. At the center of each panel (north side), fan boxes were attached to distribute the unheated or heated air (ambient air or elevated air temperature) along the corresponding zone through the perforations on the opposite layer side. The air moved longitudinally from north to south. Ambient air is pulled in by fans on the north side and distributed to the first zone through the by-layer walls, the air is then heated by electrical resistance heaters within the fan boxes to increase +2 °C just before it is dispersed evenly into zone two. The air is heated again +2 °C before it reaches zone three and is finally expelled on the south side.

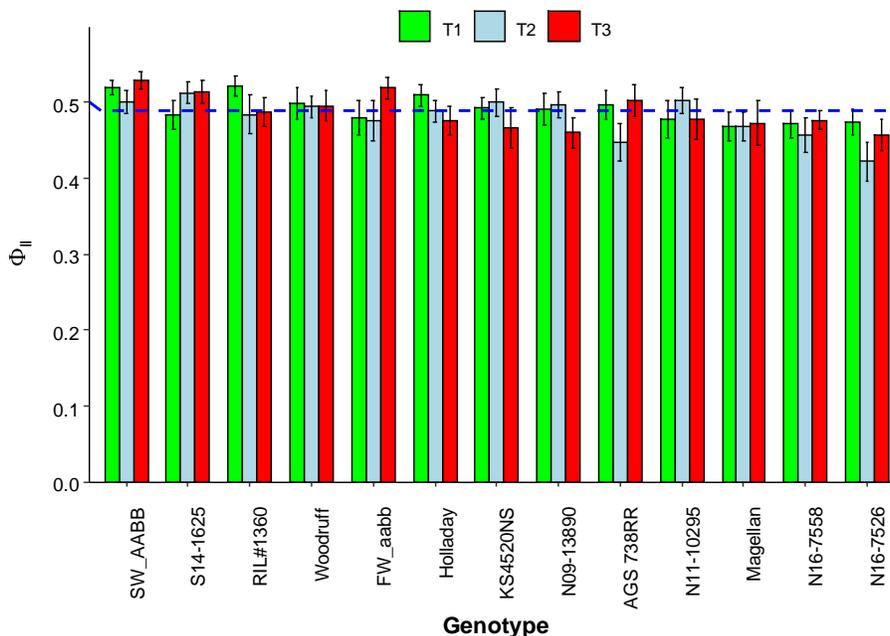


Figure 2.2. Photosystem II quantum efficiency (Φ_{PSII}) on the 13 soybean genotypes. Data from 2021. The horizontal dashed line on the graph indicates the mean Φ_{PSII} of all entries. Values are raw means ($n=12$) \pm standard error.

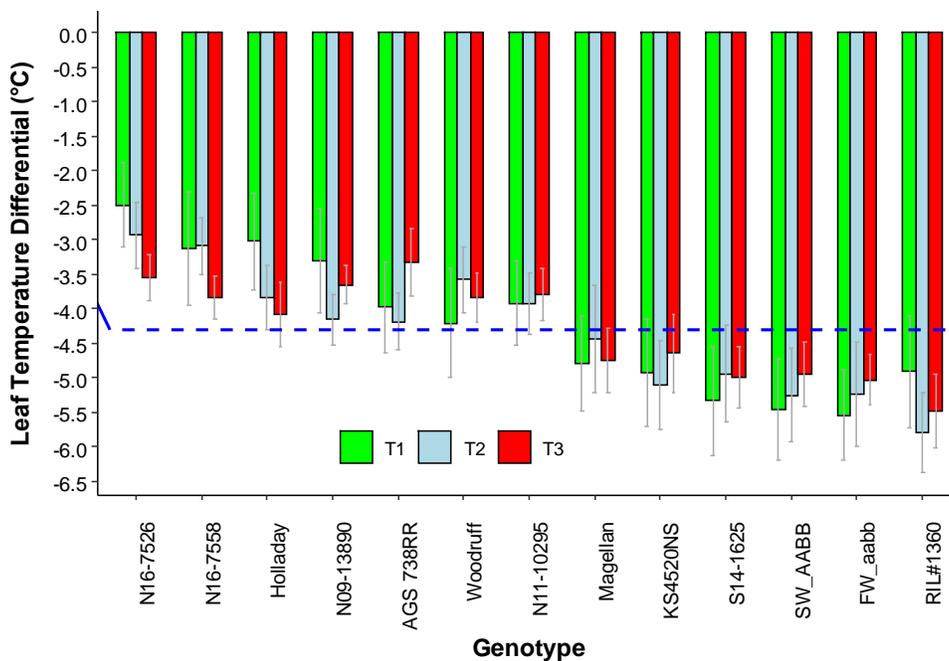


Figure 2.3. Leaf temperature differential (LTD) on the 13 soybean genotypes. Data from 2021. The horizontal dashed line on the graph indicates the mean LTD of all entries. Values are raw means ($n=12$) \pm standard error.

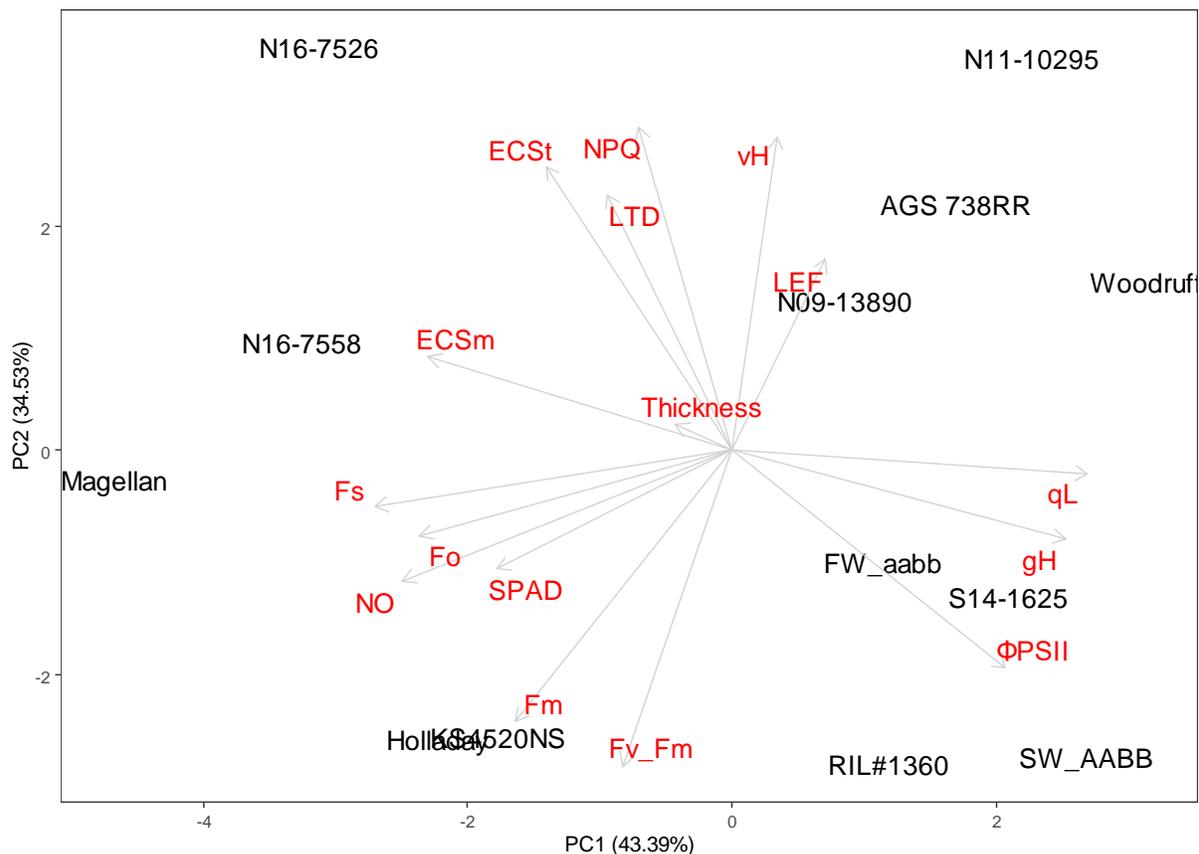


Figure 2.4. Biplot displaying relations between various fluorescence and leaf-level traits of thirteen soybean genotypes.

Biplot represents pooled data (n=9) of genotypes grown under heat stress conditions.

Variable abbreviations: photosystem II quantum efficiency (Φ PSII), non-photochemical quenching (NPQ), fraction of open Photosystem II centers (qL), non-photochemical quenching of excitons (NPQt), non-regulatory energy dissipation or other energy losses (NO), linear electron flow (LEF), maximum quantum efficiency in the light (Fv_Fm), maximum fluorescence yield (Fm), steady-state fluorescence (Fs), minimal fluorescence (Fo), proton motive force parameters (Electrochromic Shift: ECSt and ECSm; steady-state proton flux: vH; ATP synthase proton conductivity: gH), leaf temperature differential in degrees Celsius (LTD), relative chlorophyll content (SPAD units), leaf thickness (Thickness).

Chapter 3. Agronomic and developmental responses of soybean to season-long heat stress

ABSTRACT

Identification and use of regional-specific soybean genetic sources for adapting to heat stress impacts is the core of climate warming resilience. A diverse set of germplasm, 13 advanced breeding lines and 3 commercial cultivars from the southeastern region of the United States, were screened for tolerance to increased temperature using a Temperature Gradient Greenhouse (TGG) during 2021. On average, TGG achieved +1 °C, +3 °C and +4 °C increases relative to outside ambient temperatures during the growing season. Several environmental (e.g., plant height, branch number), developmental (e.g., monitoring of v-stage and r-stage), and reproductive (e.g., pod number, seed number, seed yield) plant responses were measured during and after full season heat stress. This preliminary evaluation in a greenhouse confirmed that some breeding lines may have potential as genetic materials for the development of heat-tolerant varieties. Compared with the rest of genotypes, SW_AABB showed greater seed yield, seed number, pod number, stem biomass, plant height, branch number, and short crop cycle. In contrast, N16-7526 showed high seed weight, stem biomass, and HI, but longer crop cycle, shorter plant height, and low branch number. Interestingly, S14-1625 exhibited the shortest crop cycle and low branch number but did not show seed yield reductions mainly attributed to increased main stem pod number, branch pod number, and stem biomass with elevated temperature. Although the main effects of temperature were not statistically significant, genotype response to rising temperature revealed yield losses ranging from -3% to -39% and days to maturity were delayed 7 days. The differences in sensitivity identified among genotypes could

serve as a basis for detailed field and molecular validations of multiple agronomic-related heat tolerant traits.

Keywords: heat stress, screening, soybeans, seed yield, harvest index, temperature gradient greenhouse.

INTRODUCTION

Climate warming has become a burgeoning research topic for its looming impacts on crop production systems and therefore exacerbation of global and national food security. Following the consensus of an average temperature rise of 3 °C to 5 °C by the end of the century (IPCC, 2021), research from controlled environments to field to crop modeling regard this environmental stress as a major driving force of yield reductions (Ahumada & Cornejo, 2021; Burkey et al., 2020; Geange et al., 2021; Piao et al., 2017; X. Wang et al., 2020).

Soybean growing areas began to dry and experienced abnormal temperature patterns since the 1980s (He et al., 2022), implying that temperature rise may shift soybean regions by the 2060s (Hoffman et al., 2020). In the United States, the elevated temperature alone may cause soybean yield decreases of -2.4% across the country (Mourtzinis et al., 2015) and -16% in States such as Wisconsin (Kucharik & Serbin, 2008) for each 1 °C increase during the growing season, with the largest effects in warmer months such as July and August. In the case of the Southeastern region, which includes North Carolina, a four-decade data modeling estimated soybean yield losses of -32% and -26% per 1 °C increase in daily minimum and maximum temperature, respectively (R. K. Sharma et al., 2022). The estimated losses caused by the increasing temperature and precipitation variability in an assessment of in-season weather trends from 1994 to 2013 is about 11 billion USD (Mourtzinis et al., 2015). To date, these estimates may be greater as yield tends to decrease as temperature increases (Rose et al., 2016); therefore,

there is a latent risk of major yield losses and competitive ability of growers due to warmer conditions.

To date, experiments have focused primarily on short-term heat stress or confined to a limited germplasm collection (Djanaguiraman et al., 2011; Jumrani et al., 2017, 2018; Koti et al., 2005; Ortiz et al., 2021; Siebers et al., 2015; Tacarindua et al., 2013; L. Zhang et al., 2016). Studies of isolated warming effects on crops are relatively small due to the lack of exposure systems and confounding effects of drought stress; nevertheless, in-season temperature changes have a greater impact on soybean yield than precipitation trends (Mourtzinis et al., 2015). While the combination of abiotic effects on crops certainly matters in field conditions, individual stresses may shed light on novel responses hidden in multifactor studies (Bita et al., 2013; Taiz et al., 2015; H. Zhang et al., 2022). Since the temperature factor conditions growth and development, thus will yield components (J. T. Baker et al., 1989; Hatfield & Prueger, 2015), improved infrastructure used to mimic predicted temperature throughout the crop cycle may serve as a proxy to anticipate warming effects and identify tolerant plants. Additionally, farmers need improved or new heat-tolerant varieties.

We conducted a full-season controlled-environment experiment in a large collection of soybean germplasm with contrasting backgrounds, thirteen modern breeding lines and three commercial cultivars, to test whether these genetic sources show genetic variability and to determine the extent to which warming affects yield. We utilized a state-of-the-art facility to impose warming day/night over the entire growing season and analyzed several environmental and reproductive traits to quantify the overall impact of heat stress.

METHODS

Monitoring of environmental conditions

Sensors (HOBO U23 Pro v2, Onset, Bourne, MA, USA) and data from an in-house weather station were used to monitor air temperature and relative humidity. Three sensors were installed per replicate above the canopy and were adjusted approximately every week according to the plants' height. Drip irrigation was controlled through a digital timer (Rain Bird, Tucson, AZ, USA) (Table 3.1).

Plant material and crop husbandry

Sixteen soybean genotypes, both breeding lines and commercial cultivars, were selected for favorable yield or abiotic traits from Maturity Groups 4 to 7. Seeds from selected germplasm were acquired from the United Soybean Board (USB) Drought team members in Georgia, Kansas, Missouri, and North Carolina, which represent the MGs typical of the Southeast and Midwest regions of the United States. The commercial cultivars were selected in consultation with the extension specialist at North Carolina State University. Some of the genotypes' characteristics are described in Table 3.1. After seeds were acquired, these were treated with Mefenoxam. Nursery 15-L black plastic pots were first sanitized with 10% chlorine, filled up to $\frac{3}{4}$ with commercial growing media (Sunshine Mix#8/Fafard-2), mixed with 60 g of slow-release fertilizer (Osmocote Plus 15-9-12), and finally complete filling. The pots were 0.29 m tall and 0.30/ 0.25 m (top/bottom) in diameter with bottom drain holes (~1.8 cm diameter) to drain excess water and covered/wrapped around with an insulation layer (Reflectix, Markleville, IN, USA) to maintain root zone temperature. Each replicate had 6 rows (8 pots per row) arranged perpendicular to the airflow; two border rows were next to the air distribution panels and one next to the TGG vinyl wall. Before planting, the growing media was saturated and then four

seeds were hand-sown at 2.5 cm depth on 7 or 21 June 2021 to account for MG determinacy. To leave one plant per pot, the seedlings were thinned to two plants approximately two weeks after planting and then the best plant was selected and grown to maturity. Irrigation was provided two times a day (200 and 1000 h) and records of daily water meters were maintained to ensure that the dripper time and delivery rate provided complete saturation to the growing media. Constant irrigation and well-drained growing media avoided water and soil physical limitations to focus the experiment on heat responses only rather than confounding variables. To account for possible plant bending later in the season, a stick and an antirust metal cage were installed per pot. A surface insecticide (Imidacloprid) followed by a drench fungicide solution (Mefenoxam and Pentachloronitrobenzene) was applied at ~ 27 DAS to prevent pest pressure. To control spider mites, a pesticide application was at ~63 DAP. Daily plant monitoring ensured that some larvae and weeds were removed manually.

Screening technology, research site, and experimental design

A custom-built Temperature Gradient Greenhouse (TGG) was constructed from north to south at the Lake Wheeler Farm of North Carolina State University, Raleigh, NC, USA (35°43'45'' N, 78°40'43'' W; elevation 120 m) for season-long day/night elevated temperature treatments. TGG consisted of three whole plots, one corresponding to each temperature treatment, and three subplots for replication (Figure 2.1). In a split-plot design, unheated ambient air was distributed to the first whole plot (T1= unheated ambient air), and heated air to whole plots two (T2= unheated ambient air + 2 °C) and three (T3= unheated ambient air + 4 °C). Genotypes were randomized within each subplot. The study consisted of 432 experimental plants plus borders.

Data collection

Harvest

Each experimental plant from all treatments was harvested manually at maturity (R8), which varied across genotypes from October 2 to November 22, 2021. On harvest day, plants were clipped at the growing media level, and counts of branches, pods, and unfertilized pods along with the main stem length were recorded. Tissues and pods were then dried and weighed. Stem biomass was calculated from the main stem and branch dry weight. Seeds were removed from pods, digitally counted (750-2, International Market and Design, San Antonio, TX, USA), and weighed with a digital scale for calculations of seed yield and yield components per plant. Harvest index (HI) was calculated as the ratio of seed weight to the total aboveground dry weight (pod weight + biomass). Seed yield components, pod number, and dry matter were determined separately for the main stem and branch of each experimental unit.

Statistical analysis

All statistical analyses and plots were performed in R software version 4.2.1. (R Core Team, 2022). Prior to the analysis of variance (ANOVA), data were screened for outliers and then subjected to exploratory analysis to identify hidden patterns in the data. Two-way ANOVA was performed for all measured traits using “lme4” package (Bates et al., 2015). Normality of residuals and heteroskedasticity was checked for each analysis. The data had a large and equal sample size in each group, except for one genotype with three observations missing (dead plants); thus, the central limit theorem can be applied, and normality assumptions and differences in covariance matrices across groups were relaxed for variables that seemed not to meet the assumptions. Treatment and genotype were fixed effects, replicate nested within treatment was treated as random. Mean separation was performed using Tukey honest significant difference

(HSD) at $\alpha = 0.05$ using the “emmeans” package (Lenth, 2022). To visualize and explore associations between outcome agronomic variables and the sixteen genotypes, we conducted principal component analysis and biplots.

Additionally, to examine any phenotypic traits contribution to heat stress tolerance of the thirteen genotypes analyzed for chlorophyll fluorescence, yield and fluorescence parameters were combined, and Pearson’s correlations were examined using the “rcorr” function from the “Hmisc” package (Harrell Jr, 2022). The correlation coefficient (r) was used to determine the direction and magnitude of measured traits. Correlations and biplots were based on z-scores due to different units of measurement.

RESULTS

The purpose of the study was to determine the effects of season-long heat stress (warming) on various plant growth and development and yield traits of diverse soybean germplasm and to identify best-performing genotypes under elevated temperature for future field characterizations.

Temperature treatments were achieved

Seasonal and monthly air temperature average trends indicate that increased temperature was maintained throughout the season in the Temperature Gradient Greenhouse (TGG) (Figure 3.1, hourly season average; and Figure 3.3, by month). Relative humidity figures are also provided for comparison over the growing season (Figure 3.2, seasonal average; Figure 3.4, by month). TGG provided elevated temperature treatments of approximately +1 °C, + 3 °C, and + 4 °C compared with outside temperatures (Table 3.2). This increase aligns with the global warming scenarios in the coming decades. Seasonal temperature extremes, heat shocks, and heat waves can have a greater impact on soybean growth and development, and TGG reflected the

outside seasonal temperature variability. The day/night season temperature averages were 27/20 °C, 29/22 °C, and 30/23 °C for T1, T2, and T3 respectively in 2021 (Table 3.2). The maximum temperatures recorded were 42 °C, 45 °C, and 48 °C for T1, T2, and T3, respectively. As expected, June and July experienced higher temperatures (Figure 3.3), which coincided with the early growth stages. TGG thus proved to be a reliable exposure system for mimicking warming scenarios.

Differences in yield potential

The results revealed a significant main effect of genotype for all the traits analyzed (Table 3.3; Table 3.5), and there was a significant interaction between temperature and genotype for branch number ($F_{30, 90} = 1.97$, $p = .0078$). Therefore, data were pooled across treatments for further analysis, except for branch number.

Genotypes varied significantly in seed yield. SW_AABB, N16-7526, and FW_aabb produced the highest seed yields but were not statistically different from commercial cultivars and RIL#1360, S14-1625, and Woodruff (Table 3.4; Figure 3.5). The commercial cultivar AG69XF0 and the breeding line SW_AABB both produced similar seed yield across treatments (141 g plant⁻¹). For HI, S14-1625, Woodruff and KS4520NS were significantly higher only from N16-7558 (Table 3.4). A principal component (PC) biplot helped to identify the responses of soybean genotypes to warming (Figure 3.16). This visualization demonstrated relationships between best-performing genotypes with yield-related parameters. The commercial cultivars and breeding lines SW_AABB, FW_aabb, N16-7526, RIL#1360, and S14-1625 showed superior performance across treatments, on average.

When investigating the apparent negligible main effects of temperature, we found that the seed yield and HI were negatively impacted across genotypes. For instance, N16-7558 and

Woodruff seemed to maintain their yield at T3, however, they were affected at T2 by reductions of 5% and 12%, respectively (Figure 3.6). HI decreased in either T2 or T3 for all genotypes, with reductions up to 12% in T3, except for Holladay, Magellan and Woodruff that showed minimal increases (Figure 3.7). Additionally, genotypes such as RIL#1360 and FW_aabb showed a twofold increase and N16-7558 a trifold increase in unfertilized pods from T1 to T3. Most genotypes exhibited increases in unfilled pods either in T2 or T3, except AG57XF1 and Woodruff which relatively maintained the lowest number across treatments.

Growth

The commercial cultivars AG57XF1 and AG54XF0, and breeding lines SW_AABB, FW_aabb, and S14-1625 were tallest (Table 3.6; Figure 3.9). These genotypes, AG57XF1, AG54XF0, SW_AABB, and S14-1625 exhibited height increases from T1 to T3 of 13%, 4%, 9%, and 10% respectively; except FW_aabb that decreased 10%. For other genotypes, height was relatively reduced from T1 to T3, except Magellan which increased ~10%.

Branch number was the only variable with interaction effects ($p = .0077$) (Table 3.5). Overall, SW_AABB and Woodruff consistently developed more branches in each treatment and across treatments. While SW_AABB, Woodruff, N11-10295, and FW_aabb had greater branch number, they also developed 9%, 10%, 7%, and 42 % more branches on average in T3 relative to T1. Conversely, S14-1625, Magellan, and KS4520NS had the lowest values even at T1 but also exhibited a tendency to decrease their branch number at increased temperature (Figure 3.10). Also, while SW_AABB seemed to increase its lateral branches and maintain its number of main stem branches, Woodruff and FW_aabb showed the opposite.

SW_AABB had the highest stem biomass (102 g plant^{-1}). The commercial cultivars AG57XF1 and AG69XF0 also had high biomass but were not statistically different from

breeding lines N16-7526, FW_aabb, and RIL#1360 (Figure 3.11). These greater accumulation by AG57XF1, AG69XF0, and N16-7526 is attributed to increases at T2 and T3. Interestingly, S14-1625 expressed an intermediate biomass accumulation on average ($\sim 57 \text{ g plant}^{-1}$) compared with other genotypes but had the ability to accumulate $\sim 24\%$ more stem biomass in T2 and T3 relative to T1.

Crop cycle length (days to maturity) ranged from 128 to 158 days across genotypes. The commercial cultivars and N16-7558, N16-7526, Woodruff, and N11-10295 required over 149 days to reach maturity, whereas FW_aabb and S14-1625 required 132 and 128 days, respectively (Table 3.6). All genotypes increased their cycle length with increasing temperatures, except AG69XF0 and N11-10295 which remained almost unchanged (Figure 3.12). In T2 the maturity was delayed up to 14% (20 days) and in T3 up to 9% (13 days). On average, T2 and T3 delayed harvest ~ 7 days. Vegetative (Figure 3.13) and reproductive (Figure 3.14) growth trends show the genotype changes over time, along with the overall effect of temperature on reproduction (Figure 3.15).

Multivariate analysis

The principal component analysis computed from fluorescence and yield parameters also revealed that the breeding lines with higher photosystem II quantum efficiency (Φ_{PSII}) were also grouped with seed yield, seed number, pod number, and stem biomass (Figure 3.17). These results were consistent with the mixed model analysis. Significant negative correlations were observed between seed yield and SPAD, seed yield and LTD, seed number and SPAD, seed number and LTD. Both weight per seed and HI were positively correlated with NPQ but negatively related with F_v'/F_m' and Φ_{PSII} . HI was negatively correlated to leaf temperature differential. Leaf temperature differential was only positively correlated with F_v'/F_m' (

Table 3.7; Figure 3.18).

DISCUSSION

This experiment demonstrated significant genotypic variation in all the traits analyzed, specifically in yield components, stem biomass, plant height, and crop cycle length. The sixteen genotypes evaluated include advanced breeding lines and commercial cultivars with favorable biotic or abiotic and yield traits representative of breeding programs in the Southern and Midwestern, United States. The high seed yield of AG69XF0, SW_AABB, FW_aabb, RIL#1360, and S14-1625 may be explained by the higher seed number, pod number, pod weight, seed per pod ratio, and stem biomass (Table 3.4; Table 3.6; Figure 3.16), whereas for Woodruff and N16-7526 by greatest weigh per seed (0.24 g and 22 g, respectively) and HI (Table 3.4). Although Woodruff had an average yield, it maintained a low number of unfertilized pods across treatments and a high number of branches (Figure 3.10). Overall, only the commercial cultivar AG69XF0 was superior in seed yield, seed number, and pod number to all breeding lines. However, while AG69XF0 required ~ 158 days to reach maturity, SW_AABB, FW_aabb, and S14-1625 only required 141, 132, and 128 days, respectively. These differential genotypic responses on various traits support our research hypothesis of genotypic variability and suggest that breeding lines could have potential for improvement in the case of projected increases in climate warming.

Yield components

There were no overall genotype by temperature interactions, but all genotypes showed yield reductions that ranged from -3% to -39% in T2 and -4% to -35% in T3, except AG57XF1 and S14-1625 that increased 7% and 2%, respectively (Figure 3.6). In the same way, these reductions fall between the range predicted by crop models and field experiments for soybeans

(Hoffman et al., 2020; Piao et al., 2017; X. Wang et al., 2020). Yet the reductions in HI were lower, it is clear that soybeans have a higher temperature threshold for this parameter compared with other crops and legumes (Prasad et al., 2017).

By further examining pod number and seed number on the genotypes that did not exhibit reductions at elevated temperatures, breeding lines S14-1625 and N16-7526 and the commercial cultivars AG54XF0 and AG57XF1, we found that S14-1625 and AG57XF1 were the only genotypes that showed increases in pod number and seed number at both main stem and branches in T2 and T3. Interestingly, N16-7526 and AG54XF0 increased their branch pod number and seed number but their main stem pod number and seed number decreased with higher temperature. Previously it was noted that seed yield (weight) decreased in branches whereas it increased in the main stem with elevated temperatures (J. T. Baker et al., 1989). We found either similar or the opposite responses in this germplasm collection, emphasizing the importance of evaluating various genetic sources. These shifting mechanisms in traits play an important role in germplasm improvement due to genotype- and magnitude-specific allocation of resources.

This overall negligible temperature effect on yield point out that water and acclimation may offset greater impacts. For example, a small increase of precipitation (50 mm) in field conditions during the growing season may increase yield 5-10%, which will partially compensate the adverse effects of heat stress (Kucharik & Serbin, 2008). Even though the abundant water supply in this study may have minimized the warming effect, the sensitivity to heat was evident. The genotypes such as Magellan and N11-10295 had yield reductions over 30%. These important reductions may exacerbate without water compensation throughout the season, which is often a constraint in rainfed conditions. Another reason may be that heat stress remained below

the upper limit temperature for this crop (39 °C). For instance, the no temperature by genotype interaction was found previously for yield components in warming conditions below 3 °C increments; however, significant reductions in pod number, seeds per pod, and seed number were observed at 5 °C (Choi et al., 2016). These small increases, those below 3 °C, may benefit temperate growing areas with suboptimal temperatures. However, as we expect drier and warmer conditions, screening of germplasm must continue to find genotypic sources to mitigate impacts or maintain yield gains.

Moreover, indeterminate soybeans may have compensation mechanisms such as more branches and greater plant height that allow extra stem biomass and seed number production (Prasad et al., 2015). Recently, Allen et al. (2018) found that elevated temperature increased node number, which may promote additional flowering sites and therefore extra pod number on the main stem. This compensation mechanism was observed previously, where high air temperature increased the main stem nod number and overall seed number (J. T. Baker et al., 1989).

There is a wide variation across genotype responses, and we found that high-performance genotypes had greater variation relative to lower-performance genotypes. The sensitivities in soybean traits to elevated temperatures may be due to different optimum temperature ranges for emergence, vegetative and reproductive stages (Mourtzinis et al., 2015), and thus for pod set, seed development, and stem biomass, for example (J. T. Baker et al., 1989; Choi et al., 2016; T. Zhu et al., 2021). Therefore, the high variability in traits could be attributed to temperatures above soybean growth and development requirements.

Growth

As expected, the soybean responses were complex, which reflects the polygenic nature effects of heat stress on various molecular networks, and thus in the final phenotype. For example, these changes in plant growth, increases or decreases, illustrate how intensity and duration of temperature mediate plants height and even determinacy (Allen et al., 2018). In our study, some genotypes such as SW_AABB and S14-1625 relatively increased main stem length, whereas the majority decreased. In concordance with these findings, Allen et al. (2018) studied one indeterminate soybean genotype and found that plant height tended to increase at or below 34 °C; at 38 °C plants remained short due to a greater number but shorter length of nodes. In terms of branching, SW_AABB developed the highest number and S14-1625 developed a low number, however, there were no significant differences in seed yield. Stem branching thus plays a critical role in soybean growth and yield across environments and genotypes. These contrasting responses among genotypes highlights the importance of considering various genotypic responses.

In line with our findings, elevated temperature was reported to prolong R1-R5 stages in soybeans, which resulted in delayed maturity or a longer crop cycle (Allen et al., 2018; Choi et al., 2016). Although relatively small, the time to maturity was negatively associated with seed number (Figure 3.18). In the case of yield response, there was a compensation between seed number and weight per seed (Figure 3.18).

Multivariate

Although Φ_{PSII} only showed a weak non-significant positive correlation with seed number, but not seed yield, the PC biplot further supported our analysis showing that Φ_{PSII} was clustered towards productivity (seed yield, seed number, pod number, and biomass) and

genotypes SW_AABB, S14-1625, FW_aabb, RIL#1360. The lack of correlations is understandable since correlation does not imply causation. These findings indicate that Φ_{PSII} could be a promising screening target for discriminating yield-related genotypes identification under this study conditions. Additionally, studies reported previously that SPAD was positively correlated with grain weight when measurements were at grain filling (Ergo et al., 2018), however, our study demonstrated significant negative correlations at earlier stages. The negative correlations are not entirely clear; however, the longer heat exposure may have shifted the findings of previous studies.

Limitations

Even though the recommended planting date for North Carolina, U.S. is late April to mid-May (Morris et al., 2021), the experiment was initiated in early June 2021 due to an ongoing wheat study, which is also a constraint for growers with double crop systems or rotation. Soybean root elongation/expansion may have been prevented by the pot size, however, a recent study that evaluated the genotype N09-13890, included in this study, concluded that slow wilting lines demonstrated parsimonious root systems with superior aboveground biomass, seed yield, and water use efficiency (Noh et al., 2022). Soil temperature was not recorded, but pots were well covered with a double wall insulation layer to limit the impact of root temperature in gas exchange and vapor pressure deficit. Literature suggests that root elongation is restricted over 30 °C (de Moraes & Gusmão, 2021). Our sample of germplasm was larger than previous studies and representative of the region, but we might have underestimated genetic sources tolerant to other environmental stresses; we thus suggest continuing heat screenings with different sets of genotypes. It is important to note that genotypes seemed to have increased plant height in these growing conditions relative to heights observed in field experiments. This increased stem length

was reported recently in soybeans, pointing out that UV radiation was limited by the controlled environment infrastructure (Allen et al., 2018).

A difference between this and previous research is the use of a Temperature Gradient Greenhouse that accurately mimicked outside temperature fluctuations and maintained full-season heat stress. This infrastructure sets an advantage to study the effects of elevated temperature above optimum thresholds not only in soybeans but also in other species, which will allow us to increase our understanding of warming effects on plant productivity. Besides being a reliable screening technology, TGG permits the screening of a larger set of germplasm. Previous results from the literature suggest the need to find rapid adaptation strategies to mitigate the negative effects of temperature due to location-, species-, and even genotype-specific effects; thus, the search for best-performing plants is indeed endless. Even though this is a first screening, this study lays the foundation in finding sources of heat tolerance that will aid breeding efforts to improve environmental stress.

CONCLUSIONS

This study aimed to determine the general performance of selected germplasm under warming conditions in terms of yield parameters and to identify genetic sources to use in future heat stress research. The breeding lines SW_AABB, N16-7526, FW_aabb, RIL#1360, and S14-1625 were the best performing genotypes in terms of seed yield, stem biomass, pod number, harvest index, or crop cycle length. SW_AABB, FW_aabb, and S14-1625 exhibited a shorter crop cycle, likely due to a fast-growing rate and efficient physiological machinery to harvest light, which may favor production systems under unpredictable heat events by avoiding heat effects during critical developmental stages. Overall, the commercial cultivars performed well. Warming effects did not show any statistically significant effects on seed yield and yield

parameters; however, the observed indirect seed yield reductions were within the predicted ranges, confirming that elevated temperature is a looming threat for crop production systems even with enough water supply and further warrants screening of either elite or wild germplasm. Although additional studies are needed to validate these results, this preliminary screening should pave the way for more detailed study of the identified genotypes and possibly introduce them into heat-tolerance breeding pipelines.

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Table 3.1. Breeding lines and cultivars screened under heat stress conditions.

Breeding line/cultivar	Maturity Group	Origin/Provider	Original parental source(s) of drought tolerance
Magellan	4	Kansas State University	PI 595362
KS4520NS	4	Kansas State University	Unknown
FW_aabb	4	University of Missouri	Magellan and PI 567731
SW_AABB	4	University of Missouri	Magellan and PI 567731
S14-1625	4	University of Missouri	S05-11482 x S07-5451
RIL#1360	5	University of Missouri	Pana x PI567690
Holladay	5	USDA_ARS, North Carolina	PI 572239 N85-578
N16-7526	5	USDA_ARS, North Carolina	Holladay x Fiskeby III
N16-7558	5	USDA_ARS, North Carolina	Holladay x Fiskeby III
AG54XF0	5.4	North Carolina (commercial)	Unknown
AG57XF1	5.7	North Carolina (commercial)	Unknown
AG69XF0	6.9	North Carolina (commercial)	Unknown
N09-13890	7	USDA_ARS, North Carolina	12.5% PI 416937, 25% PI 407948
N11-10295	7	USDA-ARS, North Carolina	12.5% PI 416937. Drought-Protein
Woodruff	7	University of Georgia	PI 416937
AGS-738RR	7	University of Georgia	Unknown
Jake*	5		

*Used as a border plant

Table 3.2. Season average, 12 h mean day and night, and maximum temperatures outside and inside the Temperature Gradient Greenhouse (TGG).

	Temperature (°C) in 2021			
	Season average	Day	Night	Max
Ambient air	21.9	25.2	19.8	38.1
T ₁	23.1	27.0	20.4	41.8
T ₂	25.2	29.2	22.4	44.9
T ₃	26.2	30.4	23.3	47.6

Day and night temperatures averaged over the growing season (From June 7th to November 23rd, 2021) in the TGG. Ambient air is the temperature outside the greenhouse. T₁, T₂, and T₃ correspond to the three temperature treatments inside the TGG.

Table 3.3. p-values of yield parameters.

	Pod No.	Pod Weight	Unfilled pod No.	Seed No.	Seeds per pod No.	100-seed weight	Seed Yield	HI
T	0.6346	0.1509	0.3980	0.4503	0.6935	0.3960	0.1713	0.6904
G	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0033
T x G	0.3004	0.4138	0.2075	0.5836	0.3747	0.5054	0.5484	0.3070

Effects of treatment (T), genotype (G), and treatment by genotype (T x G).

Table 3.4. Estimated marginal means for yield parameters of sixteen soybean genotypes grown in day/night warming conditions.

Genotype	Pod No.	Pod Weight (g)	Unfilled pod No.	Seed No.	Seeds per pod No.	100-seed weight (g)	Seed Yield (g)	HI
AG54XF0	285.96	183.63	8.70	635.06	2.23	21.49	135.82 A	0.52 AB
AG57XF1	257.13	170.88	1.31	551.59	2.16	23.68	128.4 A	0.5 AB
AG69XF0	368.26	185.16	12.81	883.17	2.40	16.04	141.25 A	0.52 AB
AGS 738RR	162.30	90.03	2.70	367.89	2.26	17.90	67.24 DE	0.52 AB
FW_aabb	333.93	187.02	7.67	780.11	2.37	16.66	129.91 A	0.49 AB
Holladay	243.56	91.12	21.89	466.56	1.91	15.51	72.82 CDE	0.51 AB
KS4520NS	121.15	71.56	6.52	303.31	2.40	18.68	52.64 DE	0.53 A
Magellan	99.19	55.30	2.11	240.07	2.44	17.93	41.16 E	0.51 AB
N09-13890	146.96	95.94	3.33	300.93	2.06	22.53	68.07 CDE	0.51 AB
N11-10295	236.00	111.26	3.63	418.46	1.76	21.09	83.05 BCD	0.52 AB
N16-7526	301.15	172.51	10.85	623.46	2.04	21.59	131.63 A	0.53 AB
N16-7558	203.70	95.46	35.93	358.67	1.77	19.76	69.61 CDE	0.45 B
RIL#1360	337.07	167.19	12.63	735.96	2.17	16.63	121.02 AB	0.52 AB
S14-1625	302.04	154.49	5.52	732.65	2.43	16.45	118.91 AB	0.56 A
SW_AABB	362.07	188.84	9.70	826.07	2.24	17.26	140.92 A	0.49 AB
Woodruff	244.19	139.29	1.96	467.96	1.91	23.53	106.09 ABC	0.55 A
Average	250.29	134.98	9.20	543.25	2.16	19.17	100.53	0.51

Means ($n=9$) with different letters within a column were significantly different ($\alpha=0.05$; HSD). All the estimates are on a per plant basis. Harvest index (HI).

Table 3.5. p-values of plant height, branch number, biomass, and crop cycle length.

	Plant Height	Branch No.	Stem biomass	Crop cycle
T	0.9031	0.5565	0.5337	0.2947
G	<0.001	<0.001	<0.001	<0.001
T x G	0.5293	0.0078	0.6737	0.1467

Effects of treatment (T), genotype (G), and treatment by genotype (T x G).

Table 3.6. Estimated marginal means for plant height, branch number, stem biomass, and crop cycle length of sixteen soybean genotypes grown in day/night warming conditions.

Genotype	Plant height (cm)	Branch No.	Stem biomass (g)	Crop cycle length (days)
AG54XF0	167.07	8.26	77.65 BC	152.22
AG57XF1	187.37	12.98	83.45 B	155.26
AG69XF0	120.26	12.93	82.51 B	156.37
AGS 738RR	125.48	11.93	40.22 FG	145.85
FW_aabb	182.54	13.07	79.72 B	132.44
Holladay	94.63	11.15	52.56 EF	147.15
KS4520NS	117.59	3.30	25.93 G	137.63
Magellan	111.74	5.33	22.92 G	135.89
N09-13890	114.89	12.11	38.88 FG	146.70
N11-10295	142.74	14.07	44.98 EF	148.89
N16-7526	110.26	8.67	79.85 B	155.85
N16-7558	103.70	7.67	59.67 CDE	158.41
RIL#1360	114.81	11.37	72.02 BCD	143.67
S14-1625	164.13	5.63	56.55 DEF	128.44
SW_AABB	184.89	19.48	102.01 A	141.37
Woodruff	131.07	14.85	51.99 EF	149.11
<i>Average</i>	135.82	10.80	60.68	145.95

Means ($n=9$) with different letters within a column were significantly different ($\alpha=0.05$; HSD). All the estimates are on a per plant basis. Biomass corresponds to the sum of stem and branch dry weight.

Table 3.7. Matrix of correlation coefficients between various yield and chlorophyll fluorescence traits.

	Seed No.	Pod No.	Weight per Seed	HI	Stem biomass	Crop length	Fv'/Fm'	Φ_{PSII}	NPQ	NO	SPAD	LTD	Leaf thickness
Seed weight	0.93*	0.93*	0.07	0.35*	0.82*	-0.07	-0.10	0.03	0.06	-0.18*	-0.24*	-0.22*	-0.11
Seed No.		0.94*	-0.24*	0.27*	0.82*	-0.24*	0.01	0.13	-0.06	-0.12	-0.24*	-0.28*	-0.10
Pod No.			-0.10	0.21*	0.87*	-0.04	0.00	0.10	-0.04	-0.11	-0.15	-0.18*	-0.03
Weight per seed				0.17	-0.10	0.33*	-0.29*	-0.21*	0.29*	-0.22*	0.00	0.10	0.01
HI					-0.03	-0.17	-0.29*	-0.18*	0.27*	-0.22*	-0.17	-0.23*	-0.18*
Stem biomass						0.12	0.02	0.07	-0.04	-0.06	-0.04	-0.13	0.02
Crop length							-0.05	-0.14	0.11	0.04	0.25*	0.42*	0.14
Fv'/Fm'								0.63*	-0.92*	0.74*	0.16	0.23*	-0.10
Φ_{PSII}									-0.88*	-0.04	-0.27*	-0.01	-0.10
NPQ										-0.44*	0.03	-0.14	0.11
NO											0.45*	0.31*	-0.05
SPAD												0.06	0.19*
LTD													-0.21*

Correlation coefficients were computed across genotypes and treatments. The asterisks indicate significant correlations ($p \leq 0.05$).

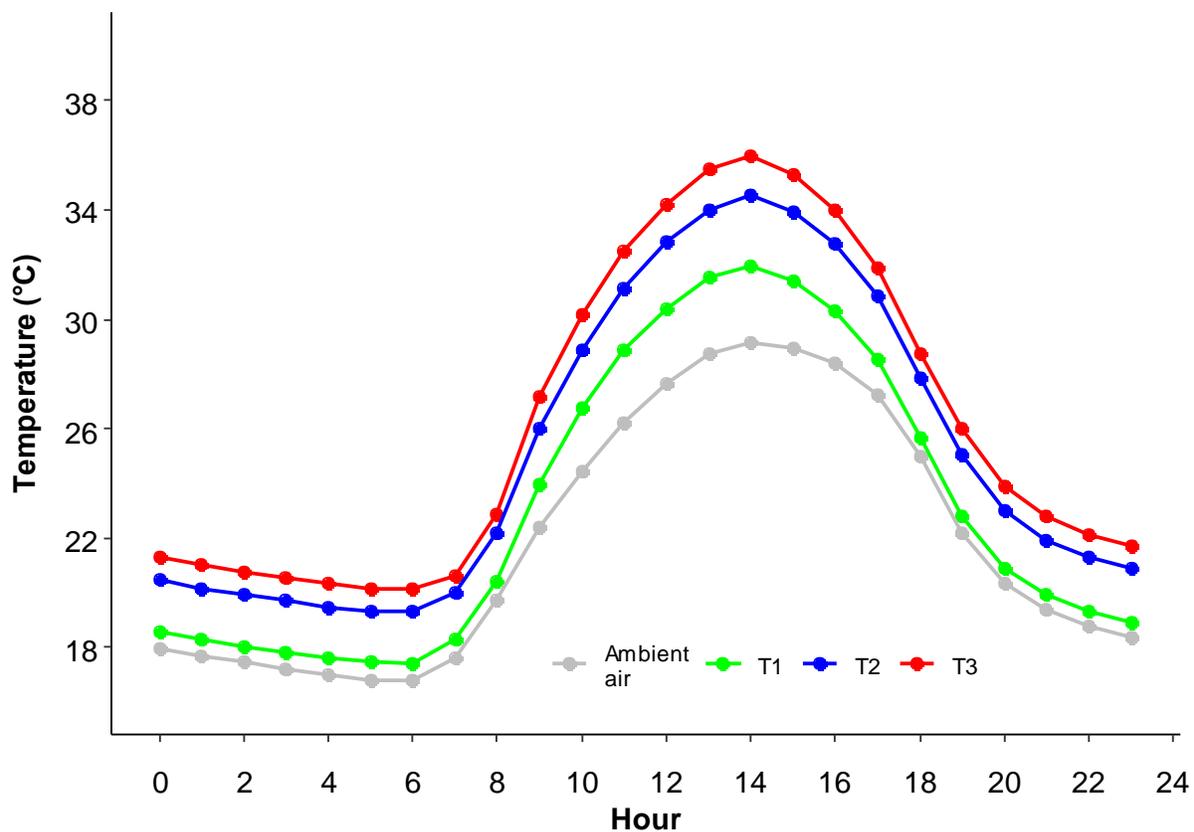


Figure 3.1. Hourly seasonal average temperature trends inside and outside the Temperature Gradient Greenhouse in 2021.

Each temperature value corresponds to the mean of nine sensors per treatment. Ambient air (Ambient air) is the temperature outside the greenhouse. T1, T2, and T3 correspond to three treatments inside the TGG.

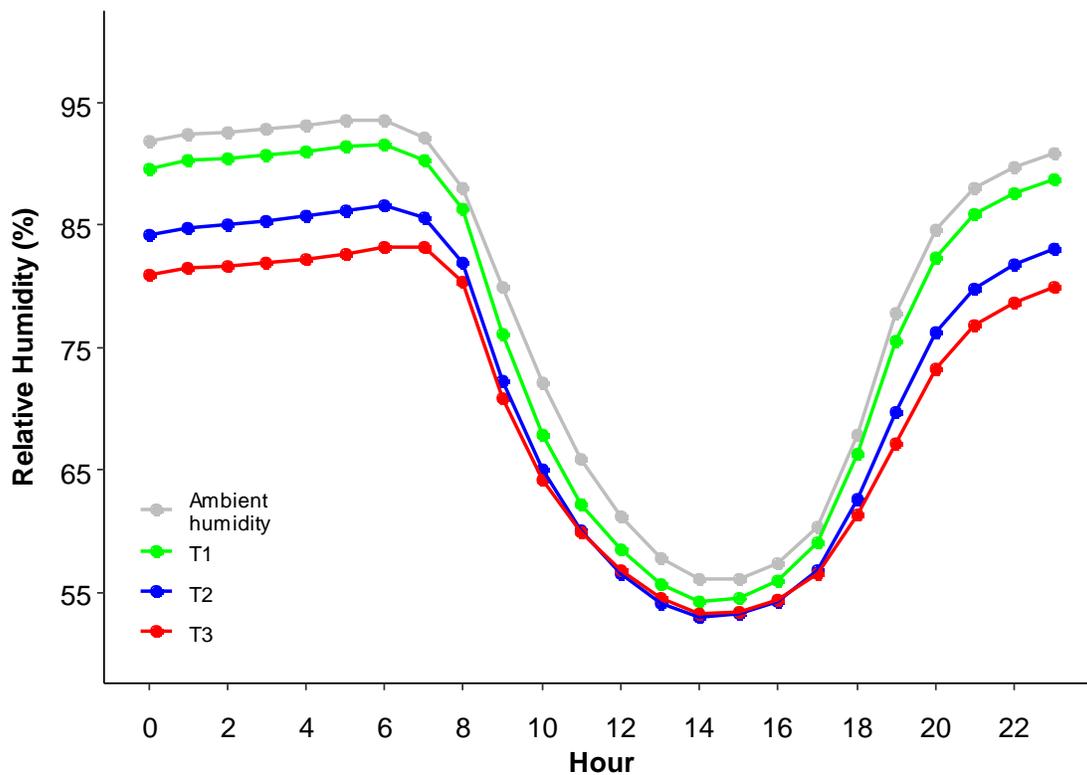


Figure 3.2. Hourly seasonal average relative humidity trends inside and outside the Temperature Gradient Greenhouse in 2021.

Each relative humidity value corresponds to the mean of nine sensors per treatment. Ambient humidity (Ambient humidity) is the relative humidity outside the greenhouse. T1, T2, and T3 correspond to the relative humidity in each temperature treatment inside the TGG.

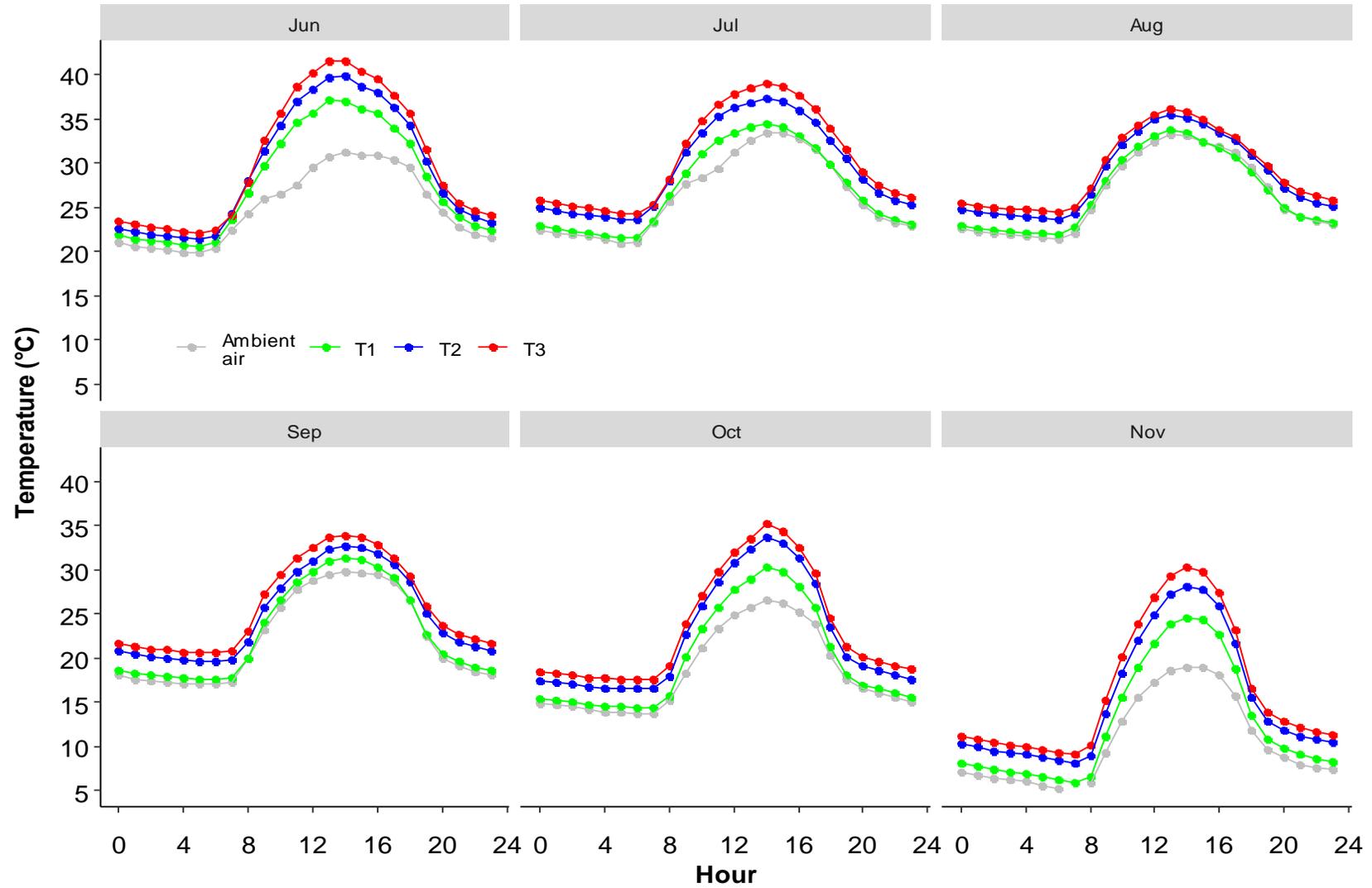


Figure 3.3. Hourly temperature trends per month inside and outside the Temperature Gradient Greenhouse in 2021.

Each temperature value corresponds to the mean of nine sensors per treatment. Ambient air (Ambient air) is the temperature outside the greenhouse. T1, T2, and T3 correspond to three treatments inside the TGG.

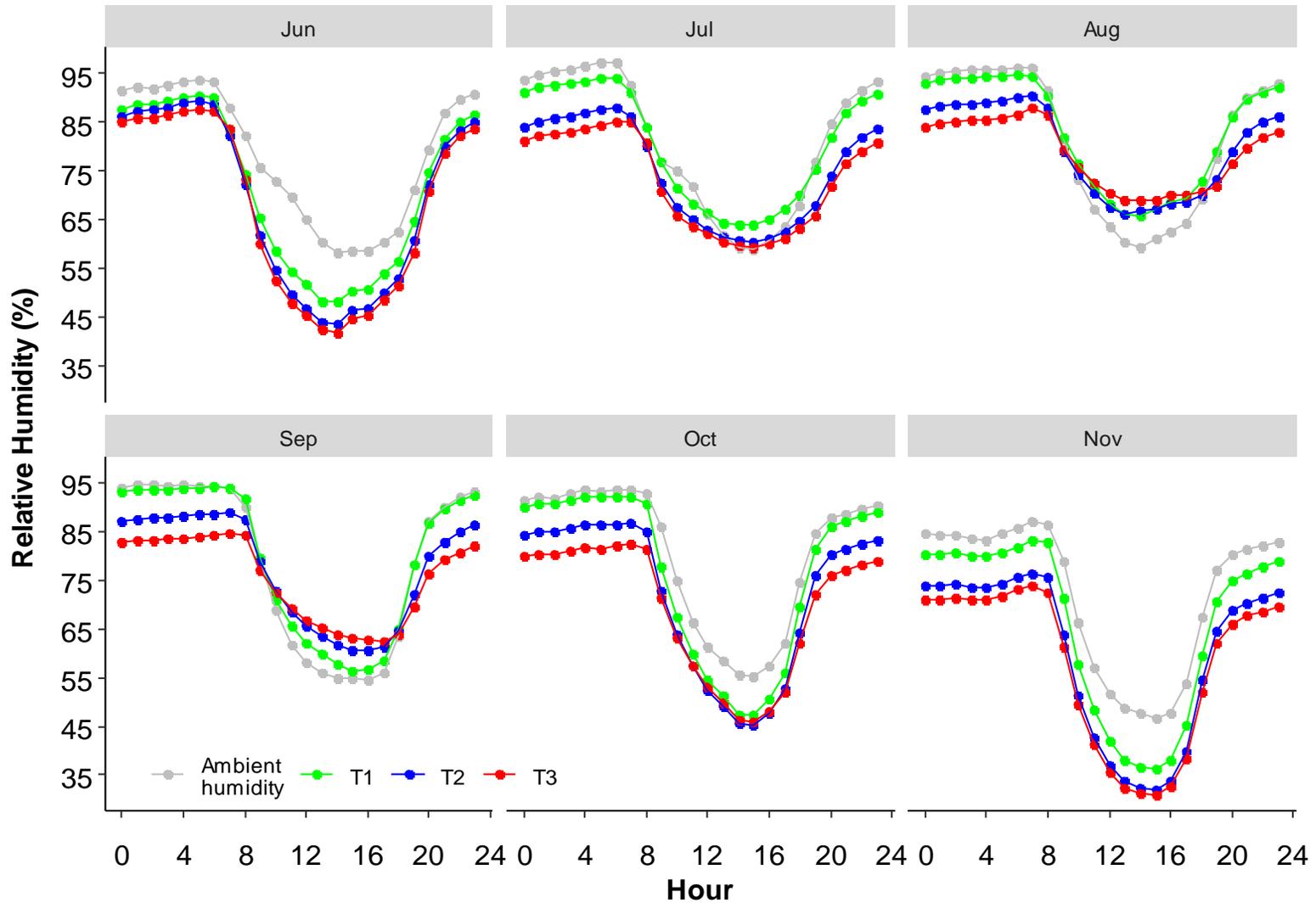


Figure 3.4. Hourly relative humidity trends per month inside and outside the Temperature Gradient Greenhouse in 2021.

Each relative humidity value corresponds to the mean of nine sensors per treatment. Ambient humidity is the relative humidity outside the greenhouse. T1, T2, and T3 correspond to the relative humidity in each temperature treatment inside the TGG.

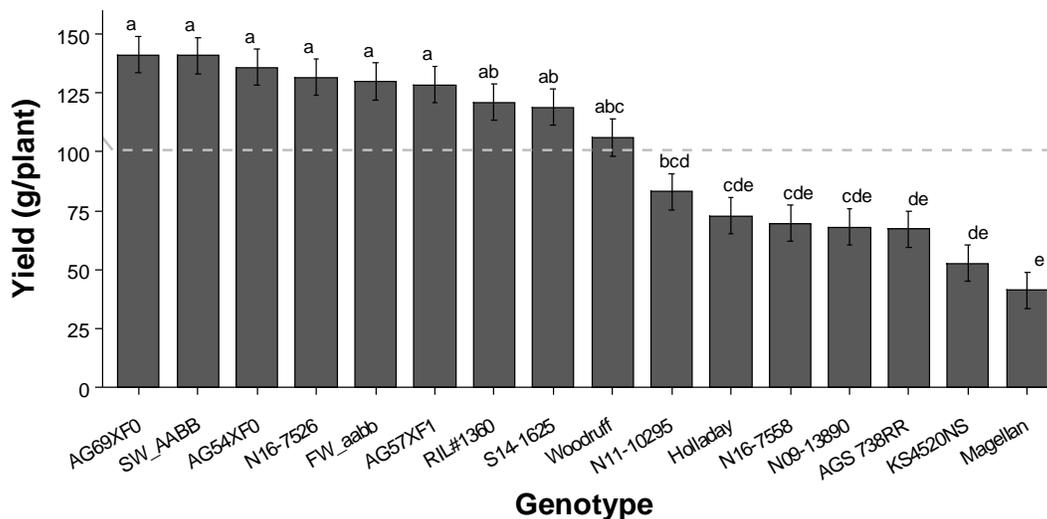


Figure 3.5. Seed yield responses in soybean genotypes grown in warming conditions.

Each bar represents the estimated marginal mean ($n=9$) \pm standard error. The horizontal dashed line on the plot indicates the pooled mean of all entries. Letters above the bars show differences among genotypes based on Tukey's test ($\alpha=0.05$; HSD).

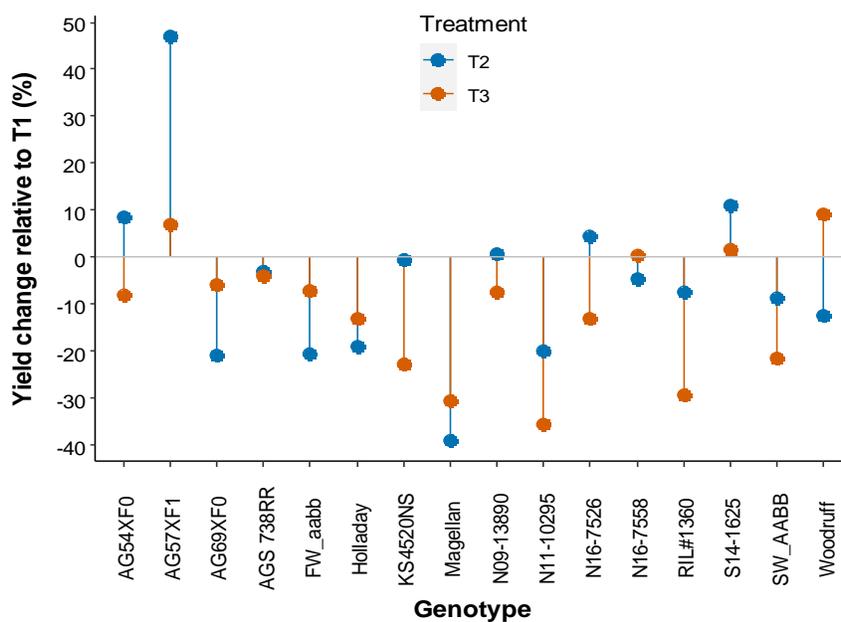


Figure 3.6. Percentage seed yield change of soybean genotypes at elevated temperatures relative to unheated ambient air conditions (T1).

Each point represents the mean change of three replicates.

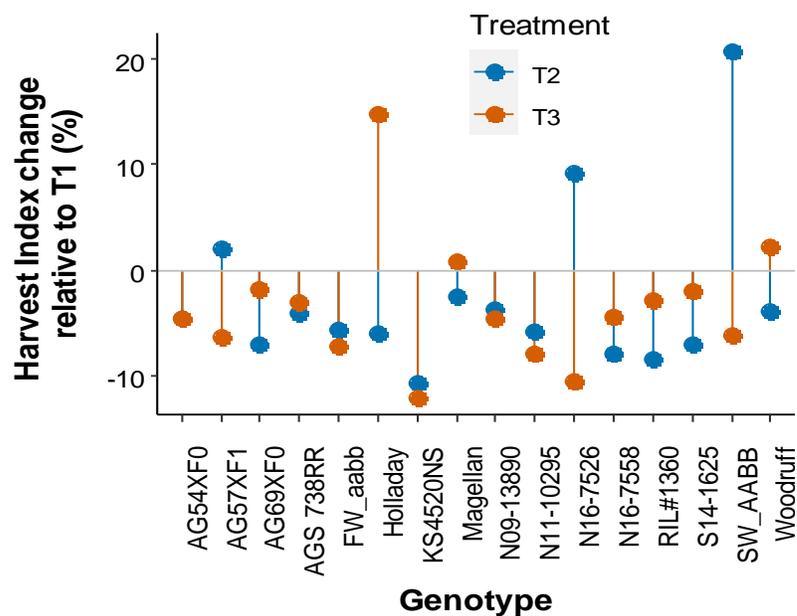


Figure 3.7. Percentage of harvest index change of soybean genotypes at elevated temperatures relative to unheated ambient air conditions (T1).

Each point represents the mean change of three replicates.

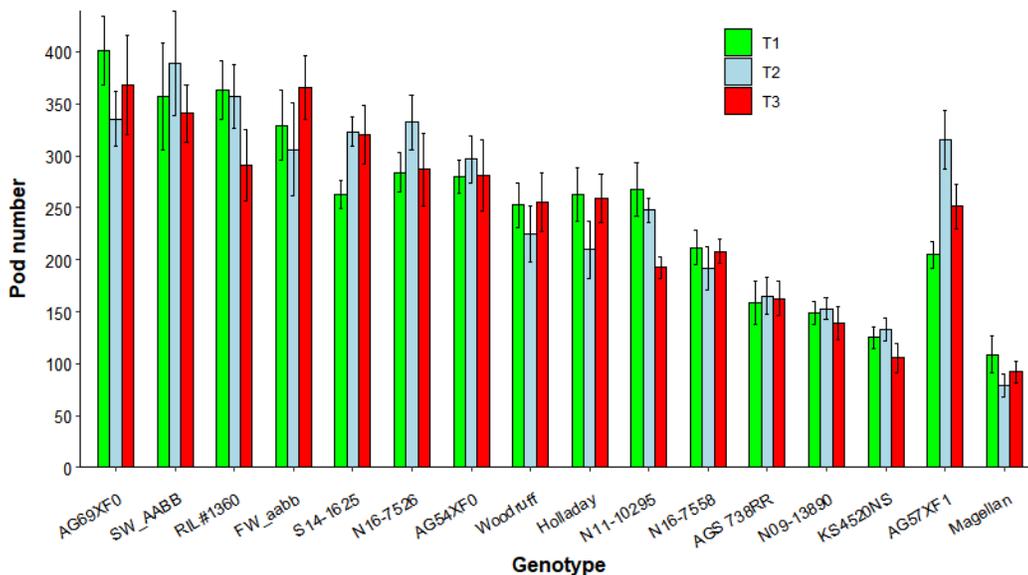


Figure 3.8. Pod number in soybean genotypes grown in warming conditions.

The bars indicate the means ($n=3$) \pm standard error. T1, T2, and T3 correspond to three treatments inside the TGG.

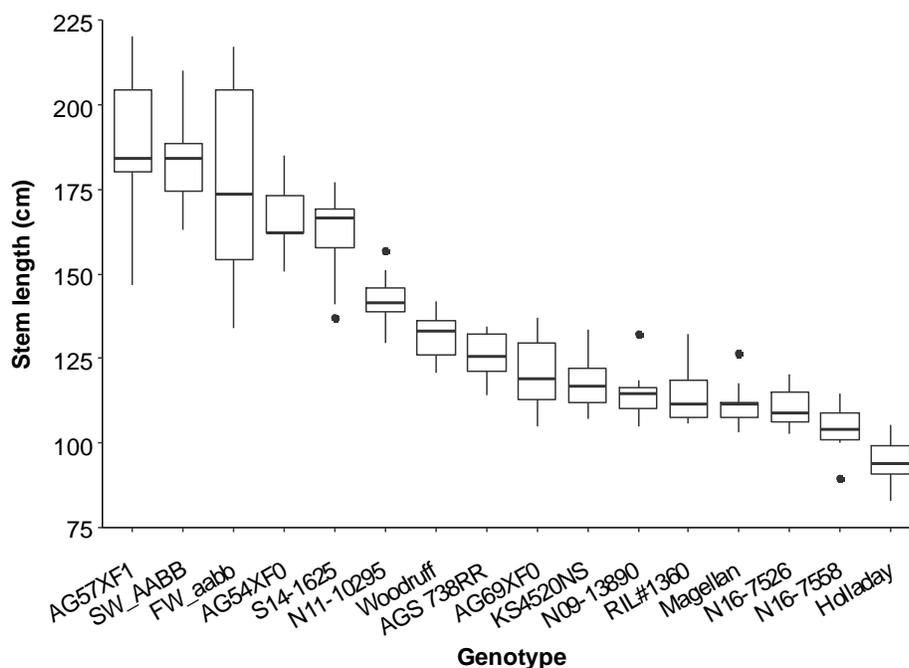


Figure 3.9. Plant height distributions for soybean genotypes grown in warming conditions. Each boxplot represents the mean of nine replicates; the horizontal line inside the boxplots indicates the median and the dots outside boxplots indicate outliers.

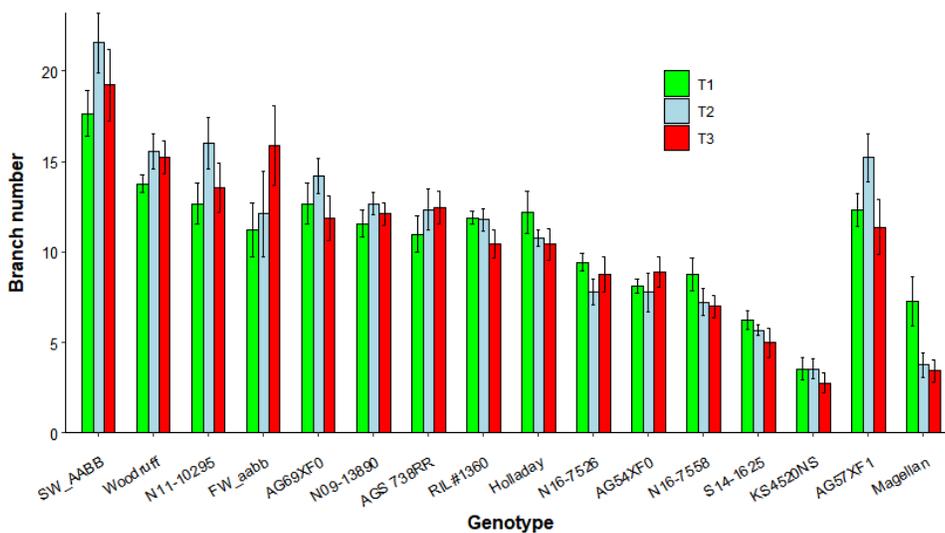


Figure 3.10. Branch number of soybean genotypes grown in warming conditions. The bars indicate the means ($n=3$) \pm standard error. T1, T2, and T3 correspond to three treatments inside the TGG.

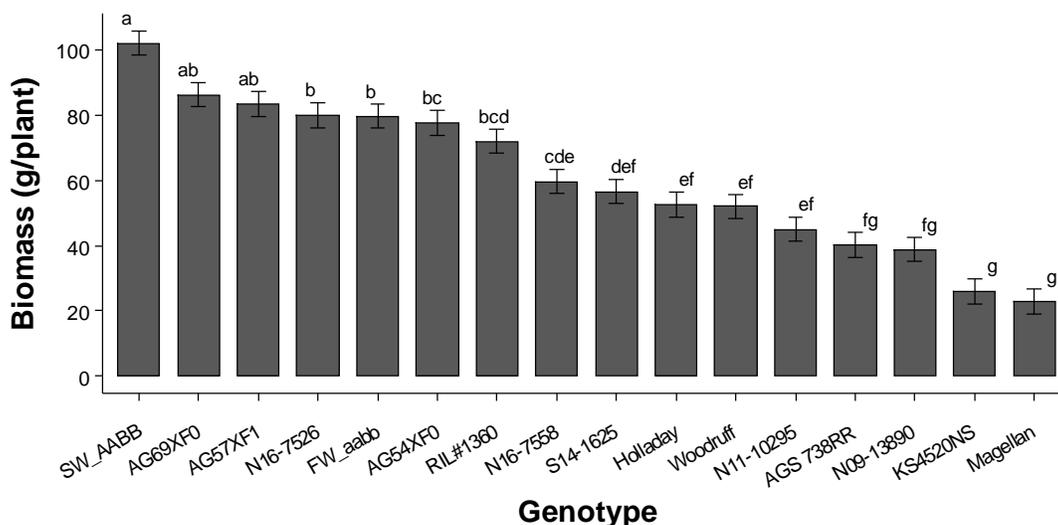


Figure 3.11. Stem biomass of soybean genotypes grown in warming conditions.

Each bar represents the estimated marginal means of nine replicates. Letters above the bars show differences among genotypes based on Tukey's test ($\alpha = 0.05$; HSD). Biomass corresponds to the sum of stem and branch dry weight.

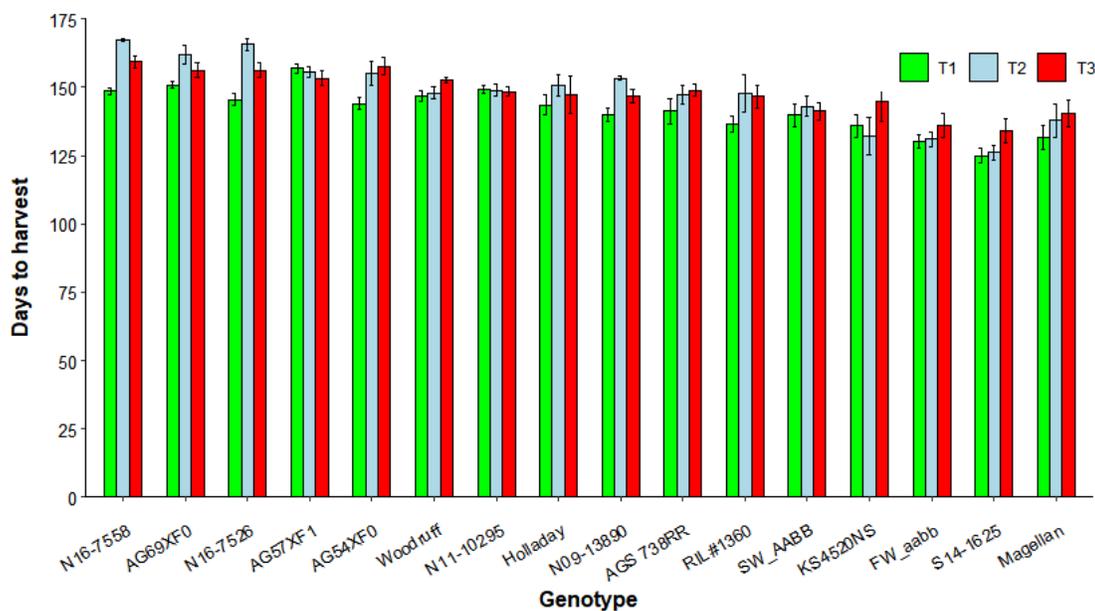


Figure 3.12. Days required to reach maturity for soybean genotypes grown in warming conditions.

The bars indicate the means ($n=3$) \pm standard error. T1, T2, and T3 correspond to three treatments inside the TGG.

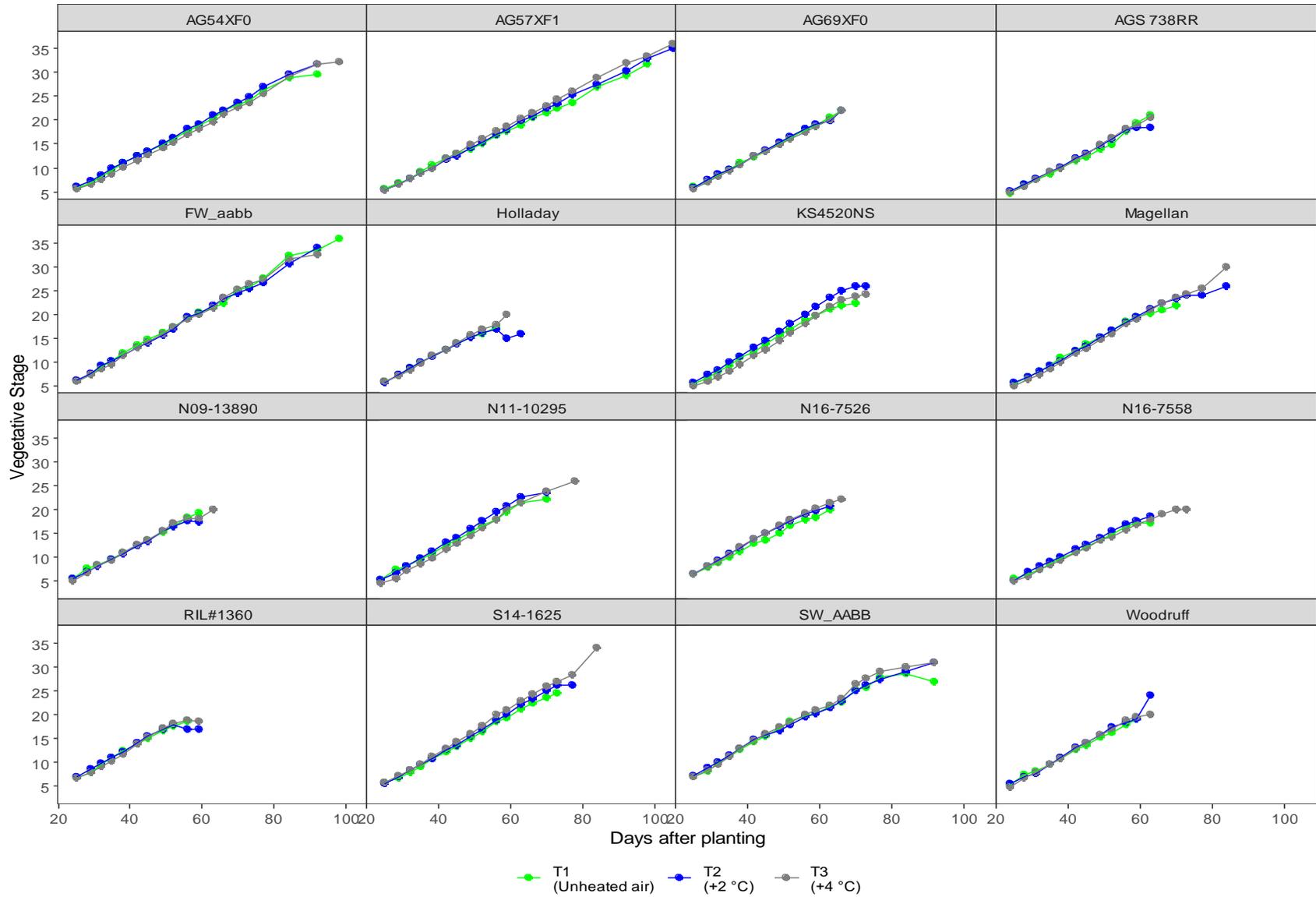


Figure 3.13. Vegetative stage changes for each genotype during the growing season in 2021.

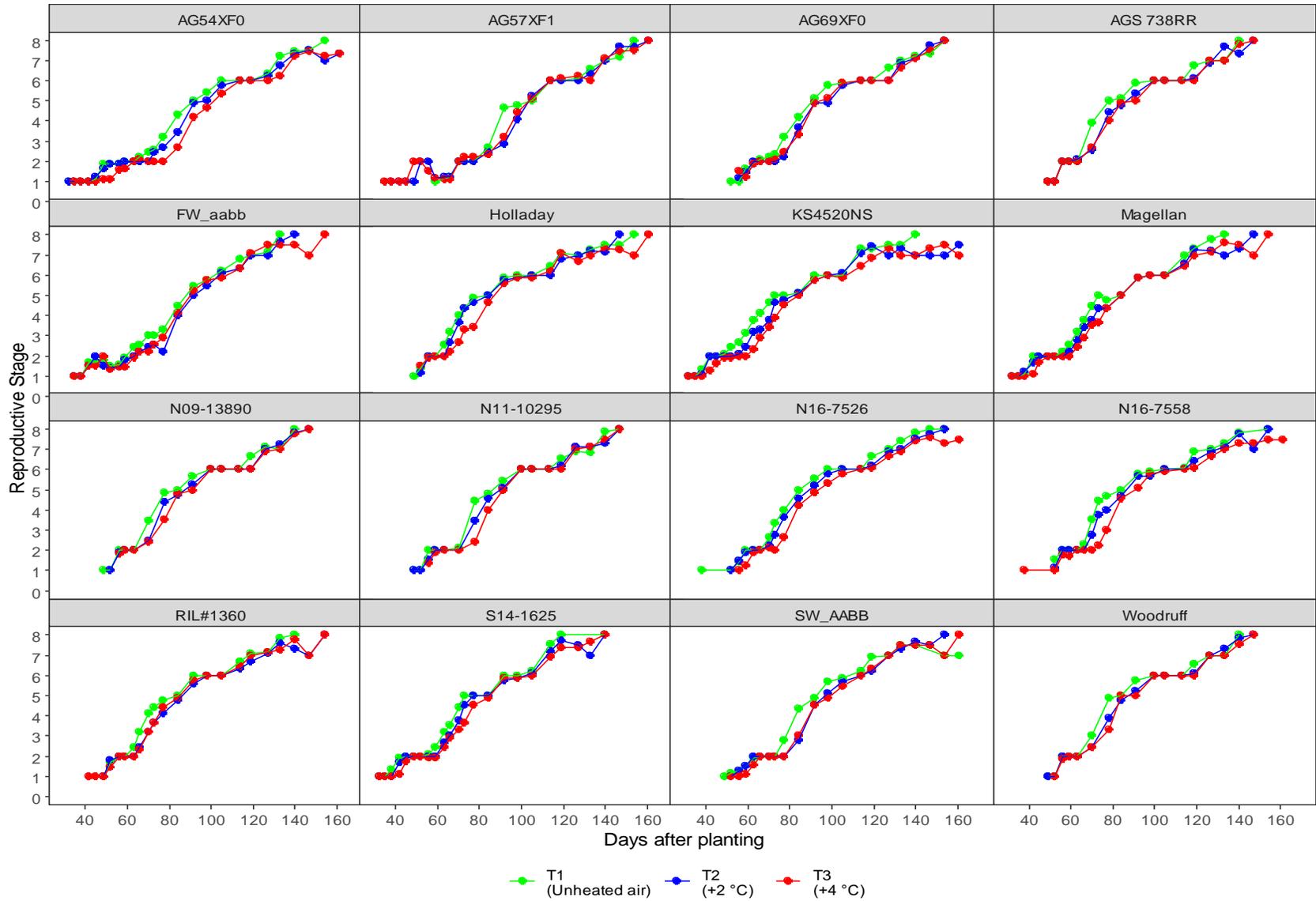


Figure 3.14. Reproductive stage changes for each genotype during the growing season in 2021.

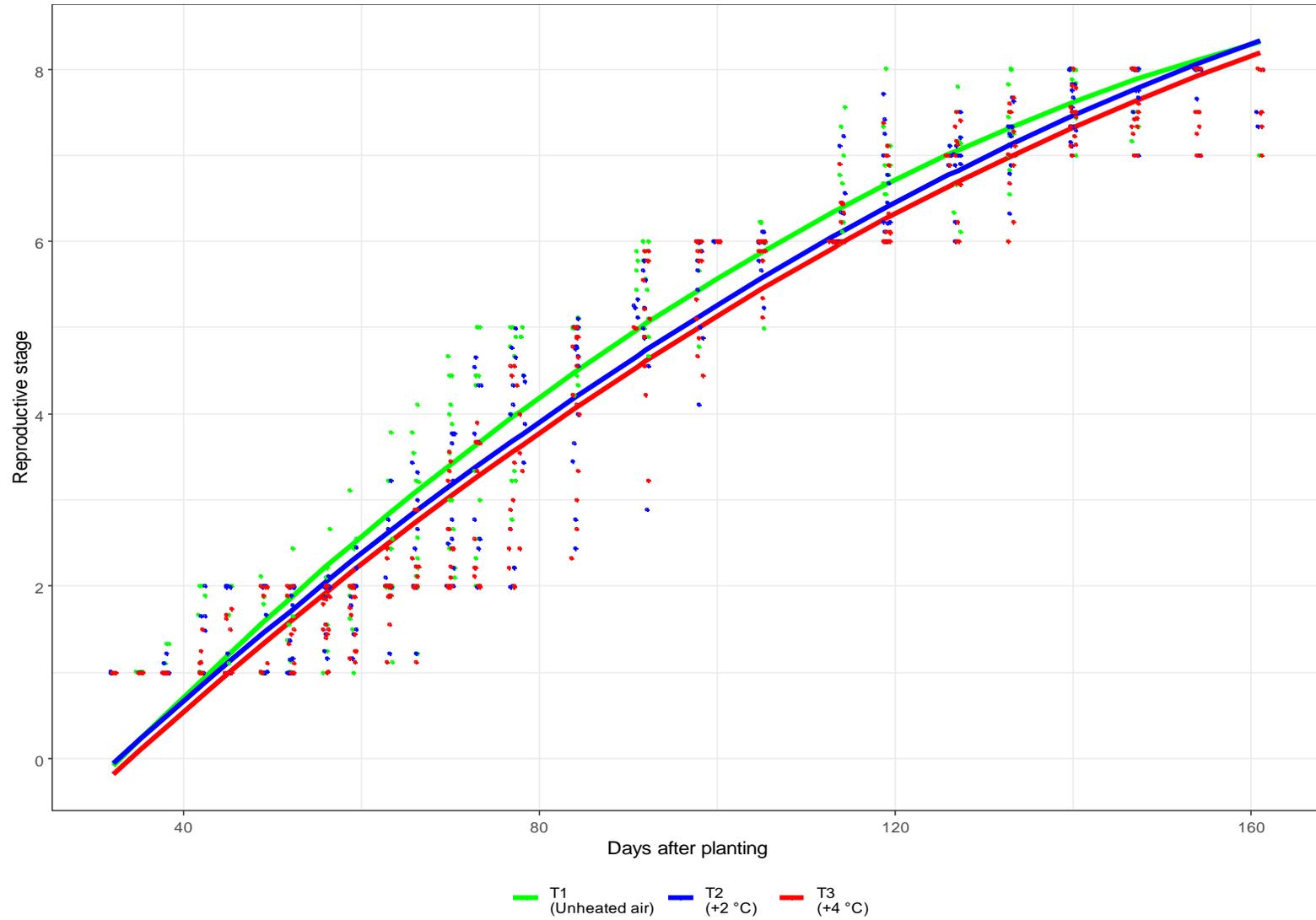


Figure 3.15. Reproductive stage changes in each temperature treatment for soybean genotypes during the growing season in 2021.

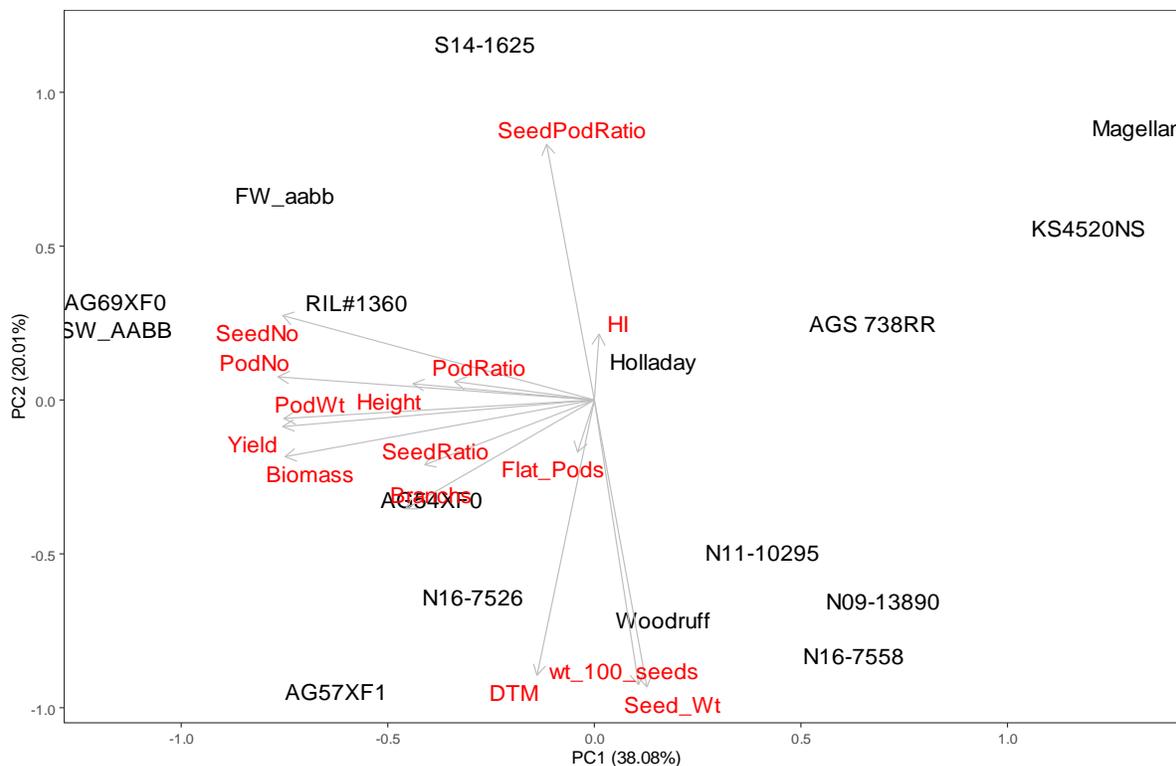


Figure 3.16. Biplot of environmental and reproductive responses for sixteen soybean genotypes exposed to warming conditions.

Variable descriptions: number of seeds per pod (SeedPodRatio), plant height in cm (Height), pod weight per plant in grams (PodWt), seed yield per plant in grams (Yield), pod number (PodNo), seed number (SeedNo), stem biomass (Biomass), branch to main stem seeds ratio (SeedRatio), branch to main stem pod ratio (PodRatio), days to maturity (DTM), number of unfertilized pods (Flat_Pods), branch number (Branches), 100-seed weight (Wt_100_Seeds), weight per seed in grams (Seed_Wt), harvest index (HI).

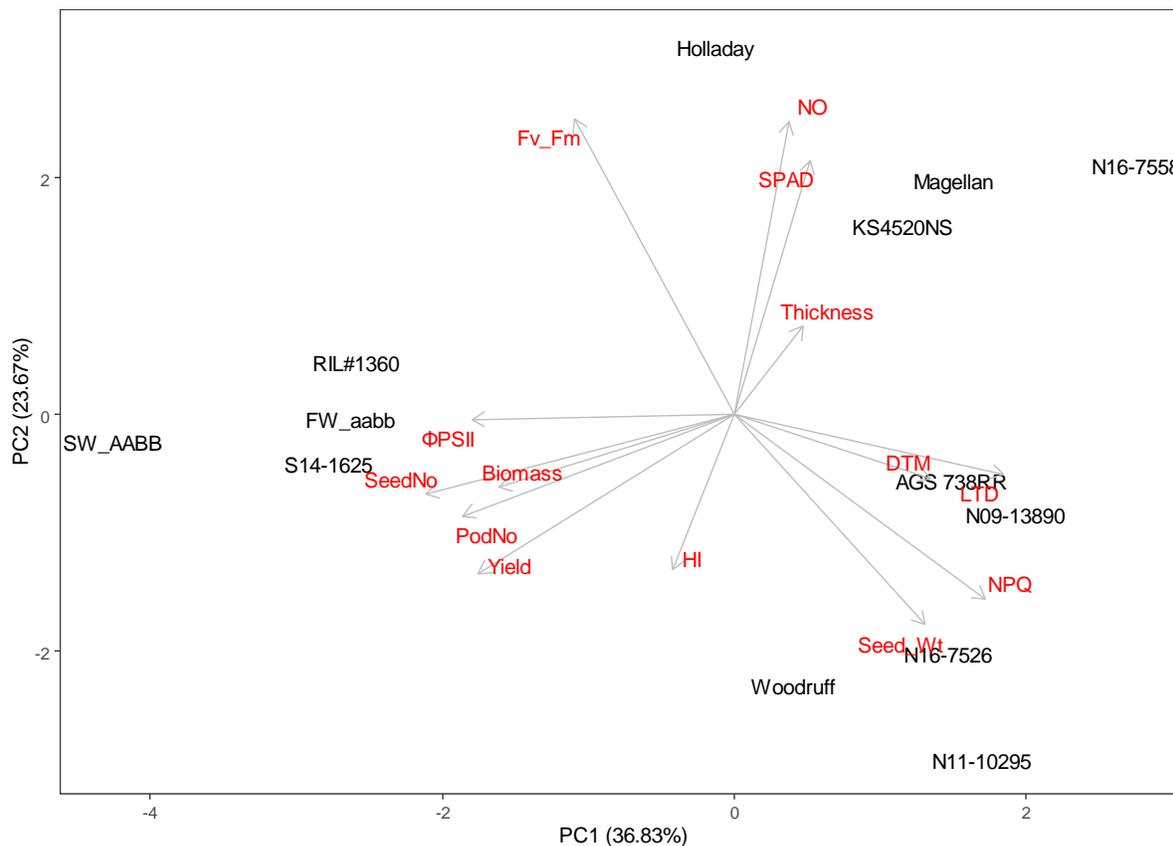


Figure 3.17. Biplot of various fluorescence, leaf temperature differential, relative chlorophyll content, and yield traits of thirteen soybean genotypes.

Biplot represents pooled data ($n=9$ for fluorescence and $n=9$ for yield-related parameters) of genotypes grown under heat stress conditions.

Variable descriptions: harvest index (HI), seed yield per plant in grams (Yield), pod number (PodNo), seed number (SeedNo), stem biomass (Biomass), days to maturity (DTM), weight per seed in grams (Seed_Wt), photosystem II quantum efficiency (Φ_{PSII}), maximum quantum efficiency in the light (Fv_Fm), non-regulatory energy dissipation or other energy losses (NO), non-photochemical quenching (NPQ), relative chlorophyll content (SPAD units), leaf temperature differential in degrees Celsius (LTD), and leaf thickness (Thickness).

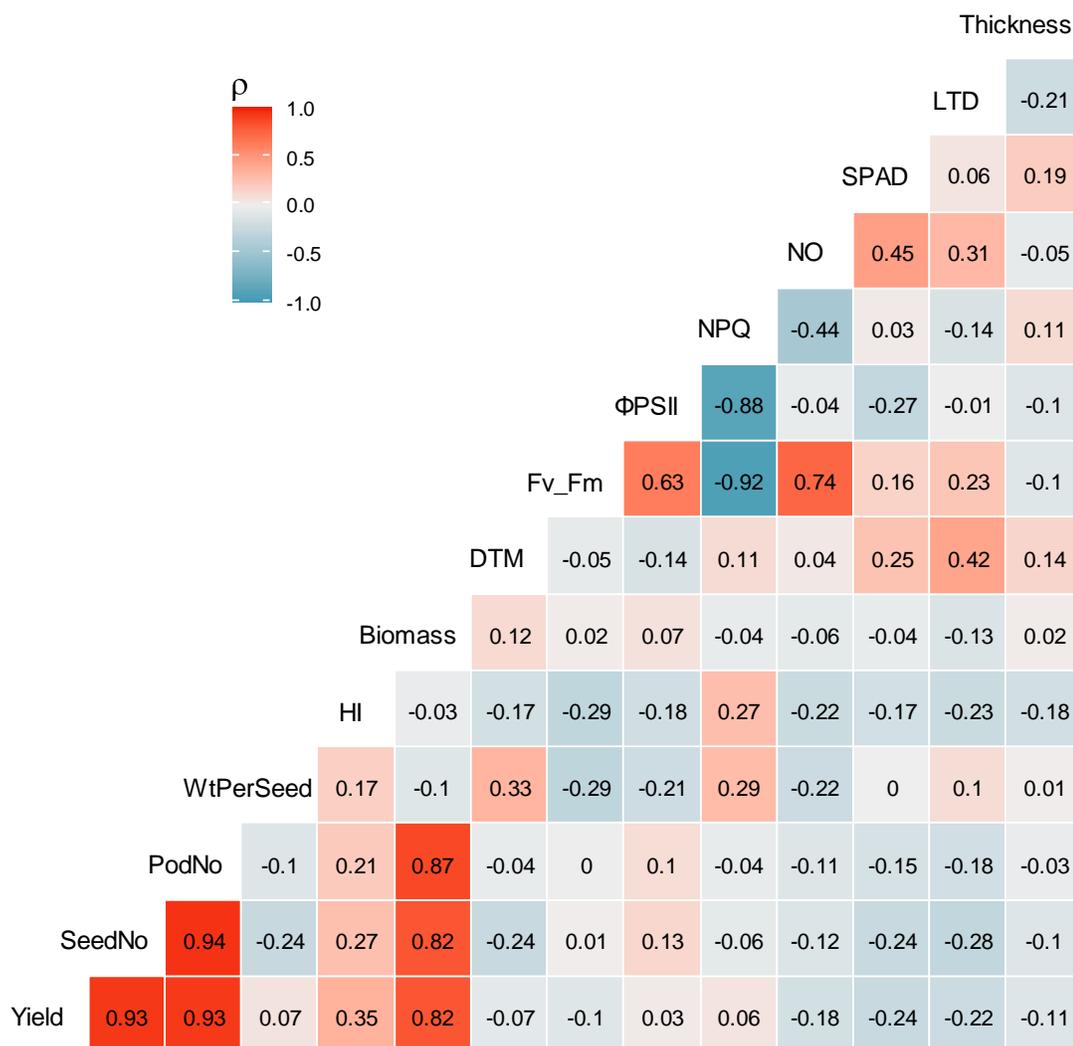


Figure 3.18. Correlation coefficients between various yield and chlorophyll fluorescence traits.

Coefficients were computed across genotypes and treatments. The color gradient represents positive (1) to negative (-1) correlations. Significant correlations ($p \leq 0.05$) are ≤ -0.18 or ≥ 0.18 .

Variable descriptions: seed yield (Yield), seed number (SeedNo), pod number (PodNo), weight per seed (WtPerSeed), harvest index (HI), stem biomass (Biomass), days to maturity (DTM), maximum quantum efficiency in the light (Fv_Fm), photosystem II operating quantum efficiency (Φ_{PSII}), non-photochemical quenching (NPQ), non-regulatory energy dissipation or other energy losses (NO), relative chlorophyll content (SPAD units), leaf temperature differential in degrees Celsius (LTD), and leaf thickness (Thickness).