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Emmalea G Ernest, Randall J Wisser. Multiple physiological mechanisms involved in reproduction could be targeted for breeding heat tolerance in lima bean. *Crop Science*, 2023, 64, pp.179 - 191. 10.1002/csc2.21167 . hal-04514952

HAL Id: hal-04514952

<https://hal.inrae.fr/hal-04514952>

Submitted on 21 Mar 2024

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ORIGINAL ARTICLE

Crop Breeding & Genetics

Multiple physiological mechanisms involved in reproduction could be targeted for breeding heat tolerance in lima bean

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Assigned to Associate Editor
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Funding information

Delaware Department of Agriculture, Specialty Crop Block, Grant/Award Numbers: 12-25-B-1661, 16SCBGPDE0040

Abstract

With global warming, the impact of high-temperature stress on crop production is one of the major issues facing agriculture. Combining a series of field and controlled environment experiments, the current study aimed at understanding causes of yield loss due to heat stress in lima bean (*Phaseolus lunatus* L.) in order to guide breeding for heat tolerance. A growth-stage analysis of multi-year field trials suggested that yields were reduced by sensitivity to heat during floral transition. High nighttime temperatures during this period were also associated with delayed pod set and subsequent harvest. These effects were validated in controlled environments, with additional tests revealing how nighttime heat stress generally reduces seeds per pod but can also reduce the number of pods set. Using an intra-plant dual temperature treatment design for vegetative and reproductive organs, it was determined that effects on pollen viability and release are primary factors underlying heat stress-associated yield losses in lima bean. Elevated nighttime temperatures did not reduce aboveground biomass, indicating the physiological basis for temperature sensitivity was not driven by a photosynthate deficit, but instead could be attributed to changes in source–sink dynamics. Still, the basis for sensitivity to heat differed among genotypes, opening new targets for a multi-mechanistic breeding approach for heat tolerance.

KEYWORDS

climate change, heat stress, pollen viability, reproductive biology, source-sink dynamics

1 | INTRODUCTION

Plants have evolved under a range of temperature conditions, with minimum, maximum, and optimum responses to temperature varying by species (Parent & Tardieu, 2012). Temperatures above a species' maximum reduce the yields

and quality of harvested products (Hatfield & Prueger, 2015). With global warming, a rise in maximum daily (daytime) temperatures will push some crops beyond their threshold. However, crop performance can also be negatively affected by elevated nighttime temperatures, even temperatures below those that would cause damage during the day (Jing et al., 2016). Breeding for tolerance to extreme temperatures is one strategy for adapting crops to climate change (Hatfield et al., 2011), but effective selection is dependent upon knowing the most heat-susceptible growth stages and understanding

Abbreviations: BFB, 'Bush Florida Butter'; CelSel, 'C-elite Select'; FAO, Food and Agriculture Organization; FH242, 'Fordhook 242'; GDD, growing degree days; HSI, heat susceptibility index; PGM, pollen germination medium; UAN, urea ammonium nitrate.

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the physiological effects of high-temperature stress (Porch & Hall, 2013).

Numerous molecular, cellular, and physiological responses to elevated temperature have been described in crops, with emergent effects on morphology, phenology, and reproduction at the whole plant scale (Bita & Gerats, 2013; Wahid et al., 2007). Effects on growth linked to changes in respiration and photosynthesis are well established, with differential sensitivities noted between species (Salvucci & Crafts-Brandner, 2004) and sometimes between genotypes within a species (Camejo et al., 2005; Coast et al., 2022). Studies on widely cultivated crops have found that the reproductive stage is particularly vulnerable to heat stress, often identifying impacts on pollen function (Lohani et al., 2020). Still, little to no information is available about responses to heat for many other crops, leaving gaps in knowledge for biological understanding and for decision-making about future cropping systems.

This study focuses on lima bean (*Phaseolus lunatus* L.), a legume species with importance to certain local economies and food systems throughout the world. As a legume crop, lima bean seeds are a good source of dietary protein, carbohydrates, fiber, and minerals, particularly iron (Bonita et al., 2020). Lima bean is grown across a wide range of environments, including humid tropical, arid, and temperate ecological zones (Adebo, 2023). Global production of lima bean is difficult to assess because many countries and the Food and Agriculture Organization (FAO) of the United Nations do not keep separate statistics on lima bean production. The FAO's legume crop reporting groups lima bean with of eight other species from the *Phaseolus* and *Vigna* genera, collectively classified as dry beans (Snapp et al., 2018). In many locations, lima bean is produced primarily on small, diversified farms for local consumption (Ibeawuchi, 2007; Lustosa-Silva et al., 2023). In the United States, production of lima bean is concentrated in California and the Delmarva Peninsula. California production constitutes approximately 8000 ha of dry lima bean and 1000 ha of beans harvested at the succulent seed stage for fresh market, canning, or freezing (USDA-NASS, 2019). Production in the Delmarva Peninsula, which comprises about 5000 ha annually, is exclusively harvested at the succulent stage, with the vast majority going to regional vegetable processors for freezing and distribution across the United States (USDA-NASS, 2019). Known as a keystone crop for the vegetable processing industry in the Delmarva Peninsula (Kee et al., 1997), lima bean also expands opportunities for farmers to diversify their cropping systems and businesses, potentiating nearly twice its production value from other frozen vegetable crops (USDA-NASS, 2015). Therefore, temperature stress is threatening this interlinked market of fresh vegetable processing in the Mid-Atlantic agricultural system of the United States, a major center for lima bean production.

Core Ideas

- Seed yield in lima bean is reduced when heat stress occurs during reproductive phase.
- Sensitivity to heat stress is greater during the night.
- High nighttime temperatures affect multiple physiological mechanisms of reproduction.
- Genotypes show mechanism-specific sensitivities to high nighttime temperature.
- Multiple physiological mechanisms may be targeted for breeding heat tolerance in lima bean.

Lima bean has a unique flowering behavior, with production of abundant flowers, indeterminately on existing racemes, until a capacity set of pods is reached (Cordner, 1933; Wootten et al., 1999). When biotic or abiotic stress prevents pods from setting, the flowering stage is prolonged to reach the capacity set, resulting in asynchronous pod maturity, as pods form over a longer period of time (Wootten et al., 1999) or delayed maturity if pod set is completely interrupted. Inferred from production and weather data in Delaware and California, high-temperature stress has been associated with delayed pod set, asynchronous pod set, and yield loss for lima bean (Wootten et al., 1999; Kee et al., 1997). Similar findings have been reported for *Phaseolus vulgaris* (Li et al., 1991), but the effects in lima bean are more pronounced because of its unique reflowering behavior. However, there have been no studies to validate this or to determine how heat stress could give rise to prolonged flowering (leading to late maturity) and yield loss.

Despite the lack of experiments to confirm the impacts of heat stress in lima bean, growers and processors have identified heat stress as the most yield-limiting factor for lima bean production in the Delmarva Peninsula, with heat tolerance rated as the highest priority trait for improvement in the crop (unpublished survey; Cooperative Extension, University of Delaware). Indeed, given that global surface temperatures have increased nearly 1°C above preindustrial levels (Stocker et al., 2014) and continue to increase further (Maldiva, 2021), prioritizing the characterization of sensitivities to heat stress and breeding for heat-tolerant varieties is now crucial.

In the only study we are aware of linking the effects of high temperature to reduced yields in lima bean, Fisher and Weaver (1974) used growth chamber experiments to demonstrate that plants exposed to identical daytime temperature produced lower yields when nighttime temperatures were high (27°C vs. 16°C). High temperatures during the night resulted in increased flower production but not increased pod set (no seed yield data were reported). The authors speculated that

this was due to carbohydrate depletion, resulting from higher respiration at higher temperatures (Fisher & Weaver, 1974). However, work in a related species, common bean (*Phaseolus vulgaris*), has shown that high nighttime temperatures directly affect reproductive structures, causing anther indehiscence and reduced pollen viability (Gross & Kigel, 1994; Porch & Jahn, 2001; Prasad et al., 2002). Similar direct effects on anthers and pollen could be present in lima bean, but this has not been investigated.

Broadly, this study aimed at dissecting physiological mechanisms underlying heat stress-associated yield loss in lima bean in order to guide breeding for heat tolerance. Although variation in heat tolerance is not well documented for lima bean, wild lima bean is adapted to warmer climates compared to wild accessions of other *Phaseolus* spp. that have also been domesticated (see Table S1 in Bitocchi et al., 2017). Therefore, diverse lima bean germplasm is expected to capture phenotypic adaptations to high-temperature stress, making breeding for heat tolerance possible. However, to develop screening methods for breeding, the susceptible growth stages and physiology of heat stress-associated yield loss must be determined. To this end, data from multi-year variety trials were analyzed to test the link between high temperatures and yield loss in field conditions. Additionally, a series of controlled environment experiments were used to characterize vegetative and reproductive responses to heat stress in lima bean, and how genotypes with contrasting sensitivities differ in these responses. Yield components were measured with a focus on pod and seed set (compared to previous studies focused on bud and flower abortion [Wootten et al., 1994] or only pod set [Fisher & Weaver, 1974]). Considering how crops will be adapted to climate change, the current study provides new insights into the mechanistic diversity of plant responses to heat stress.

2 | MATERIALS AND METHODS

2.1 | Variety trials

In breeding and extension programs, crops are routinely evaluated in variety trials to assess crop performance in representative local conditions or directly on farmers fields, typically for maturity and yield. This study used data from 8 years of variety trials for lima bean planted annually between June 7 and June 14 from 2010 to 2017 at the University of Delaware's Thurman Adams Research Farm in Georgetown, Delaware. The data from five genotypes ('Cypress', 'C-elite Select' (CelSel), 'Bert', 'Brooke', and 'DE0505002A') included in all trials were used for this study.

The experimental design and management for variety trials were as follows. Experimental units consisted of one-row plots with 76-cm spacing between rows. Plots were 7.62 m in length with 100 plants per plot (7.6-cm plant-to-plant

spacing). Cultivars were arranged in a randomized complete block design with four replications. Cypress was planted in every other plot as a border between experimental units. All plots were fertilized with potassium (0-0-60) before planting according to soil test results. A preemergence application of Dual II Magnum was made for weed control in combination with 48 kg/ha nitrogen in the form of 30% urea ammonium nitrate (UAN). Plots were cultivated one to three times, depending upon soil conditions. A sidedress application of 42 kg/ha nitrogen in the form of 30% UAN was made 35–50 days after planting. Plots were overhead irrigated as necessary. Chemical controls were applied in some years to manage stink bugs and lima bean downy mildew.

Yield data were measured on a per-plot basis, with harvest times based on visual evaluation of each plot. Each plot was harvested to maximize the yield of succulent stage seed. Therefore, not all replications of the same genotype were harvested on the same day. A 4.6-m central-section from each plot was harvested. Plants were cut off at soil level and were fed into a stationary FMC viner. Trash was removed from the shelled beans with a fan and a screen, and the cleaned beans were weighed to determine yield.

Daily maximum and minimum temperatures ($T_{\max/\min}$) during each growing season were obtained from the Delaware Environmental Observing System (<http://www.deos.udel.edu>) weather station located on the research farm. Daily accumulated growing degree days (GDD) were calculated for each season. GDD were calculated as $[(T_{\max} + T_{\min})/2] - T_{\text{base}}$ (McMaster & Wilhelm, 1997), where T_{\max} and T_{\min} are the maximum and minimum daily temperatures, respectively, and T_{base} is 10°C (based on common bean), the temperature below which growth ceases. Average maximum and minimum temperatures were calculated for each interval of 100 GDD. For the remainder of this paper, we refer to maximum temperature as daytime temperature and minimum temperature as nighttime temperature.

To determine the plant growth stages for which temperatures were associated with yield, Kendall's tau-b correlation coefficients (Bonett & Wright, 2000; appropriate for small sample size) were calculated between yield and average daily minimum and maximum temperatures for 100 GDD intervals from 0 to 1200 accumulated GDD. Correlation coefficients between days to harvest and the temperature parameters were also calculated. The Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) was used to identify correlation coefficients that were significantly different from zero with a 20% false discovery rate.

2.2 | Greenhouse chamber experiments

A set of four experiments were performed to test the effects of high nighttime temperatures on different traits, including yield, biomass, pollen release, and pollen vigor (Table S2).

TABLE 1 Characteristics of genotypes tested for responses to high nighttime temperature stress.

Genotype ^a	100 Seed weight (g)	Seed color	Origin	Improvement status
Fordhook 242 (PI 549464)	114	White	USA	Cultivar
Bush Florida Butter (PI 549509)	46	Buff/black	USA	Cultivar
Henderson Bush (PI 549466)	46	White	USA	Cultivar
PI 534918	43	Magenta	USA	Cultivated
Bridgeton (PI 549508)	40	Green	USA	Cultivar
Cypress	38	Green	USA	Cultivar
C-elite Select	32	Green	USA	Cultivar

^aPI names for accessions at the US National Plant Germplasm System indicated when possible.

Each experiment considered a specific set of genotypes and traits. The experiments included some but not all of the genotypes evaluated in the variety trials (cf. Table 1 and Table S2). The same lines were not used in each experiment because some putatively heat-tolerant genotypes were found not to be very tolerant. All of the genotypes have a determinate growth habit.

Two climate-controlled chambers with wooden frames were constructed inside a double-layer, inflated polyethylene greenhouse. The chambers were partially sheathed in double-layer polyethylene with the remainder covered in double-wall polycarbonate sheets. Each chamber was supplied with a constant circulation fan and thermostatically controlled supplemental electric heat and fan ventilation. Air temperature inside of the chambers was recorded at 10-min intervals with solar-shielded data loggers (WatchDog A150; Spectrum Technologies) placed at the canopy level. One chamber was set to maintain a high nighttime temperature (27°C) and the other a cool nighttime temperature (21°C) consistent with controlled environment studies in common bean. Daytime temperature setpoints were the same for both chambers (30°C). Average day/night temperatures were 32/27°C in the hot chamber and 28/20°C in the cool chamber. Because chamber conditions were impacted by outdoor environmental conditions (i.e., light intensity and air temperature), average temperatures were not identical across experiments (Table S2).

For each experimental unit (3.8-L nursery pots), two scarified seeds were sown into peat-based growing medium (Pro-Mix BX) and thinned to one plant per pot after emergence. Scarified seeds were used to achieve more synchronized emergence and to avoid problems with slow imbibition in peat-based growing medium. Plants were maintained in the cool nighttime temperature chamber until primary leaves had fully expanded (approximately 20 days after planting), after which they were randomly assigned to one of the two temperature regimes. Within each chamber, genotypes were arranged in a randomized complete block design with four to six plants per genotype × temperature treatment. Plants were fertilized with 15 g of controlled-release fertilizer (Nutricote Total

13-11-11 Type 100) at 20 DAP and maintained under well-watered conditions using an automated irrigation system and supplemental hand watering.

The heat susceptibility index (HSI; Fischer & Maurer, 1978) was calculated using weight of seed yield for all genotypes for each experiment:

$$HSI_i = [1 - (YH_i/YC_i)] / D,$$

where HSI_i is the heat susceptibility index for the i th genotype, YH_i is the yield (averaged across experiments) for the i th genotype under heat stress, YC_i is the yield (averaged across experiments) for the i th genotype under cool conditions, and D is stress intensity for check genotypes. Stress intensity is calculated as $[1 - (\overline{YH_i}/\overline{YC_i})]$, using the mean values among standard check genotypes. ‘Fordhook 242’ (FH242) and ‘Bush Florida Butter’ (BFB) were designated as checks included in all experiments. Following Fischer and Maurer (1978), $HSI \leq 0.5$ is considered highly heat tolerant, $0.5 < HSI \leq 1.0$ is moderately heat tolerant, and $HSI > 1.0$ is heat sensitive.

2.3 | Greenhouse chamber experiments testing heat effect on yield components and biomass

Measurements for the yield components experiment (Table S2) included days to flowering, number of mature pods, number of seeds per pod, number of mature seeds, and weight of mature seeds were determined for individual plants grown under the two temperature regimes. Measurements for the biomass experiment (Table S2) included shoot dry weight (leaves, stems, and pods, but no seeds) and total aboveground dry weight (leaves, stems, pods, and seeds).

Days to flowering and yield components were analyzed as a split-plot design in Proc-Mixed (SAS Version 9.4; SAS Institute) with temperature as the main plot treatment and genotype as the subplot treatment. The Tukey–Kramer method was used to separate treatment means at $\alpha = 0.05$.

Greenhouse chamber experiments testing heat effects on pollen release and viability. The pollen release and viability experiments (Table S2) were performed using five genotypes: (i) BFB, (ii) CelSel, (iii) FH242, (iv) Henderson Bush, and (v) PI 534918.

The same procedure was followed for tests repeated across years. Newly opened flowers were sampled on multiple dates with three flowers per genotype \times temperature combination sampled on each date. By tripping the wing petals, the stigma and style were released from the keel and removed with tweezers. Stigma and style were placed on a glass microscope slide in a drop of acetocarmine stain solution. After 3 min, a glass coverslip was placed over the drop and pressed down to flatten the style and stigma. The keel was dissected, and two stamens were removed and mounted in the same way. For style samples, the stigma and portion of the style containing the stylar brush were photographed at 40x magnification. For stamen samples, two anthers were photographed at 40x magnification. Stained pollen grains visible in the photographs were marked and counted with the aid of Fiji/ImageJ (Schindelin et al., 2012). Pollen counts were analyzed in Proc-Mixed (SAS Version 9.4; SAS Institute) with temperature, genotype, and year as fixed effects and sample date nested in year as a random effect. The Tukey–Kramer method was used to separate treatment means at $\alpha = 0.05$.

To test the effects of hot or cool night temperature conditions on pollen viability, pollen from each genotype \times temperature combination was germinated *in vitro* at 25°C. Newly opened flowers were collected in early morning from plants that had been planted in the greenhouse chambers on March 15, 2017 and November 5, 2018. Pollen germination tests were conducted using the sitting drop method in artificial pollen germination medium (PGM) (Gurusamy et al., 2007). The final concentrations of PGM components were as follows: H₃BO₃ 400 mg/L, CaNO₃ 600 mg/L, MgSO₄ 400 mg/L, KNO₃ 400 mg/L, and 40% sucrose. Humid chambers used for pollen germination consisted of 14-cm glass Petri dishes lined with 12.5-cm Whatman 4 filter paper and moistened with 4 mL distilled water. Five microscope slides were placed in each Petri dish and supported above the wet filter paper by thin strips of rigid plastic. Each of the five slides was used to conduct two separate sitting drop pollen germination assays. Two drops of PGM were placed on each slide and the covered Petri dish was placed in a germination chamber at 25°C to warm the PGM. For each test, the style and stigma were removed from a flower and swirled a prepared sitting drop of PGM on the microscope slides. After incubation for 4–5 h, one drop of acetocarmine stain was added to each drop of PGM. After 5 min, the drop was viewed with a compound microscope at 100x and 20 pollen grains were rated as germinated or ungerminated. Pollen was considered germinated if the pollen tube length exceeded the pollen grain diameter. Germination rate was measured for pollen from 23 flowers of each genotype

\times temperature combination. Germination rates were analyzed in Proc-Mixed with the following fixed effects: temperature, genotype, temperature \times genotype, temperature \times experiment, and temperature \times genotype \times experiment. Experiment was modeled as a random effect. The Tukey–Kramer method was used to separate treatment means at $\alpha = 0.05$.

2.4 | Greenhouse chamber experiment testing heat effects on pollination

To test the heat sensitivity of processes downstream of pollination, hand pollinations were made on heat-sensitive genotypes CelSel and PI 549464 FH242 grown in the hot chamber with pollen collected from CelSel or PI 534918 ‘Cave Dweller’ plants grown under ideal conditions. Self-pollinated flowers at the same developmental stage and similarly positioned on the same raceme as each hand pollination were marked as paired controls. Each tagged flower was monitored until it or the resulting pod was aborted or reached maturity. Developing pods were measured to determine the maximum length attained and the number of seeds was determined for pods reaching maturity. The total number of crosses for each pair was as follows: 70 of CelSel \times PI 534918; 60 of CelSel \times CelSel; 80 of FH242 \times PI 534918. The data from this experiment were analyzed using McNemar’s test for paired nominal data (2 \times 2 tables of pod or seed formation vs. selfed or crossed flowers).

3 | RESULTS AND DISCUSSION

3.1 | Decoupling seasonal weather data in field environments implicates growth stage-specific associations between temperature and yield

We analyzed 8 years of variety trial data (2010–2017) to search for specific stages of growth during which elevated temperatures affect yield. Across the trials, daily daytime temperatures ranged from 20.7°C (all-time low during the day) to 38.4°C (all-time high during the day), while daily nighttime temperatures ranged from 7.8°C (all-time low during the night) to 27.8°C (all-time high during the night).

To control for single-day extremes and align the cropping cycle for different years, daytime and nighttime temperatures were averaged in 100 GDD intervals (Figure 1). Although mean day and night temperatures were partially correlated within GDD intervals (Table S2), nights showed a more consistent seasonal pattern of increasing to decreasing temperatures, and the coefficient of variation (CV) in temperature was twice as large for GDD intervals during the night (median CV of 11%) than during the day (median CV of 6%)

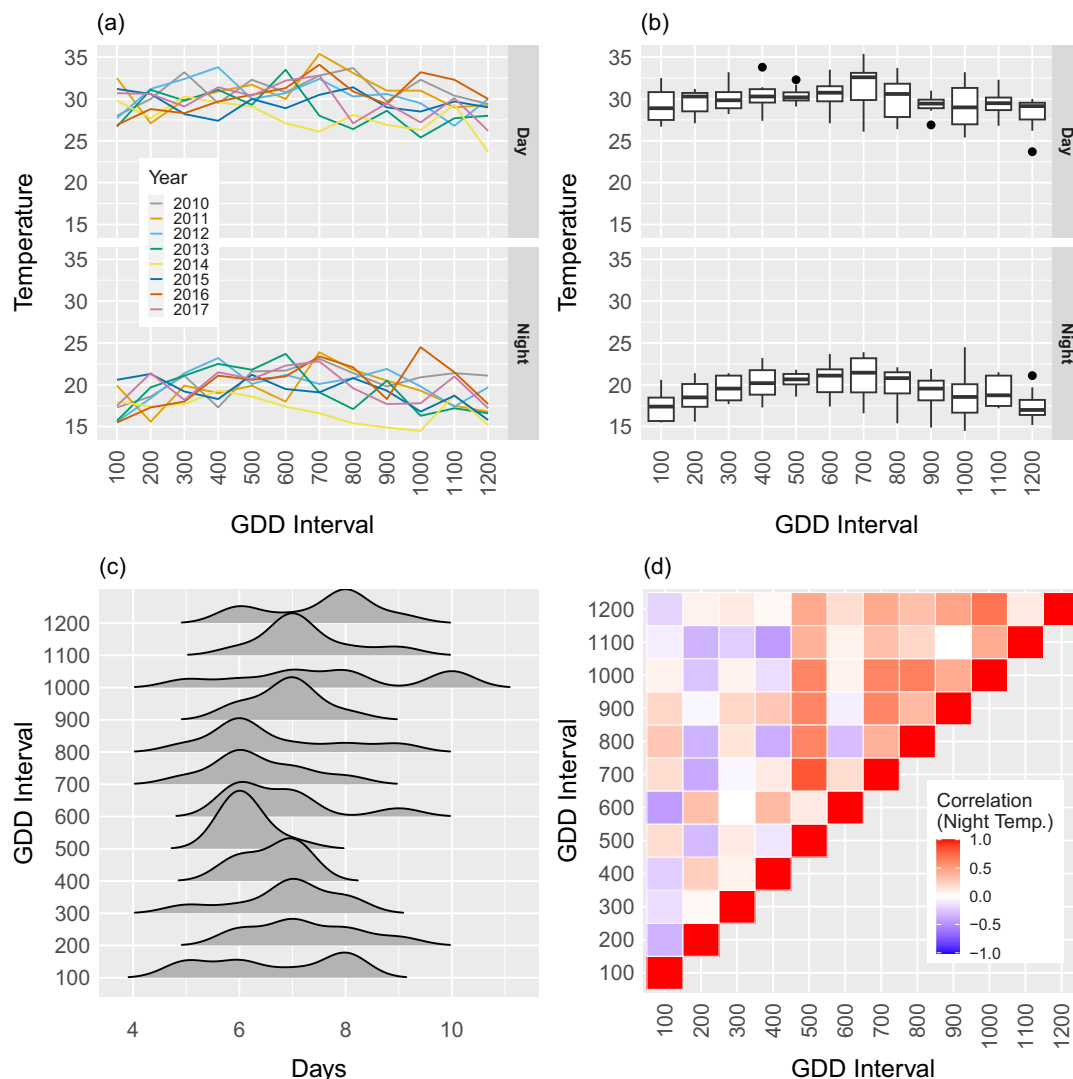


FIGURE 1 Patterns in temperature variation across field sites used for variety trials. (a) Mean temperatures across common intervals of 100 growing degree days (GDD) during the day (mean of daily maximum temperature) and night (mean of daily minimum temperature) per year. (b) Boxplot summary of the annual temperature variation shown in (a) for mean daytime and nighttime temperatures per 100 GDD interval. (c) Approximate distribution of calendar days corresponding to each of the 100 GDD intervals. (d) Pairwise correlations in mean nighttime temperatures per 100 GDD interval.

(Figure 1b). The 12 GDD intervals used to summarize these data averaged 7 calendar days per 100 GDD interval, with an interannual range of approximately 5 days (Figure 1c).

For each 100 GDD, correlations between day and night temperatures among years were all positive but varied widely, with some intervals having moderate to low correlations or no significant relationship (Table S2). Similarly, pairwise correlations for day or night temperatures between separate GDD intervals had wide variation, including negative and positive relationships (Figure 1d shows nighttime temperature relationships). Due to the temporal variation and partial correlation structure for the temperature data across years of testing, temperature–trait relationships due to day versus night effects might be distinguished for some periods of growth.

Therefore, day and night temperatures at each interval were tested for associations with maturity and yield.

Only during certain periods of the crop cycle were temperatures substantially correlated with variation in maturity and yield. In particular, the 600–700 GDD interval consistently showed the highest positive relationship with days to harvest and negative relationship with yield, that is, higher nighttime temperature during this growth stage was associated with a delayed crop cycle and reduced yield (Figure 2). Higher daytime temperature during the same period was similarly associated with reduced yield, but with somewhat weaker correlations. Across growth periods, the correlation shifted in sign between the initial and final phases of the crop cycle with the magnitudes of correlation being greater and biologically

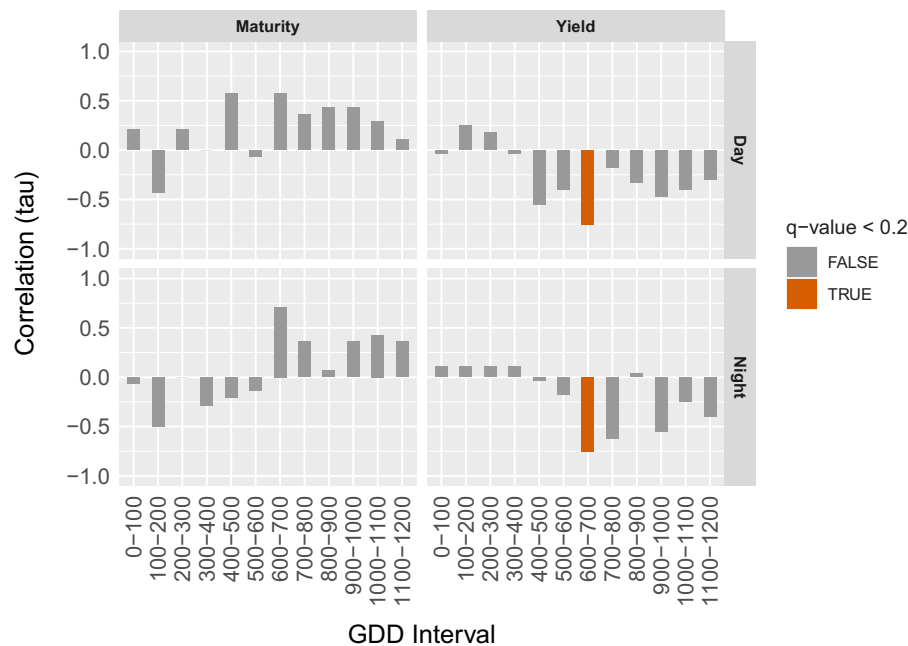


FIGURE 2 Field-based evaluation of temperature effects on maturity and yield. Kendall's tau-b correlation coefficients between average daytime (maximum) and nighttime (minimum) temperature during certain growing degree day (GDD) intervals with mean yield or maturity (days to harvest) among genotypes. A 20% FDR (false discovery rate) was used to threshold significant associations (red bars).

relevant during the latter phase. When genotypes were analyzed separately, there were only minor deviations from the average trend, suggesting shared processes during the 600–700 GDD interval were associated with phenotypic outcomes (Figure S1).

The period of 600–700 accumulated GDD occurred in the range of 39–52 days after planting (average 41–47 DAP) in the trials. This interval corresponds to when many racemes are forming buds and the first flowers are opening; typically, first and peak flowering occur approximately 35 and 60 days after planting, respectively (Kee et al., 1997). Gross and Kigel (1994) found in common bean that pollen was most sensitive to heat stress from 7 to 10 days before anthesis and that heat sensitivity decreases closer to anthesis. The data here suggested that a similar phenomenon is present in lima bean, such that high temperatures during the early reproductive phase are associated with delayed maturity, caused by delayed pod set, and lower yields, caused by low pod set. Because daytime and nighttime temperatures were correlated with one another within GDD intervals (Table S1) and both daytime and nighttime temperatures were significantly associated with yield (Figure 2), it was not possible to fully distinguish whether the effect could be due to one or the other. A prior study showed that nighttime temperature affected yield in lima bean (Fisher & Weaver, 1974). Therefore, a controlled environment experiment was performed to test the effect of nighttime temperature on yield.

3.2 | High nighttime temperatures affect some yield components more than others

In the controlled environment study, using experiments where check genotypes experienced intense heat stress ($D > 0.60$), high nighttime temperature significantly decreased the time to initial flowering for each genotype (time to initial flowering being different from time to maturity, reflected by timing of harvest). Each genotype flowered at about the same time (the genotype main effect was not significant) with no evidence of differential responses to temperature (the genotype-by-temperature interaction effect was not significant) (Figure 3; Table S3). Yet, these genotypes differed significantly in terms of yield components and the impact of temperature on yield. The effects of temperature, genotype, and genotype-by-temperature interaction were significant for all five yield components, with similar effects of temperature across tests (Table S3). Thus, independent of an overall effect of temperature on the time to initial flowering, higher nighttime temperature resulted in fewer pods per plant, fewer seeds per pod, and fewer matured seeds, leading to reduced total yields (total seed number and total seed weight). Genotype interactions with tests, as well as their interactions with temperature, were significant for total yield traits but not per seed weight or seeds per pod, suggesting that seed size and seeds per pod are less impacted by environmental conditions than the other yield components.

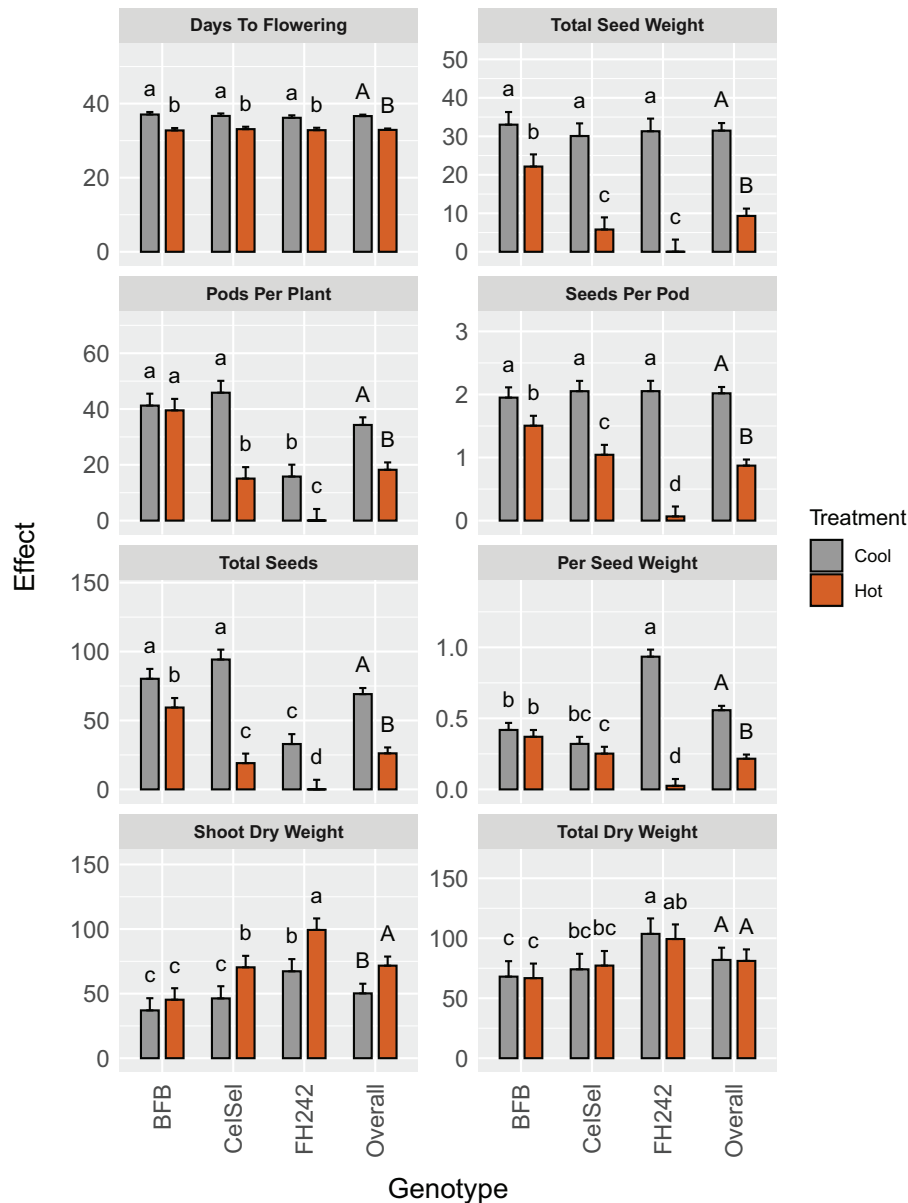


FIGURE 3 Trait effects under cool versus hot nighttime temperature treatments. Letters indicate mean separation across genotypes ('Bush Florida Butter' [BFB], 'C-elite Select' [CelSel], and 'Fordhook 242' [FH242]) and among genotypes (Overall) according to Tukey–Kramer method at $p \leq 0.05$. Error bars indicate the 95% confidence interval for the mean.

3.3 | High nighttime temperature causes split-set

Although days to initial flowering were reduced under heat stress, the high-temperature treatment prolonged the duration of flowering. Heat-sensitive genotypes grown in the high nighttime temperature chamber set very few pods (Figures 3, 4) but continued to flower profusely until the experiment was terminated. The controlled environment experiments were conducted under sustained high nighttime temperature conditions; in a field situation, where temperatures fluctuate, pods are set intermittently depending on temperature, resulting in split-sets. Thus, elevated temperatures not only affect

yield per se. It also complicates the timing of harvest, further impacting the quantity and quality of harvested seed.

3.4 | Pollination biology and not photosynthate availability determines yield under heat stress

We measured biomass to test if source-sink dynamics might explain the effects of temperature on yield. Genotypes varied significantly for both shoot dry weight and total aboveground dry weight (i.e., combined dry weight of shoots and reproductive parts), but high nighttime temperature conditions

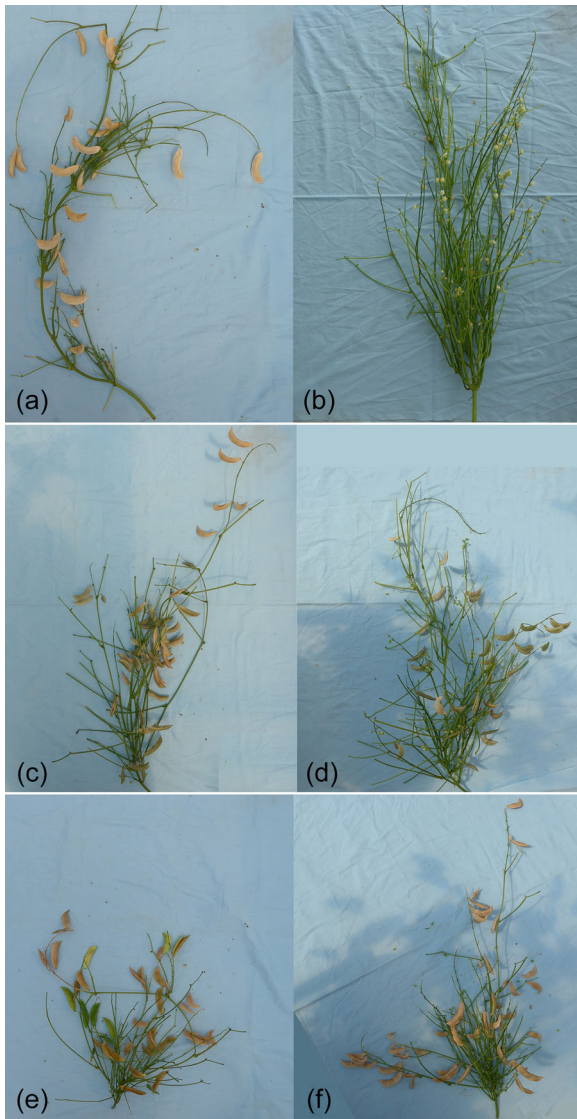


FIGURE 4 Impacts of heat stress on plant architecture. Photos of unstressed (a) and heat stressed (b) plants with leaves removed from three genotypes with differing levels of heat tolerance: ‘Fordhook 242’ (top), ‘C-elite Select’ (middle), ‘Bush Florida Butter’ (bottom).

only affected shoot dry weight (Figure 3; Table S4), which resulted in highly branched stem formation, particularly for heat sensitive genotypes (CelSel and FH242; Figure 4). Therefore, heat stress caused a repartitioning of biomass between vegetative and reproductive organs. The lack of overall differences in total aboveground biomass between heat-stressed and unstressed plants, even in heat-sensitive genotypes, suggests that carbohydrate depletion (Fisher & Weaver, 1974) is unlikely to be the cause of reduced seed yield. Rather, the reduction of total seed number and the number of seeds per pod in heat-stressed plants implicate direct effects of heat stress on pollination, fertilization, embryo development, or seed filling. This suggests similar mechanisms for heat sensitivity in lima bean and common bean, with direct effects of

heat stress on pollen quality and release (Gross & Kigel, 1994; Porch & Jahn, 2001, Prasad et al., 2002)

3.5 | Heat stress reduces pollen release and vigor

In greenhouse chamber experiments, the nighttime temperature regime had a significant effect on the amount of pollen released, reflected by substantial reductions in pollen detected on styles and stigmas (Figure 5). Excess pollen remaining in anthers post anthesis (Figure 5) indicates that anther indehiscence occurs in response to high nighttime temperature in lima bean. Fewer pollen grains maturing in heat-stressed anthers are another possible explanation for low pollen release under heat stress. This was not measured but could be assessed by examining anthers from flowers before anthesis.

Variation in the effects of heat on pollen release was related to the HSI of each genotype. Genotypes that released the least amount of pollen under heat stress, such as FH242 and CelSel, were classified as heat susceptible, while those that released more pollen under stress, such as *PI 534918*, were classified as highly tolerant (cf. Table 2; Figure 5).

Previous reports in common bean have detected effects of heat on pollen viability by quantifying pollen tube penetration of the stigma (Gross & Kigel, 1994) or with staining methods (Porch & Jahn, 2001; Prasad et al., 2002). Here, the former method proved challenging to apply in lima bean, and when using the latter, no discernable differences were detected among samples stained for pollen viability. We found that *in vitro* germination assays, as applied in other species (e.g., Pressman et al., 2002), were also effective for lima bean. Pollen from flowers of the same genotypes collected from hot and cool chambers was germinated *in vitro* at 25°C. Mean germination rates for pollen collected from the cool chamber ranged from 81% to 88%, with no significant difference among genotypes. However, hot chamber pollen germination rates ranged from 23% to 72%. As with pollen release, the germination rate of heat-stressed pollen was in relation to the HSI classification for each genotype. Thus, combined effects of elevated temperatures on pollen release and pollen vigor partially underlie heat-associated yield loss in lima bean.

3.6 | Different mechanisms of heat sensitivity implicated in a Mesoamerican versus Andean genotype

When a heat-stressed CelSel genotype (Mesoamerican background) was used as a pistillate parent, hand pollination with pollen from unstressed CelSel (heat sensitive) or *PI 534918* (heat tolerant) was significantly more likely than self-pollinated flowers to produce a pod measuring at least

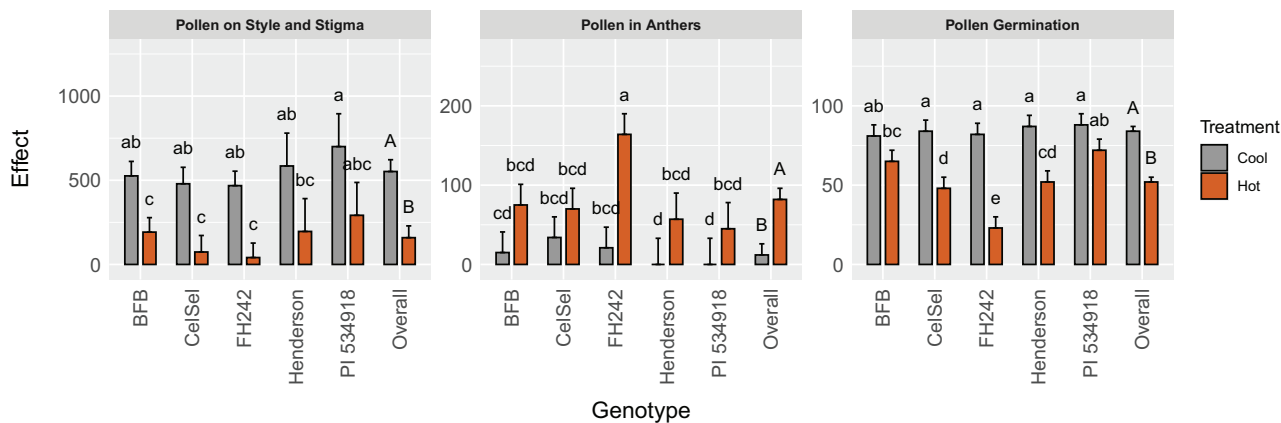


FIGURE 5 Effects of heat stress on pollen. Genotypic effects from unstressed and heat-stressed conditions on (a) counts of pollen shed onto the stigma and style, (b) counts of pollen retained in anthers, and (c) percent germinated pollen sampled from anthers. Letters indicate mean separation across genotypes ('Bush Florida Butter' [BFB], 'C-elite Select' [CelSel], 'Fordhook 242' [FH242], 'Henderson', and 'PI 534918') and among genotypes (overall) by the Tukey–Kramer method at $p \leq 0.05$. Error bars indicate the 95% confidence interval for the mean.

TABLE 2 Heat susceptibility index (HSI) values for experimental genotypes.

Genotype	2015	2016	2017	2018	Mean HSI	Tolerance ^a
PI 534918	–	–	0.28	0.48	0.38	HT
Henderson Bush	–	–	0.16	0.60	0.38	HT
Bush Florida Butter	0.56	0.61	0.34	0.49	0.50	MT
Cypress	0.53	–	–	–	0.53	MT
C-elite Select	1.39	1.24	1.03	1.26	1.23	MS
Bridgeton	1.36	–	–	–	1.36	MS
Fordhook 242	1.65	1.37	1.52	1.64	1.55	HS
Stress intensity ^b	0.61	0.73	0.65	0.40		

^aHSI < 0.5 is highly tolerant (HT), 0.5–1.0 is moderately tolerant (MT), 1.0–1.5 is moderately susceptible (MS), > 1.5 is highly susceptible (HS).

^bStress intensity was calculated using the yields for the two genotypes that were tested in all years: Bush Florida Butter and Fordhook 242.

1 cm and mature seed (Table 3). When a different heat-sensitive genotype, FH242 (Andean background), was the heat-stressed pistillate parent, pollen from the unstressed heat-tolerant genotype (*PI 534918*) was significantly more likely to form a pod, but not more likely to produce mature seed.

For CelSel, the primary factor inhibiting pod formation and seed production under heat stress was a lack of viable pollen. When good quality pollen was applied to the receptive stigma a pod formed 48% of the time compared to 12% of selfed flowers for heat-stressed plants. Furthermore, mature seed was produced 31% of the time compared to 2% of selfed flowers. For FH242, 31% of hand-pollinated flowers produced a pod compared to 8% of selfed flowers. However, mature seed was produced only 6% of the time in hand-pollinated flowers, and no selfed flowers produced mature seed.

For these contrasting genotypes, in addition to their shared effects of viable pollen impacting pod formation, other genotype-specific mechanisms linked to fertilization and seed development also appear to affect yield. For heat-stressed plants of FH242, we observed pods with underdeveloped

seeds or unfertilized seeds (no initial seed development observed). This could be explained by ineffective carbohydrate transport to developing seeds in this genotype, as observed in some common bean genotypes (Omae et al., 2007; Soltani et al., 2019). Moreover, unfertilized seeds could be explained by deformities of FH242 flowers which frequently showed excessive style length and coiling, which could interfere with pollen tube growth in this genotype. Changes to the morphology of reproductive structures may reflect more general effects of heat stress as was similarly observed for vegetative tissues (Figure 4).

3.7 | Genotypic variation in heat stress response shows potential for breeding

The few genotypes surveyed in this study captured a range of heat stress responses (Table 2), with different combinations of mechanisms conditioning tolerance or sensitivity. For example, heat-tolerant genotypes *Henderson* and *PI 534918* had

TABLE 3 Effects of using heat-stressed versus non-stressed pollen on pod set and seed production

Cross	<i>n</i> (pairs) ^a	Frequency ^b		Exact <i>p</i> -value	Odds ratio ^c	95% C.I. ^d	
		Cross + self –	Cross – self +			Lower	Upper
Formation of pod (at least 1 cm)							
CelSel × PI 534918	70	32	4	1.94E-06	8	2.84	31.14
CelSel × CelSel	60	21	2	6.60E-05	10.5	2.57	92.37
FH242 × PI 534918	80	23	4	3.11 E-04	5.8	1.96	22.87
Formation of seed							
CelSel × PI 534918	70	24	0	1.19E-07	Inf.	6.02	Inf.
CelSel × CelSel	60	13	0	2.44E-04	Inf.	3.05	Inf.
FH242 × PI 534918	80	5	0	6.25E-02	Inf.	0.92	Inf.

^aNumber of paired tests in which a flower in the hot chamber was pollinated using pollen from the cool chamber and an adjacent selfed flower was tagged for comparison.

^bFrequency of pod or seed formation; Cross +/Self–indicates that the crossed flower produced pods/seeds and the selfed flower did not (Cross–/Self + indicates the opposite).

^cOdds ratio for paired tests of crosses made on heat-stressed plants with pollen from unstressed plants versus self-pollinated flowers; Inf. indicates odds ratio or CI non-estimable.

^d95% confidence interval for the odds ratio.

similar levels of pollen release but substantially different viability of pollen in heat-stressed plants (Figure 5). Therefore, this study guides approaches surveying variation in specific mechanisms of heat tolerance and highlights the possibility of a multi-mechanistic approach for breeding heat-tolerant varieties in lima bean.

3.8 | Comparisons with other crops

High-temperature stress interferes with pollination in many plant taxa including other legumes (Warrag & Hall, 1984), solanaceous crops (Erickson & Markhart, 2002; Pressman et al., 2002; Sato et al., 2000), and grain crops (Begcy et al., 2019; Sakata et al., 2000). This study shows how high nighttime temperatures impact several processes related to pollination and seed production in lima bean, with effects on anther dehiscence and pollen viability common to many crops (Lohani et al., 2020), including the closely related crop species *P. vulgaris* (Gross & Kigel, 1994; Porch & Jahn, 2001; Prasad et al., 2002). However, some lima bean genotypes also show sensitivity in other processes occurring after pollination, leading to further reductions in yield. Evidence suggests that multiple processes in lima bean are impacted by heat stress, including effects on female reproductive development and post-fertilization processes. In certain lima bean genotypes, many flowers with abnormally elongated styles were observed on heat-stressed plants. Heat stress-induced changes to flower morphology have also been observed in tomato (Alsamir et al., 2017) and could interfere with fertilization. In addition, the formation of small, partially developed seeds in heat-stressed lima bean could be a sign of disrupted photosynthate partitioning to pods and seeds, which has also

been observed in some common bean genotypes (Omae et al., 2007; Soltani et al., 2019).

3.9 | Prospects for lima bean in heat-stressed environments

Genotype differences in yield and physiological response to heat suggest that there is an opportunity to develop cultivars of lima bean with superior heat tolerance. The few genotypes surveyed in this study captured a range of heat stress responses (Table 2), with different combinations of mechanisms conditioning tolerance or sensitivity. For example, heat-tolerant genotypes *Henderson* and *PI 534918* had similar levels of pollen release but substantially different viability of pollen in heat-stressed plants (Figure 5). A survey of a wider diversity of lima bean germplasm is likely to identify genotypes that are more heat tolerant than those tested here. Because lima bean is adapted to a warmer and more humid climate than other domesticated *Phaseolus* species (Bitocchi et al., 2017), it has potential value as a legume crop in heat-stressed environments, where it is currently underutilized. Lima bean's potential for productivity in such conditions could exceed that of common bean.

The reflowering ability of lima bean can lead to the avoidance or masking of heat stress responses. Therefore, selection for progress in yield will be most effective under sustained high nighttime temperature conditions. The current study identified pollen release, pollen vigor, and seed filling under heat stress as potential targets for germplasm screening and selection for adaptation to heat stress in lima bean. This study guides approaches for surveying variation in specific mechanisms of heat tolerance and highlights the possibility

of a multi-mechanistic approach for breeding heat-tolerant varieties in lima bean.

AUTHOR CONTRIBUTIONS

Emmalea G. Ernest: Conceptualization; formal analysis; funding acquisition; investigation; methodology; writing—original draft. **Randall J. Wisser:** Conceptualization; supervision; writing—review and editing.


ACKNOWLEDGMENTS

This work was supported by the Delaware Department of Agriculture, Specialty Crop Block Grants No. 12-25-B-1661 and 16SCBGPDE0040. The authors thank Danielle Watkins for assistance with the controlled chamber experiments.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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How to cite this article: Ernest, E. G., & Wisser, R. J. (2024). Multiple physiological mechanisms involved in reproduction could be targeted for breeding heat tolerance in lima bean. *Crop Science*, 64, 179–191. <https://doi.org/10.1002/csc2.21167>