

**PHYSIOLOGICAL EFFECTS OF
HIGH TEMPERATURES AND THE GENETIC
ARCHITECTURE OF HEAT STRESS RESPONSE
IN LIMA BEAN**

by

Emmalea Garver Ernest

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant and Soil Sciences

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ABSTRACT

Heat stress reduces yields of lima bean (*Phaseolus lunatus*) in the Mid-Atlantic Region of the US. High night temperatures during flowering reduce or delay pod set, resulting in delayed harvest, split pod sets and lower yield. Breeding heat tolerant small- and large-seeded lima beans is a high priority for the University of Delaware lima bean breeding program, but the physiological and genetic basis for lima bean's heat stress response was poorly understood. The purpose of this project was to determine the physiological bases for heat stress induced yield loss in lima bean, identify tolerant genotypes and develop heat response screening methods to use in breeding.

High nighttime temperatures reduce yield in some lima bean genotypes by reducing the number of pods set and the number of seeds per pod. High night temperatures do not reduce overall aboveground biomass, indicating that this type of temperature stress may have little effect on photosynthate availability. High night temperature has a significant effect on pollen release and viability, and in some genotypes results in changes to flower morphology that could interfere with pollination and fertilization. There is evidence that other aspects of reproduction, such as stigma receptivity or seed development are impaired by heat stress in some sensitive genotypes, but not others. The correlation of pollen quantity and yield under heat stress is present gene pool-wide. Heat sensitivity is not isolated to large-seeded Andean genotypes, but

such genotypes exhibit heat sensitivity in the post pollination stage, which was not apparent in Mesoamerican types.

Lanceolate leaflets are present in the lima bean gene pool and have been hypothesized to offer heat and drought stress tolerance advantages over the typical ovate leaflet shape. Shape descriptors, measured via image analysis, were used to characterize the leaflet shape for a diverse collection of lima bean germplasm. A few loci may be involved in determining leaflet shape, which is under strong genetic control. Lanceolate leaflet shape is conferred by one locus and could be incorporated into commercial germplasm if it is found to offer a stress tolerance advantage.

Chapter 1

INTRODUCTION

Lima bean (*Phaseolus lunatus*) is a minor legume crop originating in the Americas but having a worldwide distribution with importance to certain local economies and food systems. Total US lima bean production area in 2017 was 20,991 hectares (51,870 acres) and 58% of the production area is harvested at the succulent seed stage for processing (freezing or canning) or fresh market sales (USDA-NASS, 2019). The remaining 42% of the production area is harvested at the dry stage. Lima bean production area in the US has declined since 2007 with decreases in both dry and processing acreage (Figure 1). The number of farms producing dry limas has decreased slightly since 2007 but the number of farms producing processing limas has increased (Figure 2). The number of farms growing fresh market limas nearly doubled, although acreage remained steady, suggesting that there is increasing interest in growing lima bean on a small scale for fresh market sales.

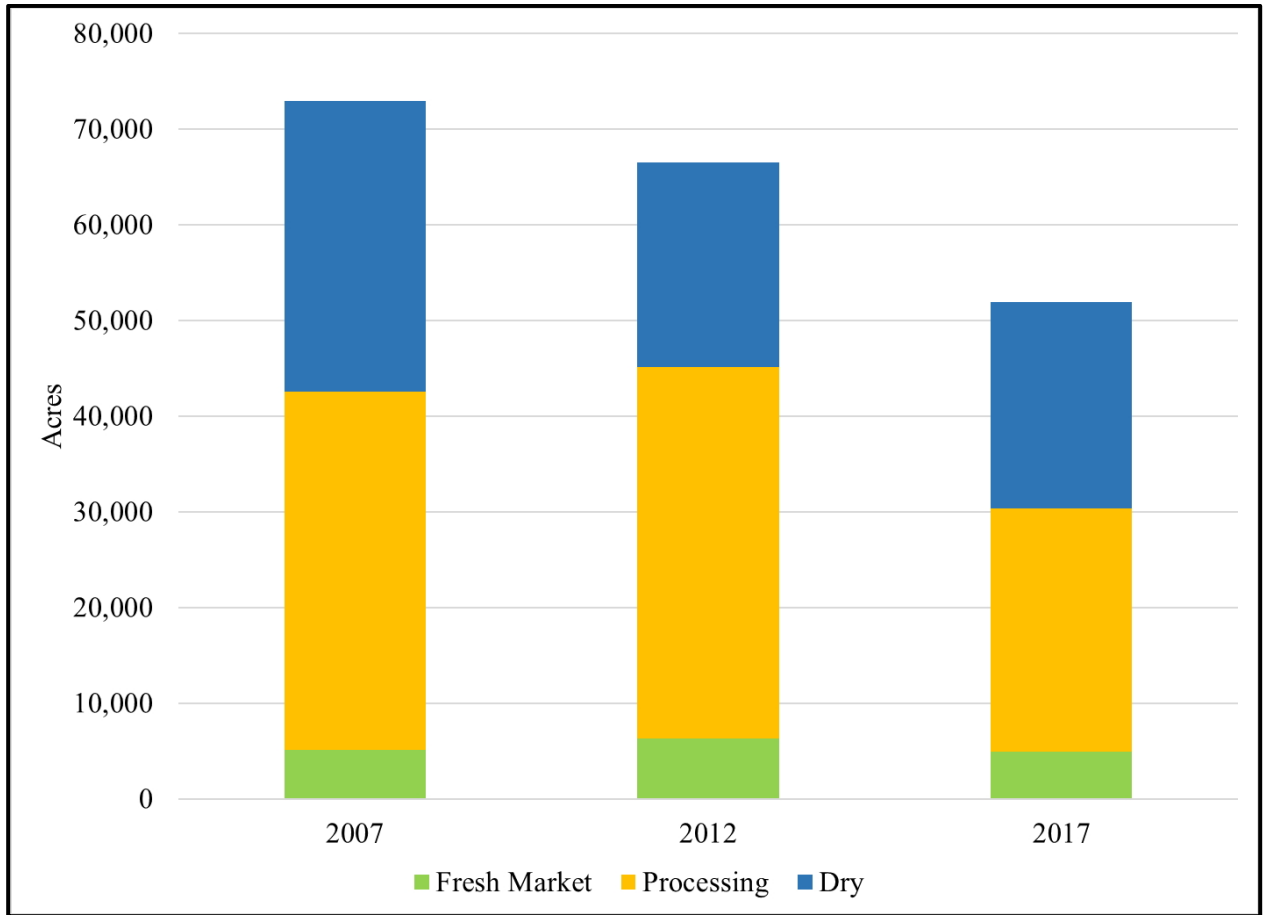


Figure 1 Lima bean production acreage by market type. Data from U.S. Censuses of Agriculture (USDA-NASS, 2009; USDA-NASS, 2014; USDA-NASS, 2019).

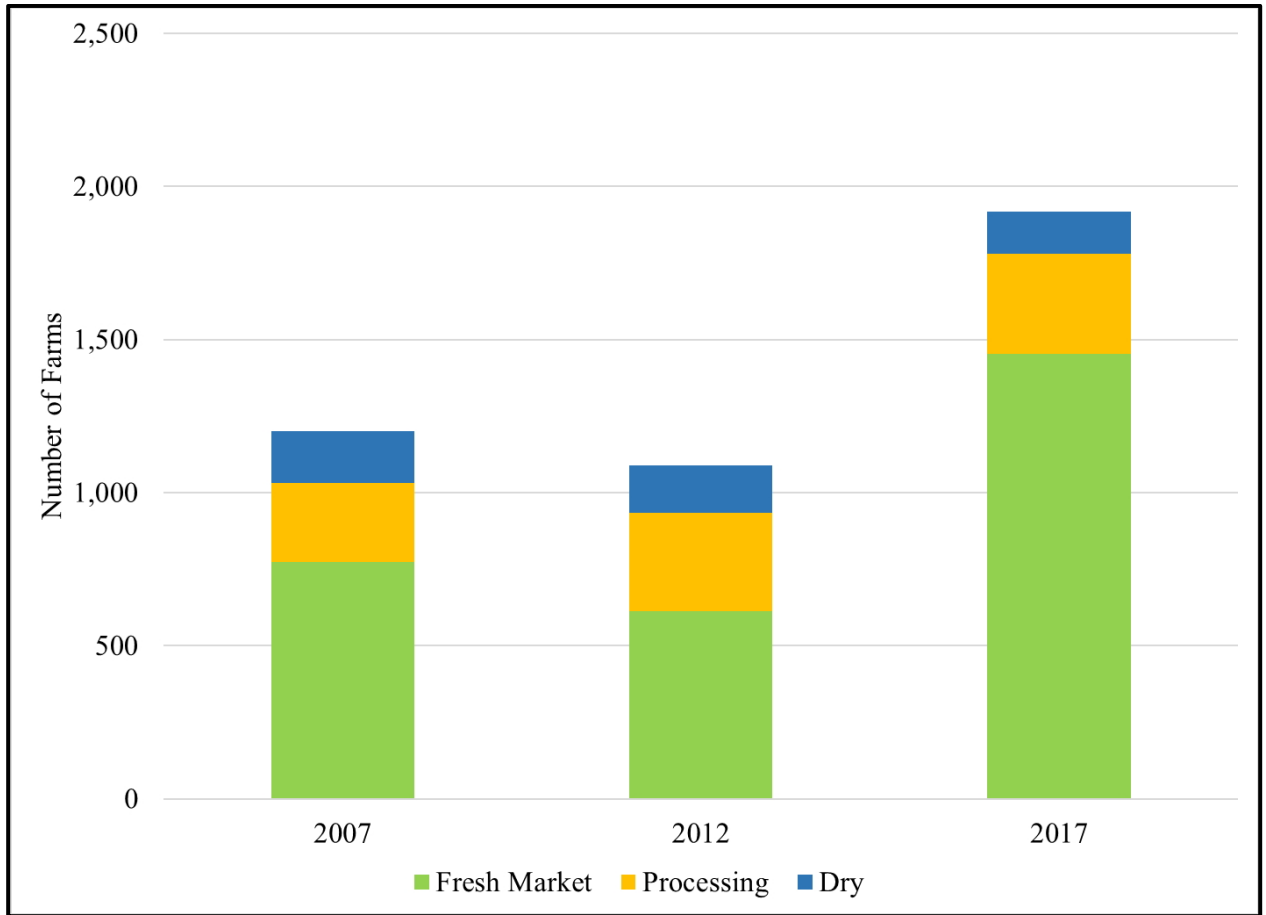


Figure 2 Lima bean producing farms by market type. Data from U.S. Censuses of Agriculture (USDA-NASS, 2009; USDA-NASS, 2014; USDA-NASS, 2019).

Fresh market lima bean production is located throughout the US with all but six states (Kansas, Nevada, North Dakota, Rhode Island, South Dakota and Wyoming) reporting production in 2017 (USDA-NASS, 2019). The largest number of producing farms is in the South (Figure 3). Processing production is concentrated in the Mid-Atlantic region with some production in the West and Northeast. Acreage is not reported for many of the Midwestern and Southern states reporting processing

production and a third of the processing production cannot be tied to a particular region from the 2017 Ag Census data (Figure 4). Dry lima production is nearly all located in the West with 89% of the production area in California.

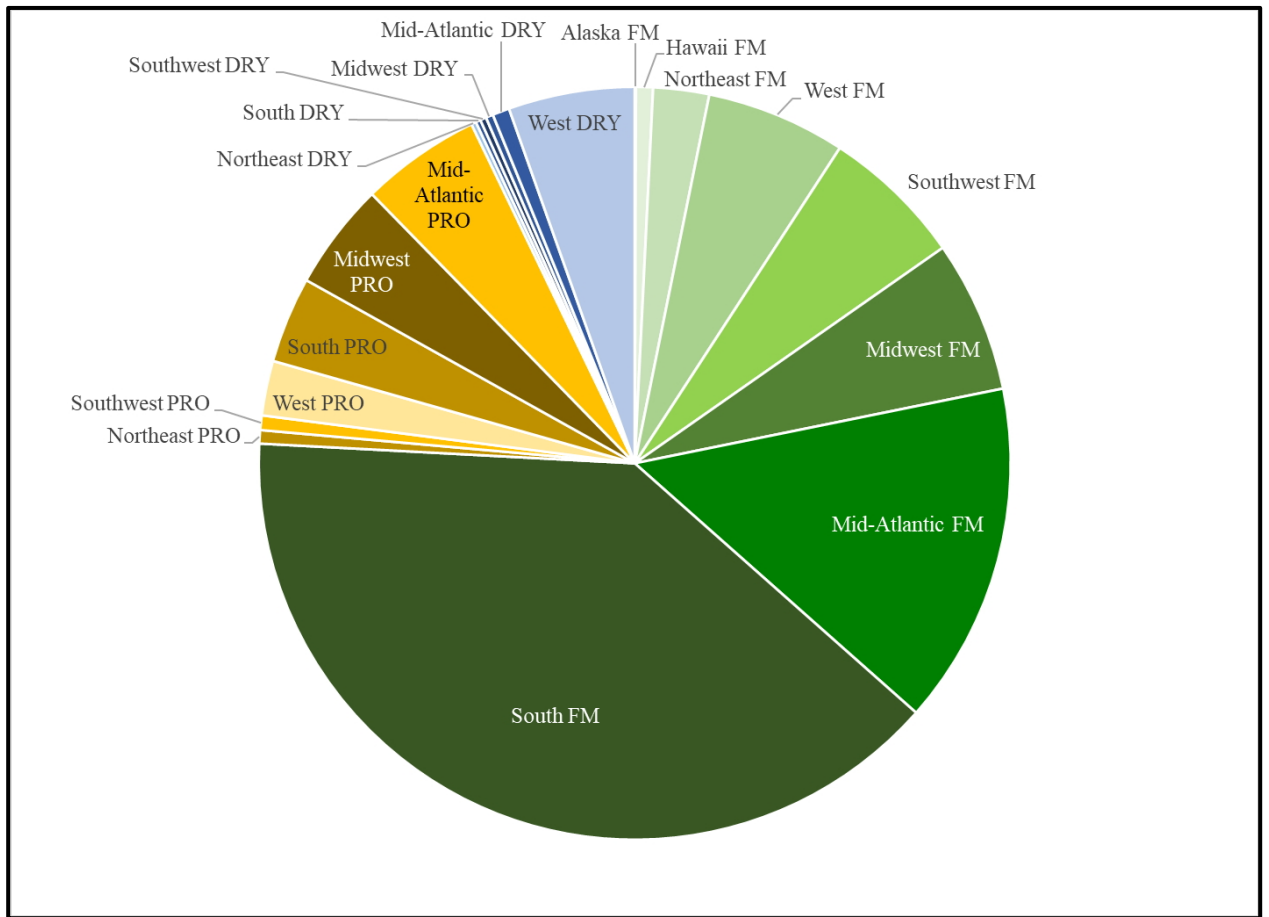


Figure 3 Lima bean producing farms by region and market type: fresh market (FM) is green, processing (PRO) is yellow/brown, dry is blue. Data from 2017 U.S. Census of Agriculture (USDA-NASS, 2019).

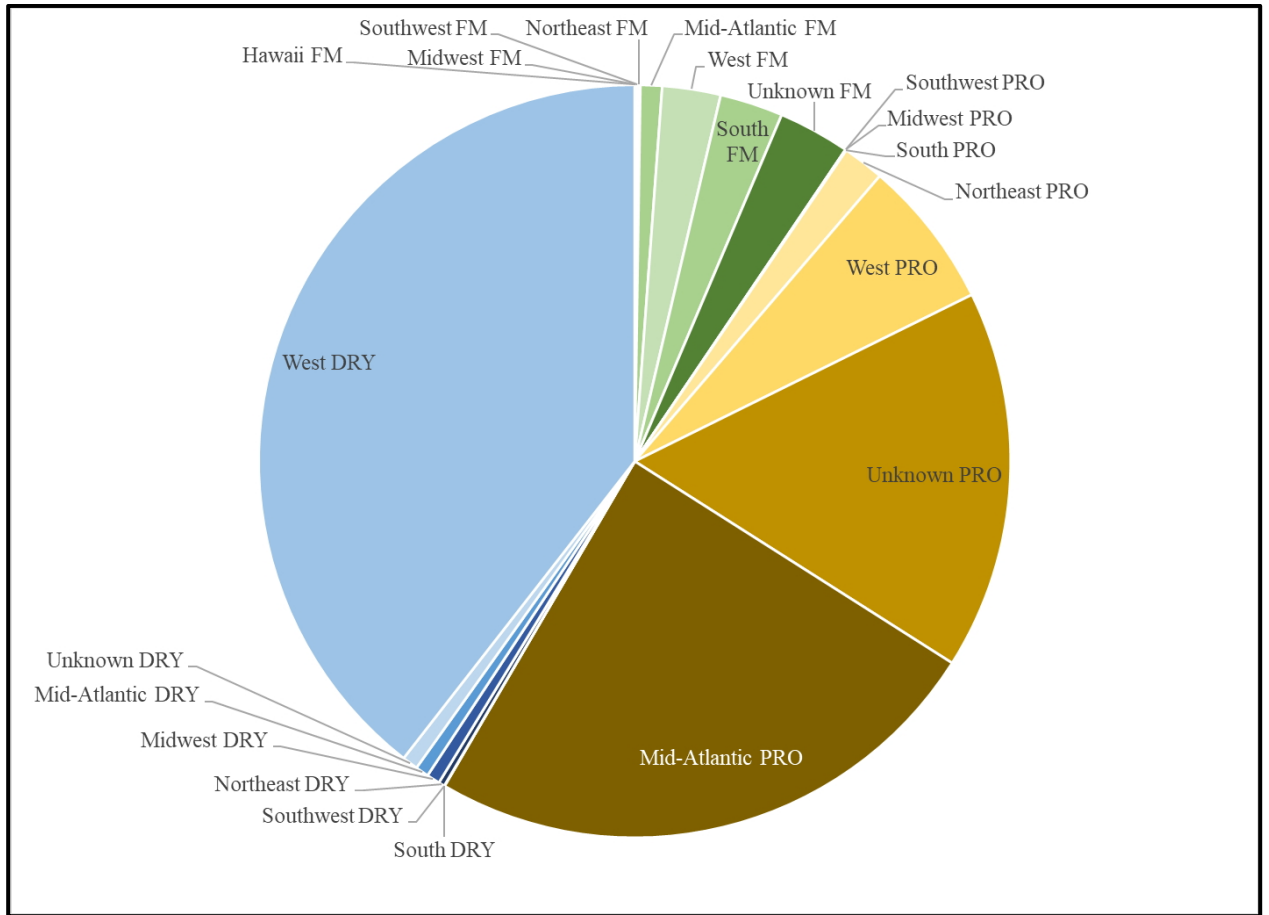


Figure 4 Lima bean production area by region and market type: fresh market (FM) is green, processing (PRO) is yellow/brown, dry is blue. Data from 2017 U.S. Census of Agriculture (USDA-NASS, 2019).

Domesticated lima bean exhibits a wide range of seed sizes (20-240 g/hundred seeds), shapes and seed coat colors and patterns, but only a few types are of commercial importance for US production. Most of the production in the Mid-Atlantic region (MAR) is green baby lima, which has a flat seed of 30-35 g/ hundred seeds with seed coat and cotyledons that retain green color at maturity. Two other small seeded types are grown on a limited scale: a buff and black speckled type with flat

seed (Jackson Wonder) and a plum, round white type (Dixie Butterpea). Fordhook types, which have large plump seeds, (80-110 g/hundred seeds) have historically been grown in the MAR and on the southern coast of California. Large, flat-seeded vining types are grown on trellises for fresh market sales. Most have green or white seed that is 170-240 g/hundred seeds. All the aforementioned types are harvested at the succulent stage. Dry lima production is concentrated in California, where white seeded baby and large limas are produced. The California climate allows vining types to be grown in the field without trellising, so both bush and vining cultivars of these two types are used (Long, *et al.*, 2014).

Processing in MAR

Currently, lima bean is produced the Mid-Atlantic region for both processing and fresh market sales. Baby and Fordhook lima beans are produced on a large scale and machine harvested for freezing or canning. Baby and Fordhook types, as well as vining types with large, flat seeds are produced for local fresh market sale. Green seeded types predominate but some speckled and white types are produced on a limited scale for processing.

Processing production of lima beans has been practiced in the MAR since the late 19th century (Kee, 2006). The Delmarva Peninsula's level fields and sandy soils can accommodate the large mobile viners used to harvest lima beans. The availability of irrigation and a long growing season continue to make the Delmarva an attractive location for lima bean production. To fully utilize their facilities, processors also contract with Delmarva farmers for production of green peas, which are harvested with the same equipment as lima beans, and a variety of other processing vegetables (Kee *et al.*, 1997). The opportunity to contract processing vegetables benefits farmers

by allowing them to diversify their crop rotations and business models, thereby managing financial and crop loss risks. The economics of production in the MAR are affected by prices of competing crops (corn and soybean); input costs; changes in weed, insect and disease pressures; consumer demand; and availability of lima beans from other regions. There are currently three vegetable processing companies contracting with farmers for lima bean production in the MAR. One other long-time lima processor ceased operations in 2019. Lima beans have been viewed as a stabilizing factor in the Mid-Atlantic processing vegetable industry (Kee *et al.*, 1997) however their continued production in the region is at risk because high temperatures, weed and disease pressure, and competition from other production regions.

Lima bean acreage in Delaware was at its peak from the late 1940s through the late 1960s (Figure 5). More recently, acreage was declining from a peak in the late 1990s. The USDA, National Agricultural Statistics Service has not reported annual acreage, yield or price statistics for Delaware since 2012, but based on 2017 Census of Agriculture data, Delaware lima bean acreage had further declined to 9,880 acres (USDA-NASS, 2019).

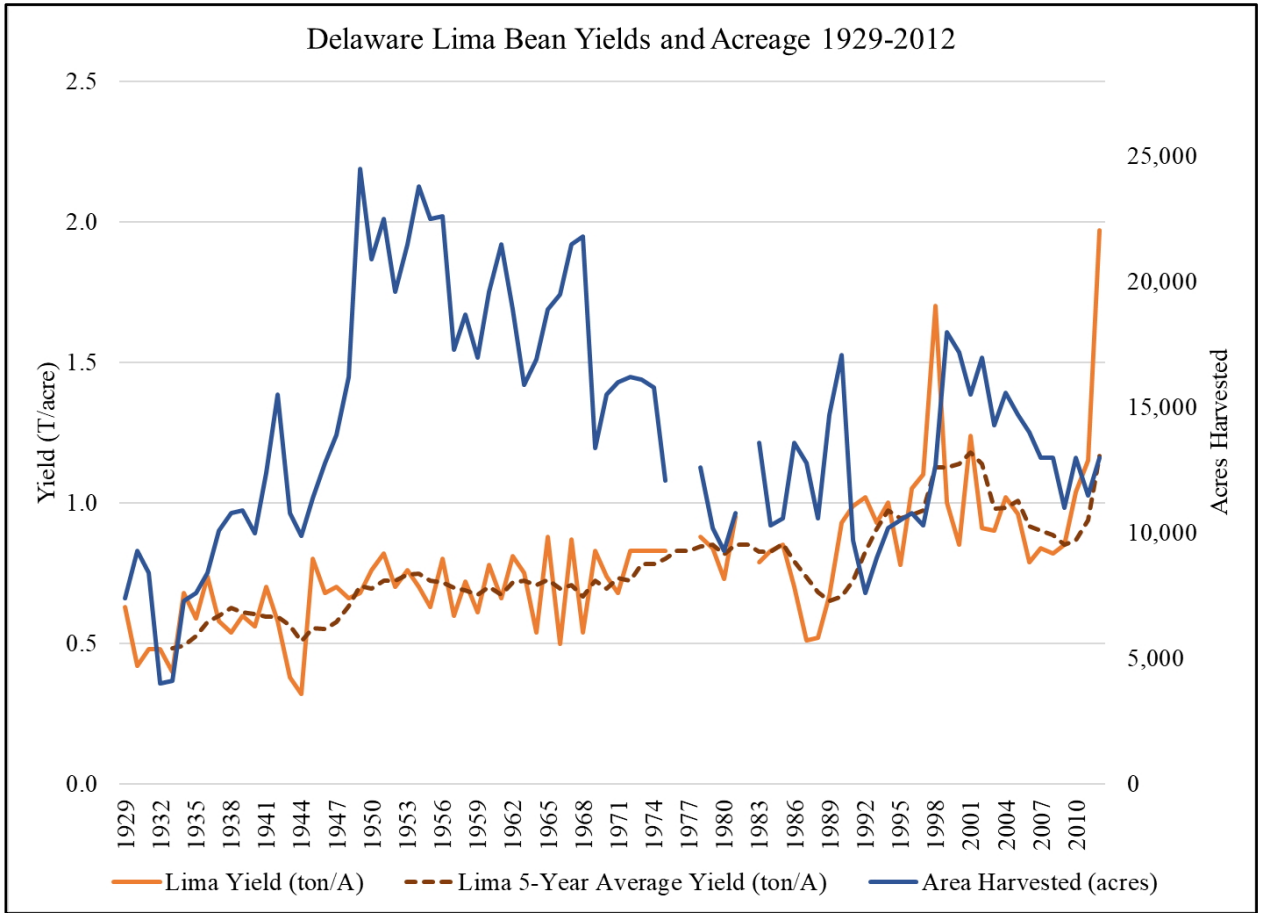


Figure 5 Delaware lima bean yields and acreage from 1929 to 2012 compiled from Smith & Witzig, 1976 and Delaware Agricultural Statistics Summaries from 1984 through 2012-2013.

Producing a high-quality product, free of beans that are over mature or affected by pathogens, is a challenge for processors and growers. High temperatures have been observed to cause quality problems by interrupting pod set, causing some pods to mature much earlier than others (split set) (Kee *et al.*, 1997). Seeds that have reached physiological maturity do not have the preferred taste profile and can sprout or become infected with fungi when exposed to moisture from rain, irrigation or dew.

Harvest lots of mixed quality beans must be subjected to costly cleaning and sorting by processors. If pod set has not begun before heat exposure a split set may be avoided, but pod set is delayed, causing many fields to mature at the same time and wreaking havoc with harvest and processing schedules and resulting in lost revenue for growers and processors.

Additionally, being a minor crop, lima bean has not received much attention from plant breeders. Contrasted with yields of green pea, which is supported by several active private breeding programs, lima yields in Delaware have been stagnant for about 50 years, whereas pea yields in the state have increased (Figure 6).

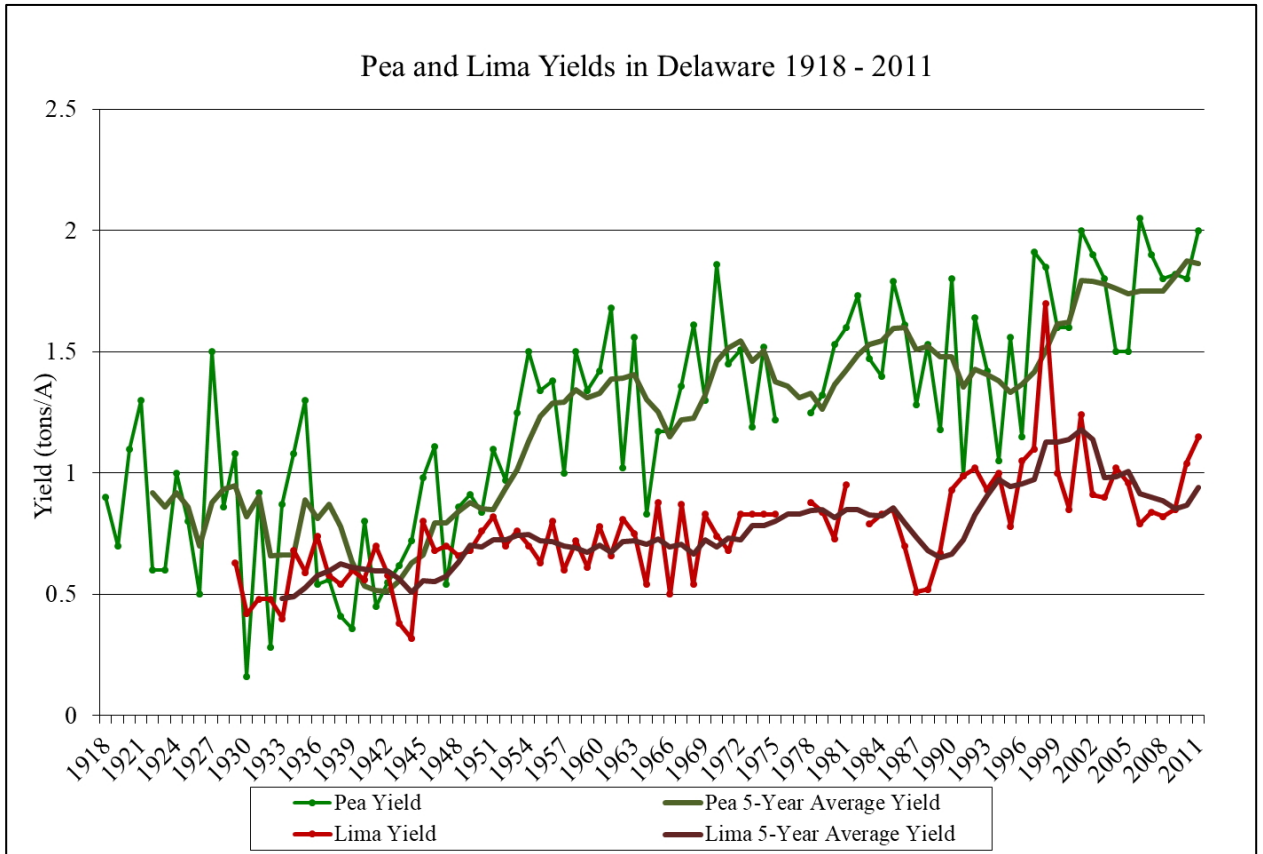


Figure 6 Delaware lima bean and pea yields from 1918 to 2011 compiled from Smith & Witzig, 1976 and Delaware Agricultural Statistics Summaries from 1984 through 2012-2013.

Lima Bean Breeding Programs

USDA, Beltsville

USDA scientists based in Beltsville, Maryland developed and released improved lima bean cultivars and germplasm from the 1930s through 1989 (Stavelly, 1991). The combination of high yield and green seed coats for Fordhook and baby lima types was one aim of the breeding program (Stavelly, 1991). From 1948 onward, downy mildew (*Phytophthora phaseoli*) resistance became a major goal of the

breeding program for both baby and Fordhook types. New races of the pathogen continued to emerge as multiple resistance genes were identified and incorporated into commercially acceptable lines. After resistance to four races of downy mildew was incorporated into cultivars, no new races were reported for 20 years and the disease was managed by the use of resistant varieties (Santamaria *et al.*, 2018). Several of the green baby lima cultivars currently used in US production were selected from USDA's downy mildew resistant germplasm releases, B2C and C171, or are derived from this material (Ben Fish & Son, PVP 76TQ001, 1977; and Ben Fish & Son, PVP 7700005, 1978). Even though the USDA germplasm and derived cultivars no longer offer complete resistance to downy mildew with the emergence of downy mildew races E and F (Evans *et al.*, 2002; Santamaria *et al.*, 2018), this material still has desirable yield and quality attributes.

University of California

The University of California has an active lima bean breeding program which has released white-seeded cultivars for harvest at the dry seed stage. Both baby and large-flat seeded lima beans are being improved in the California program. Recent cultivars released were selected for resistance to root-knot nematode (*Meloidogyne incognita* and/or *M. javanica*) and lygus bugs (*Lygus* spp.) (Long *et al.*, 2014).

University of Delaware

The University of Delaware scientists collaborated with the USDA lima breeding program when it was active, but lima breeding and selection in the eastern US ceased when the USDA program ended in 1989. The current University of Delaware lima bean breeding program was initiated in 2004 by the author.

Improvement efforts are focused on three seed types: green baby, Fordhook and large-flat. Experimental lines with other seed types have been developed and tested incidentally as they result from making genetically diverse crosses.

The breeding goals and approach vary depending on seed type, but a pedigree breeding system with two generations per year (one in the greenhouse and one in the field) forms the backbone of the program. Generally, biparental crosses are made in the greenhouse in December and F_1 seed is planted in the greenhouse in March. The F_2 seed is planted in the field in June with selections made in October. Subsequent odd generations are grown in the greenhouse and even generations in the field for selection. F_7 single seed selections are grown in the greenhouse to obtain F_8 seed for initial replicated yield trials. Lines that perform well in first-year trial are increased by a winter nursery in Puerto Rico for yield trials in subsequent years. Breeder seed of experimental lines and varieties is maintained in the greenhouse because of the difficulty of producing quality seed in the field in Delaware and the high frequency of outcrossing by insects.

The number of baby and Fordhook breeding lines grown during the field season each year of the UD breeding program is shown in Table 1. An average of 23 baby lima crosses and nine Fordhook crosses were made each year, although this number varied widely from year to year as new germplasm became available and priorities and projects shifted. The number of baby lima lines being yield tested each year reached 67 in 2015 and has remained at this level as this is the capacity of the current yield data collection system. No breeding lines were planted in the field in 2018 because extremely wet field conditions prevented planting in the mid to late June time period.

Table 1 Number of Breeding Lines Grown in the Field Each Year of the University of Delaware Lima Bean Breeding Program

Year	Baby Lima Breeding Lines					Fordhook Lima Breeding Lines			
	F ₂	F ₄	F ₆	1 st Year Trial	Advanced Trial	F ₂	F ₄	F ₆	Trial
2005	25								
2006	23	47				27			
2007	0	11	38			20	35		
2008	0	0	5	12		7	17	26	
2009	22	0	0	3	9	14	25	36	8
2010	18	37	0	0	11	4	12	43	28
2011	25	49	55	0	9	4	27	16	28
2012	43	75	71	14	5	13	6	5	19
2013	36	117	108	26	12	15	54	5	14
2014	36	135	124	24	21	4	29	61	18
2015	20	42	119	32	35	0	15	30	37
2016	7	23	87	22	31	0	0	38	33
2017	37	47	5	35	33	5	0	0	26
2018	0	0	0	29	33	0	0	0	0
2019	47	73	113	39	31	9	13	0	15
Average	23	47	56	20	21	9	18	22	21

Much of the breeding program's effort is devoted to improving green baby limas, which occupy the majority of the acreage in the Mid-Atlantic region. Germplasm used in baby lima breeding was obtained from the USDA germplasm collection, the UC breeding program, commercial seed suppliers and James Beaver at University of Puerto Rico, Mayaguez. Green baby limas developed by the USDA and landraces from the southern and southwestern U.S. have been particularly useful. Aside from yield, breeding goals for green baby limas are heat tolerance, upright plant habit to improve harvest efficiency and possibly disease avoidance, concentrated pod set, early maturity, downy mildew resistance and root-knot nematode (*M. incognita*) resistance. Greenhouse and field screening methods are being used to select for downy

mildew and root-knot nematode resistance. Single seed descent has been used in populations being selected for heat tolerance and downy mildew and nematode resistance.

Fordhook limas continue to interest processors in the Mid-Atlantic region but reliable production has not been achieved due to heat sensitivity. Heat tolerance is presently the primary goal of Fordhook breeding activities, but some downy mildew resistant experimental varieties have been selected. Green seed is also prioritized in selection as this trait is preferred by processors. Landrace germplasm from the Andean region and Africa, and the USDA's green-seeded and downy mildew resistant Fordhook releases have been useful in improving this seed type.

Large flat seeded vining types are grown for fresh market sales throughout the Mid-Atlantic region. About fifteen regional landraces have been donated to the program by growers. Other varieties and landraces of this type were obtained from the USDA germplasm collection or seed suppliers. As with Fordhooks, heat sensitivity is the major limitation for this type and heat tolerance is the primary breeding goal. Green seed is again a market requirement.

Lima Bean Diversity Panel

In order to better understand and utilize diverse lima bean germplasm in the Delaware breeding program a panel of diverse lima bean germplasm was assembled. Much of the material was obtained from the USDA germplasm collection, but some was collected in the MAR from seed savers, submitted by the UC breeding program, purchased from home garden seed suppliers or obtained from a recent collection of Caribbean landraces (Montero-Rojas *et al.*, 2013).

Approximately half of the panel (51%) is US cultivars or landraces, with half of those lines obtained from western locations and half from eastern ones (Figure 7). The other half of the panel is from outside the US with 18% from South American, 13% from Central America and Mexico, 13% from the Caribbean and 4% from Africa, Asia or Europe. Except for 9 accessions, the 125 lines from outside of the US are photoperiod sensitive and will not flower in time to produce pods under summer field conditions in Delaware. This creates additional challenges when using this material in the breeding program or screening it for traits of interest.

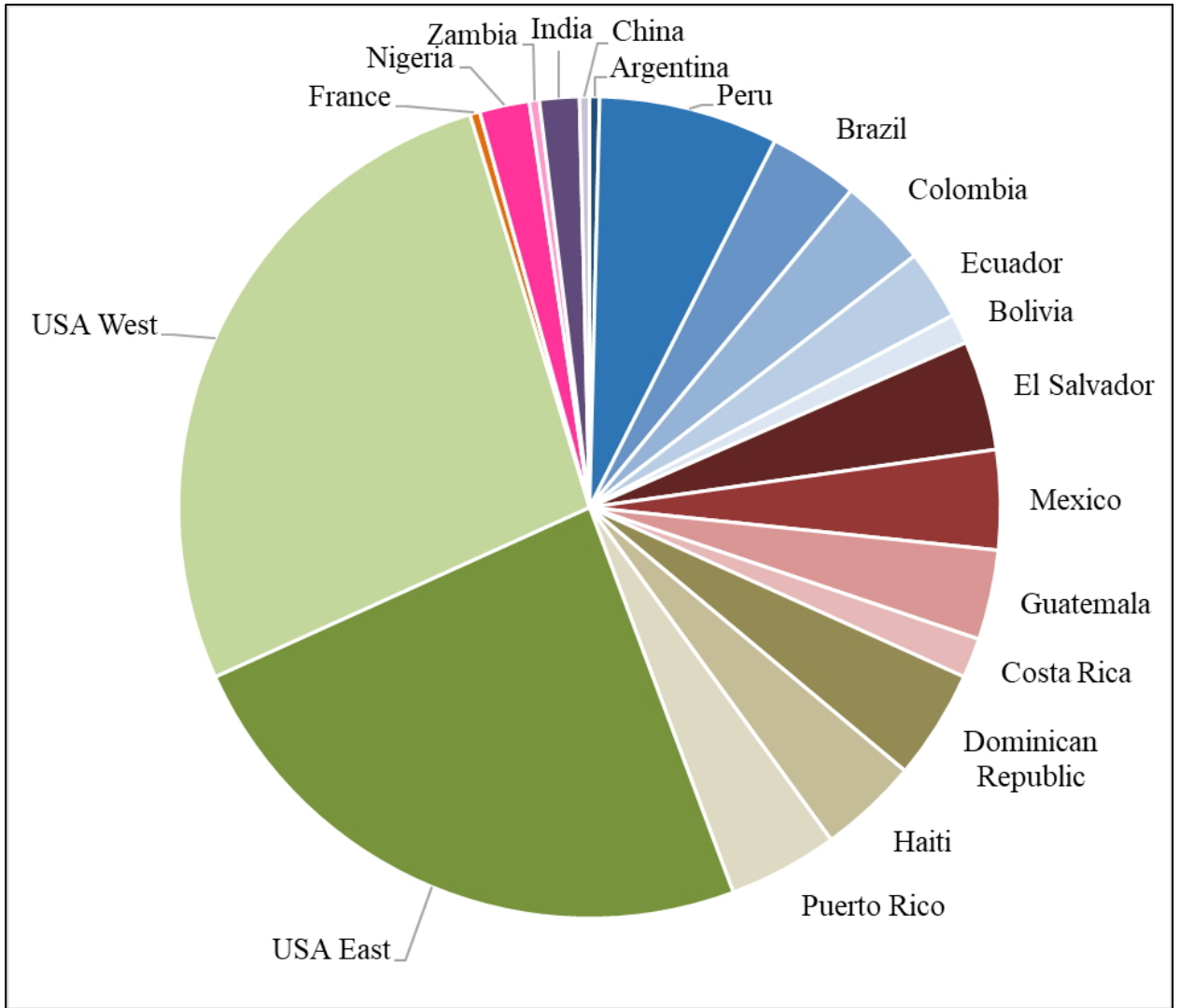


Figure 7 Country of origin for the 255 accessions that comprise the lima bean diversity panel.

Most of the diversity panel accessions (232) have been genetically characterized using Genotyping-by-sequencing (GBS) with the goal of using the genotype data for association studies in traits of interest (Mhoro, 2018). The diversity panel was used as a part of this project to understand the distribution of heat

tolerance/sensitivity across the lima bean gene pool and explore the genetic basis for this trait. The panel was also used to test methods to characterize leaflet shape and identify loci associated with this trait.

Heat Stress Response in Lima Bean and Other Legume Crops

As discussed in previous sections, heat stress is a major threat to current and continued lima bean production in the MAR. At a forum held on December 11, 2012, lima bean growers and processors identified heat and split sets as the top yield limiting factor for the crop's production in Delaware, and heat tolerance as the highest priority trait for improvement in the crop. Thereafter, heat tolerance became the priority trait for improvement in the Delaware lima bean breeding program and a major focus of the author's research and Extension program.

The inheritance or mechanism of heat tolerance in lima bean has not been studied, but a few lima bean genotypes are reported to be heat tolerant. Wester (1961) reported PI 347786 (Hopi 5989) to be heat tolerant. In a greenhouse experiment comparing yield of lima bean genotypes grown under cool conditions and hot conditions Dixie Butterpea, a landrace, and 'UC Luna' were identified as highly heat tolerant cultivars (Kleiner and Frett, 1996). The landrace Jackson Wonder was also reported to be heat tolerant (Keeler, *et al.*, 1996).

Heat stress tolerance has been more thoroughly investigated in other legume crops, including cowpea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*). For cowpea, yield under heat stress has been substantially improved through breeding (Hall, 2004) and the work in this crop may provide insight as to how to achieve heat tolerance in lima bean. For cowpea, tolerance to heat during anther development was associated with a single dominant gene in populations derived from crosses involving

two different heat tolerant and three different heat sensitive genotypes (Marfo and Hall, 1992). More recent work with a broader range of germplasm has revealed both major gene and minor gene involvement in cowpea heat tolerance (Lucas *et al.*, 2013; Angria, 2016). Progress in breeding cowpea for heat tolerance during reproductive development has been made through several generations of selection of families with nearly all plants exhibiting high pod sets under high night temperatures, followed by single plant selections from within highly heat tolerant families (Hall, 2004).

Similar to cowpea, high night temperatures are damaging to reproductive structures of common bean. Physiological effects of high night temperatures before anthesis include anther indehiscence and pollen inviability (Gross and Kigel, 1994; Porch and Jahn, 2001).

Rainey and Griffiths (2005b) investigated the inheritance of reproductive heat tolerance in common bean. They observed heterosis for heat tolerance in the F1 generation, which has been reported by other researchers working with this trait in common bean (Shonnard and Gepts, 1994; Arndt and Gepts, 1990). Additionally, genes from both the heat sensitive and the heat tolerant parent positively contributed to yield component measurements (pod number and seeds per pod) under heat stress. Inheritance of heat tolerance of reproductive structures, however, (as measured by percent of reproductive organs abscised under stress) was conditioned by a single dominant gene (Rainey and Griffiths, 2005b). Screening and selection for heat tolerance may be more successful in later generations, because of the increased heat tolerance observed in early generations and attributed to heterozygosity may not be heritable. Wasonga *et. al* (2012) report successful development of heat tolerant snap bean lines with heat tolerance screening done in the F3 generation in the greenhouse

and in F5 generation using multiple screening locations. In some common bean genotypes high temperatures also interfere with seed maturation and pod filling, resulting in small seed size (Rainey and Griffiths, 2005a; Shonnard and Gepts, 1994).

In some genotypes, the effect of heat stress on common bean yield results from interruption of photosynthate transport, not direct effects on photosynthesis (Soltani *et al.*, 2019). Direct effects on photosynthesis have not been ruled out as a heat stress effect in common bean because a limited range of germplasm has been characterized for photosynthetic rate and carbon accumulation. Also, heat stress results in physiological changes to common bean leaf area, stomatal density, chlorophyll content and expression of genes related to thermotolerance and oxidative stress, with genotype differences observed for some of these responses (Lizana *et al.*, 2006; Wentworth *et al.* 2006; Soltani *et al.*, 2019). Drought stress, which often accompanies heat stress, suppresses photosynthesis directly (Miyashita *et al.*, 2004). In many environments, heat and drought stress occur together and for such situations tolerance traits for both heat and drought must be combined to achieve high temperature tolerant varieties (Mittler, 2006). Heat and drought stress tolerance of reproductive and vegetative structures will be important to realize yield gains in genotypes with reproductive heat tolerance in lima bean.

Project Objectives, Hypotheses and Approaches

Objective 1: Characterize heat stress associated yield loss in lima bean

Hypothesis: High night temperatures reduce yield in lima bean.

Approach: Use controlled greenhouse chamber experiments to determine which yield components are affected by heat stress. Use multiple years of field trial data to determine if there is temperature associated yield loss and delayed maturity under field conditions in Delaware.

Hypothesis: Genotypes differ in their heat tolerance.

Approach: Expose diverse lima bean genotypes to heat stress and unstressed conditions in controlled greenhouse chamber experiments and characterize their yield response.

Hypothesis: Growth stages and/or plant organs differ in their heat tolerance.

Approach: Use multiple years of field trial data to determine the growth stage most sensitive to high temperatures. Determine physiological effects of heat stress on plants in greenhouse experiments.

Objective 2. Determine heat stress effects on reproductive structures in lima bean

Hypothesis: Heat stress reduces pollen quality and quantity

Approach: Determine heat effects on lima bean reproductive structures in controlled greenhouse chamber experiments that test: (1) Quantity of pollen shed onto pistil, (2) Anther dehiscence/indehiscence, and (3) In vitro pollen germination

Hypothesis: Heat stress impairs stigma, style and/or ovule function.

Approach: Apply high quality pollen to the flowers of heat stressed plants to test stigma/style/ovule function under heat stress.

Hypothesis: Heat stress effects on reproductive structures will be correlated with heat stress response across the gene pool and can be used to select for heat tolerance

Approach: Characterize heat stress effects on yield and reproductive structures for a set of diverse genotypes.

Hypothesis: Heat tolerance/susceptibility is genetically controlled.

Approach: Use genome-wide association analysis of a diversity panel to identify genome regions associated with heat stress responses.

Objective 3: Characterize leaflet shape for a lima bean diversity panel and its genetic basis.

Hypothesis: Diverse leaflet shapes in lima bean can be efficiently characterized using shape descriptors measured using image processing.

Approach: Develop protocol to use image processing to measure length to width ratio (aspect ratio), roundness, and circularity of scanned lima bean leaflets and describe leaflet shapes of the 255-member lima bean diversity panel.

Hypothesis: Leaflet shape is heritable and loci controlling leaflet shape characteristics can be identified.

Approach: Use association mapping based on GBS data to identify possible loci associated with aspect ratio, roundness and circularity measurements.

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Chapter 2

GROWTH STAGE AND GENOTYPE DIFFERENCES IN HIGH TEMPERATURE STRESS IMPACT ON YIELD COMPONENTS AND DAYS TO HARVEST IN LIMA BEAN (*PHASEOLUS LUNATUS* L.)

Introduction

Plants have evolved under a wide range of temperature conditions, with minimum, maximum and optimum temperatures for growth varying by species, genotype and stage of development. Temperatures above the maximum initiate an array of physiological and biochemical changes which can result in yield and quality loss in crop species (Hatfield and Prueger, 2015). Crop germplasm improvement is one strategy for reducing the impacts of temperature on agricultural systems (Hatfield *et al.*, 2011), but effective selection is dependent upon identifying the most heat susceptible growth stages and understanding the physiological effects of high temperature stress (Porch and Hall, 2013).

Lima bean (*Phaseolus lunatus*) is a minor legume crop with importance to certain local economies and food systems throughout the world. Based on heat killing time for leaf tissue, lima bean and tepary bean (*P. acutifolius*) are considered more heat tolerant than common bean (*P. vulgaris*) and runner bean (*P. coccineus*) (Marsh & Davis, 1985). However, lima bean germplasm has not been screened extensively for heat tolerance and yield loss has been attributed to heat stress in some production areas (Kee *et al.*, 1997). Lima bean production in the United States is concentrated in California and on the Delmarva Peninsula. California production includes 20,000

acres of dry lima bean and about 2,400 acres harvested at the succulent seed stage for fresh market, canning or freezing (USDA-NASS, 2019). Production on the Delmarva Peninsula, which comprises about 12,000 acres annually, is exclusively for harvest at the succulent stage, with the vast majority going to three regional vegetable processors for freezing (USDA-NASS, 2019). On Delmarva, lima bean is a key component of the regional processing vegetable industry (Kee *et al.*, 1997). In Delaware, total processing vegetable production was valued at \$30 million in 2012 and lima bean at \$11 million (USDA-NASS, 2015). Processing vegetable production on Delmarva offers farmers profitable opportunities to diversify their cropping systems and their businesses.

On the Delmarva Peninsula, the planting window for lima bean is from mid-May through mid-July. Harvest at the succulent stage is 70 to 90 days after planting. Time to maturity is dependent upon cultivar differences and whether temperature and drought conditions cause a prolonged flowering period, commonly described as split-set. Split sets result in asynchronous pod maturity, making timing of harvest difficult and causing an overall reduction in yield. (Kee *et al.*, 1997). Growers and processors have identified split sets and yield loss attributed to heat stress as the most yield limiting factors for lima bean production on Delmarva, and heat tolerance as the highest priority trait for improvement in the crop. Development of heat tolerant lima bean cultivars, which producers can use to mitigate yield loss from high temperatures is timely given that global surface temperatures have increased by nearly 1°C over preindustrial levels and are likely to increase by an additional 1°C by 2100 (Stocker *et al.*, 2013).

Lima bean produces abundant flowers, indeterminately on existing racemes, until a capacity set of pods is reached. When pods do not set, flowering is prolonged which can result in a split set as pods form over a longer period (Cordner, 1933). In growth chamber experiments, lima bean plants exposed to identical daytime temperature produced lower yields when nighttime temperatures were hot (27°C) rather than cool (16°C). High night temperatures result in increased flower production but not increased pod set (Fisher & Weaver, 1974). Depletion of carbohydrates due to higher respiration at higher temperatures was hypothesized as the explanation for reduced pod set under high night temperature conditions (Fisher & Weaver, 1974). Work in a related species, common bean, has shown that high temperatures directly affect reproductive structures causing anther indehiscence and pollen inviability (Gross and Kigel, 1994; Porch and Jahn, 2001, Prasad *et al.*, 2002). Soltani *et al.* (2019) found no detrimental effects of temperatures of 35/25 °C (day/night) on photosynthesis when comparing heat sensitive and heat tolerant kidney bean genotypes. Rather, there was evidence for heat induced disruption of photosynthate transport from leaves to flowers and pods. Omae *et al.* (2007) had previously described heat induced changes to photosynthate partitioning after pod set, with different responses in heat sensitive and tolerant genotypes.

Compared to other domesticated *Phaseolus* species, lima bean is adapted to warmer, more humid climates (Bitocchi *et al.*, 2017) and could have potential for genetic improvement for adaptation to hot, humid climates beyond that of common bean. To develop screening methods for breeding heat stress tolerant lima bean, the susceptible growth stages and physiology of heat stress associated yield loss must be determined. To this end, yield, maturity and weather data from eight years of cultivar

trials was analyzed to confirm the anecdotal link between high temperatures and yield loss and high temperatures and delayed maturity in lima bean under field conditions. Additionally, greenhouse chamber experiments were used to identify heat tolerant and heat susceptible lima bean genotypes and to characterize their yield response to high night temperature stress.

Materials and Methods

Field Trials

Yield trials were 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. Each trial included 23 to 57 genotypes. Five genotypes that were included in all eight trials ('Cypress', 'C-elite Select', 'Bert', 'Brooke', and DE0505002A) were used in the analysis presented. Genotypes were planted in one-row plots with 76 cm between row spacing and 7.6 cm in-row spacing. Plots were 7.62 m in length. The cultivar 'Cypress' was planted in every other row as a border between experimental plots. Plots were arranged in a randomized complete block design with four replications. 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% UAN. Plots were cultivated 1 to 3 times, depending upon soil conditions. A sidedress application of 42 kg/ha nitrogen in the form of 30% UAN was made 35 to 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.

Per-plot harvest timing decisions were made based on visual evaluation. 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 section from each plot was harvested. The 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 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 base 10 °C growing degree days (GDD) were calculated for each season and average maximum and minimum temperatures were calculated for each interval of 100 GDD.

Kendall's Tau-b correlation coefficients (Bonett and Wright, 2000) 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 and Hochberg, 1995) was used to identify correlation coefficients that were significantly different from zero with a 20% false discovery rate.

Germplasm

Eight lima bean inbred lines were evaluated for yield response to sustained high night temperatures in greenhouse chambers (Table 2). All genotypes had a determinate growth habit. Seven of the lines were cultivars or cultivated material from the USA. The eighth was a landrace from Haiti. 'Cypress' and 'C-elite Select' are the

standard green baby lima cultivars grown for processing on the Delmarva Peninsula and ‘Fordhook 242’ is the standard Fordhook cultivar grown in the region.

Table 2 Genotypes tested for response to high night temperatures stress, their seed size, seed color, origin and improvement status

Accession Number and/or Name	100 Seed Weight (g)	Seed Color	Origin	Improvement Status
PI 549464 ‘Fordhook 242’	114	white	USA	cultivar
PI 549509 ‘Bush Florida Butter’	46	buff/black	USA	cultivar
PI 549466 ‘Henderson Bush’	46	white	USA	cultivar
PI 534918 Cave Dweller	43	magenta	USA	cultivated
PI 549508 ‘Bridgeton’	40	green	USA	cultivar
‘Cypress’	38	green	USA	cultivar
‘C-elite Select’	32	green	USA	cultivar
G27525	28	buff/magenta	Haiti	landrace

Temperature Treatment

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 ten-minute intervals with data loggers (WatchDog A150, Spectrum Technologies, Plainfield, IL) at canopy level and shielded from the sun. One chamber was set to maintain hot nighttime temperature and the other cool nighttime temperature. Night temperature averaged 28 °C in the hot chamber and 21°C in the cool chamber. Target daytime temperatures for both

chambers were 31 °C and averaged 30 °C in the cool night chamber and 32 °C in the hot night chamber.

For each experimental unit, two scarified seeds were sown into peat based growing medium (Pro-Mix BX) in 3.8 liter nursery pots 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 night temperature chamber until primary leaves had fully expanded (20 days after planting) then were randomly assigned to one of the two temperature regimes. Within each chamber, genotypes were arranged in a randomized complete block design with 4 to 6 plants per genotype x temperature treatment. Plants were fertilized with 15g of controlled release fertilizer (Nutricote Total 13-11-11 Type 100) at 20 DAP.

Five experiments were conducted with planting dates of 3 Nov. 2014, 12 Mar. 2015, 11 Mar. 2016, 15 Mar. 2017, and 5 Nov 2018. 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 of three genotypes grown under the two temperature regimes. Shoot dry weight (including weight of threshed pods) and total aboveground dry weight (shoot dry weight + seed weight) was determined for the 2016 and 2017 experiments.

Data Analysis

Three genotypes were included in all three years: Fordhook 242, C-elite Select and Bush Florida Butter. Yield components and days to flowering for all years for these genotypes were analyzed as a split-plot design in Proc-Mixed (SAS Version 9.4; SAS Institute, Cary, NC) with temperature for the main plot and genotype for the

subplot. The Tukey-Kramer method was used to separate treatment means at $\alpha=0.05$. Shoot dry weight and total aboveground dry weight were analyzed in the same way except that year was not included in the model because only two years of data were available. Heat Susceptibility Index (HSI), according to the formula of Fischer and Maurer (1978), was calculated using weight of seed yield for all genotypes for each year. Only the three genotypes tested in all years were used to calculate the stress intensity.

$$\text{HSI}=(1 - \text{YH}/\text{YC})/\text{D where}$$

YH=mean yield under heat stress, YC=mean yield under cool conditions and D= stress intensity = (1 – mean yield of standard genotypes under heat stress/mean yield of standard genotypes under cool conditions). $\text{HSI} \leq 0.5$ is considered highly heat tolerant, $0.5 < \text{HSI} \leq 1.0$ is moderately heat tolerant and $\text{HSI} > 1.0$ is heat sensitive.

Results and Discussion

Correlation Between Temperature and Yield, Days to Harvest in Field Trials

Maximum daily temperatures from 0 to 1200 accumulated GDD during the eight trials ranged from 38.4 to 20.7 C. Minimum daily temperatures in the same period ranged from 27.8 to 7.8 C. Overall, the average maximum and minimum temperatures were positively correlated, although in some GDD intervals the correlation between the two was not significant (Table 3).

Table 3 Kendall's Tau-b correlation coefficients for average maximum versus minimum temperatures for 100 GDD intervals

Accumulated GDD	Max vs Min	
	τ -b	p-value
0-100	0.71	0.0133
100-200	0.50	0.0833
200-300	0.36	0.2160
300-400	0.71	0.0133
400-500	0.07	0.8046
500-600	0.79	0.0065
600-700	0.86	0.0030
700-800	0.57	0.0478
800-900	0.36	0.2160
900-1000	0.79	0.0065
1000-1100	0.86	0.0030
1100-1200	0.57	0.0478
<i>All Intervals</i>	<i>0.62</i>	<i><.0001</i>

Highlighted correlation coefficients are significantly different than 0 at $\alpha=0.05$. Bold correlation coefficients are significantly different than 0 with Benjamini-Hochberg correction for multiple comparisons with a 20% false discovery rate.

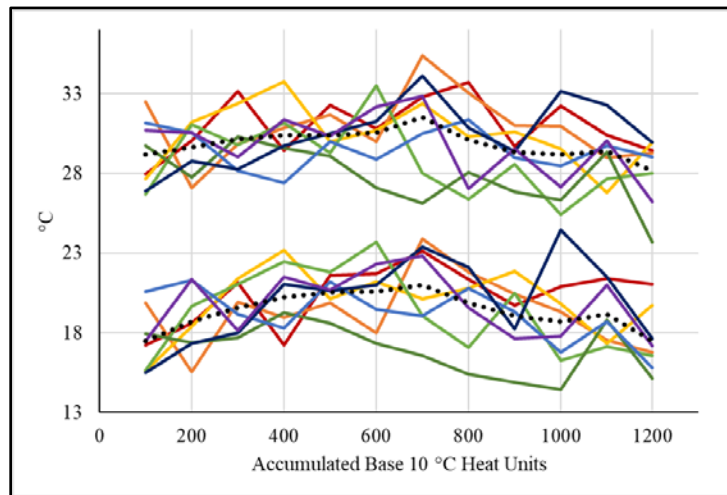


Figure 8 Average maximum and minimum temperatures for 100 heat unit intervals from 100 to 1200 accumulated heat units for the eight years of field trials. Max and min temperatures for each year are the same color. Dotted lines are overall average max and min temperatures.

High daytime and nighttime temperatures are present across the growing season (Figure 8) but only certain periods with high temperatures were correlated with effects on yield and maturity. Average maximum and minimum temperatures during the 600-700 GDD interval were negatively correlated with yield (Table 4, Figure 9); higher temperatures in this time period were correlated with lower yields. Average minimum temperature during the 600-700 GDD interval is positively correlated with days to harvest; higher minimum temperatures in this growth stage were correlated with longer days to maturity.

Table 4 Kendall's Tau-b correlation coefficients for average maximum or minimum temperatures for 100 GDD intervals versus yield or days to harvest of field grown lima bean

Accumulated GDD	Max x Yield		Min x Yield		Max x DTH		Min x DTH	
	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value
0-100	-0.04	0.9008	0.11	0.7084	0.21	0.4579	-0.07	0.8046
100-200	0.25	0.3828	0.11	0.7084	-0.43	0.1376	-0.50	0.0833
200-300	0.18	0.5330	0.11	0.7084	0.21	0.4579	0.00	1.0000
300-400	-0.04	0.9008	0.11	0.7084	0.00	1.0000	-0.29	0.3223
400-500	-0.55	0.0615	-0.04	0.9008	0.57	0.0478	-0.21	0.4579
500-600	-0.40	0.1702	-0.18	0.5330	-0.07	0.8046	-0.14	0.6207
600-700	-0.76	0.0088	-0.76	0.0088	0.57	0.0478	0.71	0.0133
700-800	-0.18	0.5330	-0.62	0.0340	0.36	0.2160	0.36	0.2160
800-900	-0.33	0.2618	0.04	0.9008	0.43	0.1376	0.07	0.8046
900-1000	-0.47	0.1051	-0.55	0.0615	0.43	0.1376	0.36	0.2160
1000-1100	-0.40	0.1702	-0.25	0.3828	0.29	0.3223	0.43	0.1376
1100-1200	-0.30	0.3149	-0.40	0.1702	0.11	0.7084	0.36	0.2160

Highlighted correlation coefficients are significantly different than 0 at $\alpha=0.05$. Bold and highlighted correlation coefficients are significantly different than 0 with Benjamini-Hochberg correction for multiple comparisons with a 20% false discovery rate.

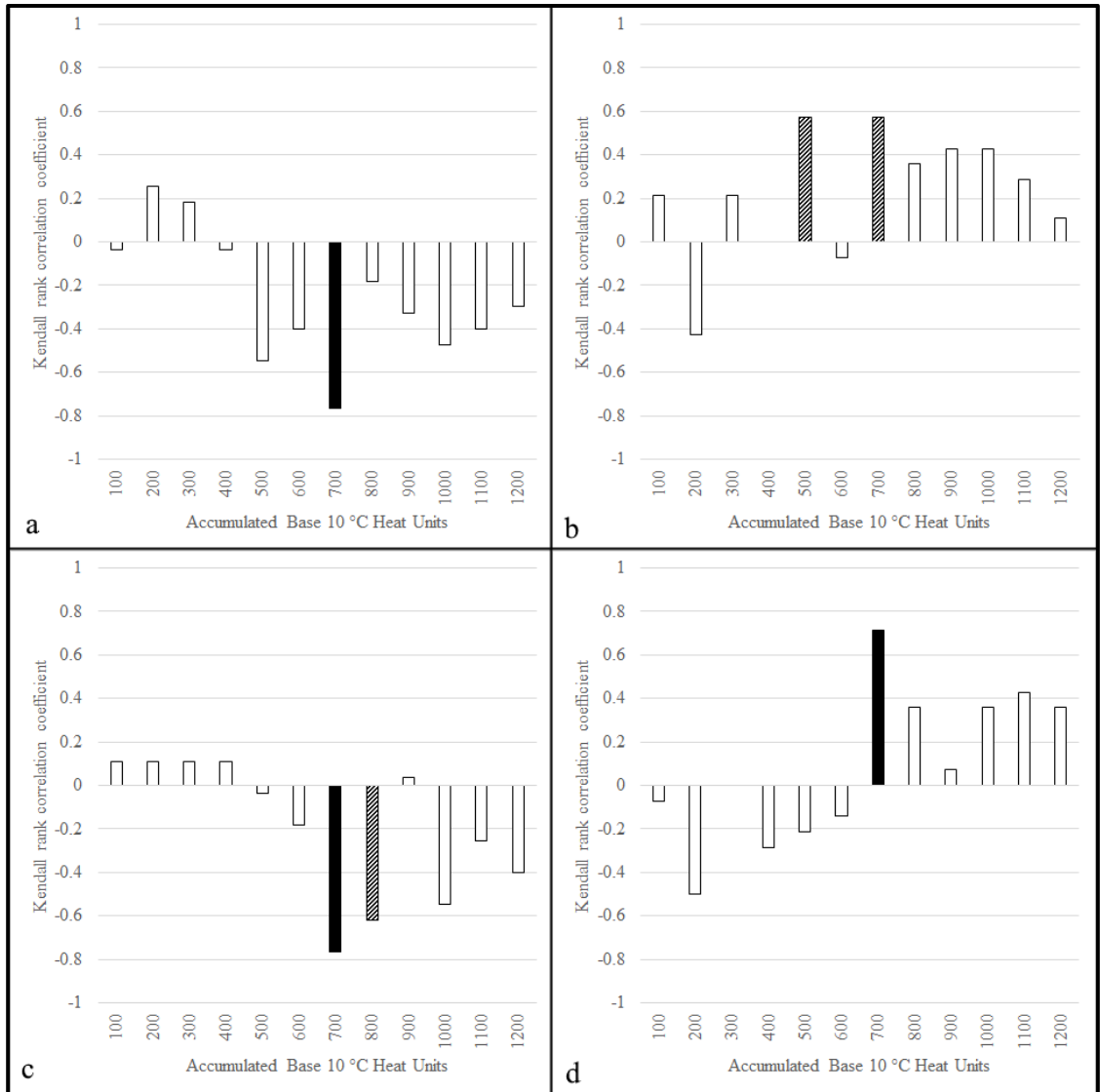


Figure 9 Kendall's Tau-b correlation coefficients for average maximum or minimum temperature over 100 GDD intervals versus yield or days to harvest (a) average maximum vs yield (b) average maximum vs days to harvest (c) average minimum vs yield and (d) average minimum vs days to harvest

When considered individually, the correlation between yield and temperature followed a similar pattern for the five genotypes. The highest magnitudes of negative

correlation were between 500 and 1000 accumulated heat units and there was a lower magnitude of correlation between temperature and yield at the beginning and end of the growing season (Tables 4 & 5, Figure 10). Correlation between minimum temperature and yield was more discrete than correlation between maximum temperature and yield, with the highest magnitude of correlation and six of eight significant correlation coefficients in the 600 to 800 GDD interval.

The five genotypes also shared a similar pattern of correlation for temperature and days to harvest, with the highest magnitude of positive correlation between 500 and 800 accumulated heat units and a lower magnitude of correlation early in the season (Tables 6 & 7, Figure 10). For one genotype, Bert, significant correlation between minimum temperature and days to harvest extended in to the 800-1100 heat unit range. Compared to correlation with maximum temperature, correlation between minimum temperature and days to harvest was more discretely aligned with the 600 to 700 GDD interval in terms of magnitude and statistical significance.

The period of 600-700 accumulated GDD occurred in the range of 39-52 days after planting (average 41-47 DAP) in these trials. This interval corresponds to the early reproductive phase of lima bean, when many racemes are forming buds and the first flowers are opening. First flowering is at about 35 DAP and peak flowering at 60 DAP (Kee, *et al.*, 1997). Gross and Kigel (1994) found that in common bean, pollen was most sensitive to heat stress from 7-10 days before anthesis and that heat sensitivity decreases closer to anthesis. The data here suggest that a similar phenomenon is present in lima bean, such that high temperatures in the early reproductive phase are associated with lower yields and delayed maturity. Daily maximum and minimum temperatures are significantly correlated in most GDD

intervals, including the 600-700 GDD interval (Table 3) so from the field data it is not possible to distinguish separate day and night temperature effects that have been reported in controlled environment studies in lima bean. However, the pattern of correlation coefficients observed for the genotypes analyzed individually had a more discrete pattern for correlations with daily minimum temperatures versus daily maximum temperatures.

Table 5 Kendall's Tau-b correlation coefficients for average maximum temperatures over 100 GDD intervals versus days to harvest of field grown lima bean by genotype

Accumulated GDD	DE0505002A		C-Elite Select		Cypress		Brooke		Bert	
	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value
0-100	0.00	1.0000	0.00	1.0000	0.25	0.3828	0.25	0.3828	0.07	0.8046
100-200	-0.36	0.2160	-0.50	0.0833	-0.47	0.1051	-0.47	0.1051	-0.43	0.1376
200-300	0.14	0.6207	0.14	0.6207	0.18	0.5330	0.18	0.5330	0.07	0.8046
300-400	0.07	0.8046	-0.07	0.8046	-0.04	0.9008	-0.04	0.9008	0.00	1.0000
400-500	0.64	0.0260	0.36	0.2160	0.69	0.0178	0.62	0.0340	0.57	0.0478
500-600	0.14	0.6207	0.00	1.0000	-0.11	0.7084	-0.11	0.7084	0.07	0.8046
600-700	0.64	0.0260	0.50	0.0833	0.69	0.0178	0.62	0.0340	0.71	0.0133
700-800	0.29	0.3223	0.29	0.3223	0.47	0.1051	0.40	0.1702	0.36	0.2160
800-900	0.50	0.0833	0.21	0.4579	0.55	0.0615	0.47	0.1051	0.43	0.1376
900-1000	0.36	0.2160	0.50	0.0833	0.55	0.0615	0.47	0.1051	0.71	0.0133
1000-1100	0.21	0.4579	0.36	0.2160	0.33	0.2618	0.33	0.2618	0.43	0.1376
1100-1200	0.18	0.5330	0.18	0.5330	0.22	0.4510	0.15	0.6153	0.40	0.1702

Highlighted correlation coefficients are significantly different than 0 at $\alpha=0.05$. Bold correlation coefficients are significantly different than 0 with Benjamini-Hochberg correction for multiple comparisons with a 20% false discovery rate.

Table 6 Kendall's Tau-b correlation coefficients for average minimum temperatures over 100 GDD intervals versus days to harvest of field grown lima bean by genotype

Accumulated GDD	DE0505002A		C-Elite Select		Cypress		Brooke		Bert	
	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value
0-100	-0.14	0.6207	-0.14	0.6207	-0.04	0.9008	-0.04	0.9008	-0.21	0.4579
100-200	-0.29	0.3223	-0.57	0.0478	-0.40	0.1702	-0.47	0.1051	-0.36	0.2160
200-300	0.07	0.8046	-0.21	0.4579	-0.04	0.9008	-0.04	0.9008	-0.14	0.6207
300-400	-0.21	0.4579	-0.21	0.4579	-0.33	0.2618	-0.33	0.2618	-0.14	0.6207
400-500	0.00	1.0000	-0.29	0.3223	-0.25	0.3828	-0.25	0.3828	-0.21	0.4579
500-600	0.07	0.8046	-0.21	0.4579	-0.18	0.5330	-0.18	0.5330	-0.14	0.6207
600-700	0.79	0.0065	0.64	0.0260	0.69	0.0178	0.69	0.0178	0.71	0.0133
700-800	0.43	0.1376	0.43	0.1376	0.47	0.1051	0.40	0.1702	0.64	0.0260
800-900	0.14	0.6207	-0.14	0.6207	0.04	0.9008	0.04	0.9008	-0.07	0.8046
900-1000	0.43	0.1376	0.43	0.1376	0.47	0.1051	0.40	0.1702	0.64	0.0260
1000-1100	0.21	0.4579	0.50	0.0833	0.47	0.1051	0.47	0.1051	0.57	0.0478
1100-1200	0.43	0.1376	0.29	0.3223	0.33	0.2618	0.33	0.2618	0.36	0.2160

Highlighted correlation coefficients are significantly different than 0 at $\alpha=0.05$. Bold correlation coefficients are significantly different than 0 with Benjamini-Hochberg correction for multiple comparisons with a 20% false discovery rate.

Table 7 Kendall's Tau-b correlation coefficients for average maximum temperatures over 100 GDD intervals versus yield of field grown lima bean by genotype

Accumulated GDD	DE0505002A		C-Elite Select		Cypress		Brooke		Bert	
	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value
0-100	-0.07	0.8046	-0.07	0.8046	0.14	0.6207	-0.21	0.4579	0.00	1.0000
100-200	0.43	0.1376	0.14	0.6207	0.07	0.8046	0.29	0.3223	-0.07	0.8046
200-300	0.21	0.4579	0.07	0.8046	0.00	1.0000	0.21	0.4579	0.43	0.1376
300-400	0.00	1.0000	-0.29	0.3223	0.21	0.4579	0.00	1.0000	-0.21	0.4579
400-500	-0.43	0.1376	-0.57	0.0478	-0.50	0.0833	-0.71	0.0133	-0.36	0.2160
500-600	-0.21	0.4579	-0.07	0.8046	-0.29	0.3223	-0.21	0.4579	-0.29	0.3223
600-700	-0.57	0.0478	-0.86	0.0030	-0.21	0.4579	-0.86	0.0030	-0.64	0.0260
700-800	-0.07	0.8046	-0.36	0.2160	-0.43	0.1376	-0.36	0.2160	0.00	1.0000
800-900	-0.14	0.6207	-0.71	0.0133	-0.21	0.4579	-0.43	0.1376	-0.36	0.2160
900-1000	-0.29	0.3223	-0.57	0.0478	-0.50	0.0833	-0.57	0.0478	-0.36	0.2160
1000-1100	-0.43	0.1376	-0.14	0.6207	-0.21	0.4579	-0.43	0.1376	-0.21	0.4579
1100-1200	-0.11	0.7084	-0.55	0.0615	-0.47	0.1051	-0.40	0.1702	-0.33	0.2618

Highlighted correlation coefficients are significantly different than 0 at $\alpha=0.05$. Bold correlation coefficients are significantly different than 0 with Benjamini-Hochberg correction for multiple comparisons with a 20% false discovery rate

Table 8 Kendall's Tau-b correlation coefficients for average minimum temperatures over 100 GDD intervals versus yield of field grown lima bean by genotype

Accumulated GDD	DE0505002A		C-Elite Select		Cypress		Brooke		Bert	
	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value	τ -b	p-value
0-100	0.07	0.8046	0.21	0.4579	0.29	0.3223	0.07	0.8046	0.29	0.3223
100-200	0.21	0.4579	0.36	0.2160	0.14	0.6207	0.07	0.8046	0.00	1.0000
200-300	0.29	0.3223	-0.14	0.6207	-0.36	0.2160	0.14	0.6207	0.21	0.4579
300-400	0.14	0.6207	0.00	1.0000	0.21	0.4579	0.14	0.6207	-0.21	0.4579
400-500	0.07	0.8046	0.36	0.2160	-0.43	0.1376	0.07	0.8046	0.14	0.6207
500-600	0.00	1.0000	0.14	0.6207	-0.21	0.4579	0.00	1.0000	-0.07	0.8046
600-700	-0.57	0.0478	-0.71	0.0133	-0.36	0.2160	-0.71	0.0133	-0.50	0.0833
700-800	-0.50	0.0833	-0.64	0.0260	-0.57	0.0478	-0.79	0.0065	-0.43	0.1376
800-900	0.21	0.4579	-0.21	0.4579	-0.29	0.3223	0.07	0.8046	0.00	1.0000
900-1000	-0.36	0.2160	-0.64	0.0260	-0.43	0.1376	-0.64	0.0260	-0.43	0.1376
1000-1100	-0.43	0.1376	-0.14	0.6207	-0.07	0.8046	-0.43	0.1376	-0.21	0.4579
1100-1200	-0.21	0.4579	-0.36	0.2160	-0.43	0.1376	-0.36	0.2160	-0.29	0.3223

Highlighted correlation coefficients are significantly different than 0 at $\alpha=0.05$. Bold correlation coefficients are significantly different than 0 with Benjamini-Hochberg correction for multiple comparisons with a 20% false discovery rate.

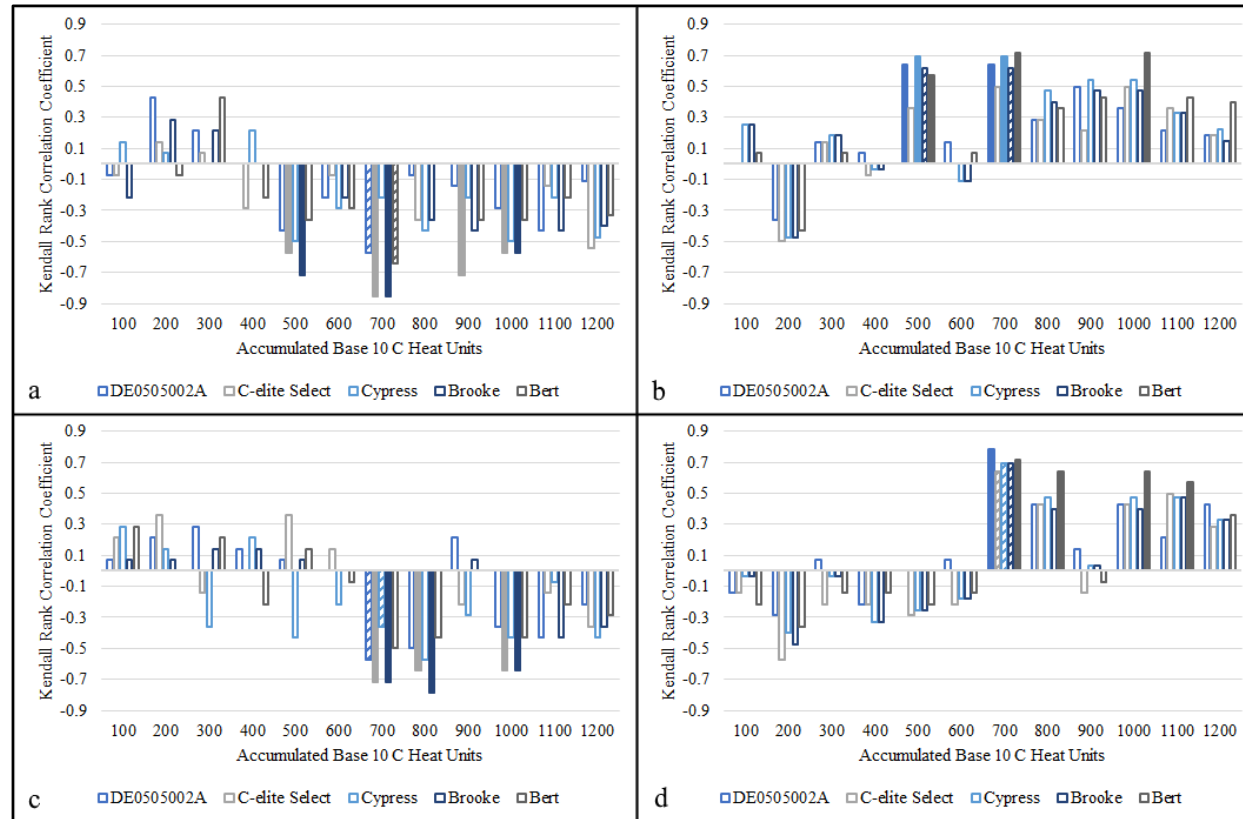


Figure 10 Kendall's Tau-b correlation coefficients by genotype for average maximum or minimum temperature over 100 GDD intervals versus yield or days to harvest (a) average maximum vs yield (b) average maximum vs days to harvest (c) average minimum vs yield and (d) average minimum vs days to harvest. Solid bars indicate coefficients that are significantly different than 0 at $\alpha=0.05$ and with Benjamini-Hochberg correction for multiple comparisons with a 20% false discovery rate. Diagonally striped bars indicate coefficients that are significantly different than 0 at $\alpha=0.05$. White filled bars indicate coefficients that are not significantly different than 0.

High Night Temperature Effects on Yield Components

The effect of temperature, genotype and genotype x temperature interaction (G x T) were significant for the five yield components measured in the greenhouse chamber experiments: yield in weight of seeds and number of seeds, mean per seed weight, number of pods per plant and number of seeds per pod (Table 9). The effect of temperature was also significant for days to flower but the effects of genotype and G x T were not. Year x temperature interaction was only significant for days to flower, indicating that the temperature effects on yield components were stable across years. Year x genotype interaction and year x temperature x genotype interaction were significant for days to flower, seed yield and pods per plant.

Table 9 P-values for type 3 tests of fixed effects for days to flower, yield in weight of seeds and number of seeds, mean per seed weight, number of pods per plant and number of seeds per pod

Effect	Days to Flower	Yield (wt of seed)	Yield (no. of seeds)	Per Seed Weight	Pods/ Plant	Seeds/ Pod
Temperature (T)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Genotype (G)	0.3776	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G x T	0.3132	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Year (Y) x T	0.0174	0.1316	0.2576	0.8106	0.3754	0.2268
Y x G	<0.0001	0.003	0.0003	0.9573	0.0001	0.8437
Y x T x G	0.0023	0.0009	0.0075	0.8386	0.0483	0.3796

For all three genotypes high night temperature stress reduced the weight of seeds produced and the number of seeds (Figure 11). Heat stress had less of an effect on the yield of Bush Florida Butter than it did on C-elite Select or Fordhook 242, indicating that Bush Florida Butter is the most heat tolerant of the three genotypes. Heat stress did not significantly affect per seed weight in either Bush Florida Butter or

C-elite Select but heat stressed Fordhook 242 produced significantly smaller seeds. In C-elite Select and Fordhook 242, heat stress reduced the number of pods per plant, but the same effect was not present in Bush Florida Butter, again indicating that this cultivar is more heat tolerant. The number of seeds per pod was reduced in all three cultivars under heat stress, but there were significant differences separating each of the genotypes from the other two in the number of seeds per pod under heat stress with Bush Florida Butter having more seeds per pod than C-elite Select and C-elite Select having more seeds per pod than Fordhook 242. Heat stress induced reduction in the number of seeds per pod suggests that pollination and/or fertilization are being affected in heat sensitive lima bean genotypes, similar to heat effects in common bean (Gross and Kigel, 1994; Porch and Jahn, 2001, Prasad *et al.*, 2002).

Days to flowering was reduced under heat stress in all genotypes (Figure 11). It does not appear that heat stress delays flowering in these genotypes of lima bean. Heat sensitive genotypes grown in the high night temperature chamber continued to flower profusely until the experiment was terminated. Plants that reached their capacity set ceased flowering.

The effects of temperature and genotype x temperature interaction were significant for shoot dry weight but not for total aboveground dry weight (Table 10). The effect of genotype was significant for both shoot dry weight and total aboveground dry weight — different genotypes produced different size plants.

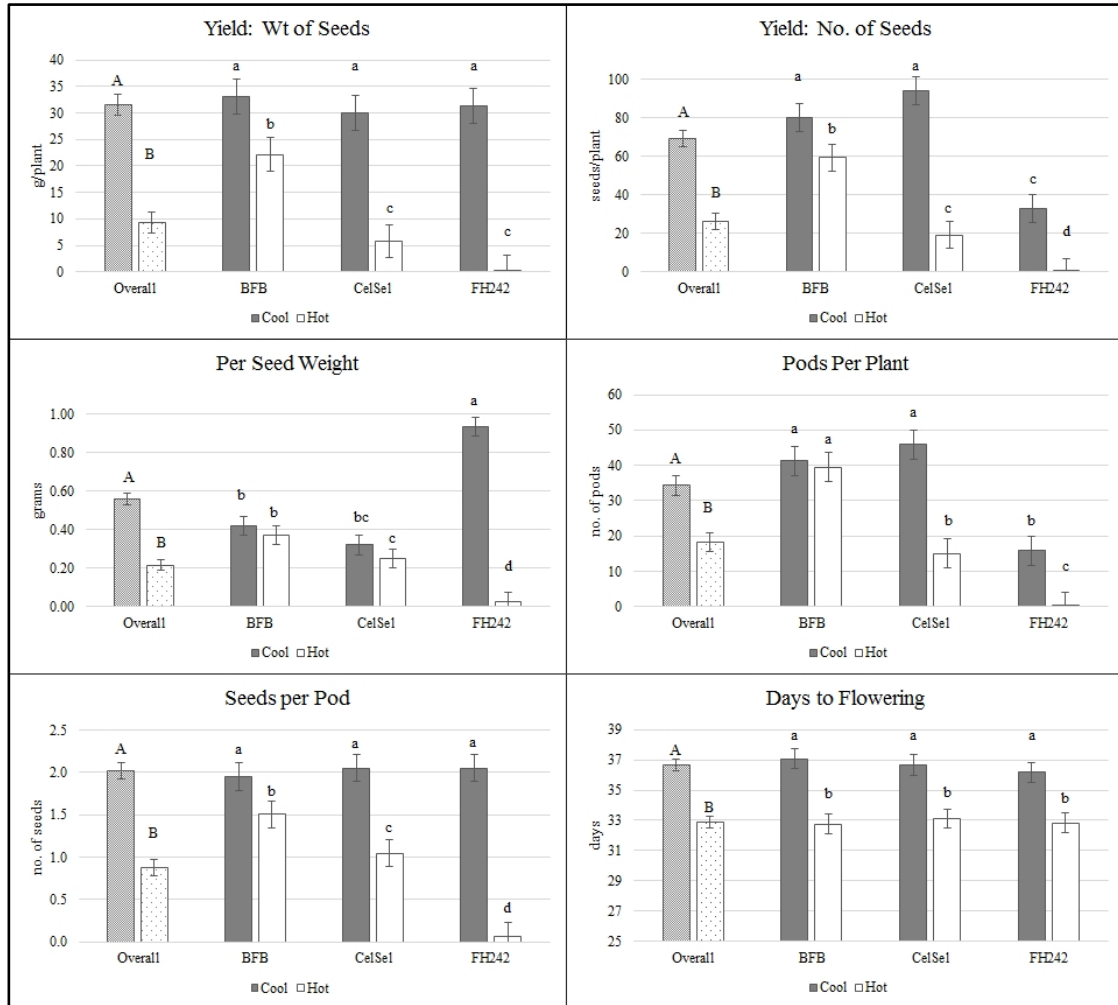


Figure 11 Three year means of yield components and days to flowering under heat stress and unstressed by genotype and for all genotypes combined. Mean separation is by the Tukey-Kramer method at $P \leq 0.05$. Error bars indicate the 95% confidence interval for the mean.

Shoot dry weight increased under heat stress in the more heat sensitive genotypes, C-elite Select and Fordhook 242 (Figure 12). Under heat stress, branching increased and plants produced a greater number of stems (Figure 13). This effect was particularly notable in the more heat sensitive genotypes, Fordhook 242 and C-elite

Select. When seed weight was included in the aboveground dry weight there were no significant differences between heat stressed and unstressed plants of the same genotype (Figure 12).

Table 10 P-values for type 3 tests of fixed effects for shoot dry weight and total aboveground dry weight

Effect	Shoot Wt	Total Wt
Temperature (T)	0.0044	0.8796
Genotype (G)	<0.0001	<0.0001
G x T	0.0381	0.8368

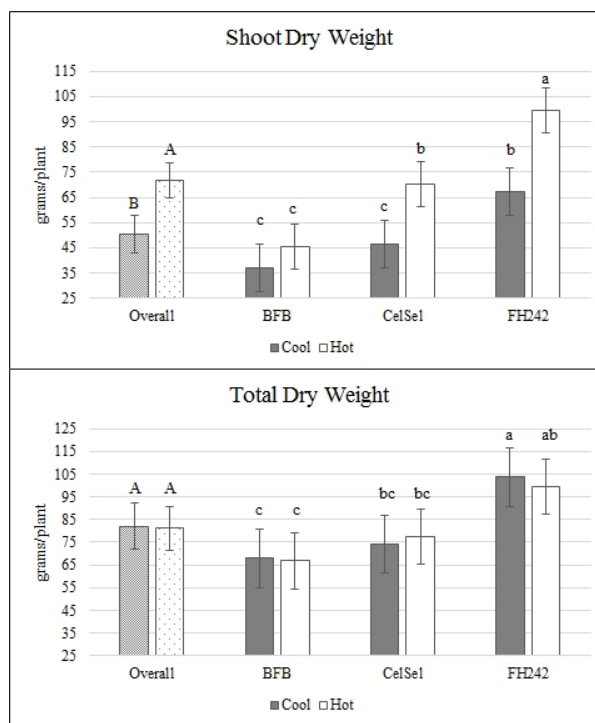


Figure 12 Mean shoot dry weight and total aboveground dry weight under heat stress and unstressed by genotype and for all genotypes combined. Mean separation is by the Tukey-Kramer method at $P \leq 0.05$. Error bars indicate the 95% confidence interval for the mean.

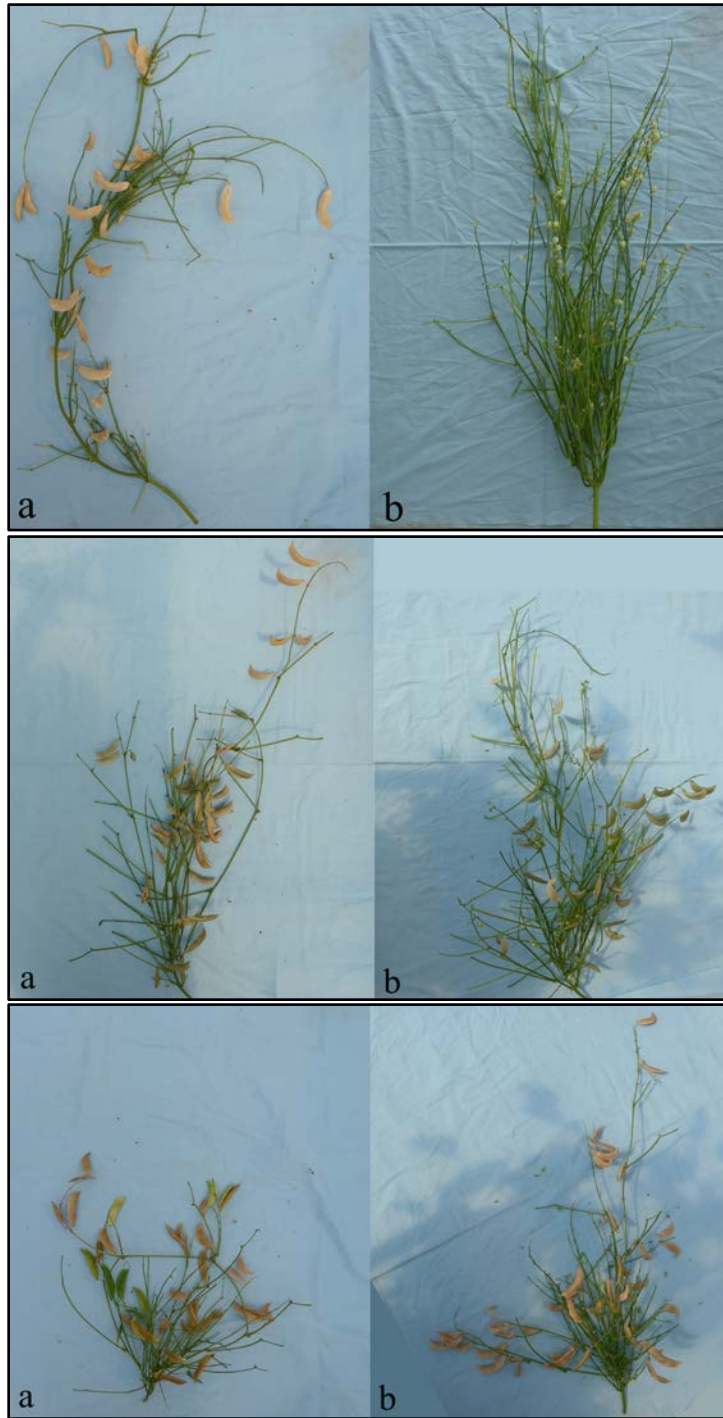


Figure 13 Photos of unstressed (a) and heat stressed (b) plants with leaves removed from three genotypes with differing levels of heat tolerance: Fordhook 242 (top), C-elite Select (middle), Bush Florida Butter (bottom).

Genotype Differences in Yield Response to High Night Temperature

Heat Susceptibility Index (HSI) for weight of seeds produced per plant differed among the eight genotypes tested (Table 11). Henderson Bush, Bush Florida Butter, and PI 534918 were rated highly tolerant and Cypress was rated moderately tolerant. G27525, Bridgeton and C-elite Select were rated moderately susceptible and Fordhook 242 was rated highly susceptible.

Table 11 Heat Susceptibility Index for all genotypes tested in all years.

Genotype	Year					Avg	Rating ^y
	2014	2015	2016	2017	2018		
Henderson Bush				0.16	0.24	0.20	HT
Bush Florida Butter	-0.26	0.56	0.61	0.34	0.49	0.35	HT
PI 534918				0.26	0.47	0.37	HT
Cypress	0.98	0.53				0.75	MT
G27525	1.27	0.67	0.67		2.00	1.15	MS
Bridgeton	1.03	1.36				1.20	MS
C-elite Select		1.39	1.24	1.03	1.23	1.22	MS
Fordhook 242	1.91	1.65	1.37	1.52	1.61	1.61	HS
Stress Intensity^z	0.51	0.61	0.73	0.65	0.40		

^yHSI < 0.5 is highly tolerant (HT), HSI 0.5-1.0 is moderately tolerant (MT), HSI 1.0-1.5 is moderately susceptible (MS) and HSI > 1.5 is highly susceptible (HS)

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

Conclusions

Analysis of eight years of yield trial data from Delaware demonstrates that under field conditions periods of high temperature in the early flowering period of lima bean, (which occurs at 600-700 accumulated heat units and from 39-52 days after planting) are correlated with decreased yield and delayed maturity. High temperatures during the vegetative phase and after pod set did not correlate with decreased yield and delayed maturity. The early reproductive phase is when lima bean is susceptible to

yield loss or delayed maturity caused by high temperatures. Identification of this growth stage as susceptible to heat stress allows growers to target these periods for irrigation or other practices to mitigate heat stress. It also allows breeders to more effectively select for heat tolerance by targeting this growth stage for heat stress exposure in germplasm screens.

When grown under continuous high night temperature (28 °C) conditions in a controlled environment, lima bean begins flowering earlier than at a moderate night temperature (21 °C). Heat sensitive genotypes set fewer pods and produce fewer seeds per pod under high night temperatures. Heat tolerant genotypes set equivalent numbers of pods but still produce fewer seeds per pod. The overall result is fewer seeds produced under high night temperatures, resulting in yield loss. In one very heat sensitive genotype (Fordhook 242) per seed weight was also reduced by high night temperatures. Carbohydrate depletion due to higher respiration at higher temperatures had been hypothesized as the explanation for reduced lima bean pod set under high night temperature conditions (Fisher and Weaver, 1974) but the results reported here do not indicate a reduction of carbohydrate availability in heat stressed plants. Even in heat sensitive genotypes the total aboveground biomass accumulation was equivalent in stress and unstressed conditions. Rather, the reduction in the number of seeds per pod in high night temperature conditions suggests that pollination and/or fertilization are affected in heat sensitive genotypes. Heat effects on pollen release and viability have been described in other crops including common bean (Gross and Kigel, 1994), cowpea (Warrag and Hall, 1984), tomato (Sato *et al.*, 2000).

The eight genotypes that were grown under high and moderate night temperature conditions differed in their response to heat stress with some producing

nearly equivalent yield at continuous 28 °C nights and others producing 20% or less of normal yield. Three of the genotypes, C-elite Select, Cypress and Fordhook 242, are cultivars that are used in Delaware. C-elite Select is moderately heat susceptible and Cypress is moderately heat tolerant based on the HSI rating. For both cultivars, high temperatures during the flowering period were correlated with delayed harvest and yield loss (Figure 10), indicating that even the moderate heat tolerance present in Cypress is not sufficient to protect it from heat effects under field conditions in Delaware. Fordhook 242 is highly heat susceptible, as has been observed in field conditions. The current production practice is to delay planting of Fordhook 242 until late June or early July to avoid high temperatures during flowering.

Differences between genotypes in their yield response to heat suggests that there is opportunity for development of cultivars of lima bean with superior heat tolerance and a survey of a wider diversity of lima bean germplasm is likely to identify genotypes that are more heat tolerant than those used in this study. Because lima bean is adapted to warmer and more humid climate than other domesticated *Phaseolus* species (Bitocchi *et al.*, 2017), it has potential value as an alternative legume crop in hot, humid climates, where it is currently underutilized. Lima bean's potential for productivity in such conditions could exceed that of common bean.

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Chapter 3

HEAT STRESS EFFECTS ON LIMA BEAN REPRODUCTIVE STRUCTURES AND PROSPECTS FOR IMPROVING REPRODUCTIVE HEAT STRESS TOLERANCE

Introduction

When grown under continuous high night temperature (28 °C) conditions in a controlled environment, heat sensitive lima bean genotypes set fewer pods and produced fewer seeds per pod than under more moderate night temperature (21 °C) conditions. More heat tolerant genotypes set equivalent numbers of pods but still produced fewer seeds per pod. The overall result is fewer seeds produced under high night temperatures, resulting in yield loss. In one very heat sensitive genotype (Fordhook 242) seed weight is also reduced by high night temperatures. Carbohydrate depletion due to higher respiration at higher temperatures has been hypothesized as the explanation for reduced pod set under high night temperature conditions (Fisher and Weaver, 1974) but even in heat sensitive genotypes the total aboveground biomass accumulation is equivalent in stress and unstressed conditions. Rather, the reduction in the number of seeds per pod in high night temperature conditions suggests that pollination and/or fertilization are affected in heat sensitive genotypes.

The objective of this research was to evaluate the physiological effects of high night temperatures on reproductive structures in lima bean and then evaluate a larger diversity of germplasm to determine if the observed physiological effects of heat stress are widespread in cultivated lima bean. Heat related yield loss has been linked to

anther indehiscence or loss of pollen quality in several crop plants, including cowpea (*Vigna unguiculata*), common bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*).

In cowpea high night temperatures in the first four weeks after germination cause complete suppression of floral buds (Ahmed and Hall, 1993) and high night temperatures during anther development cause pollen sterility and sometimes anther indehiscence (Warrag and Hall, 1984; Mutters and Hall, 1992). The most sensitive stage of floral development is 9-7 days before anthesis (Ahmed et al., 1992) and pollen development is most affected by high temperatures during the time between midnight and dawn (Mutters and Hall, 1992). Artificial pollination of heat stressed plants with pollen from non-stressed plants demonstrated that stigma receptivity in cowpea is not diminished by high night temperatures (Warrag and Hall, 1984).

Similar to cowpea, high night temperatures are damaging to reproductive structures of common bean. For pollen, the most sensitive stage of development is sporogenesis, which occurs 7-10 days before anthesis. Heat sensitivity decreases closer to anthesis, and immature pods are not affected by high temperatures (Gross and Kigel, 1994). Physiological effects of high night temperatures before anthesis include anther indehiscence and pollen inviability (Gross and Kigel, 1994; Porch and Jahn, 2001). Unlike cowpea, high temperature also affects stigma function in heat sensitive common bean genotypes by reducing stigma secretions (Gross and Kigel, 1994). Effects on the stigma result from high temperatures at anthesis, and other high temperature effects at this stage, such as impairment of pollen tube growth, might also inhibit pod set (Gross and Kigel, 1994).

In tomato, high temperatures decrease starch accumulation in developing pollen grains. The starch is converted to soluble sugars as the pollen matures. Thus, pollen produced under heat stress contains less soluble sugar at anthesis, resulting in fewer viable pollen grains and lower pollen germination rates (Pressman et al, 2002). Carbohydrate content of pollen from heat tolerant tomato cultivars is not reduced under high temperature conditions, which suggests that interruption of carbohydrate accumulation in developing pollen is a major factor limiting fruit set in heat stressed tomato (Firon *et al.*, 2006).

Flower anatomy and flowering biology will affect the way high temperatures impact pollination, fertilization and, ultimately, yield. Lima bean flowers are hermaphroditic and primarily self-pollinated, with the pistil and 10 stamens coiled in the spiral-shaped keel. Anthers dehisce, releasing pollen as flowers open in the early morning. The stigmatic pad is within the keel of the open flower but can be extruded by depression of the wing petals through the action of insect visitors (Webster *et al.*, 1979). Flowers do not close but the corolla is shed about three days after opening. Lima bean flowers are produced on an indeterminate raceme with three flowers at each node of the raceme. Additional flowers may form at a node if some or all of the original structures abscise, termed “reflowering”. Reflowering has been observed to occur up to four times in annual field cultivation (Wootten, 1994). Lima bean produces abundant flowers, indeterminately on existing racemes, until a capacity set of pods is reached. Remaining reproductive structures are then abscised (Cordner, 1934).

As described in the previous chapter, lima bean genotypes exhibit different levels of heat tolerance. The range of heat adaptation observed in lima bean is impacted by the environments in which the wild species evolved and in which

landraces and cultivars were selected. Wild and cultivated lima bean grows under a range of environmental conditions. Wild lima bean has a wide distribution with its northern limit in Sinaloa, Mexico (Freytag and Debouck, 2002) and its southern distribution extending to the eastern slope of the Andes in Northern Argentina and Paraguay (Serrano-Serrano *et al.*, 2009). Within wild lima accessions three gene pools have been identified: one Andean (AI) and two Mesoamerican (MI and MII). AI is larger seeded type with a range restricted to Ecuador and northern Peru that is found at elevations of 470-1810 masl. MI and MII are both smaller seeded with MI occupying the northern range of wild lima in northwest Mexico and MII distributed from the Yucatan Peninsula in southern Mexico, through Central America, the Caribbean, Columbia, Peru and Boliva to the species southern limit in northern Argentina. Accessions from the two Mesoamerican gene pools were collected at elevations ranging from 3 to 1750 masl (Serrano-Serrano *et al.*, 2009).

Cultivated lima bean was domesticated from the wild species at least twice, based on morphological, genetic and archeological evidence (Motta-Aldana *et al.*, 2010; Gutiérrez Salgado *et al.*, 1995). One domestication event from the AI wild gene pool occurred in Ecuador or northern Peru. Landraces derived from this domestication event were later distributed in the Andean region from Colombia to Argentina and the southern Pacific coastal region of Peru (Motta-Aldana *et al.*, 2010). One or two domestications occurred from the Mesoamerican gene pools with either domestication occurring from each gene pool (Andueza-Noh *et al.*, 2015) or domesticated material from MI crossing with MII wild material and further selection occurring in the MII gene pool region of Central America (Chacón-Sánchez and Martínez-Castillo, 2017). Lima bean landraces from the Caribbean group with Mesoamerican derived material

(Castiñeiras *et al.*, 2007; Montero-Rohas *et al.*, 2013) while Brazilian material includes Andean, Mesoamerican and mixed gene pool derived landraces (Oliveira-Silva *et al.*, 2017).

US cultivars and landraces can be grouped into small seed and large seeded types with the small seeded types associating with the Mesoamerican gene pool and large seeded types associated with the Andean gene pool. However, large seeded US cultivars, such as the Fordhook type formed a distinct group in an analysis of genetic relationships based on RAPD markers (Nienhuis *et al.*, 1995). Mesoamerican lima beans were likely cultivated in the southwestern and southeastern U.S. before European contact. Andean lima beans were introduced into North America by post-contact trade (Mackie, 1943).

An examination of lima bean yield components in a stressed and unstressed environment suggested that pollination or fertilization may be affected by high night temperatures. The objective of this study was to determine which aspects of reproduction are affected by heat stress. Pollen release, pollen quality and pistil function under heat stress were examined in a set of five genotypes with a range of heat stress tolerance. A larger set of 131 genotypes was screened for quantity of pollen released and yield under heat stress to determine if the connection between these two characteristics was widespread among genotypes. This also allowed identification of additional heat tolerant genotypes and regions from which additional heat tolerant lima bean germplasm might be available.

Materials and Methods

Greenhouse Chamber Experiments

Lima bean plants grown as described previously in the two climate-controlled greenhouse chambers were used in experiments to determine the effects of high night temperatures on reproductive structures and processes. Five genotypes were used in these experiments: PI 549509 'Bush Florida Butter', 'C-elite Select', PI 549464 'Fordhook 242', PI 549466 'Henderson Bush' and PI 534918 Cave Dweller. Night temperature averaged 28 °C in the hot chamber and 21°C in the cool chamber. Target daytime temperatures for both chambers were 31 °C and averaged 30 °C in the cool night chamber and 32 °C in the hot night chamber.

Pollen Quantity

Newly opened flowers were collected from plants seeded on 12 Mar. 2015, 11 Mar. 2016, and 15 Mar. 2017. Flowers were sampled on multiple dates per experiment with three flowers per genotype x 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 three minutes a glass coverslip was placed over the drop and pressed down to flatten the style and stigma. The keel was dissected, and 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, anthers were photographed at 40x magnification. Stained pollen grains visible in the photographs were marked and counted with the aid of Image J. Pollen counts were analyzed in Proc-Mixed (SAS Version 9.4; SAS Institute, Cary, NC) with temperature, genotype and experiment as fixed effects and

sample date, nested with in experiment, as a random effect. Fisher's LSD was used to separate treatment means at $\alpha=0.05$.

In Vitro Pollen Germination

Artificial pollen germination medium (PGM) was prepared according to the methods of Gurusamy *et al.* (2007). The final concentrations of PGM components were: H_3BO_3 400 mg/l, CaNO_3 600 mg/l, MgSO_4 400 mg/l, KNO_3 400 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. Two separate drops of PGM were placed on each slide and the covered petri dish was placed in a preheated germination chamber until the petri dish reached the desired pollen germination temperature. Newly opened flowers were collected in early morning from plants planted in the greenhouse chambers on 15 Mar. 2017 and 5 Nov. 2018. Style and stigma were removed from each flower as described and swirled in the prepared sitting drops of PGM on the microscope slides. Lidded petri dishes were incubated in a germination chamber for 4-5 hours.

After incubation one drop of acetocarmine stain was added to each drop of pollen germination medium. After 5 minutes the drop was viewed with a compound microscope at 100x and twenty pollen grains were rated as germinated or ungerminated. Pollen was considered germinated if the pollen tube exceeded the pollen grain diameter.

To test the effects of ambient temperature on pollen germination, pollen from flowers of the five genotypes collected from the cool chamber was germinated in vitro

at 25°C, 28°C, 31°C, 34 °C, 37°C and 40°C. To test the effects of pollen development under sustained hot or cool night temperature conditions, pollen from flowers of the five genotypes collected from the hot and cool chambers was germinated in vitro at 25°C. Pollen germination rates for incubation temperature treatments were analyzed in Proc-Mixed (SAS Version 9.4; SAS Institute, Cary, NC) with temperature, genotype and temperature x genotype interaction as fixed effects. The tests of temperature exposure during pollen development were analyzed in Proc-Mixed with the following fixed effects: temperature (temp), genotype (gen), temp x gen, temp x experiment (exp), temp x exp x gen. Experiment was modeled as a random effect. The Tukey-Kramer method was used to separate treatment means at $\alpha=0.05$.

Crossing Experiments

To test the heat sensitivity of processes downstream of pollination, hand pollinations were made on heat sensitive genotypes C-elite Select and Fordhook 242 grown in the hot chamber with pollen collected from C-elite Select or PI 534918 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. Crossing experiments were performed on plants planted in the greenhouse chambers, as previously described, on 15 Mar. 2017, 6 Nov. 2017 and 6 Mar. 2018. The total number of crosses for each pair was as follows: 70 of C-Elite Select x PI 534918; 60 of C-Elite Select x C-Elite Select; 80 of Fordhook 242 x PI

534918. The data from this experiment were analyzed using McNemar's test for paired nominal data.

Diversity Panel Heat Screen

A set of 131 diverse lima bean genotypes was screened for traits associated with heat tolerance under field and greenhouse conditions. Yield under heat stress was determined in two greenhouse experiments planted on 6 Jul. 2016 and 13 Jul. 2018. Night temperature in the greenhouse was maintained at 27 °C. Two scarified seeds were sown into peat based growing medium (Pro-Mix BX) in 3.8 liter nursery pots and thinned to one plant per pot after emergence. Vining and bush genotypes were separately arranged in a randomized complete block design with 3 replications in 2016 and 2 replications in 2018. Plants were fertilized with 15g of controlled release fertilizer (Nutricote Total 13-11-11 Type 100) at 20 DAP. The number of pods and seeds and weight of seeds produced by each plant was determined. Seed appearance and quality characteristics were assessed for seeds produced by the heat screened plants, compared to seed produced under cool greenhouse conditions.

Pollen release and release under heat stress was determined for the 131 genotypes in a field trial. Bush and vining genotypes were planted in separate but adjacent areas with each type planted in a randomized complete block design with three replications. Rows were spaced at 76 cm with 'Queen Anne' bush blackeye pea on outside rows and in every other row as a border. Plots were 60 cm long with 8 seeds per plot for small seeded genotypes and 5 seeds per plot for large seeded genotypes. Bush genotypes were planted on 22 Jun. 2018 with 60 cm alleys between plots. Vining genotypes were planted on 25 Jun. 2018 with 90 cm alleys between plots. Ten flowers per plot were visually evaluated for the quantity of pollen on the

stigma and style using a three-level rating scale: no pollen, some pollen, or abundant pollen. The number of flowers with deformed keels was also noted. Bush genotypes were rated on 17 Aug. 2018 (56 DAP) and vining genotypes on 20 and 21 Aug. 2018 (56 and 57 DAP). Average daytime highs and lows in the 10 days prior to rating date were 32 and 21°C for the bush genotypes and 31 and 21°C for the vining genotypes. Bush genotypes in the 2018 greenhouse heat screen were rated for pollen quantity and keel deformity on 27 Aug. 2018 (50 DAP).

Pollen quantity rating data were analyzed nonparametrically according to the methods described by Shah and Madden (2004) using Proc Mixed (SAS Version 9.4; SAS Institute, Cary, NC). Relative treatment effects (REpollen) and confidence intervals were calculated using a SAS macro, LD_CI. Yield under heat stress was analyzed and least squares means obtained for each genotype using Proc GLM. Regression analysis for the model $\text{yield} = \text{REpollen}$ was performed using Proc Reg.

Diversity Panel Population Structure Analysis and Marker-Trait Associations

The 131 genotypes that were screened for heat tolerance are members of a larger lima bean diversity panel that has been characterized using genotyping-by-sequencing (GBS). The original dataset includes genotype information for 258 individuals and 17,997 SNPs. Missing genotype data was imputed using the entire dataset with the FILLIN approach (Swarts *et al.*, 2014) implemented in TASSEL 5.0 (Bradbury *et al.*, 2007). The sequence dataset was filtered to remove duplicated individuals, individuals with >70% missing data, and sites with > 50% missing data. SNPs were further filtered for minor allele frequency (minimum 0.05). After filtering the dataset was comprised of 232 individuals and 7835 SNPs.

Multidimensional scaling (MDS) implemented in TASSEL was used to evaluate population structure. Three MDS axes were used as covariates to correct for population structure in association analysis. To correct for relatedness in association analysis, a kinship matrix was calculated using the centered-identity-by-descent method in TASSEL. SNP-trait associations were estimated for yield and pollen release under heat stress based on 120 individuals with genotype and phenotype data using a mixed linear model (MLM) in TASSEL (Zhang *et al.*, 2010). The Benjamini and Hochberg (1995) procedure with a false discovery rate of 0.25 was used to identify potentially significant SNPs.

Results and Discussion

Pollen Release in Chamber Experiments

In greenhouse chamber experiments, both genotype and nighttime temperature regime had a significant effect on the amount of pollen released onto the style and stigma (Table 12). Overall, and for each genotype significantly less pollen was present on the stigma and stylar brush of flowers from plants grown in the hot night temperature chamber than on those of plants grown in the cool night temperature chamber (Figure 14). Under cool night temperatures PI 534918 released significantly more pollen than C-elite Select and Fordhook 242, but all other genotypes released equivalent amounts of pollen. Under hot night conditions PI 534918 released significantly more pollen than Fordhook 242 and C-elite Select. Bush Florida Butter also released significantly more pollen than Fordhook 242, but not more than C-elite Select. The heat susceptibility index (HSI) based on yield data from the chamber experiments indicates that the genotypes producing the least pollen under heat stress, Fordhook 242 and C-elite Select, are heat susceptible, and those producing the most, PI 534189, Bush Florida Butter and Henderson, are highly tolerant. Within this small sample of genotypes, heat stress reduces the amount of pollen released and genotypes that release more pollen under heat stress have higher yield under heat stress.

Table 12 P-values for type 3 tests of fixed effects for pollen released onto the stigma and style and pollen remaining in anthers

Effect	Style & Stigma	Anther
Temperature (T)	<0.0001	<0.0001
Genotype (G)	0.0080	<0.0001
G x T	0.8431	0.0007
Year	0.0297	0.0856

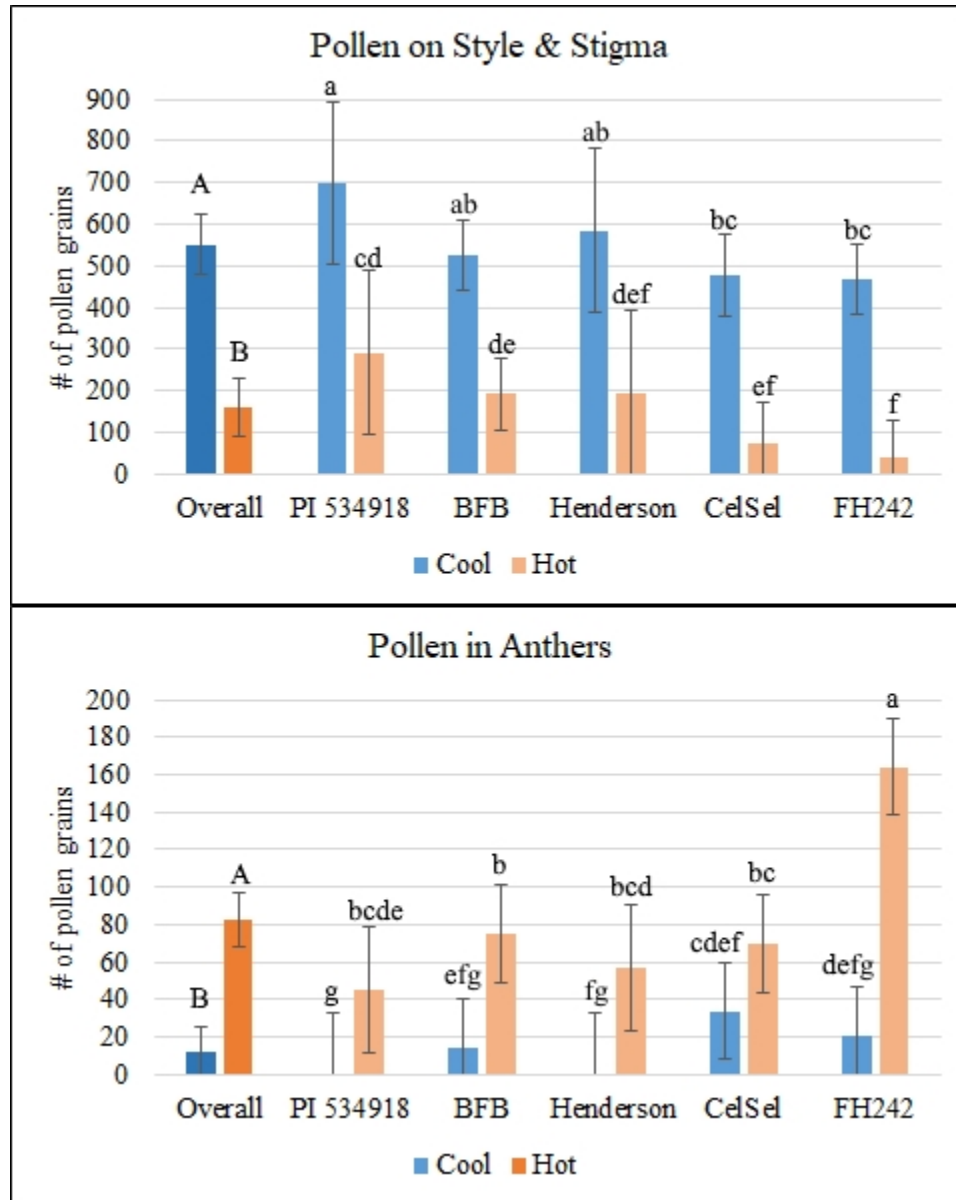


Figure 14 Counts of pollen shed onto the stigma and style or retained in anthers under heat stress and unstressed by genotype and for all genotypes combined. Mean separation is by Fisher's LSD. Error bars indicate the 95% confidence interval for the mean.

Both genotype and nighttime temperature regime had a significant effect on the amount of pollen remaining in anthers after anthesis (Table 12). Counts of pollen grains retained in the anthers indicate that anthers are more completely emptied of pollen under cool night conditions (Figure 14). Under heat stress, the very heat sensitive genotype Fordhook 242 retained significantly more pollen in anthers than the other four genotypes. Lower quantities of pollen present on the style and stigma in heat stressed lima bean is partly due to anther indehiscence as observed in *P. vulgaris*. Fewer pollen grains maturing in heat stressed anthers is another possible explanation for less pollen released under heat stress. The total number of pollen grains produced under stressed and unstressed conditions was not measured but could be assessed by examining anthers from flowers one day before anthesis.

Pollen Germination in Chamber Experiments

Tests of in vitro pollen germination at temperatures of 25°C, 28°C, 31°C, 34°C, 37°C and 40°C indicated a small but statistically significant decrease in pollen germination between 28 and 31°C (Figure 15). There was a second small but significant decrease in germination between 31 and 34°C. A large and significant decrease in germination occurred between 37 and 40°C from 60% to 7% germination respectively.

The optimal temperature for lima bean pollen germination is less than 31°C. The lower limits of temperature for lima bean pollen germination were not explored in this experiment. Germination at the two lowest temperatures tested, 25 and 28°C, was 85% and 89%, respectively. Pollen germination was decreased at temperatures between 28 and 37°C, so daytime temperatures in this range could impact pod set in lima bean. However, the germination percentage is still high enough (60% or greater)

to achieve fertilization if high quality pollen is present on the stigma. Between 37 and 40°C there is a large enough decrease in pollen germination to effectively stop fertilization. Daytime temperatures above 37°C could significantly impact pod set in lima bean. Temperatures rarely reach this level in the Mid-Atlantic region, but high daytime temperatures could impact lima bean pod set in some production areas.

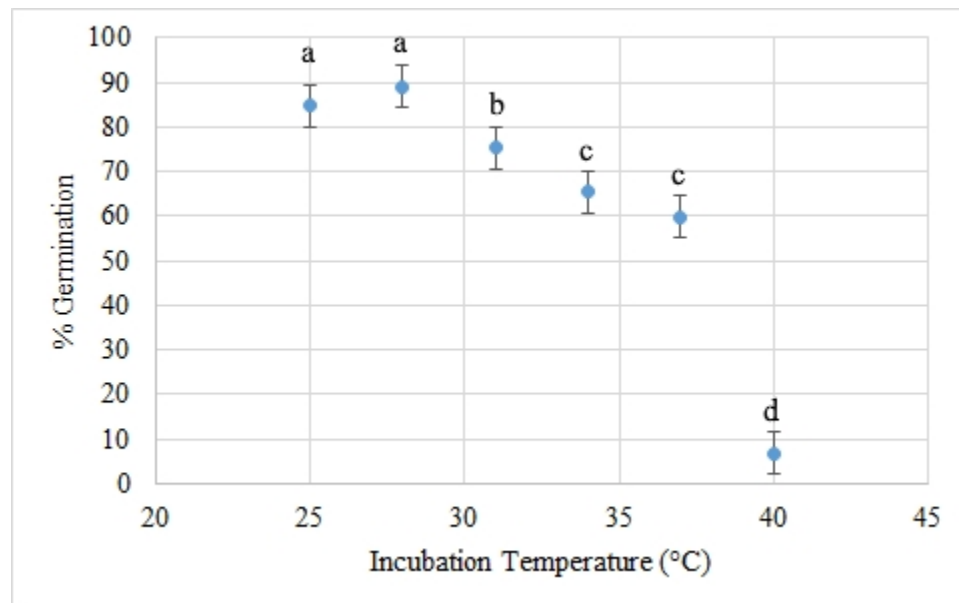


Figure 15 Percent in vitro germination of pollen from unstressed plants incubated at different temperatures for all genotypes combined. Mean separation is by the Tukey-Kramer method at $P \leq 0.05$. Error bars indicate the 95% confidence interval for the mean.

In addition to incubation temperature, percent in vitro germination was affected by genotype and genotype x temperature interaction (Table 13). C-elite Select had the highest percent germinated pollen at all temperatures (Figure 16). Genotypes followed the same general ranking pattern in percent germination at all temperatures

except 34 °C, where Fordhook 242 had an unexpectedly high germination rate and Bush Florida Butter had an unexpectedly low germination rate. Additional experiments of this sort would be necessary to understand how genotype and genotype x temperature interactions related to pollen germination might affect heat tolerance in lima bean.

Table 13 P-values for type 3 tests of fixed effects for in vitro pollen germination of five genotypes at six temperatures

Effect	p-value
Temperature (T)	<0.0001
Genotype (G)	<0.0001
G x T	<0.0001

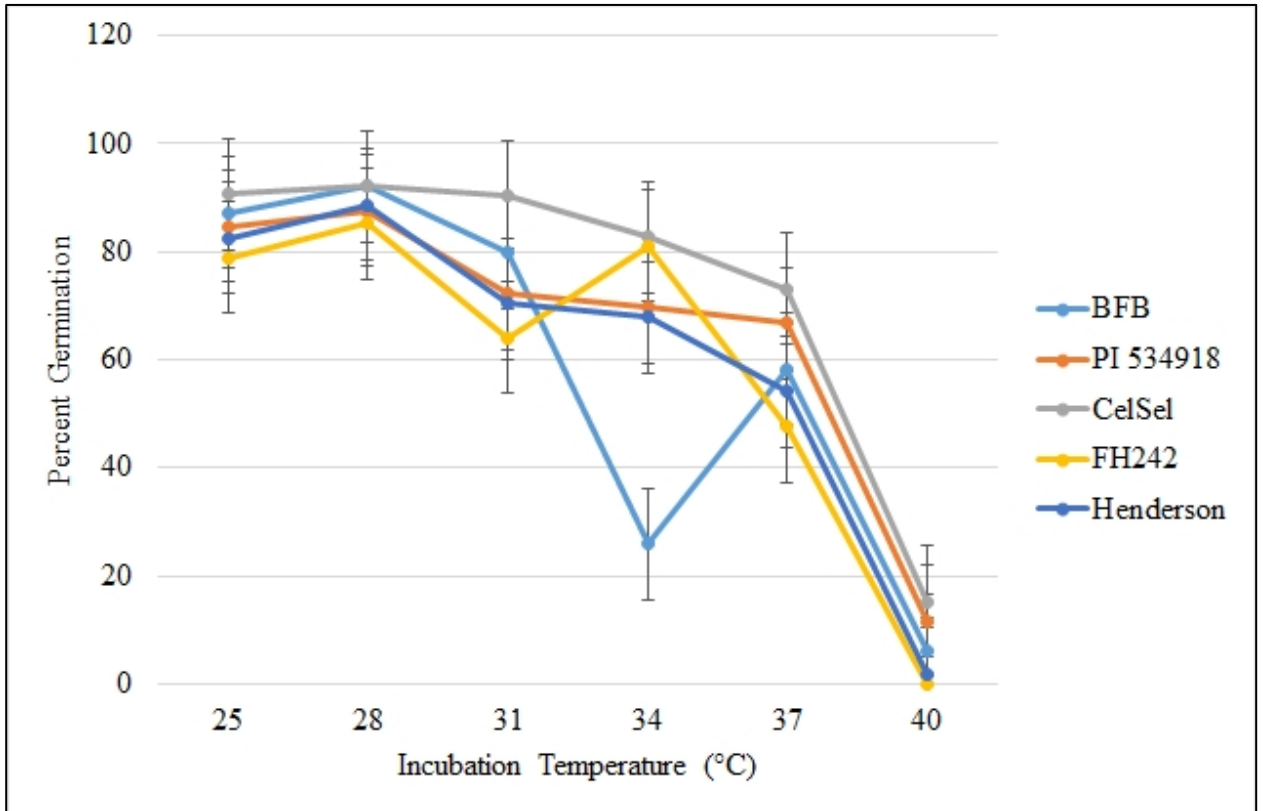


Figure 16 Percent in vitro germination of pollen from unstressed plants incubated at different temperatures for the five genotypes. Error bars indicate the 95% confidence interval for the mean.

In order to understand how pollen germination (quality) is affected by development under sustained hot or cool night temperature conditions, pollen from flowers of the five genotypes collected from the hot and cool chambers was germinated in vitro at 25°C. Genotype average germination rates for pollen collected from the cool chamber ranged from 81 to 88% and there were no statistically significant differences in these means (Figure 17). Genotype averages for hot chamber pollen ranged from 23 to 72% and there were differences between these means with Fordhook 242 having the lowest rate and PI 534918 and Bush Florida Butter the

highest rates. For each genotype, germination was significantly reduced in pollen collected from the hot chamber.

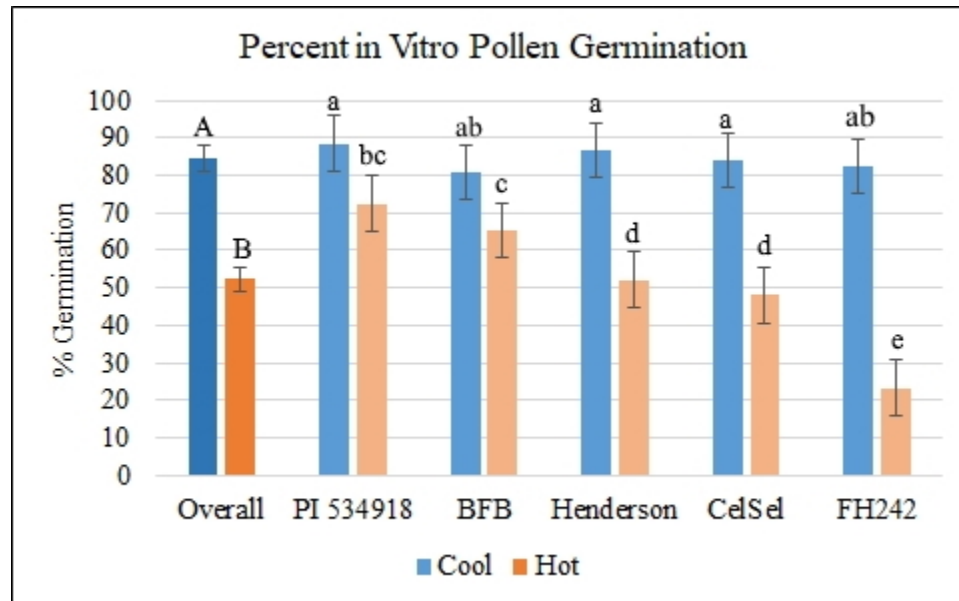


Figure 17 Percent in vitro germination of pollen collected from flowers under heat stress and unstressed by genotype and for all genotypes combined. Mean separation is by the Tukey-Kramer method at $P \leq 0.05$. Error bars indicate the 95% confidence interval for the mean.

Crossing Experiments

When a heat stressed C-elite Select plant was the pistillate parent, hand pollination with pollen from an unstressed C-elite Select or PI 534918 plant was significantly more likely than self-pollinated flowers to produce a pod measuring at least 1 cm and mature seed (Table 14). When a heat stressed Fordhook 242 was the pistillate parent, pollen from an unstressed PI 534918 plant was significantly more likely to form a pod, but not more likely to produce mature seed.

For C-elite Select, the primary factor inhibiting pod formation and seed production under heat stress is a lack of viable pollen. When quality pollen was applied to the receptive stigma a pod formed 48% of the time compared to 12% of selfed flowers. Furthermore, mature seed was produced 31% of the time compared to 2% of selfed flowers. For Fordhook 242, 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. No selfed flowers produced mature seed. Additional factors, beyond the presence of viable pollen is inhibiting pod formation, and, especially, seed production in heat stressed Fordhook 242. Flower deformity resulting in excessive style length and coiling was frequently observed in heat stressed Fordhook 242 and could be one factor interfering with fertilization in this genotype.

Table 14 Pedigree, number of tests, frequency of pod or seed formation, exact p-value, odds ratio and 95% confidence intervals for the odds ratio for paired tests of crosses made on heat stressed plants with pollen from unstressed plants versus self-pollinated flowers.

Cross	n (pairs)	Frequency		Exact p-value	Odds Ratio	95% C. I.	
		Cross = + Self = -	Cross = - Self = +			Lower	Upper
<i>formation of pod measuring at least 1 cm</i>							
CelSel x PI 534918	70	32	4	1.94E-06	8	2.84	31.14
CelSel x CelSel	60	21	2	6.60E-05	10.5	2.57	92.37
FH242 x PI 534918	80	23	4	0.000311	5.75	1.96	22.87
<i>formation of mature seed</i>							
CelSel x PI 534918	70	24	0	1.19E-07	∞	6.02	∞
CelSel x CelSel	60	13	0	0.000244	∞	3.05	∞
FH242 x PI 534918	80	5	0	0.0625	∞	0.92	∞

Heat Tolerance Evaluation of Diversity Panel Members

Small and large seeded types and lines with bush and vining growth habit were among the 131 accessions that were evaluated for yield under sustained high night temperatures in summer greenhouse conditions and rated for quantity of pollen released under high night temperature conditions. Most of the genotypes (93%) were of U.S. origin because much of the germplasm available from outside the U.S. is photoperiod sensitive and does not flower under summer conditions in Delaware. Germplasm was classified as either Andean or Mesoamerican background based on genotype data (Table 15). The relative effect for genotype on pollen quantity rating ranged from 0.084 to 0.735 with statistically significant differences between genotypes (Table 15). Additionally, genotypes produced significantly different yields under heat stress ($p < 0.0001$). Based on linear regression the relative effect for pollen quantity rating (RE_{pollen}) under heat stress could be used to predict yield under heat stress; $\text{yield} = 46.0 \times RE_{\text{pollen}} - 5.7$, $R^2 = 0.539$ (Figure 11).

Genotypes derived from the Andean gene pool generally produced lower yields under heat stress, with many genotypes producing little or no yield. Two Andean genotypes (PI 549493 and PI 549497) produced moderate yields under heat stress and had moderate pollen ratings (Figure 18). Two Andean genotypes had pollen ratings that were in the range of that of the best yielding Mesoamerican genotypes, but they did not produce equivalent yields (PI 549461 and PI 249040). As suggested by the greenhouse experiments, factors beyond the quantity of pollen released, such as pollen germination rate, pistil function, or photosynthate partitioning also play a role in determining yield under heat stress. Nonetheless some Andean genotypes did have higher yield and pollen ratings under heat stress and this germplasm may be source of useful heat tolerance traits. Based on genotype, PI 549461, PI 249040, PI 549493 and

PI 549497 group together with accessions from Peru, while the remainder of the U.S.-collected large seeded limas were in a separate group that consisted of only U.S. accessions. Screening a wider diversity of Andean germplasm may reveal additional sources of heat tolerance that could be used to improve U.S. Fordhook and large seeded lima cultivars.

Among the Mesoamerican derived genotypes, the three accessions with the highest yield and pollen ratings were PI 347829, PI 347786 and PI 347787. These three lines were collected in California and Arizona and suggests that some germplasm from the western U.S. may be useful in improving heat tolerance in baby lima types. Other heat tolerant genotypes were collected from India, Haiti and other regions of the U.S. Some Mesoamerican genotypes were not very heat tolerant (Figure 19). PI 549507, a cultivar called ‘Mackie’, was classified as Mesoamerican based on genotype, but it has large seed and resembles the Fordhook and large lima types. No pedigree information is available for this line, but it is likely the result of crosses between Andean and Mesoamerican genotypes. PI 347790 is another Mesoamerican type that had low yields and is probably the result of similar crossing between gene pools. This line was very low yielding under heat stress and has relatively small seeds, but it is designated a “large lima” in the USDA database; additionally, it has a blunted pod shape and large flower size, which are present in Andean derived genotypes. PI 347812, PI 451782 and Virginia Butterbean are Mesoamerican accessions with low yield under heat stress that do not have any indications of having outcrossed with Andean germplasm, which suggests that heat susceptibility is not absent from the Mesoamerican gene pool.

Table 15 Diversity panel genotype identifiers, growth habit, gene pool grouping, seed weight, yield under heat stress, relative genotype effect for pollen rating, 95% confidence interval for relative effect and location of collection.

Entry #	Name	Accession #	Growth Habit	Group	Seed Wt (g)	Heat Stress Yield (g/plant) (rank)	Pollen Rating		Origin
							Relative Genotype Effect (rank)	95% CI for Relative Effect	
215		PI 347816	vine	Meso*	0.38	38 (1)	0.652 (35)	(0.589, 0.710)	U.S..
145	L 136	PI 347829	vine	Meso	0.40	38 (1)	0.721 (4)	(0.693, 0.748)	U.S.
139	Hopi 5989	PI 347786	vine	Meso	0.40	37 (3)	0.708 (10)	(0.668, 0.744)	U.S.
161		PI 347834	vine	Meso	0.35	36 (4)	0.619 (51)	(0.524, 0.707)	U.S.
138	Wilbur		vine	Meso	0.33	35 (5)	0.587 (57)	(0.500, 0.668)	U.S.
158	Bal	PI 180461	vine	Meso	0.39	34 (6)	0.624 (45)	(0.556, 0.688)	India
38	Hopi 2000	PI 347787	vine	Meso	0.44	34 (6)	0.708 (10)	(0.668, 0.744)	U.S.
253	Brown Crower Pole 118	PI 550301	vine	Meso*	0.33	33 (8)	0.658 (31)	(0.591, 0.719)	U.S.
143	Dixie Speckled Butterpea		bush	Meso	0.33	33 (8)	0.650 (41)	(0.576, 0.718)	U.S.
18	PA German Red Lima		bush	Meso	0.46	33 (8)	0.603 (53)	(0.526, 0.675)	U.S.
2	1102-6		bush	Meso	0.33	33 (8)	0.658 (31)	(0.591, 0.719)	Haiti
66	Henderson Bush	PI 549466	bush	Meso	0.42	32 (12)	0.680 (22)	(0.626, 0.729)	U.S.
47	Sieva	PI 549469	vine	Meso	0.41	32 (12)	0.480 (79)	(0.385, 0.577)	U.S.
188		PI 257381	vine	Meso	0.34	32 (12)	0.658 (31)	(0.591, 0.719)	Colombia
58	Westan	PI 347777	vine	Meso	0.35	31 (15)	0.658 (31)	(0.591, 0.719)	U.S.
246	Easy Shell	PI 549484	vine	Meso	0.50	31 (15)	0.524 (70)	(0.432, 0.613)	U.S.
141	Violet's Multicolored Butterbean		vine	Meso	0.39	31 (15)	0.660 (27)	(0.607, 0.720)	U.S.
135	UC Haskell		vine	Meso	0.46	30 (18)	0.721 (4)	(0.693, 0.748)	U.S.

Entry #	Name	Accession #	Growth		Seed Wt (g)	Heat Stress	Pollen Rating		Origin
			Habit	Group		Yield (g/plant) (rank)	Relative Genotype Effect (rank)	95% CI for Relative Effect	
37	Hopi 155	PI 347784	vine	Meso	0.33	30 (18)	0.708 (10)	(0.668, 0.744)	U.S.
137	Mezcla		vine	Meso	0.36	30 (18)	0.721 (4)	(0.693, 0.748)	U.S.
35	Hopi 13	PI 347779	vine	Meso	0.33	30 (18)	0.673 (25)	(0.603, 0.736)	U.S.
54	Manteca Ramas	PI 162688	vine	Meso	0.42	30 (18)	0.589 (55)	(0.511, 0.662)	Argentina
131	Pat		vine	Meso*	0.36	30 (18)	0.721 (4)	(0.693, 0.748)	U.S.
211		PI 347801	vine	Meso	0.52	30 (18)	0.694 (14)	(0.646, 0.737)	U.S.
132	Henderson		bush	Meso	0.39	30 (18)	0.589 (55)	(0.511, 0.662)	U.S.
300	Hopi 5989	PI 347786	vine	Meso*	0.41	29 (26)	0.510 (71)	(0.419, 0.599)	U.S.
212		PI 347807	vine	Meso	0.45	29 (26)	0.694 (14)	(0.646, 0.737)	U.S.
207	Hopi 5986	PI 347785	vine	Meso	0.37	28 (28)	0.721 (4)	(0.693, 0.748)	U.S.
36	Hopi 15	PI 347781	vine	Meso	0.31	28 (28)	0.680 (22)	(0.626, 0.729)	U.S.
70	Baby Potato	PI 549494	bush	Meso	0.37	28 (28)	0.437 (84)	(0.341, 0.538)	U.S.
136	UC Luna		bush	Meso	0.48	28 (28)	0.694 (14)	(0.646, 0.737)	U.S.
218		PI 347842	vine	Meso	0.31	28 (28)	0.652 (35)	(0.589, 0.710)	U.S.
237	Cowey	PI 534913	bush	Meso	0.58	28 (28)	0.638 (42)	(0.572, 0.699)	U.S.
239	Half & Half	PI 534915	vine	Meso	0.39	27 (34)	0.652 (35)	(0.589, 0.710)	U.S.
210		PI 347799	vine	Meso	0.43	27 (34)	0.431 (86)	(0.339, 0.528)	U.S.
209	Sierra	PI 347798	vine	Meso	0.53	27 (34)	0.652 (35)	(0.589, 0.710)	U.S.
16	Jackson Wonder		bush	Meso	0.41	26 (37)	0.567 (63)	(0.483, 0.647)	U.S.
6	C-elite Select		bush	Meso	0.39	26 (37)	0.587 (57)	(0.500, 0.668)	U.S.
62	Bixby	PI 549460	bush	Meso	0.39	25 (39)	0.623 (49)	(0.544, 0.695)	U.S.
125	Cariblanco Norte		vine	Meso	0.37	25 (39)	0.680 (22)	(0.626, 0.729)	U.S.

Entry #	Name	Accession #	Growth		Seed Wt (g)	Heat Stress	Pollen Rating		Origin
			Habit	Group		Yield (g/plant) (rank)	Relative Genotype Effect (rank)	95% CI for Relative Effect	
142	Alabama Blackeyed Butterbean		vine	Meso	0.47	25 (39)	0.561 (64)	(0.481, 0.637)	U.S.
41		PI 347826	vine	Meso	0.41	24 (42)	0.652 (35)	(0.589, 0.710)	U.S.
222	Climbing Speckled	PI 638826	vine	Meso	0.43	24 (42)	0.735 (1)	(0.729, 0.742)	U.S.
15	Improved Kingston		bush	Meso*	0.32	24 (42)	0.280 (107)	(0.211, 0.362)	U.S.
224		PI 427217	vine	Meso	0.58	24 (42)	0.624 (45)	(0.556, 0.688)	U.S.
3	184-85		bush	Meso	0.35	24 (42)	0.638 (42)	(0.572, 0.699)	U.S.
57		PI 256820	vine	Meso	0.39	23 (47)	0.616 (52)	(0.511, 0.712)	Ecuador
68	Nemagreen	PI 549481	bush	Meso	0.38	23 (47)	0.308 (101)	(0.229, 0.400)	U.S.
190		PI 347836	vine	Meso	0.43	23 (47)	0.454 (81)	(0.369, 0.542)	U.S.
11	Dixie Butterpea	PI 549462	bush	Meso	0.27	23 (47)	0.525 (68)	(0.442, 0.608)	U.S.
238	Ganymede	PI 534914	vine	Meso	0.48	23 (47)	0.581 (59)	(0.498, 0.659)	U.S.
46	Cave Dweller	PI 534918	bush	Meso	0.45	23 (47)	0.660 (27)	(0.607, 0.720)	U.S.
53	Hopi 50	PI 347782	vine	Meso	0.30	22 (53)	0.694 (14)	(0.646, 0.737)	U.S.
206	Hopi 14	PI 347780	vine	Meso	0.38	22 (53)	0.672 (26)	(0.609, 0.729)	U.S.
40	Willow Leaf, White	PI 347819	vine	Meso	0.39	22 (53)	0.620 (50)	(0.550, 0.686)	U.S.
5	Bush Florida Butter	PI 549509	bush	Meso*	0.51	22 (53)	0.708 (10)	(0.668, 0.744)	U.S.
17	Maffei 15		bush	Meso	0.42	21 (57)	0.575 (61)	(0.496, 0.650)	U.S.
49	Thaxter	PI 549454	bush	Meso	0.41	21 (57)	0.322 (95)	(0.239, 0.418)	U.S.
151		PI 347831	vine	Meso	0.37	21 (57)	0.660 (27)	(0.607, 0.720)	U.S.
229	L 59	PI 451776	vine	Meso	0.53	21 (57)	0.715 (9)	(0.672, 0.754)	U.S.

Entry #	Name	Accession #	Growth		Seed Wt (g)	Heat Stress	Pollen Rating		Origin
			Habit	Group		Yield (g/plant) (rank)	Relative Genotype Effect (rank)	95% CI for Relative Effect	
236		PI 502183	vine	Meso	0.31	21 (57)	0.492 (77)	(0.414, 0.570)	U.S.
61	Dover Bush	PI 549455	bush	Meso	0.37	21 (57)	0.316 (97)	(0.230, 0.417)	U.S.
255	Black and Buff Bush	PI 347822	vine	Meso	0.47	20 (63)	0.446 (83)	(0.359, 0.537)	U.S.
124	UC Beija Flor		bush	Meso	0.32	20 (63)	0.735 (1)	(0.729, 0.742)	U.S.
248	Giant Florida Pole	PI 549493	vine	Andean	0.89	19 (65)	0.502 (73)	(0.417, 0.587)	U.S.
72	Maffei 76	PI 549512	bush	Meso	0.39	19 (65)	0.624 (45)	(0.556, 0.688)	U.S.
240	Red Calico	PI 534917	vine	Meso*	0.52	18 (67)	0.686 (20)	(0.628, 0.738)	U.S.
247	Buttergreen	PI 549485	vine	Meso	0.44	18 (67)	0.158 (126)	(0.105, 0.232)	U.S.
234		PI 477041	vine	Meso	0.48	18 (67)	0.531 (66)	(0.444, 0.617)	U.S.
223		PI 427216	vine	Meso	0.54	18 (67)	0.623 (48)	(0.549, 0.692)	U.S.
214		PI 347813	vine	Meso*	0.41	17 (71)	0.569 (62)	(0.494, 0.641)	U.S.
213		PI 347811	vine	Meso	0.52	17 (71)	0.525 (68)	(0.442, 0.608)	U.S.
105	1102-9		bush	Meso	0.34	17 (71)	0.694 (14)	(0.646, 0.737)	Haiti
228	Natiquo	PI 451715	vine	Meso	0.47	17 (71)	0.247 (113)	(0.171, 0.343)	U.S.
140	Worcester Indian Red Pole		vine	Meso	0.50	16 (75)	0.694 (14)	(0.646, 0.737)	U.S.
231	L 120A	PI 451780	vine	Meso	0.29	16 (75)	0.652 (35)	(0.537, 0.752)	U.S.
250	Giant Christmas	PI 549497	vine	Andean	1.05	16 (75)	0.486 (78)	(0.412, 0.559)	U.S.
225		PI 427218	vine	Meso	0.39	16 (75)	0.682 (21)	(0.604, 0.751)	U.S.
8	Cypress		bush	Meso	0.37	15 (79)	0.603 (53)	(0.526, 0.675)	U.S.
1	1102-10 (Beseba)		bush	Meso	0.28	15 (79)	0.456 (80)	(0.378, 0.536)	Haiti

Entry #	Name	Accession #	Growth		Seed Wt (g)	Heat Stress	Pollen Rating		Origin
			Habit	Group		Yield (g/plant) (rank)	Relative Genotype Effect (rank)	95% CI for Relative Effect	
12	Dover Tucker	PI 549456	bush	Meso	0.36	13 (81)	0.311 (100)	(0.227, 0.410)	U.S.
244	Willow Leaf	PI 549474	vine	Meso	0.30	12 (82)	0.735 (1)	(0.729, 0.742)	U.S.
169	Seven-year Bean	PI 221202	vine	Andean	1.01	12 (82)	0.327 (94)	(0.264, 0.398)	Zambia
243	Triumph	PI 549473	bush	Meso	0.34	11 (84)	0.539 (65)	(0.455, 0.621)	U.S.
194		PI 347839	vine	Meso	0.27	9 (85)	0.527 (67)	(0.349, 0.698)	U.S.
245	Wasatch	PI 549482	vine	Meso	0.32	9 (85)	0.393 (88)	(0.299, 0.496)	U.S.
63	Christmas Lima	PI 549461	vine	Andean	0.86	9 (85)	0.627 (44)	(0.548, 0.699)	U.S.
85	Zerbe		vine	Andean	2.00	9 (85)	0.176 (124)	(0.128, 0.237)	U.S.
84	Susie		vine	Andean	0.63	9 (85)	0.225 (116)	(0.186, 0.269)	U.S.
87	Lineberger - Layton		vine	Andean	1.15	8 (90)	0.178 (123)	(0.132, 0.235)	U.S.
65	Fordhook Bush ind	PI 549465	vine	Andean	1.10	8 (90)	0.321 (96)	(0.265, 0.383)	U.S.
133	Lee		vine	Andean	0.78	7 (92)	0.432 (85)	(0.347, 0.522)	U.S.
201	Purple Spray	PI 249040	vine	Andean	1.01	7 (92)	0.660 (27)	(0.607, 0.720)	Nigeria
86	Lineberger - Warren		vine	Andean	1.28	6 (94)	0.201 (118)	(0.159, 0.251)	U.S.
83	Pete's Dr. Martin		vine	Andean	1.82	6 (94)	0.229 (115)	(0.172, 0.299)	U.S.
252	F - 169	PI 549514	vine	Andean*	0.73	6 (94)	0.502 (74)	(0.408, 0.595)	U.S.
106	King Louie		vine	Andean	1.16	6 (94)	0.166 (125)	(0.122, 0.223)	U.S.
77	Dodd		vine	Andean	1.04	5 (98)	0.272 (108)	(0.203, 0.356)	U.S.
59	Hopi Red/ Pala Hatiquo	PI 476859	vine	Meso	0.44	5 (98)	0.504 (72)	(0.417, 0.590)	U.S.
129	White Ventura	PI 549503	vine	Andean	0.83	5 (98)	0.198 (120)	(0.141, 0.270)	U.S.

Entry #	Name	Accession #	Growth		Seed Wt (g)	Heat Stress	Pollen Rating		Origin
			Habit	Group		Yield (g/plant) (rank)	Relative Genotype Effect (rank)	95% CI for Relative Effect	
50	Virginia Butterbean		vine	Meso	0.67	4 (101)	0.452 (82)	(0.361, 0.548)	U.S.
75	Big Momma		vine	Andean	1.37	4 (101)	0.201 (118)	(0.128, 0.302)	U.S.
71	Large White	PI 549496	bush	Andean	1.14	3 (103)	0.193 (121)	(0.155, 0.240)	U.S.
249	Carpinteria	PI 549495	vine	Andean	0.95	2 (104)	0.231 (114)	(0.182, 0.289)	U.S.
251	Mackie	PI 549507	vine	Meso	1.14	2 (104)	0.581 (59)	(0.498, 0.659)	U.S.
130		PI 347812	vine	Meso	0.83	1 (106)	0.411 (87)	(0.325, 0.503)	U.S.
81	Jones		vine	Andean	0.96	1 (106)	0.347 (90)	(0.274, 0.429)	U.S.
9	DE0501102B		bush	Andean	0.74	1 (106)	0.330 (93)	(0.248, 0.424)	U.S.
60	Sprigg	PI 549516	vine	Andean	0.75	1 (106)	0.259 (109)	(0.195, 0.335)	U.S.
64	Fordhook Bush	PI 549465	bush	Andean	1.25	1 (106)	0.308 (101)	(0.229, 0.400)	U.S.
80	Kuvilek		vine	Andean	1.64	1 (106)	0.182 (122)	(0.123, 0.260)	U.S.
79	DSU Big 6		vine	Andean	1.54	1 (106)	0.313 (99)	(0.243, 0.394)	U.S.
82	Moser		vine	Andean	1.27	1 (106)	0.123 (130)	(0.064, 0.222)	U.S.
78	Rohrer's Dr. Martin		vine	Andean	2.04	1 (106)	0.084 (131)	(0.079, 0.089)	U.S.
128	White Ventura 63	PI 549502	vine	Andean	1.05	1 (106)	0.369 (89)	(0.293, 0.452)	U.S.
76	Coverdale		vine	Andean	1.34	1 (106)	0.248 (112)	(0.203, 0.300)	U.S.
4	90-1		bush	Andean	1.15	1 (106)	0.339 (92)	(0.265, 0.424)	U.S.
232	L 122A	PI 451782	bush	Meso	0.29	1 (106)	0.146 (128)	(0.113, 0.188)	U.S.
48	Sussex		bush	Andean	0.99	0 (119)	0.502 (74)	(0.408, 0.595)	U.S.
235		PI 502182	vine	Andean	1.22	0 (119)	0.154 (127)	(0.112, 0.209)	U.S.
10	DE0504103A		bush	Andean	0.91	0 (119)	0.215 (117)	(0.166, 0.275)	U.S.
67	King of the Garden	PI 549468	vine	Andean	0.97	0 (119)	0.253 (111)	(0.196, 0.319)	U.S.

Entry #	Name	Accession #	Growth		Seed Wt (g)	Heat Stress Yield (g/plant) (rank)	Pollen Rating		Origin
			Habit	Group			Relative Genotype Effect (rank)	95% CI for Relative Effect	
127	White Ventura	PI 549499	vine	Andean	0.85	0 (119)	0.303 (104)	(0.237, 0.380)	U.S.
14	FH 242	PI 549464	bush	Andean	1.00	0 (119)	0.298 (105)	(0.238, 0.366)	U.S.
73	Dompe 95	PI 549518	bush	Andean	0.98	0 (119)	0.345 (91)	(0.265, 0.436)	U.S.
7	Concentrated Fordhook		bush	Andean	1.06	0 (119)	0.305 (103)	(0.249, 0.369)	U.S.
13	FH 1072	PI 549519	bush	Andean*	1.11	0 (119)	0.294 (106)	(0.220, 0.381)	U.S.
126	UC 92		bush	Andean	1.19	0 (119)	0.254 (110)	(0.207, 0.309)	U.S.
134	459-1		vine	Andean	1.16	0 (119)	0.314 (98)	(0.253, 0.383)	U.S.
200	Fordhook 861 Charter	PI 549457	bush	Andean*	0.98	0 (119)	0.137 (129)	(0.094, 0.195)	U.S.
208		PI 347790	bush	Meso	0.47	0 (119)	0.496 (76)	(0.406, 0.586)	U.S.

* designation based on seed size rather than genotype

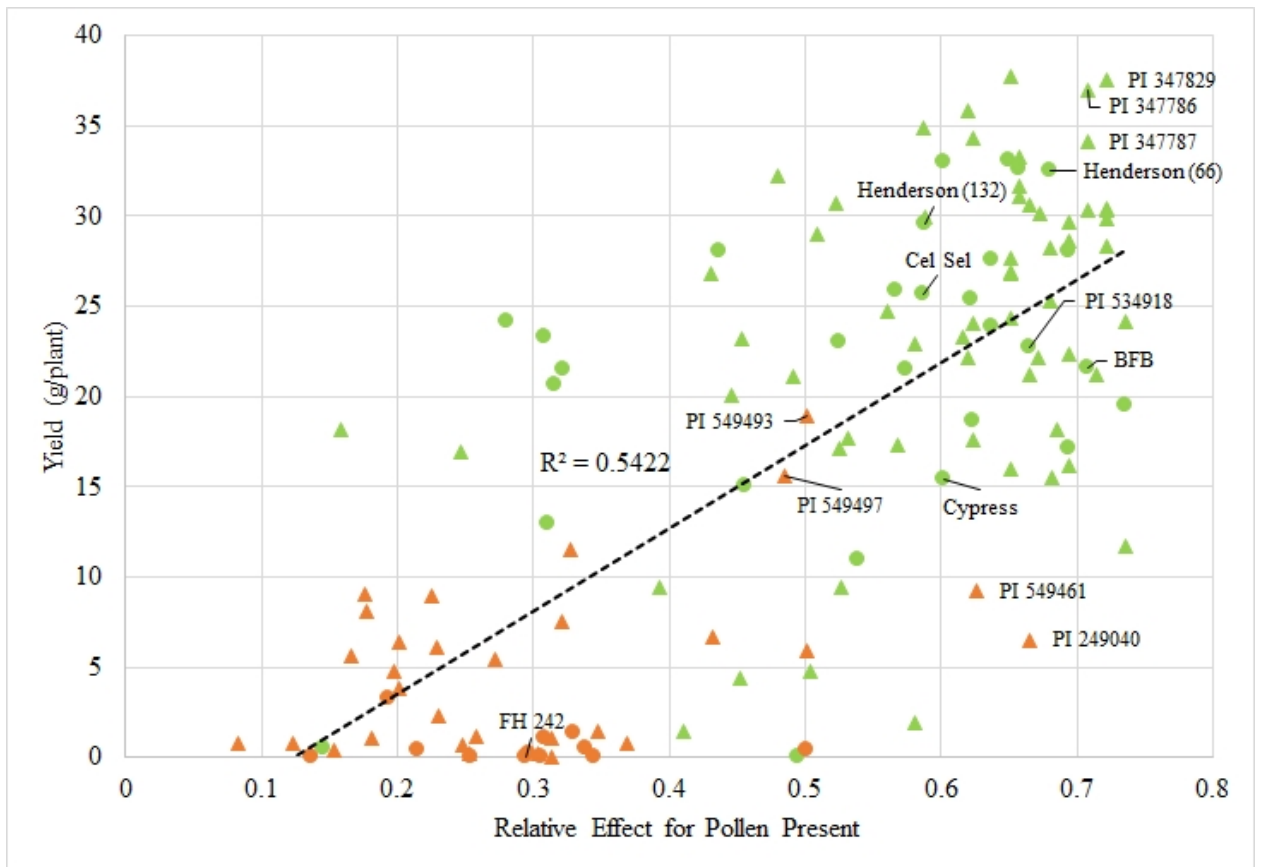


Figure 18 Yield in grams per plant versus relative effect for rating of pollen present on the style and stigma. Triangle markers indicate vining genotypes, circles indicate bush genotypes. Green markers are Mesoamerican genotypes and orange markers are Andean genotypes.

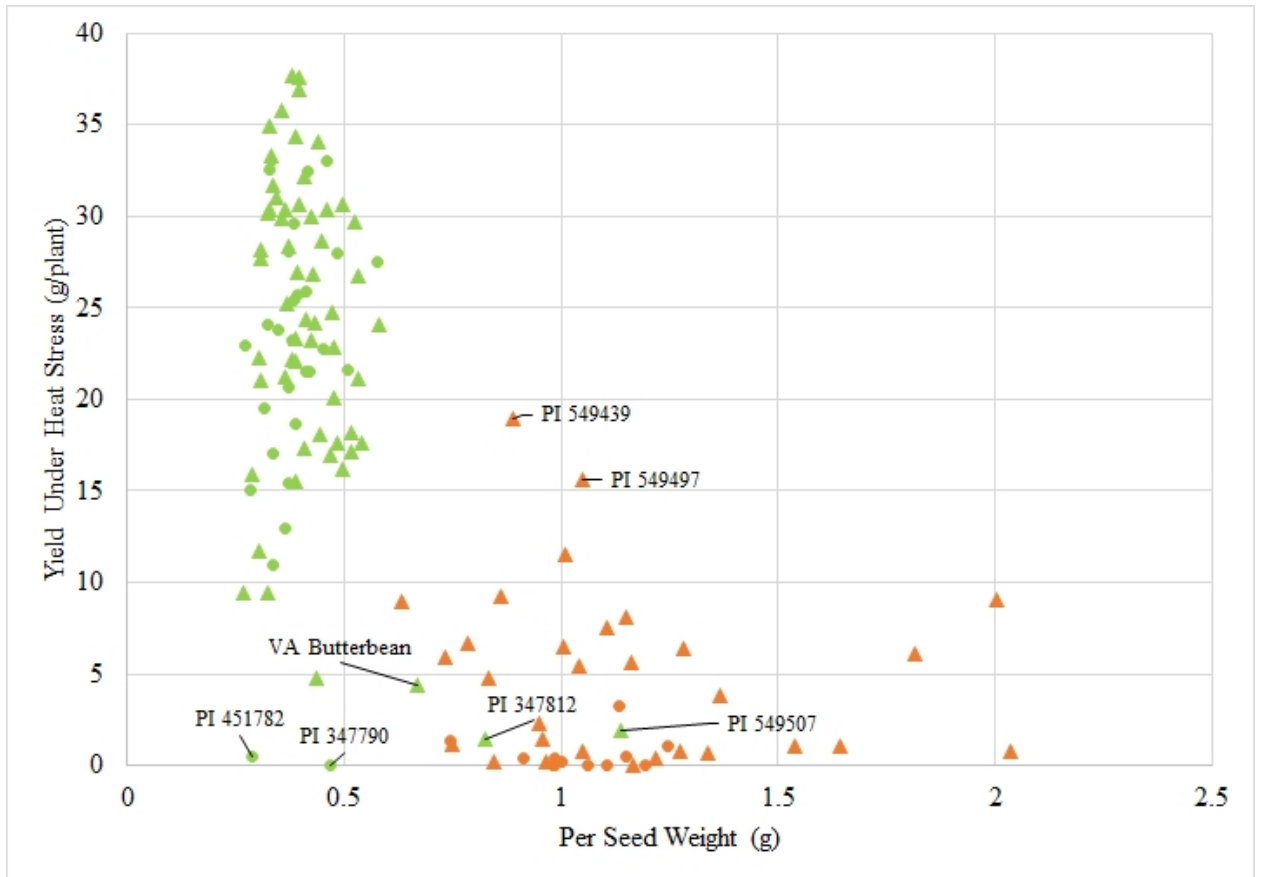


Figure 19 Yield in grams per plant versus per seed weight. Triangle markers indicate vining genotypes, circles indicate bush genotypes. Green markers are Mesoamerican genotypes and orange markers are Andean genotypes.

Heat Stress Effects on Seed Characteristics

The genotypes screened for heat tolerance have a variety of seed coat patterns and colors. When the appearance of seed from the greenhouse heat tolerance screen was compared to seed grown under cool greenhouse conditions, differences in seed coat pattern expression and seed quality were apparent (see Appendix).

Eleven of the genotypes have seed coat pattern similar to that of Bush Florida Butter (PI 549509), a buff background with a darker color around the hilum and

speckling that extends from the micropyle along the dorsal side of the seed. For all genotypes with this pattern the darker secondary color was more restricted under heat stress. This was also the case with Alabama Blackeyed Butterbean, which has a similar pattern, but with the secondary color only around the hilum. Four genotypes have a pattern like that of Christmas Lima (PI 549461), a white background with broken maroon stripes. In all four genotypes with this pattern, the maroon stripes were wider and more continuous under heat stress. Fifteen genotypes had a pattern like that of Jackson Wonder, most seeds with dark speckles on a lighter background, but some seeds having the reverse pattern with light speckles on a dark background. Eight of these genotypes had similar pattern expression in stressed and unstressed conditions, the remaining seven had more of the darker color expressed under heat stress. Two genotypes appear to be self (solid) colored under normal conditions, but under heat stress have small darker colored flecks (PI 347812 and PI 451715). Three genotypes appear to be self-colored under heat stress but under normal conditions have small lighter colored flecks (PI 347836, PI 534913 and PI 534918). PI 249040 has a unique pattern of tiny dark and light purple dots on a white background, which was similarly expressed in stressed and unstressed conditions. The expression of genes controlling seed coat pattern is affected by temperature conditions with some having restricted secondary color expression under heat stress (hilum ring patterns) and some having greater secondary color expression under heat stress (striping and speckling).

While the changes in pattern expression described above are not likely to be of concern for lima bean production, some genotypes did have reduced seed quality under heat stress. Yellow, brown or orange discoloration was present on seed from 51 genotypes (39%) when grown under heat stress (Appendix). All but one of these

genotypes was self-colored green or white, which may be because the discoloration is less observable on dark colored seeds or because the patterned and dark colored genotypes are less susceptible to this discoloration. Seed coat discoloration caused by heat stress is also reported in cowpea (Patel and Hall, 1988) where three QTL associated with this response have been identified (Pottorff *et al.*, 2014). Candidate genes in the QTL regions are involved in ethylene formation and response (Pottorff *et al.*, 2014). In cowpea the mechanisms controlling seed coat discoloration and their inheritance are independent of those controlling reproductive heat stress tolerance (Patel and Hall, 1988) and this seems to be the case in lima bean as well. Consequently, absence of heat induced seed discoloration will have to be selected for independently of reproductive heat stress tolerance.

Twenty-three genotypes (18%) had incompletely formed testae on some seeds (Appendix). This was observed only in genotypes with self-colored white or green seed but was present in both Andean and Mesoamerican genotypes. Shrunken seeds that failed to fully mature were observed in 31 genotypes (24%), all of which were of Andean origin (Appendix). This phenomenon was widespread in the Andean genotypes, with 89% of the Andean lines that produced seed under heat stress having small seeds. In some common bean genotypes, high temperatures interfere with seed maturation and pod filling, resulting in small seed size (Rainey and Griffiths, 2005a; Shonnard and Gepts, 1994). There is evidence that heat stress disrupts photosynthate transport to flowers and pods in certain common bean genotypes (Soltani *et al.*, 2019; Omae, *et al.*, 2007) although this phenomenon has not been specifically linked to failed seed maturation. A similar disruption of photosynthate transport could be the

cause of failed seed maturation in lima bean that appears frequently in Andean derived germplasm.

Thirteen Mesoamerican genotypes with white or light green seed produced high quality seed without discoloration or incomplete testae under heat stress: PI 347786, PI 347777, PI 347779, PI 347785, PI 347842, PI 549460, PI 347782, PI 347780, PI 549474, PI 549473, Wilbur, UC Luna, and Cariblanco Norte. Of these lines, the highest yielding under heat stress were: PI 347786, Wilbur, PI 347777 and PI 347779. These lines may be important for breeding for heat tolerance in green and white seeded lima beans so that seed quality, as well as yield, is maintained under heat stress.

Population Structure of Diversity Panel Members and Genetic Architecture of Heat Tolerance

Analysis of the diversity panel population structure using multidimensional scaling (MDS) separated the panel members into Andean and Mesoamerican gene pools on the first axis (Figure 20). The second axis divides the Andean group in two, with one group containing the US derived large-seeded genotypes and the second containing large-seeded genotypes collected from South America, Africa and three genotypes from the southern US. Small seeded US genotypes grouped with accessions from Central America, Mexico and the Caribbean.

As observed when using seed size as an indicator of gene pool, Andean genotypes that were screened for heat stress were all low yielding (Figure 21a) while the Mesoamerican genotypes exhibited a range of yield response under heat stress with many producing high yields, but some producing low yields. Pollen release under heat stress was high for most Mesoamerican genotypes but a few had low pollen

release (Figure 21b). Most Andean genotypes had low pollen release under heat stress, but a few had high or very high pollen release. Andean genotypes with high pollen release had relatively higher yield under heat stress, but their yield was low compared to the most heat tolerant Mesoamerican genotypes.

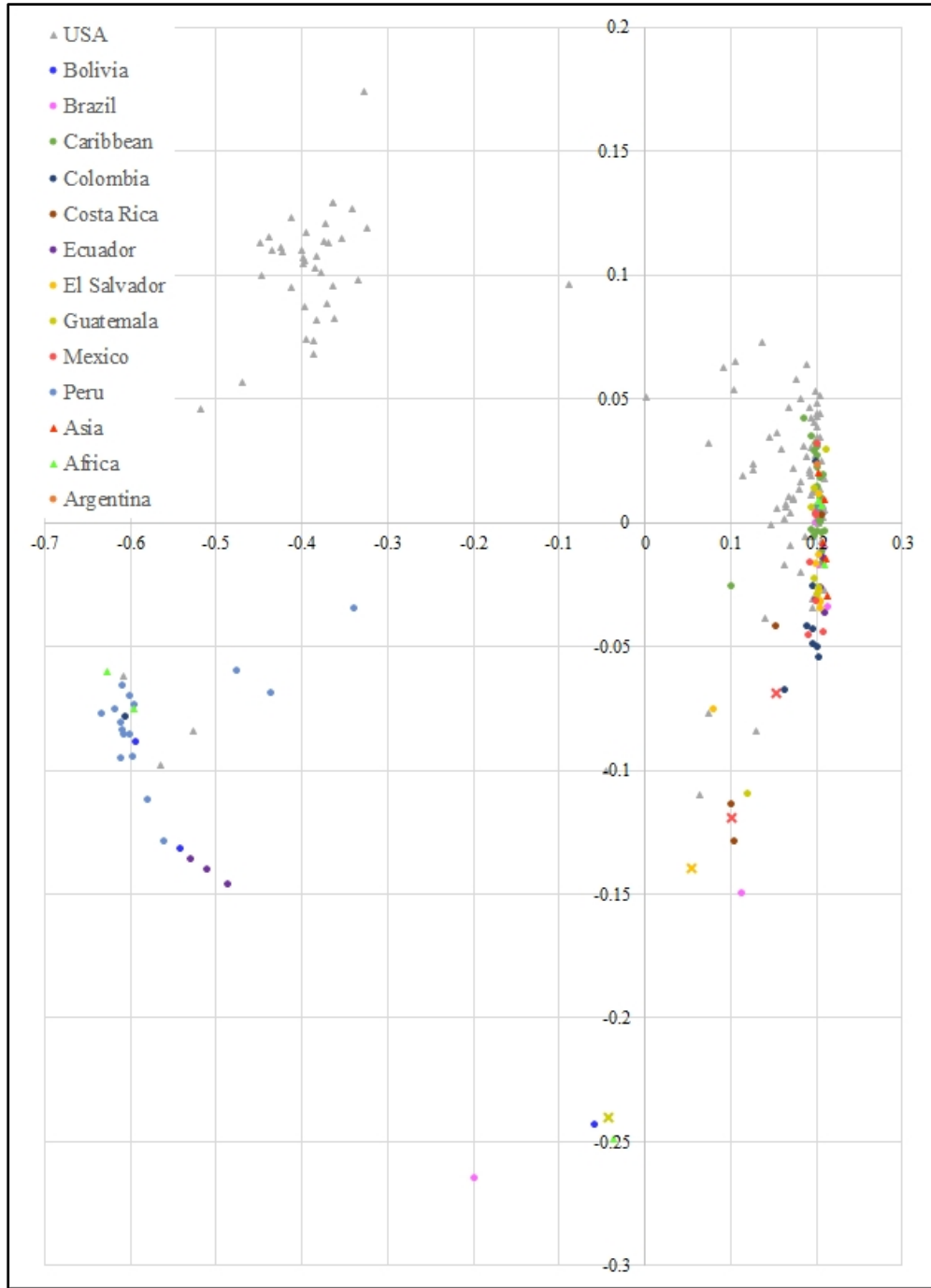


Figure 20 Plot of the first two MDS axes with diversity panel lines color coded according to country or region of collection. Triangle markers indicate collections from outside of the centers of origin. X markers indicate wild accessions.

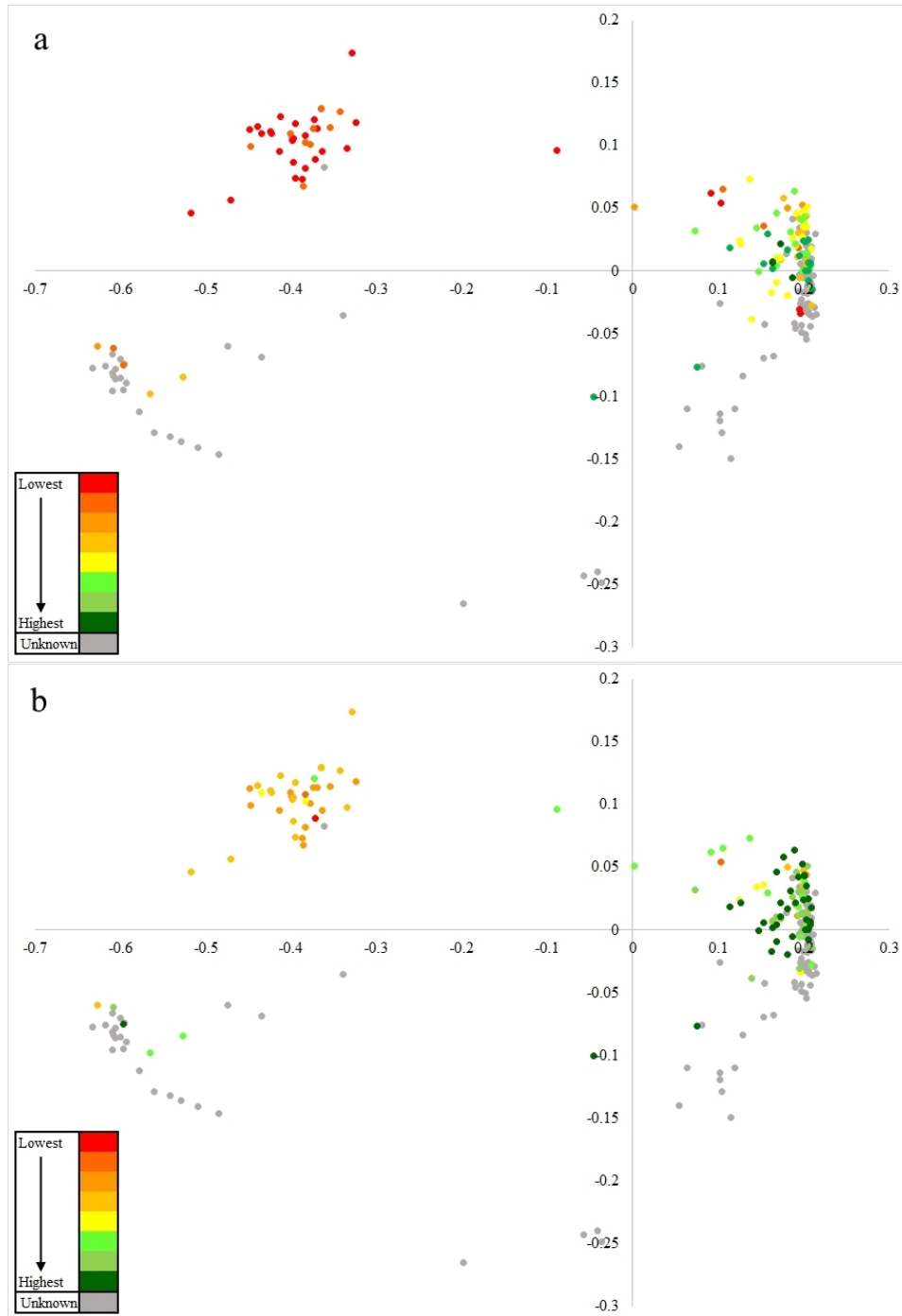


Figure 21 Plots of the first two MDS axes with diversity panel lines color coded according to yield under heat stress (a) and pollen quantity under heat stress (b). Diversity panel members with unknown heat stress reactions are shown in gray.

Mixed linear model analysis of SNP-trait associations for yield and pollen release under heat stress did not reveal any significant associations for yield under heat stress (Figure 22). For pollen release under heat stress, six potentially significant associations were identified for SNPs mapped to linkage groups 1 (1 SNP), 2 (2 SNPs), and 6 (2 SNPs) and one unmapped SNP (Figure 22). The research described previously in this chapter indicates that yield under heat stress is related to pollen release under heat stress, however yield under heat stress is also influenced by other traits under genetic control, including pollen quality, plant size, carbohydrate partitioning, and pistil function, all of which could be affected by heat stress. The association analysis indicates that pollen release under heat stress may be under the control of a few loci and could be effectively selected for in breeding populations in order to improve heat tolerance.

The current study involved a diversity panel rather than a population derived from two parents, as has been used in studies of heat tolerance in cowpea and common bean where single dominant genes conditioning heat tolerance of reproductive structures have been identified in populations derived from heat tolerant x susceptible crosses (Marfo and Hall, 1992; Rainey and Griffiths, 2005b). By screening a diversity panel there was opportunity to identify loci affecting pollen release under heat stress from across the gene pool. Since genotypes releasing pollen under heat stress are present in both the Andean and Mesoamerican gene pools it would be useful to determine if this phenotype is conditioned by different loci that are gene pool specific. The QTLs identified in this study are on different linkage groups and it is possible that the loci involved in pollen release could function synergistically if favorable alleles were combined.

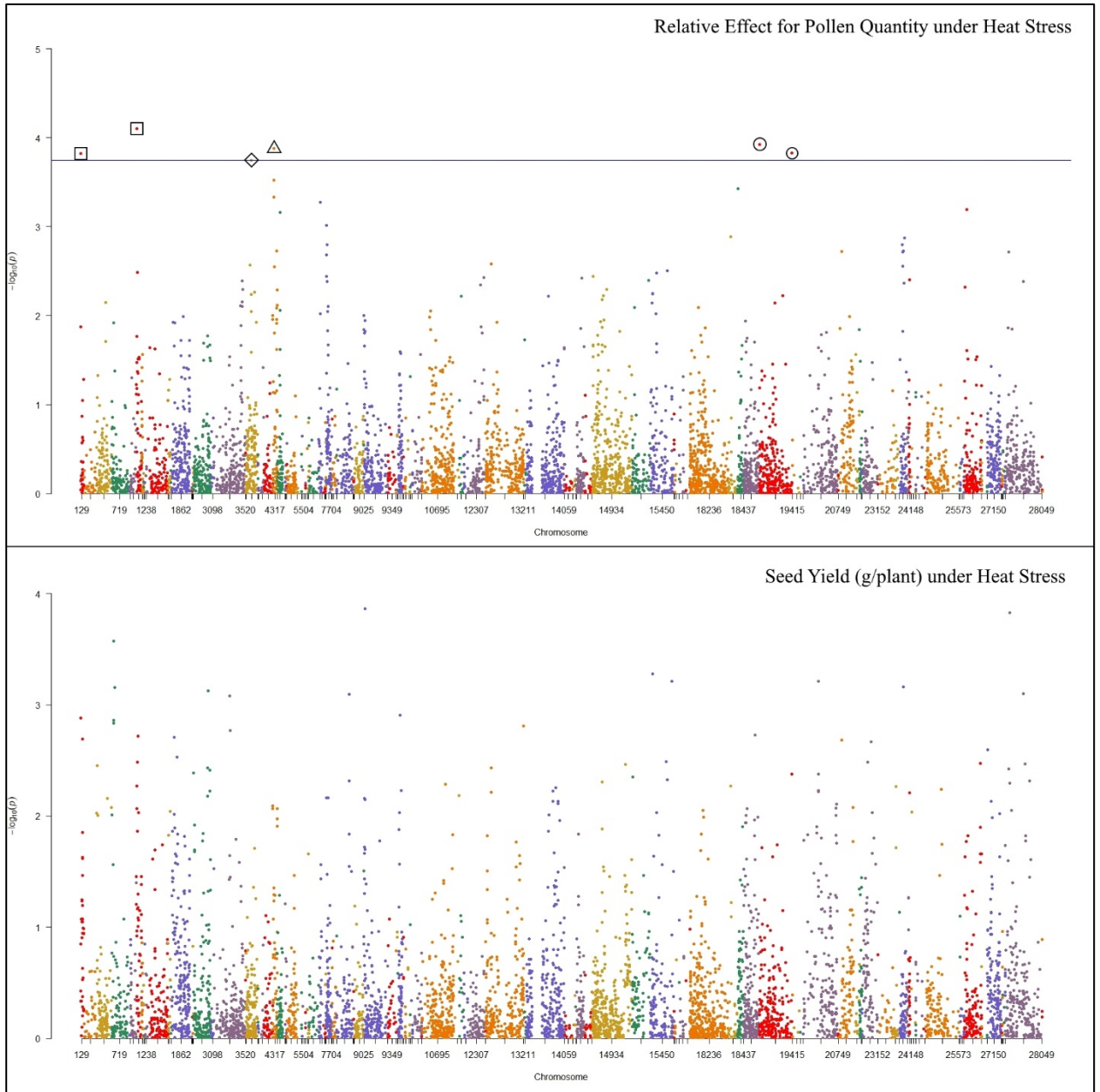


Figure 22 Manhattan plots of p-values for association between SNPs and relative effect for pollen quantity under heat stress (top) and yield under heat stress (bottom). Line indicates the cutoff for 25% FDR by the Benjamini-Hochberg method. Potentially significant SNPs for pollen quantity are mapped to linkage group 1 (diamond), 2 (circles), 6 (squares) and unmapped (triangle).

Conclusion

Heat stress effects on reproductive structures are observed in lima bean and can result in yield loss. Some of the effects observed have also been reported in common bean, a related species with a parallel domestication history.

In some heat sensitive lima bean genotypes, anthers do not dehisce and less pollen is released onto the stigma and stylar brush. This is a yield limiting effect that has also been reported in common bean (Porch and Jahn, 2001), cowpea (Warrag and Hall, 1984; Mutters and Hall, 1992), tomato (Sato *et al.*, 2000) and rice (Kobayashi, 2011).

In vitro germination of pollen from unstressed plants was reduced beginning at a temperature between 28 and 37 °C. Therefore, daytime temperatures, especially early morning temperatures, in this range could decrease yield. At 37-40 °C pollen germination is reduced to less than 10%, effectively shutting down fertilization and pod set. Genotype impacts pollen germination response to temperature and should be further investigated to determine how this trait might impact development of heat tolerant varieties.

Pollen from heat stressed plants had a lower *in vitro* germination rate than pollen from unstressed plants. This reduction was observed in all five genotypes tested, including those considered heat tolerant based on yield. Heat stressed pollen from the heat tolerant genotypes did, however, have comparatively higher germination rates than that of heat sensitive genotypes. Anther dehiscence and pollen quality under heat stress may be independently controlled. One genotype, Henderson, released pollen under heat stress, but pollen its germination rates were significantly lower than other heat tolerant genotypes and equivalent to that of the heat sensitive genotype C-elite Select. Additionally, when pollen release of the 131-member diversity panel was

measured, some genotypes with high pollen release ratings did not produce high yields under heat stress. Loss of pollen quality under heat stress is one possible explanation for this disparity.

Stigma function and seed development was not impaired by heat stress in the Mesoamerican heat sensitive genotype, C-elite Select, but seed production in Fordhook 242, the Andean heat sensitive genotype, was affected by factors beyond the presence of viable pollen on the stigma. Possible factors include reduced stigma secretions (Gross and Kigel, 1994) or interrupted photosynthate partitioning (Omae, *et al.*, 2007; Soltani *et al.*, 2019) which have been observed in heat stressed common bean. Another possible cause is the flower deformity resulting in excessive style length and coiling that was observed in heat stressed Fordhook 242. The combination of reduced pollen quantity and reduced germination rate caused by heat stress are major contributors to reduced pod set and yield in heat stressed lima bean. For some genotypes additional factors are also impacting heat stress related yield loss.

The quantity of pollen released under heat stress can be visually evaluated in the field using a three-level ordinal scale and was predictive of yield under heat stress in a broad range of lima bean germplasm. This technique can be used to field screen germplasm for heat stress tolerance where interpreting yield data is often complicated by presence of pods set during more favorable temperature conditions. Evaluation of *in vitro* germination rate of field-collected pollen could be a useful additional screening approach for genotypes producing ample pollen under heat stress.

Within a larger set of photoperiod insensitive lima bean genotypes, mostly collected from the U.S., Andean-derived genotypes were more heat sensitive than Mesoamerican-derived genotypes. Andean genotypes did have a range of yield and

pollen ratings under heat stress and those with higher yield and pollen ratings may be useful for improving heat tolerance in the Andean-derived Fordhook and large lima types. Among the Mesoamerican genotypes, there were heat sensitive accessions, but some of these are suspected to be the result of crosses between Andean and Mesoamerican germplasm based on seed size, flower size and pod shape. To improve heat tolerance in Andean types it remains to be seen whether it is more effective to look for and employ heat tolerance in Andean germplasm or to attempt transfer of tolerance genes from Mesoamerican germplasm. Both strategies are being attempted.

Heat stress effects on seed characteristics were observed in some genotypes, including seed coat pattern expression, dark discoloration, shrunken seeds and incomplete testa development. Seed quality reducing effects (i.e. discoloration, shrunken seeds and incomplete testae) of heat stress were absent in several genotypes with high yield under heat stress and these are of particular interest for heat tolerance breeding.

In cowpea, seed coat discoloration caused by heat stress is independent of reproductive heat stress tolerance (Patel and Hall, 1988) and may be related to ethylene formation and response (Pottorff *et al.*, 2014). If a similar mechanism underlies seed discoloration in lima bean, seed quality under heat stress will have to be selected for independently of reproductive heat stress tolerance.

Small seeds and incomplete testae are possibly related to photosynthate transport to the developing seeds and pod, which could be controlled independently from photosynthate transport during flower (pollen and anther) development. This is suggested by hand pollination with quality pollen inducing production of pods and

normal seed in 'C-elite Select' while in "Fordhook 242" inducing pod set but not production of normal seed.

Mixed linear model analysis of SNP-trait associations for yield and pollen release under heat stress did not reveal any significant associations for yield under heat stress, but six significant SNPs associated with pollen release under heat stress were identified. Yield under heat stress is related to pollen release under heat stress however yield under heat stress is influenced by multiple factors influenced by genotype, including pollen quality, photosynthetic efficiency, carbohydrate partitioning, and pistil function, all of which interact with environmental factors including heat stress. The association analysis indicates that pollen release under heat stress may be under the control of a few loci and could be effectively selected for in breeding populations to improve heat tolerance. Genotypes that released high levels of pollen under heat stress were identified from both gene pools, which presents the opportunity to compare the mechanism and control of anther heat tolerance in these contrasting populations which evolved and were domesticated separately.

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Chapter 4

DESCRIBING LIMA BEAN LEAFLET SHAPE VARIABILITY AND ITS GENETIC CONTROL IN A DIVERSITY PANEL

Introduction

Lima bean (*Phaseolus lunatus* L.) is a minor legume crop with importance in certain regional agricultural systems around the world. The wild and domesticated members of the species have a wide distribution across climatic conditions and, compared to other domesticated *Phaseolus* species, lima bean is adapted to warmer, more humid climates (Bitocchi *et al.*, 2017). Direct heat stress effects on lima bean reproductive structures have been documented to cause yield loss but photosynthetic efficiency may also be affected by heat stress. Additionally, drought stress, which often accompanies and exacerbates heat stress, impacts yield by directly impairing photosynthetic efficiency (Miyashita *et al.*, 2004). Leaflet shape may impact lima bean's ability to maintain photosynthetic efficiency under heat and drought stress. The cultivated and wild genotypes that make up the 255-member lima bean diversity panel have a range of leaflet shapes which could influence their responses to these stresses.

One gene controlling leaflet shape has been described in lima bean, Wl/wl (willow leaflet). The homozygous condition produces leaflets that are either lanceolate (Wl/Wl) or ovate (wl/wl) while heterozygotes (Wl/wl) have an intermediate leaflet shape (Allard, 1953). The Wl locus is linked to the D locus (1.8 cM), which controls bush or vining growth habit (Bemis, 1958). In lima bean the willow leaflet phenotype

resulted in lower yields in a study of near-isogenic lines with lanceolate versus ovate leaflet shapes (Solh *et al.*, 1997).

In soybean, where the Ln locus has a similar effect on leaflet shape as W1 in lima bean, narrow leaflet shape has been proposed as a way to increase canopy light penetration, improve photosynthetic efficiency, and increase water use efficiency (Hiebsch *et al.*, 1976). Soybean has been demonstrated to produce more leaves than necessary to maximize yield and mechanically reducing leaf area toward an optimal level increases yield (Srinivasan, *et al.*, 2017). In soybean, the narrow leaflet phenotype did not affect yield in some studies (Hartwig and Edwards, 1970; Mandl and Buss, 1981; Dinkins *et al.*, 2002), but in others narrow leaflet phenotypes had reduced yields (Kilen, 1990). In one study the effect of the heterozygous intermediate leaflet shape was determined, and these plants produced yields 12% higher than ovate leaflet plants and 19% higher than narrow leaflet plants (Dinkins, *et al.*, 2002). Possibly the heterozygous intermediate leaflet shape in soybean achieves a more optimal canopy structure and leaf area index than either homozygous condition of Ln. Because lima bean exhibits a range of leaflet shape phenotypes a more efficient and climate resilient canopy which approaches the leaf area optimum might be obtained in genotypes with a leaflet shape between the phenotypic extremes.

To inform development of populations for investigation of the genetic control and effects of leaflet shape in lima bean this study was undertaken to empirically describe the leaflet shapes of the 255 members of the diversity panel and to assess the genetic control of leaflet shape through a genome wide association study.

Leaf and leaflet shapes in various species have been efficiently and meaningfully described using shape descriptors obtained through image analysis (Migicovsky *et al.*, 2018; Chitwood *et al.*, 2014).

The shape descriptors measured in this study were (Figure 23):

Circularity: $4\pi \cdot \text{area} / \text{perimeter}^2$, where a value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape or a more lobed shape.

Aspect Ratio: $\text{major_axis} / \text{minor_axis}$ for a fitted ellipse

Roundness: $4 \cdot \text{area} / (\pi \cdot \text{major_axis}^2)$, or the inverse of the aspect ratio.

Materials and Methods

The leaflet shapes of 255 lima bean diversity panel members were analyzed using image processing to determine circularity, aspect ratio and roundness. Fully expanded leaves from plants sown in the greenhouse in Nov 2017 and Nov 2018 were collected and scanned in black and white with an Epson Perfection 4180 flatbed scanner at 600 dpi. Six leaves (18 leaflets) were scanned from the 2017 planting and three leaves (9 leaflets) were scanned from the 2018 planting. ImageJ (Schneider *et al.*, 2012) was used to batch process the scanned images using a custom macro to run Analyze Particles with options to measure shape descriptors (including circularity, aspect ratio and roundness). The macro saved masks of the analyzed leaflets which were then reviewed to confirm that leaflets had been accurately detected and measured by ImageJ.

Best linear unbiased estimators (BLUEs) and heritability for leaflet circularity and aspect ratio were calculated for each genotype using META (Vargas *et al.*, 2013), a suite of SAS programs for analyzing multienvironment trials.

Most members of the lima bean diversity panel have been characterized using genotyping-by-sequencing (GBS). The original dataset includes genotype information for 258 individuals and 17,997 SNPs. Missing genotype data was imputed using the entire dataset with the FILLIN approach (Swarts *et al.*, 2014) implemented in TASSEL 5.0 (Bradbury *et al.*, 2007). The sequence dataset was filtered to remove duplicated individuals, individuals with >70% missing data, and sites with > 50% missing data. SNPs were further filtered for minor allele frequency (minimum 0.05). After filtering the dataset was comprised of 232 individuals and 7835 SNPs.

Multidimensional scaling (MDS) implemented in TASSEL was used to evaluate population structure. Three MDS axes were used as covariates to correct for population structure in association analysis. To correct for relatedness in association analysis, a kinship matrix was calculated using the centered-identity-by-descent method in TASSEL. SNP-trait associations were estimated for circularity, aspect ratio and roundness for 232 individuals with genotype and phenotype data using a mixed linear model (MLM) in TASSEL (Zhang *et al.*, 2010). Growth habit associated SNPs were also identified using the same individuals with a binary phenotype of determinate or indeterminate. The Benjamini and Hochberg (1995) procedure with a false discovery rate of 0.10 was used to identify potentially significant SNPs.

Results and Discussion

Heritability values for leaflet aspect ratio, roundness and circularity were very high (0.979, 0.950 and 0.910 respectively) indicating that leaflet shape is under strong genetic control. The diversity panel accessions have a range of leaflet shapes from lanceolate to ovate (Figure 23). Most diversity panel accessions have ovate leaflets, with higher circularity and roundness values and lower aspect ratio values. Some

genotypes have the willow leaflet phenotype described by Allard (1953); and some have a leaflet shape similar to the intermediate (Wl/wl) phenotype, but not the result of heterozygous condition (i.e. it is present in inbred lines). Distribution of the three shape descriptors, (aspect ratio, roundness and circularity) within the diversity panel is very skewed with aspect ratio having the most skewed distribution (Figure 24).

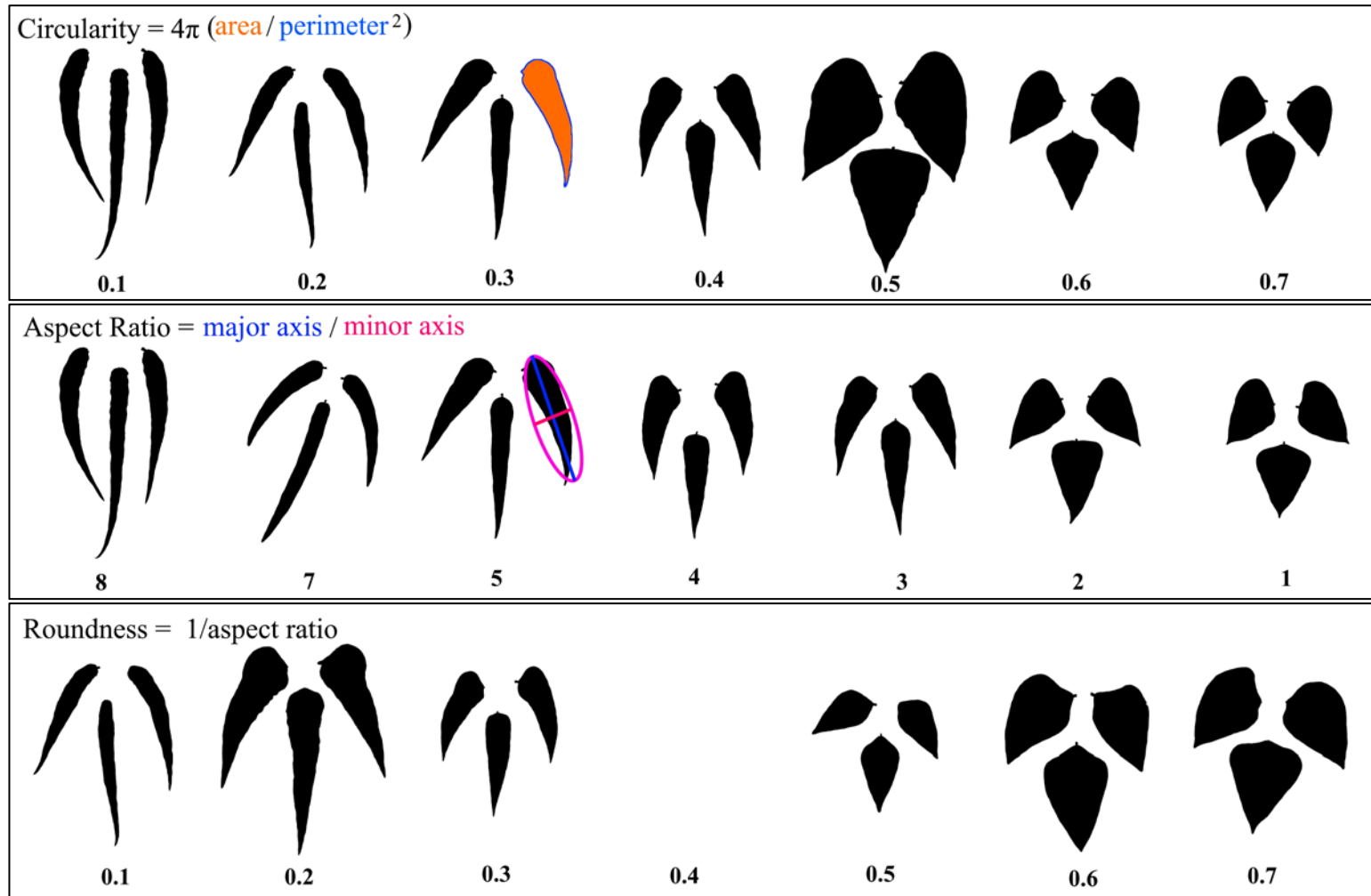


Figure 23 Representative leaflets for the range of circularity, aspect ratio and roundness values among diversity panel accessions.

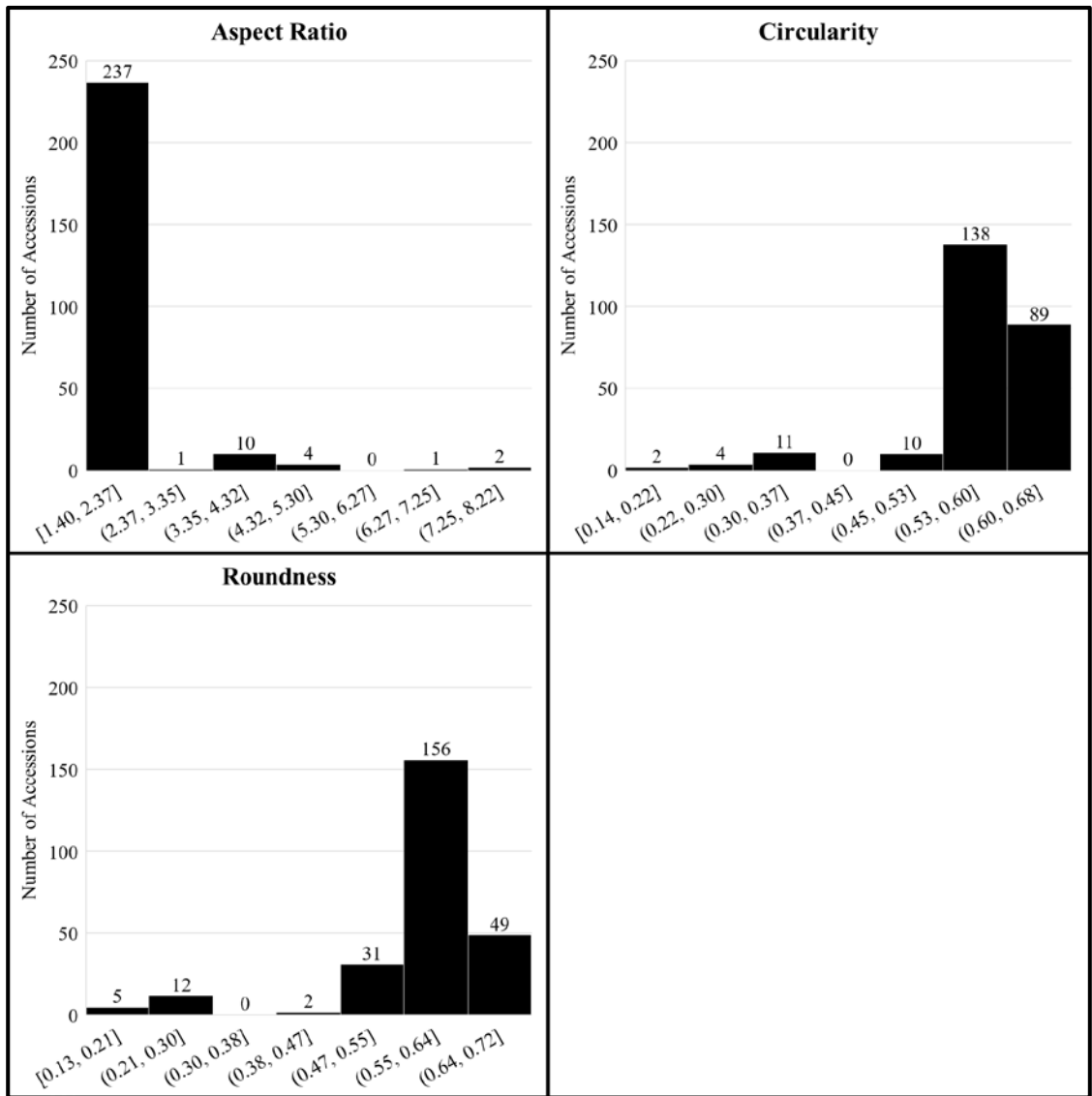


Figure 24 Distribution of leaflet shape descriptor values among accessions in the diversity panel for aspect ratio, circularity and roundness.

Narrow leaflet types with low circularity and roundness values were present only in Mesoamerican gene pool accessions which are separated from the Andean

accessions by the first MDS axis (Figure 25). Ovate leaflet shapes are present in both gene pools. One wild Mesoamerican accession had a narrow leaflet shape. Possibly, the narrow leaflet phenotype is not present in the Andean gene pool.

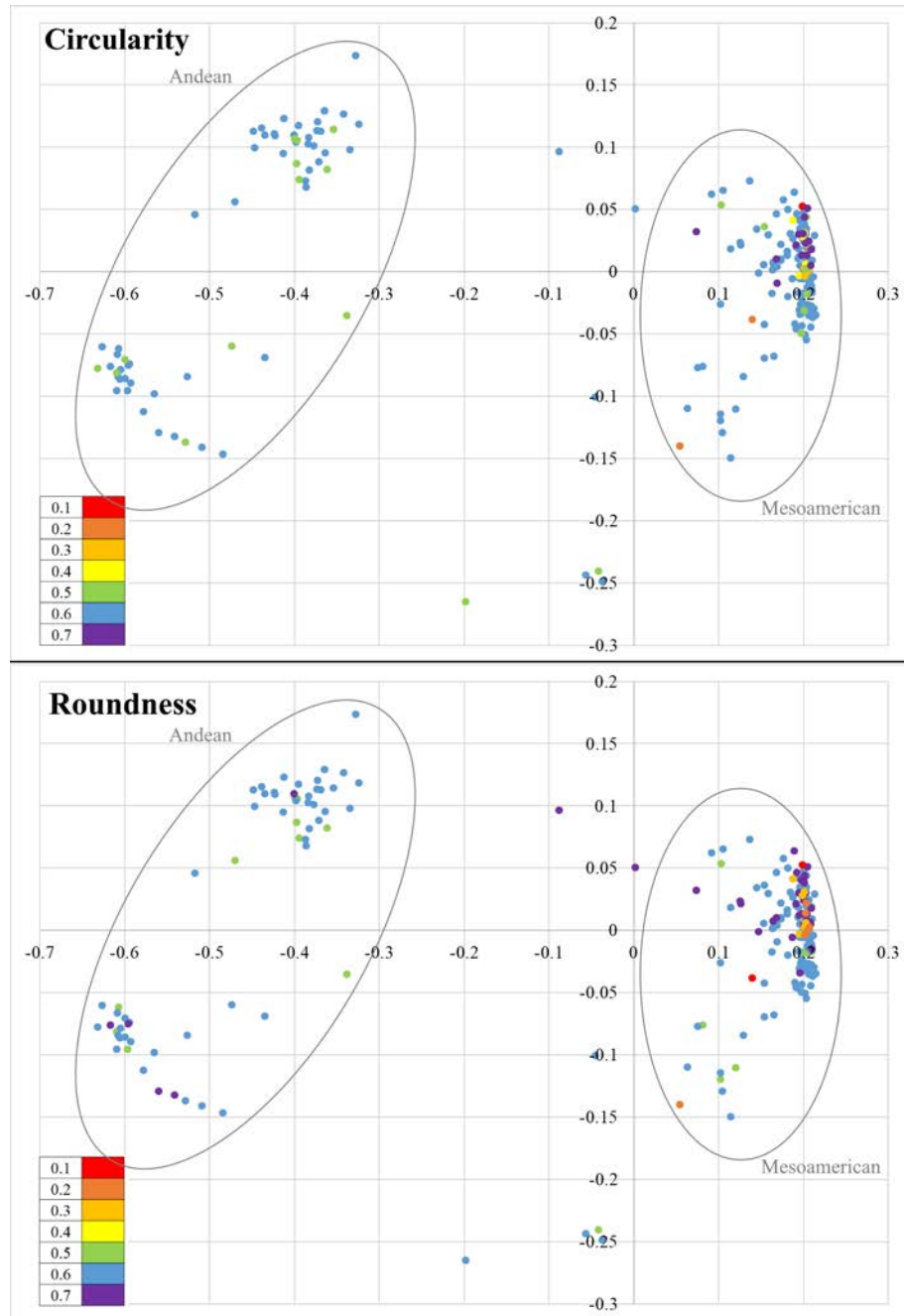


Figure 25 Plot of the first two MDS axes with diversity panel lines color coded according circularity (top) or roundness (bottom) value for leaflets. Andean and Mesoamerican gene pools are indicated by ellipses.

Mixed linear model analysis of SNP-trait associations with leaflet shape descriptors at 10% FDR indicated 151 significant associations for aspect ratio, 45 significant associations for circularity and 34 significant associations for roundness (Figure 26). The extremely skewed distribution of aspect ratio among the diversity panel members might make it a less reliable indicator of SNPs associated with leaflet shape loci. Of SNPs significantly associated with circularity and roundness, the majority (32) are significant for both descriptors.

Four SNPs were significantly associated with plant growth habit (bush vs vining) (Figure 27). Two are on scaffold 21362, which is syntenous with common bean chromosome 1 (Pv01), the location of a determinant growth habit gene, *fin* or *PvTFL1y*, in common bean (Koinange *et al.*, 1996; Repinski *et al.*, 2012). The other two are on scaffold 14807, which remains unmapped, but SNPs on this scaffold were associated with the three leaflet shape descriptors. The two particular growth habit-associated SNPs on scaffold 14807 were also significantly associated with roundness and one was also associated with circularity. The most significantly associated SNPs for circularity and roundness on scaffolds 27150 and 13072 map to the same linkage group (5), which also has regions that are syntenous with Pv01.

The association analysis seems to have accurately detected the one previously described locus controlling leaflet shape (*W1*), which is linked to the growth habit locus and is located on a chromosome syntenous with Pv01. Other loci that affect leaflet shape are indicated by the analysis and are located on other linkage groups (4, 7, 9, 11) which are syntenous with common bean chromosomes other than Pv01 (Pv04, Pv06 and Pv07).

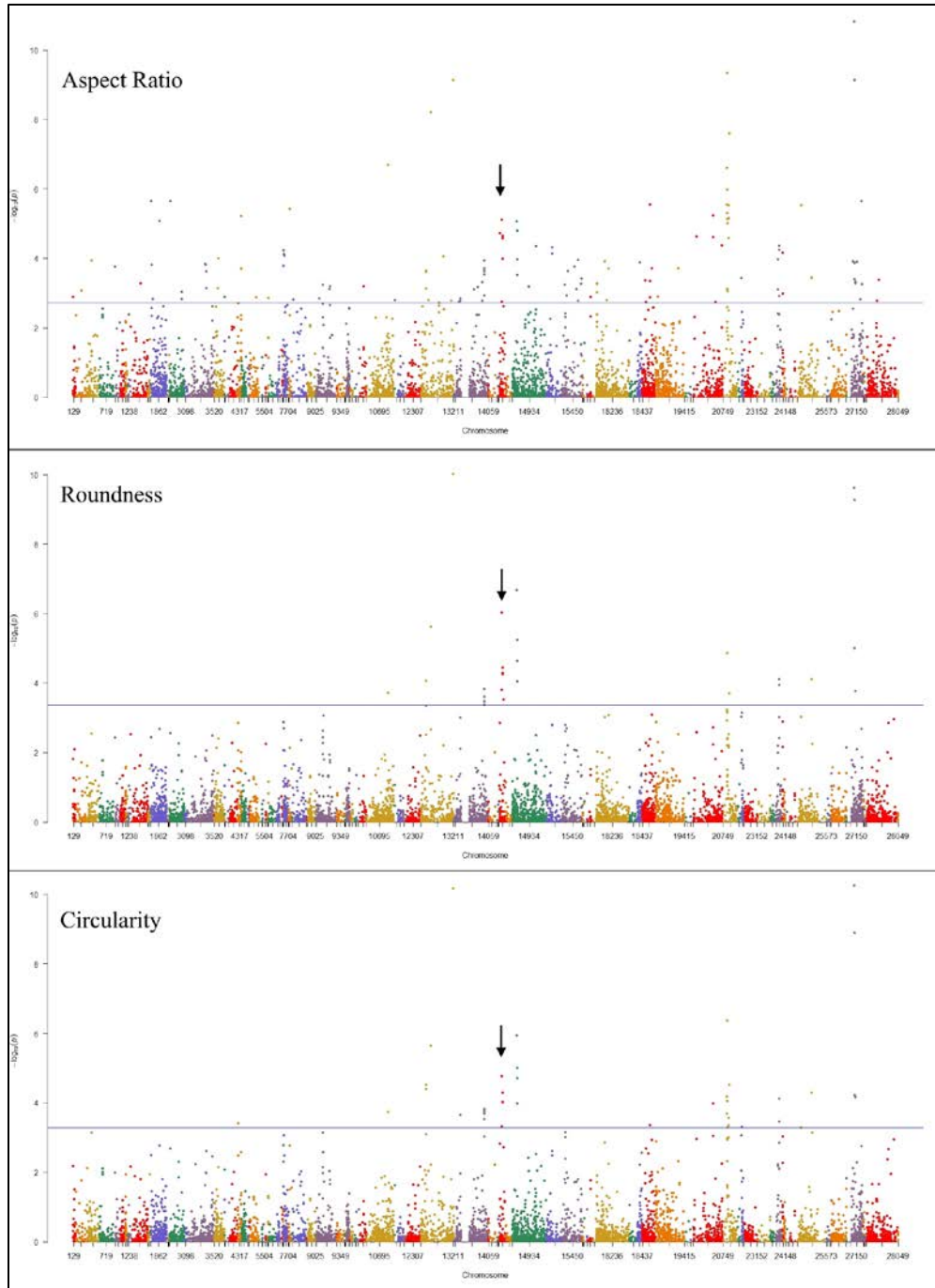


Figure 26 Manhattan plots of p-values for association between SNPs and aspect ratio, circularity and roundness. Line indicates the cutoff for 10% FDR by the Benjamini-Hochberg method. Black arrow indicates scaffold 14807, site of SNPs associated with all three shape descriptors and growth habit.

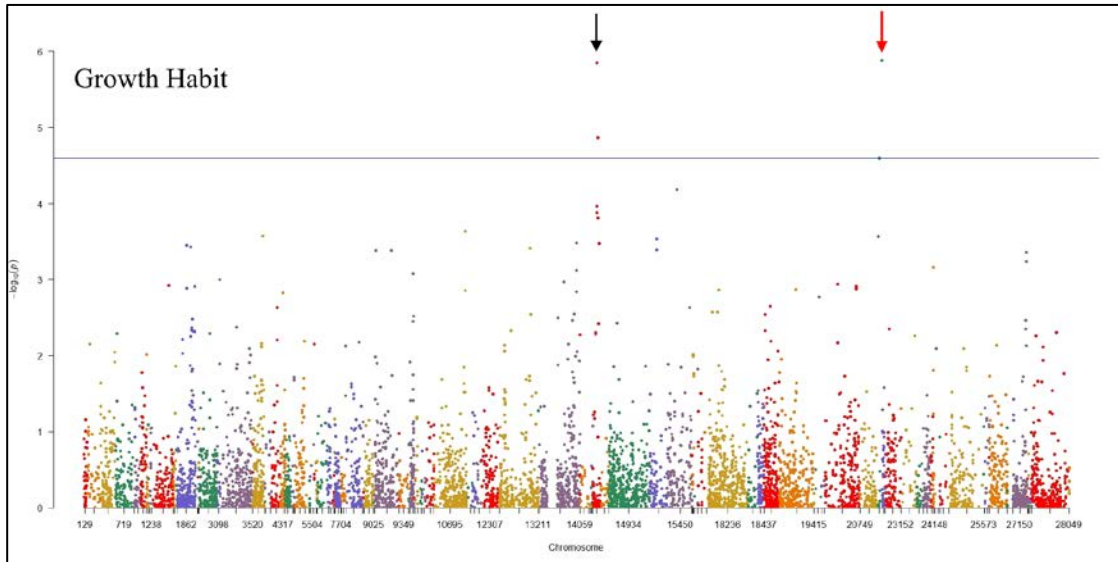


Figure 27 Manhattan plot of p-values for association between SNPs and growth habit. Line indicates the cutoff for 10% FDR by the Benjamini-Hochberg method. Black arrow indicates scaffold 14807, site of SNPs associated with all three shape descriptors and growth habit. Red arrow indicates scaffold 21362, which is syntenous with *Phaseolus vulgaris* chromosome 1.

Conclusion

Leaflet shape in lima bean was effectively described by shape descriptors: circularity, aspect ratio and roundness. Because lima bean leaflets are mostly without lobes, circularity is highly correlated with roundness and aspect ratio and either circularity or roundness alone can be used to describe leaflet shape. Image analysis could be used to describe other shape traits of interest using similar techniques. For example, in horticultural crops the shape of fruits, pods, seeds, tubers or storage roots are important to consumers and therefore breeders. Image analysis may be an efficient way to describe, differentiate and categorize such characteristics so that their underlying genetic control may be determined.

Several loci are involved in determining leaflet shape, which is under strong genetic control. One QTL identified for all three shape descriptors is on the same scaffold as a QTL for growth habit. This QTL is probably the site of the willow leaflet (Wl/wl) gene described by Allard (1953) that is linked to the D locus for growth habit (Bemis, 1958). One of the two QTL associated with growth habit was located on a scaffold syntenous with common bean chromosome Pv01, the site of a determinant growth habit gene, *fin* or *PvTFL1y*, in common bean (Koinange *et al.*, 1996; Repinski *et al.*, 2012). There is high synteny between lima bean and common bean, but gaps and/or rearrangements are present on each chromosome (Almeida and Pedrosa-Harand, 2013; Dohle, 2017). Based on a mapping population of 135 RILs Dohle (2017) also found a QTL associated with growth habit on a lima bean linkage group syntenous with Pv01. Compared to lima bean, much more is known about the genetic architecture of common bean including locations of loci controlling seed characteristics, biotic and abiotic stress tolerance, yield and plant architecture. Lima bean's high level of synteny with common bean could be helpful in confirming locations of major loci and QTL related to traits of interest and the gene function underlying them.

To determine the physiological effects of leaflet shape on yield, biotic and abiotic stress tolerance, pairs of near isogenic lines (NILs) with the lanceolate and ovate leaflet shapes have been developed and are being yield tested in the field. NILs with the intermediate leaflet shape are being produced. Parents of additional populations to determine the genetic control of the intermediate leaflet shape phenotypes will be chosen based on circularity and roundness classification and relatedness to maximize genetic diversity represented in the populations.

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Chapter 5

CONCLUSIONS, ONGOING AND FUTURE WORK

Summary of Conclusions

Heat stress is a major threat to lima bean production in the Mid-Atlantic region and a problem that is best addressed through the development of heat tolerant cultivars. Under field conditions, periods of high temperature in the early flowering period of lima bean correlated with decreased yield and delayed maturity. High temperatures during the vegetative phase before flowering and in the period after pod set did not correlate with decreased yield or delayed maturity. This indicates that the flowering period is the most heat susceptible growth stage of lima bean.

In the genotypes tested, there was no indication that high temperatures inhibit flowering in lima bean as observed in cowpea (Ahmed and Hall, 1993) and common bean (Shonnard and Gepts, 1994). Rather high temperatures decreased days to flowering in the genotypes tested. In greenhouse experiments, heat sensitive genotypes set fewer pods and produce fewer seeds per pod under high night temperatures. More heat tolerant genotypes set similar numbers of pods under stressed and unstressed conditions but still produce fewer seeds per pod. The overall result is fewer seeds produced under high night temperatures, resulting in yield loss. Carbohydrate depletion due to higher respiration at higher temperatures was hypothesized as the explanation for reduced pod set under high night temperature conditions (Fisher & Weaver, 1974) but in the genotypes tested there was no evidence of reduced carbohydrate availability in heat stressed plants. Even in heat sensitive genotypes the

total aboveground biomass accumulation was equivalent in stress and unstressed conditions. Similarly, in common bean, photosynthesis was not negatively impacted in a study involving a few genotypes with differing yield under heat stress (Soltani *et al.*, 2019). However, photosynthate partitioning to reproductive structures is affected by high temperatures in common bean, with differences between genotypes for this trait (Omae *et al.*, 2007; Soltani *et al.*, 2019). A similar mechanism may be present in lima bean.

Heat stress effects on reproductive structures are observed in lima bean and can result in yield loss. In heat sensitive genotypes anthers do not dehisce and less pollen is released onto the stigma and stylar brush. Anther indehiscence resulting in less pollen released is a yield limiting effect that has also been reported in other species, including common bean (Porch and Jahn, 2001), cowpea (Warrag and Hall, 1984; Mutters and Hall, 1992), tomato (Sato *et al.*, 2000) and rice (Kobayashi, 2011).

Germination of pollen from unstressed lima bean plants was reduced *in vitro* beginning at a temperature between 28 and 37 °C. Therefore, daytime temperatures, especially early morning temperatures, in this range could decrease yield. At 37-40 °C pollen germination is reduced to less than 10%, effectively shutting down fertilization and pod set. Genotypic variation in the germination of pollen in response to temperature could be further investigated to determine how this trait might impact development of heat tolerant varieties.

Pollen from heat stressed plants has a lower *in vitro* germination rate than pollen from unstressed plants. This reduction was observed in all five genotypes tested, including those considered heat tolerant based on yield. Heat stressed pollen from the heat tolerant genotypes did, however, have comparatively higher germination

rates than that of heat sensitive genotypes. One genotype that was heat tolerant based on yield and had high pollen release in chamber and field experiments had low *in vitro* pollen germination, equivalent of the moderately heat susceptible variety. This suggests that the anther indehiscence response to heat stress is independent of pollen quality under heat stress.

Stigma function and seed development was not impaired by heat stress in a Mesoamerican-derived heat sensitive genotype, C-elite Select, but seed production in Fordhook 242, an Andean-derived heat sensitive genotype, was affected by factors beyond the presence of viable pollen on the stigma. Possible factors include reduced stigma secretions (Gross and Kigel, 1994) or interrupted photosynthate partitioning (Omae, *et al.*, 2007; Soltani *et al.*, 2019) as observed in common bean. Another possible cause is the flower deformity resulting in excessive style length and coiling that was present in heat stressed Fordhook 242. Flower deformity was observed in many Andean genotypes during the diversity panel heat screen (Appendix).

The combination of anther indehiscence and reduced pollen germination rate caused by heat stress are major contributors to reduced pod set and yield in heat stressed lima bean. For some genotypes additional factors, possibly changes in flower morphology or altered photosynthate partitioning, are also contributing to heat stress related yield loss.

In the field and greenhouse evaluation of the diversity panel a three-level ordinal scale was used to visually rate the amount of pollen released. This method was effective in separating genotypes by anther indehiscence response and was predictive of yield under heat stress in a broad range of lima bean germplasm. This technique could be used to field screen germplasm for heat stress tolerance where interpreting

yield data is often complicated by presence of pods set during more favorable temperature conditions. Evaluation of *in vitro* germination rate of field-collected pollen could also be a useful additional screening approach for genotypes producing ample pollen under heat stress.

Within a larger set of photoperiod insensitive lima bean genotypes, mostly collected from the U.S., Andean-derived genotypes were more heat sensitive than Mesoamerican-derived genotypes. Andean genotypes did have a range of yield and pollen ratings under heat stress and those with higher yield and pollen ratings might be useful for improving heat tolerance in the Andean-derived Fordhook and large lima types. Among the Mesoamerican genotypes, there were heat sensitive accessions, but some of these are suspected to be the result of crosses between Andean and Mesoamerican germplasm based on seed size, flower size and pod shape. To improve heat tolerance in Andean types it remains to be seen whether it is more effective to look for and employ heat tolerance derived from Andean germplasm or to attempt transfer of tolerance from Mesoamerican germplasm. Both strategies are being attempted.

Heat stress affected seed characteristics in some genotypes. Some seed coat patterns expressed more secondary coloration under heat stress and some less. More problematic quality problems observed were dark discoloration, unfilled seeds and incomplete testa development. Seed coat discoloration caused by heat stress is also reported in cowpea (Patel and Hall, 1988). Candidate genes in three QTL regions associated with cowpea seed coat discoloration are involved in ethylene formation and response (Pottorff *et al.*, 2014). The mechanisms controlling cowpea seed coat discoloration and their inheritance are independent of those controlling reproductive

heat stress tolerance (Patel and Hall, 1988) and this seems to be the case in lima bean as well. Consequently, absence of heat induced seed discoloration will have to be selected for independently of reproductive heat stress tolerance. Seed quality reducing effects (i.e. discoloration, unfilled seeds and incomplete testae) of heat stress were absent in certain genotypes and these are of particular interest for heat tolerance breeding.

Mixed linear model analysis of SNP-trait associations for yield and pollen release under heat stress did not reveal any significant associations for yield under heat stress, but six significant SNPs associated with pollen release under heat stress were identified. Yield under heat stress is related to pollen release under heat stress however yield under heat stress is also influenced by other traits under genetic control which could be affected by heat stress and other environmental factors. The association analysis indicates that pollen release under heat stress may be under the control of a small number of loci and could be effectively selected for in breeding populations to improve heat tolerance.

Leaflet shape in lima bean is diverse and highly heritable. This trait may affect heat, drought and disease tolerance. Shape descriptors such as circularity or roundness can be used to efficiently characterize scanned leaf images and could be applied to other shape traits of interest to plant breeders. A few loci are involved in determining leaflet shape, which is under strong genetic control. One QTL identified for all three shape descriptors is on the same scaffold as a QTL for growth habit. This QTL is probably the site of the willow leaflet (Wl/wl) gene described by Allard (1953) that is linked to the D locus for growth habit (Bemis, 1958). One of the two QTL associated with growth habit was located on a scaffold syntenous with common bean

chromosome Pv01, site of a determinant growth habit gene called *fin* or *PvTFL1y* (Koinange *et al.*, 1996; Repinski *et al.*, 2012). Dohle (2017) also reported a QTL associated with growth habit on a lima bean linkage group syntenous with Pv01. A high level of synteny has been reported between lima bean and common bean, but with gaps and/or rearrangements are present on each chromosome (Almeida and Pedrosa-Harand, 2013; Dohle, 2017). Because more is known about the genetic architecture of common bean than that of lima bean, the high level of synteny between the two species could be helpful in confirming locations of major loci and QTL in lima bean that are related to traits of interest and the gene function underlying them.

Based on the work described here there is evidence for several distinct physiological effects of high night temperature stress on lima bean, each of which could be under individual genetic control: anther indehiscence, pollen quality, flower morphology, seed filling/maturation and seed discoloration. Achieving a high level of heat tolerance in lima bean will require combining favorable alleles for all these traits. Addition of narrower leaflet shapes and drought tolerance-related traits could enhance photosynthetic efficiency under heat stress and contribute to overall abiotic stress tolerance.

Ongoing and Future Work

Breeding for Heat Tolerance in Lima Bean

Heat tolerance breeding populations have been generated and are being screened for heat tolerance using field and greenhouse screening techniques. For baby limas, heat tolerant genotypes have been crossed with green seeded, high yielding

types and selected for heat tolerance and desirable agronomic and quality traits. Heat tolerant genotypes have been intercrossed with each other and are being selected for superior heat tolerance, regardless of seed coat color, to develop maximally heat tolerant lines. For large seeded vining and Fordhook types, crosses have been made between high quality green seeded types and heat tolerant Andean genotypes. Crosses have also been made between the high quality large seeded types and Mesoamerican heat tolerant germplasm.

Additional Germplasm Screening and Development of Screening Techniques

It would be beneficial to identify additional heat tolerant germplasm from both the Mesoamerican and Andean gene pools by screening photoperiod sensitive germplasm. This would have to be done in collaboration with cooperators in a suitably hot location at a tropical latitude. Additional U.S. germplasm, especially that collected in the Southwest, should also be screened, which could be done in Delaware.

In vitro pollen germination should be tested for additional heat tolerant germplasm to determine if it is valuable in discerning heat tolerance in diverse germplasm and whether there are genotypes exhibiting a higher level of pollen heat tolerance than those already characterized.

Post pollination transport of photosynthate to developing seeds may be affected by heat in some genotypes and it would be useful to be able to screen for this trait separately from anther and pollen heat tolerance. A possible technique is to expose plants to heat after initial pod set and measure partitioning to pods/seeds vs vegetative tissues. Photosynthate transport could also be affected by high daytime temperatures, and this should be investigated.

Characterization of Pollen Release QTL

QTL associated with pollen release under heat stress mapped to several different linkage groups. Pollen release could be further investigated in biparental populations to determine whether these loci are gene pool specific and whether they act synergistically when combined.

Effects of Leaflet Shape

The effects of leaflet shape on yield, biotic and abiotic stress tolerance should be explored to determine if narrow leaflets offer any advantages. Pairs of near isogenic lines (NILs) with the lanceolate and ovate leaflet shapes have been developed and are being yield tested and screened for disease avoidance in the field. NILs with the intermediate leaflet shape and the ovate leaflet shape are being produced. Parents of additional populations to determine the genetic control of the intermediate leaflet shape phenotypes will be chosen based on circularity and roundness classification and relatedness to maximize genetic diversity represented in the populations.

Further Application of Image Processing and Shape Descriptors

Lima bean exhibits a wide variety of sizes and shapes with variations in length to diameter ratios, angularity and plumpness. Certain size and shape characteristics are associated with each commercial class and must be maintained in new cultivars. An understanding of the genetic basis for seed shape characteristics in lima bean would be helpful in maintaining desired qualities in breeding material. Image analysis and association mapping of seed shape in the diversity panel would be a starting place for understanding inheritance of seed size and shape traits.

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Appendix

SUPPLEMENTAL TABLE

Diversity panel genotype identifiers, gene pool grouping, mean % deformed flowers, 95% confidence interval for % deformed flowers, primary seed coat color, secondary seed coat color, seed coat pattern, conditional color expression, presence of seed coat discoloration under heat stress, presence of split testa under heat stress and presence of small seeds under heat stress.

Entry #	Name (Accession #)	Group	% Def	95% CI for % Def	1° Color	2° Color	Pattern	More 2° Color in...	Dis-coloration	Split Testa	Small Seed
1	1102-10 "Beseba"	Meso	1	(-7.5, 8.9)	white	NA	self	equal	moderate	no	no
2	1102-6	Meso	4	(-4.1, 12.3)	buff	magenta	hilum	cool	moderate	no	no
3	184-85	Meso	1	(-7.5, 8.9)	green	NA	self	equal	moderate	no	no
4	90-1	Andean	11	(2.4, 18.9)	green	NA	self	equal	moderate	no	yes
5	Bush Florida Butter (PI 549509)	Meso*	3	(-5.5, 10.9)	buff	black	hilum	cool	no	no	no
6	C-elite Select	Meso	3	(-5.5, 10.9)	green	NA	self	equal	moderate	no	no
7	Concentrated Fordhook	Andean	26	(17.8, 34.3)	lt green	NA	self	-	no seed	-	-
8	Cypress	Meso	5	(-3.5, 12.9)	green	NA	self	equal	moderate	no	no
9	DE0501102B	Andean	7	(-1.5, 14.9)	lt green	NA	self	equal	moderate	no	yes
10	DE0504103A	Andean	5	(-3.5, 12.9)	lt green	NA	self	-	no seed	-	-
11	Dixie Butterpea	Meso	1	(-7.5, 8.9)	white	NA	self	equal	moderate	yes	no

Entry #	Name (Accession #)	Group	% Def	95% CI for % Def	1° Color	2° Color	Pattern	More 2° Color in...	Dis-coloration	Split Testa	Small Seed
	(PI 549462)										
12	Dover Tucker (PI 549456)	Meso	3	(-6.7, 11.7)	green	NA	self	equal	moderate	no	no
13	FH 1072 (PI 549519)	Andean*	21	(12.4, 28.9)	green	NA	self	-	no seed	-	-
14	FH 242 (PI 549464)	Andean	9	(0.4, 16.9)	lt green	NA	self	-	no seed	-	-
15	Improved Kingston	Meso*	1	(-7.5, 8.9)	green	NA	self	equal	moderate	no	no
16	Jackson Wonder	Meso	1	(-7.5, 8.9)	buff	black	speckle	hot	no	no	no
17	Maffei 15	Meso	7	(-2.5, 15.9)	green	NA	self	equal	minor	no	no
18	PA German Red Lima	Meso	5	(-3.5, 12.9)	red	black	speckle	hot	no	no	no
35	Hopi 13 (PI 347779)	Meso	8	(-4.7, 21.5)	white	NA	self	equal	no	no	no
36	Hopi 15 (PI 347781)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	moderate	no	no
37	Hopi 155 (PI 347784)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	moderate	yes	no
38	Hopi 2000 (PI 347787)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	minor	no	no
40	Willow Leaf, White (PI 347819)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	yes	no
41	(PI 347826)	Meso	7	(-3.9, 17.5)	buff	purple	speckle	hot	no	no	no
46	Cave Dweller (PI 534918)	Meso	1	(-7.5, 8.9)	maroon	buff	flecks	cool	no	no	no
47	Sieva (PI 549469)	Meso	13	(2.6, 24.1)	white	NA	self	equal	minor	no	no
48	Sussex	Andean	7	(-1.5, 14.9)	green	NA	self	equal	no	yes	yes
49	Thaxter (PI 549454)	Meso	1	(-7.5, 8.9)	green	NA	self	equal	moderate	yes	no
50	Virginia Butterbean	Meso	20	(9.3, 30.8)	red	black	speckle	equal	no	no	no
53	Hopi 50 (PI 347782)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
54	Manteca Ramas (PI 162688)	Meso	7	(-3.9, 17.5)	white	NA	self	equal	moderate	no	no
57	(PI 256820)	Meso	3	(-9.7, 16.5)	white	NA	self	equal	moderate	no	no

Entry #	Name (Accession #)	Group	% Def	95% CI for % Def	1° Color	2° Color	Pattern	More 2° Color in...	Dis-coloration	Split Testa	Small Seed
58	Westan (PI 347777)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
59	Hopi Red/ Pala Hatiqo (PI 476859)	Meso	3	(-7.3, 14.1)	magenta	NA	self	equal	no	no	no
60	Sprigg (PI 549516)	Andean	10	(-0.6, 20.8)	white	NA	self	equal	no	no	yes
61	Dover Bush (PI 549455)	Meso	3	(-4.7, 11.7)	green	NA	self	equal	moderate	no	no
62	Bixby (PI 549460)	Meso	3	(-5.5, 10.9)	white	NA	self	equal	no	no	no
63	Christmas Lima (PI 549461)	Andean	3	(-7.3, 14.1)	white	maroon	stripe	hot	no	no	yes
64	Fordhook Bush det (PI 549465 det)	Andean	5	(-3.5, 12.9)	lt green	NA	self	equal	moderate	no	yes
65	Fordhook Bush ind (PI 549465 ind)	Andean	3	(-7.3, 14.1)	lt green	NA	self	equal	no	no	yes
66	Henderson Bush (PI 549466)	Meso	1	(-7.5, 8.9)	white	NA	self	equal	no	yes	no
67	King of the Garden (PI 549468)	Andean	7	(-3.9, 17.5)	lt green	NA	self	equal	no	no	yes
68	Nemagreen (PI 549481)	Meso	1	(-7.5, 8.9)	green	NA	self	equal	moderate	no	no
70	Baby Potato (PI 549494)	Meso	10	(2.2, 18.7)	white	NA	self	equal	moderate	yes	no
71	Large White (PI 549496)	Andean	23	(14.4, 30.9)	lt green	NA	self	equal	moderate	no	yes
72	Maffei 76 (PI 549512)	Meso	7	(-1.5, 14.9)	green	NA	self	equal	major	no	no
73	Dompe 95 (PI 549518)	Andean	7	(-1.5, 14.9)	white	NA	self	equal	no	no	yes
75	Big Momma	Andean	8	(-4.7, 21.5)	lt green	NA	self	equal	moderate	yes	yes
76	Coverdale	Andean	8	(-4.7, 21.5)	lt green	NA	self	equal	moderate	no	yes
77	Dodd	Andean	17	(6.0, 27.5)	lt green	NA	self	equal	moderate	yes	yes
78	Rohrer's Dr. Martin	Andean	3	(-15, 21.9)	lt green	NA	self	equal	moderate	yes	yes
79	DSU Big 6	Andean	43	(32.6, 54.1)	lt green	NA	self	equal	moderate	no	yes
80	Kuvilek	Andean	43	(30.2, 56.5)	white	NA	self	equal	moderate	no	yes

Entry #	Name (Accession #)	Group	% Def	95% CI for % Def	1° Color	2° Color	Pattern	More 2° Color in...	Dis-coloration	Split Testa	Small Seed
81	Jones	Andean	3	(-7.3, 14.1)	white	NA	self	equal	moderate	no	yes
82	Moser	Andean	73	(60.2, 86.5)	white	NA	self	equal	no	no	yes
83	Pete's Dr. Martin	Andean	37	(26.0, 47.5)	white	NA	self	equal	no	no	yes
84	Susie	Andean	3	(-7.3, 14.1)	white	NA	self	equal	moderate	yes	yes
85	Zerbe	Andean	7	(-3.9, 17.5)	white	NA	self	equal	moderate	no	yes
86	Lineberger - Warren	Andean	40	(29.3, 50.8)	lt green	NA	self	equal	moderate	no	yes
87	Lineberger - Layton	Andean	18	(5.2, 31.5)	lt green	NA	self	equal	moderate	no	yes
105	1102-9	Meso	1	(-7.5, 8.9)	buff	magenta	hilum	cool	no	no	no
106	King Louie	Andean	23	(10.2, 36.5)	white	NA	self	equal	no	yes	yes
124	UC Beija Flor	Meso	1	(-7.5, 8.9)	white	NA	self	equal	minor	no	no
125	Cariblanco Norte	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
126	UC 92	Andean	7	(-1.5, 14.9)	white	NA	self	-	no seed	-	-
127	White Ventura (PI 549499)	Andean	17	(6.0, 27.5)	white	NA	self	equal	moderate	no	yes
128	White Ventura 63 (PI 549502)	Andean	7	(-3.9, 17.5)	white	NA	self	equal	moderate	yes	yes
129	White Ventura 65 (PI 549503)	Andean	13	(2.6, 24.1)	white	NA	self	equal	no	yes	no
130	(PI 347812)	Meso	7	(-3.9, 17.5)	magenta	black	flecks	hot	no	no	no
131	Pat	Meso*	3	(-7.3, 14.1)	white	NA	self	equal	no	yes	no
132	Henderson	Meso	1	(-7.5, 8.9)	white	NA	self	equal	minor	no	no
133	Lee	Andean	3	(-7.3, 14.1)	white	NA	self	equal	no	yes	yes
134	459-1	Andean	13	(2.6, 24.1)	white	NA	self	-	no seed	-	-
135	UC Haskell	Meso	3	(-7.3, 14.1)	white	NA	self	equal	moderate	yes	no
136	UC Luna	Meso	1	(-7.5, 8.9)	white	NA	self	equal	no	no	no
137	Mezcla	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	yes	no

Entry #	Name (Accession #)	Group	% Def	95% CI for % Def	1° Color	2° Color	Pattern	More 2° Color in...	Dis-coloration	Split Testa	Small Seed
138	Wilbur	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
139	Hopi 5989 (PI 347786)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
140	Worcester Indian Red Pole	Meso	13	(2.6, 24.1)	black	NA	self	equal	no	no	no
141	Violet's Multicolored Butterbean	Meso	7	(-3.9, 17.5)	magenta	purple	speckle hilum	hot	no	no	no
142	Alabama Blackeyed Butterbean	Meso	3	(-7.3, 14.1)	buff	black	ring	cool	no	no	no
143	Dixie Speckled Butterpea	Meso	5	(-3.5, 12.9)	magenta	black	speckle	equal	no	no	no
145	L 136 (PI 347829)	Meso	3	(-7.3, 14.1)	maroon	NA	self	equal	no	no	no
151	(PI 347831)	Meso	7	(-3.9, 17.5)	buff	lavender	speckle	hot	no	no	no
158	Bal (PI 180461)	Meso	3	(-7.3, 14.1)	buff	maroon	hilum	cool	no	no	no
161	(PI 347834)	Meso	8	(-4.7, 21.5)	white	NA	self	equal	moderate	yes	no
169	Seven-year Bean (PI 221202)	Andean	3	(-7.3, 14.1)	white	maroon	stripe	hot	no	no	no
188	(PI 257381)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	moderate	no	no
190	(PI 347836)	Meso	3	(-7.3, 14.1)	black	buff	flecks	cool	no	no	no
194	(PI 347839)	Meso	3	(-15, 21.9)	purple	NA	self	equal	no	no	no
200	Fordhook 861 Charter (PI 549457)	Andean*	39	(30.4, 46.9)	green	NA	self	-	no seed	-	-
201	Purple Spray (PI 249040)	Andean	7	(-3.9, 17.5)	white	lavender + purple	spray	equal	no	no	no
206	Hopi 14 (PI 347780)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
207	Hopi 5986 (PI 347785)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
208	(PI 347790)	Meso	15	(6.4, 22.9)	white	maroon	hilum	-	no seed	-	-
209	Sierra (PI 347798)	Meso	3	(-7.3, 14.1)	buff	purple	speckle	equal	no	no	no

Entry #	Name (Accession #)	Group	% Def	95% CI for % Def	1° Color	2° Color	Pattern	More 2° Color in...	Dis-coloration	Split Testa	Small Seed
210	(PI 347799)	Meso	3	(-7.3, 14.1)	green	NA	self	equal	no	yes	no
211	(PI 34780)	Meso	10	(-0.6, 20.8)	buff	lavender	hilum	cool	no	no	no
212	(PI 347807)	Meso	3	(-7.3, 14.1)	buff	black	hilum	cool	no	no	no
213	(PI 347811)	Meso	10	(-0.6, 20.8)	red	black	speckle	equal	no	no	no
214	(PI 347813)	Meso*	13	(2.6, 24.1)	buff	maroon	hilum	cool	no	no	no
215	(PI 347816)	Meso*	3	(-7.3, 14.1)	maroon	NA	self	equal	no	no	no
218	(PI 347842)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	no	no	no
222	Climbing Speckled (PI 638826)	Meso	3	(-7.3, 14.1)	buff	black	hilum	cool	no	no	no
223	(PI 427216)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	moderate	yes	no
224	(PI 427217)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	moderate	no	no
225	(PI 427218)	Meso	3	(-9.7, 16.5)	white	NA	self	equal	moderate	no	no
228	Natiquo (PI 451715)	Meso	10	(-0.6, 20.8)	magenta	black	flecks	hot	no	no	no
229	L 59 (PI 451776)	Meso	3	(-9.7, 16.5)	red	black	speckle	equal	no	no	no
231	L 120A (PI 451780)	Meso	3	(-15, 21.9)	purple	NA	self	equal	no	no	no
232	L 122A (PI 451782)	Meso	33	(24.4, 40.9)	buff	lavender	speckle	equal	no	no	no
234	(PI 477041)	Meso	3	(-7.3, 14.1)	yellow	black	speckle	hot	no	no	no
235	(PI 502182)	Andean	28	(15.2, 41.5)	maroon	NA	self	equal	no	no	yes
236	(PI 502183)	Meso	3	(-7.3, 14.1)	buff	black	speckle	equal	no	no	no
237	Cowey (PI 534913)	Meso	3	(-5.5, 10.9)	maroon	buff	flecks	cool	no	no	no
238	Ganymede (PI 534914)	Meso	3	(-7.3, 14.1)	buff	red + black	hilum + speckle	cool	no	no	no
239	Half & Half (PI 534915)	Meso	7	(-3.9, 17.5)	buff	purple	hilum	cool	no	no	no
240	Red Calico (PI 534917)	Meso*	3	(-7.3, 14.1)	red	black	speckle	equal	no	no	no
243	Triumph (PI 549473)	Meso	9	(0.4, 16.9)	lt green	NA	self	equal	no	no	no

Entry #	Name (Accession #)	Group	% Def	95% CI for % Def	1° Color	2° Color	Pattern	More 2° Color in...	Dis-coloration	Split Testa	Small Seed
244	Willow Leaf (PI 549474)	Meso	3	(-9.7, 16.5)	white	NA	self	equal	no	no	no
245	Wasatch (PI 549482)	Meso	3	(-7.3, 14.1)	green	NA	self	equal	moderate	no	no
246	Easy Shell (PI 549484)	Meso	7	(-3.9, 17.5)	white	NA	self	equal	no	yes	no
247	Buttergreen (PI 549485)	Meso	10	(-0.6, 20.8)	green	NA	self	equal	moderate	no	no
248	Giant Florida Pole (PI 549493)	Andean	3	(-7.3, 14.1)	white	maroon	stripe	hot	no	no	no
249	Carpinteria (PI 549495)	Andean	7	(-3.9, 17.5)	green	NA	self	equal	moderate	no	yes
250	Giant Christmas (PI 549497)	Andean	7	(-3.9, 17.5)	white	maroon	stripe	hot	no	no	yes
251	Mackie (PI 549507)	Meso	3	(-7.3, 14.1)	white	NA	self	equal	moderate	yes	no
252	F - 169 (PI 549514)	Andean*	10	(-0.6, 20.8)	lt green	NA	self	equal	moderate	no	yes
253	Brown Crower Pole 118 (PI 550301)	Meso*	7	(-3.9, 17.5)	magenta	black	speckle	hot	no	no	no
255	Black and Buff Bush (PI 347822)	Meso	13	(2.6, 24.1)	white	NA	self	equal	moderate	no	no