IMPACT OF ENVIRONMENT, PLANT MORPHOLOGY, AND RESISTANCE GENES ON DISEASE SEVERITY OF DOWNY MILDEW IN LIMA BEAN IN DELAWARE

by

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ABSTRACT

Lima bean is the cornerstone crop of the Delaware vegetable processing industry and a major source of income in the mid-Atlantic region (MAR) as a whole. Lima bean is planted mid-summer and harvested during early fall. Unfortunately, the cool, humid conditions near harvest provide conducive conditions for the destructive oomycete pathogen, *Phytophthora phaseoli*, the causal agent of downy mildew of lima bean. Control options utilized by growers include using resistant cultivars and preventative/curative oomyceticide applications, though both have drawbacks. Currently, physiological race F of *P. phaseoli* is dominant in MAR lima bean fields. It is unclear whether race E is still present, and there are no commercial cultivars resistant to both races. Oomyceticides, although useful, are expensive and often select for resistant individuals. Research has been conducted on alternate methods of disease inhibition, including altering leaf shape and architecture, probing new sources of race E/F resistance, and applying regiments of red light, all with the express purpose of stopping downy mildew of lima bean. Results have shown applications of red wavelengths of light and thinner lanceolate leaf shapes both correspond to lower disease severity. In addition, new sources of resistance have been uncovered, though their apparent recessive natures were not expected. Continued research on these disease inhibition methods is needed, however, results of this project indicate potential for implementation into the commercial grower's disease toolbox to allow for healthier, cheaper lima beans.

Chapter 1

LITERATURE REVIEW

Origin. Lineages of current lima bean (*Phaseolus lunatus* L.) can be traced back to Central and South America, specifically the Andean and Mesoamerican regions (Quat Ng, 2013; Nienhuis et al., 1995). DNA analyses have led researchers to believe that lima bean was domesticated at two different times giving rise to the lineages that we see today (Tolomeo et al., 2013). Small-seeded lima beans, such as today's baby lima bean, were discovered in Guatemala in the 1930's and were estimated to have been domesticated 1,200 years ago in the Mesoamerican region, specifically southern Mexico (Motta-Aldana et al., 2010). The large-seeded landrace of lima bean is believed to have been domesticated 6,000 years ago in the Andean region, current day Ecuador and Peru (Motta-Aldana et al., 2010). At present, both lima bean morphologies are found throughout Central and South America (Motta-Aldana et al., 2010). Lima bean is believed to have been introduced to North America by Native Americans before 1492AD via trade routes with Central and South America (de Araujo et al., 2017; Mackie, 1943). Lima bean was introduced to countries in Africa, Asia, and Europe in the Columbian Exchange (de Araujo et al., 2017).

Production. Lima bean is an economically important crop for the Delaware vegetable processing industry and the mid-Atlantic region (MAR) of the United States. Approximately 10,000 acres of baby lima beans are produced in Delaware annually, accounting for almost a third of the nation's overall lima bean acreage (Kee et al. 1997; Hawks, 2019; USDA-NASS, 2019). A vast majority of the lima beans grown in Delaware and the MAR are processed by freezing or canning, where Delaware accounts for 60% of the nation's total frozen and canned lima bean industry (Evans et al., 2007). Between 2014 and 2017, Delaware overtook California as the country's top succulent lima bean producer with Delaware having approximately 5 times more succulent lima bean acreage (USDA, 2017; CDFA, 2018).

Profits from Delaware's lima bean production are approximately $7.8M, with national frozen stocks equaling $59.313 million per year (National Agriculture Statistics Services; USDA-NASS, 2019). Production nation-wide has declined in recent years from a peak in 1997 at 900,000 Cwt to approximately 300,000 Cwt in 2014 (NASS USDA). This decline has been associated with increases in lima bean disease and poor climate adaptability (Ernest et al., 2011). The current commercial types of lima bean in the mid-Atlantic region include Fordhook which originated in the Andean region of Central America, and green baby lima beans and Speckled which have been traced back to the Mesoamerican region of South America (Figure 1) (Evans et al., 2007; Motta-Aldana et al., 2010).

Figure 1 Commercial Fordhook 242 lima bean seeds (left), speckled lima bean (middle), and green baby lima bean (right) (photo taken by Dr. Emmalea Ernest).

Lima bean growing season and life cycle. Lima bean is generally planted in Delaware between June 1st and July 10th depending on the cultivar (Whalen et al., 2006). Planting dates also generally follow the harvest of grain or pea crops (Evans et al., 2007). This timeframe corresponds with climate conditions such as sufficient rainfall, high humidity, and cooler temperatures approaching harvest that make Delaware an ideal location for high yield lima bean crops. (Whalen et al., 2006; Wooten, 1995). Harvest dates fall between 80 and 100 days post planting in mid-August and early September (Evans et al., 2007). Lima beans prefer sandy, acidic soils ranging from 5.8-6.5 pH and germination temperatures of approximately 65°F (Whalen et al., 2006). They are planted in 76-cm rows and will germinate within 6 days of planting (Evans et al., 2007). Flowers will develop 35-60 days post planting, with pins shortly thereafter. (Evans et al., 2007). Pod harvest occurs around 45 days after and is accomplished using mechanical pod-stripper combines, generally done by a processing company (Evans et al., 2007).

Phytophthora phaseoli

History. The causal agent of downy mildew, *Phytophthora phaseoli* Thaxter, has been observed on lima bean within the mid-Atlantic region (henceforth referred to as MAR) of the United States for over a century (Thaxter, 1889; Agrios, 2005**;** Leonian, 1925). The pathogen was first observed by Thaxter in a lima bean crop in Hamden, Connecticut in September of 1889, and five years later was in 1904 was recorded for the first time in Delaware (Thaxter, 1889; Smith, 1904). Downy mildew has since been found throughout the MAR, along with several instances of reports overseas including the Philippines, Congo, Italy, and Puerto Rico (Clara, 1928; Hendrikx, 1939; Casarini et al., 1960; Henricksen, 1907). A total of six physiological races, labeled A-F, have been observed since 1889 (Evans et al., 2007). A physiological race constitutes a subdivision of a pathogen that has a unique method of pathogenicity that sets itself apart from others within its taxonomic group (Kirk et al., 2001). Initially race A of *P. phaseoli* was the primary pathogen of lima bean, driving production from Connecticut further south on the east coast until 1958 when the first resistant lima bean cultivar, Thaxter, was introduced by the USDA (Wester, 1968; Hyre and Cox, 1952; Wester and Cetas, 1959). In that same year, Race B of downy mildew was isolated for the first time in New Jersey (Wester and Jorgensen, 1959). Race C was discovered in 1969 followed shortly thereafter by Race D in 1975 (Wester, 1970; Thomas and Blount, 1976). Race D was the dominant race until the observation and isolation of Race E in 1995 near Milton, Delaware (Evans et al., 2002). The current physiological race that is affecting the MAR lima bean crop is Race F and was first observed in 2000 (Evans et al., 2002).

Economic losses attributed with *P. phaseoli* epidemics are not well recorded, though in 2000, Race E caused over $3,000,000 in yield loss in Delaware by destroying upwards of 40% of Delaware's lima bean crop (Davidson et al., 2008).

Lifecycle. The homothallic oomycete pathogen *P. phaseoli* infects primarily lima bean (*Phaseolus lunatus*) in the MAR (Gallegly et al., 2004). As a polycyclic pathogen, it infects plants using both primary and secondary inoculum. Oospores are the primary inoculum, made by the fusing of oogonia and antheridia (Figure 2) (Gallegly et al., 2004). They appear as thick-walled spheres approximately 24 µm in diameter (Gallegly et al., 2004). These are downy mildew's overwintering structure, remaining viable in eastern soils for upwards of two years, generally surviving on plant debris from previous seasons (Leonian, 1925). Oospores are not often observed unless temperature fluctuations are present or tissue death is occurring (Evans et al., 2007). The oospores can infect directly via germ tube or can give rise to the asexual secondary inoculum, sporangia (Walker, 1952). The sporangia are oblong lemon-shaped vessels approximately 28x18µm that are filled with zoospores (Figure 2) (Gallegly et al., 2004). These sporangia can again infect directly via germ tube or can release the motile zoospores, which will infect susceptible tissues of the lima bean plant (Figure 2) (Walker, 1952; Cox and Hyre, 1951). Non-septate hyphae give rise to new sporangia, repeating the cycle (Agrios, 2005). This pathogen prefers cool temperatures and humid environments between 15-20°C (Gallegly et al., 2004; Evans et al., 2002). *Phytophthora phaseoli* is capable of being maintained on agar with high amino-nitrogen sources, such as lima bean agar and potato dextrose agar at 20°C (Gallegly et al., 2004; Evans et al., 2002).

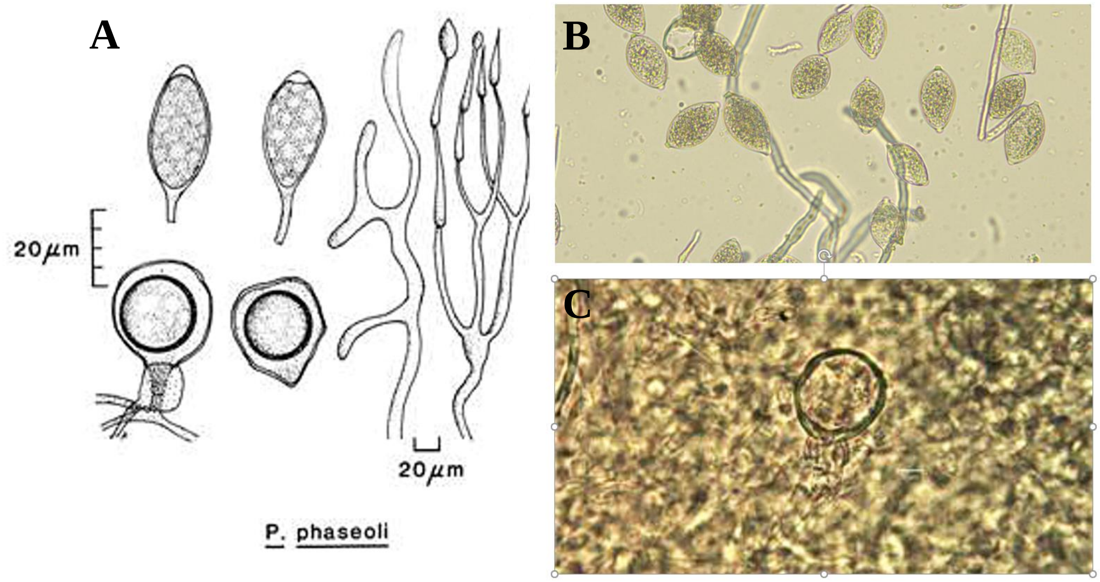

Figure 2 (**A**) Diagram of sporangia (top left), sporangiophores and hyphae (right), oogonium with attached antheridia (bottom left), and an oospore (bottom middle) (Erwin and Ribeiro, 1996); (**B**) sporangia; (**C**) oogonium with attached amphigynous antheridia (Courtesy of Nancy Gregory, University of Delaware).

Signs and Symptoms. Susceptible tissue (infection courts) of lima bean includes racemes, pins, petioles, and shoot tips, while leaves are rarely effected under natural inoculation circumstances (Figure 3) (Davidson et al., 2008; Evans et al., 2007). Signs and symptoms of this pathogen manifest themselves as white fluffy mycelial growth on the susceptible tissues (Figure 3) (Evans et al., 2007). Closer inspection of the mycelia growth will show non-septate hyphae, thus alluding towards an oomycete pathogen (Agrios, 2005). Infection on pods appears as white mycelial growth often with a red band surrounding the infected tissue during late stages of infection (Figure 4) (Evans et al., 2007). At later stages of infection, the pods will become necrotic, shriveling and eventually falling off the plant entirely (Evans et al., 2007).

Figure 3 *Phytophthora phaseoli* mycelial growth on lima bean raceme (left), and pod (right).

Figure 4 Lima bean pods inoculated with *Phytophthora phaseoli* at various stages of infection displaying a red infection band around mycelial growth.

Disease Management. Management strategies for *P. phaseoli* depend heavily on resistance as well chemical controls (Evans et al., 2007). Resistant cultivars of lima bean have been employed since 1958 when the USDA released ‘Thaxter’, a cultivar with resistance to Race A of downy mildew (Wester and Cetas, 1959). The current physiological races E and F affect the MAR lima bean crop and have corresponding resistant cultivars; Bridgeton and 184-85 for Race E, and M-15 and 8-78 for Race F. As of 2018, there are no cultivars resistant to both races (Santamaria et al., 2018). The resistance in all of these cultivars is controlled by a single dominant resistance gene (R-gene) (Santamaria et al., 2018).

Predictive models are also a viable method of knowing when a lima bean crop is at high risk for developing downy mildew. A model developed by Hyre in 1958 outlines the environmental conditions most correlated to emergence of downy mildew (Hyre, 1958). These conditions include 7 days of temperatures averaging 79°F and over 3.05cm of rainfall (Hyre, 1958). If these conditions are met, it is advised that farmers preemptively preventatively spray their crop with the appropriate oomyceticide (Hyre, 1959, Evans et al., 2007). Early fungicides included the Bordeaux Mixture and various copper fungicides, but as the dominant cultivars transitioned from Fordhook to baby lima bean varieties, these fungicides proved to be less effective (Sturgis, 1897; Evans et al., 2007). New races of *P. phaseoli* necessitated new oomyceticides leading to the University of Delaware's discovery that metalaxyl was effective in inhibiting the growth of this pathogen (Evans et al., 2007; Mulrooney, 1981; Mulrooney, 1983; Mulrooney and Carrol, 1982). The effectiveness of metalaxyl allowed for mefenoxam to be labeled for treating downy mildew on lima bean crop (Evans et al., 2007). Other oomyceticides such as Phostrol and various copper fungicides are available to farmers, but Ridomil Gold SL (mefenoxam) is the most widely used product to combat Races E and F of downy mildew (Kee et al., 2006; Evans et al., 2007). Mefenoxam's mode of action involves inhibiting RNA synthesis by blocking RNA polymerase I (https://www.frac.info). While effective, mefenoxam poses a high risk of selecting for resistant individuals through repeated applications (https://www.frac.info). This resistance has been observed in various isolates, all of which were collected from Milton, Delaware (Mhora et al., not published) (Figure 5).

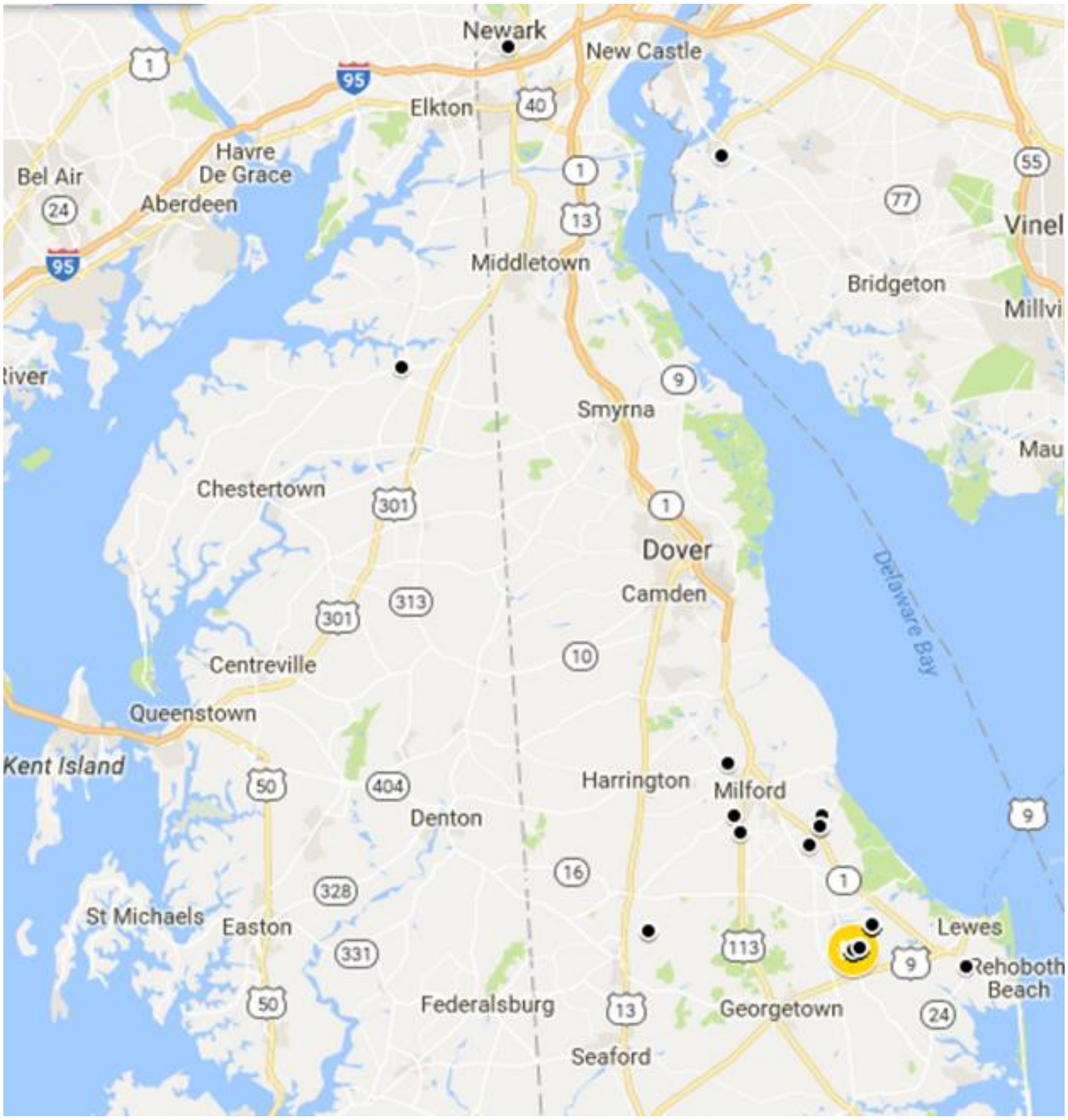

Figure 5 Location of *Phytophthora phaseoli* isolates collected in Delaware, New Jersey, and Maryland. Note: The yellow circle indicates where *P. phaseoli* isolates with resistance to mefenoxam were discovered.

Project Hypotheses and Objectives

Several hypotheses have been formed due to the growing instances of *P. phaseoli* insensitivity to current chemical treatments and the need for disease verification. Hypotheses along with their objectives have been separated by project, though all relate to the need for alternative management methods that reduce the risk of fungicide resistance.

Exploring Effects of Leaf Shape and Plant Architecture on Lima Bean Disease Tolerance and Succulent Seed Quality

<u>Hypothesis 1:</u> Lanceolate leaf shape will reduce humidity within the lima bean canopy thereby decreasing the infection severity of Downy Mildew.

<u>Hypothesis 2:</u> Increased canopy height will reduce the severity of infection caused by *Phytophthora phaseoli*.

<u>Objectives</u>

1. Screen 6 pairs of leaf shape near isogenic lines (NILs) in the field and rate for severity of *P. phaseoli* visually and via qPCR.
2. Screen 6 of lima bean lines with contrasting plant architectures (3 sprawling, 3 upright) in the field and rate for severity of *P. phaseoli* visually and via qPCR.

Characterization and Deployment of Newly Identified Genes for Durable Resistance to Downy Mildew in Lima Bean

<u>Hypothesis 1:</u> The resistance to downy mildew races E and F in PI 256417 is controlled by a dominant gene.

Hypothesis 2: The resistance to race E in PI 256417 is independent of the resistance to race F.

<u>Objectives</u>

1. Observe and characterize resistance to *Phytophthora phaseoli* in the race E and F resistant landrace, PI 256417 in order to ascertain underlying resistance genetic architecture by observing resistance reaction to both races in an $F_{2:3}$ resistant x susceptible population (PI 256417 x PI 534918).

Effects of Red Light on Sporangial Growth and Development of Downy Mildew on Lima Bean

<u>Hypothesis 1:</u> Application of a red wavelength of light to *in planta* and *in vitro* isolates of *P. phaseoli* will inhibit growth and sporulation of the pathogen.

<u>Objectives</u>

1. Collect preliminary data on the effects that red light has on *P. phaseoli* both *in vitro* and *in planta.*
2. Determine the most effective, yet practical regiment of light treatments for inhibiting pathogen growth and production.
3. Understand the methods of inhibition by red light on downy mildew of lima beans.

REFERENCES

"United States Department of Agriculture." USDA, 2014, www.nass.usda.gov/Charts_and_Maps/Dry_Beans,_Dry_Peas,_and_Lentils/.

Agrios, George N. "Diseases Caused by Fungal-Like Organisms." Plant Pathology, 5th ed., Elsevier, 2005, p. 409-414.

Casarini, B., Quaglia, A., and Silvestri, G. 1960. "La 'Peronospora' del 'Fagiolo di lima' (A Peronospora of lima bean)." Phytopathology. Mediterranean. 1:47-48.

CDFA. California Agricultural Statistics Review 2017-2018. California: CDFA, 2018. Web. 15 Aug. 2020.

Clara, F. M. 1928. "A Phytophthora disease of Santol seedlings." Phillip. J. Sci. 35:411-425.

Clarke, C. R., Cai, R., Studholme, D. J., Guttman, D. S., Vinatzer, B. A., "Pseudomonas syringae strains naturally lacking the classical P. syringae hrp/hrc Locus are common leaf colonizers equipped with an atypical type III secretion system." Molecular Plant Microbe Interactions. 2010; 23:198–210. [PubMed: 20064063]

Cox, R. S., and Hyre, R. A. 1951. "Downy mildew of lima bean: Overwintering of the causal organism, *Phytophthora phaseoli* Thaxter, in Delaware." Trans. Peninsula Horticultural Society. 41(1):58-64.

Davidson, C. R., Evans, T. A., Mulrooney, R. P., Gregory, N. F., Carroll, R. B., & O'Neill, N. R. (2008). "Lima bean downy mildew epiphytotics caused by new physiological races of *Phytophthora phaseoli*." Plant Disease, 92(5), 670–674. https://doi.org/10.1094/PDIS-92-5-0670

de Araujo, A. S. F., de Almeida Lopes, A. C., Teran, J. C. B. M. y., Palkovic, A., and Gepts, P. (2017). "Nodulation ability in different genotypes of *Phaseolus lunatus* by rhizobia from California agricultural soils." Symbiosis, 73(1), 7–14. https://doi.org/10.1007/s13199-016-0465-0

Ernest, E. (2011). "Fordhook Lima Bean Production." 1972–1975.

Evans, T. A., Davidson, C. R., Dominiak, J. D., Mulrooney, R. P., Carroll R. B., and Antonius S. H. 2002. T"wo new races of *Phytophthora phaseoli* from lima bean in Delaware." Plant Disease. 86:813.

Evans, T. A., et al. “Two New Races of *Phytophthora phaseoli* from Lima Bean in Delaware.” Plant Disease, vol. 86, no. 7, July 2002, pp. 813–813., doi:10.1094/pdis.2002.86.7.813b.

Evans, T. A., Mulrooney, R. P., Gregory, N. F., and Kee, E. (2007). “Lima Bean Downy Mildew: Impact, Etiology, and Management Strategies for Delaware and the Mid-Atlantic Region, U.S.” Plant Disease, *91*(2), 128–135. https://doi.org/10.1094/pdis-91-2-0128

Gallegly, M. E., and Chuanxue H. *Phytophthora*: Identifying Species by Morphology and DNA Fingerprints. American Phytopathological Society, 2008.

Garrido-Sanz D, et al. “Genomic and genetic diversity within the *Pseudomonas fluorescens* Complex.” PLoS One. 2016; 11:e0150183. [PubMed: 26915094]

Hawks, D. (2019, June 21). “Delaware: A Small State that is Big in Agriculture.” Retrieved August 16, 2020, from https://www.usda.gov/media/blog/2019/06/21/delaware-small-state-big-agriculture

Hendrikx, F. L. 1939. “Phytopathological observations at the station of Mulungu in 1938.” Reviewed Applied Mycology. 19:329-330.

Henricksen, H. C. 1907. “El cultivo de legum- bres en Puerto Rico.” Puerto Rico Agricultural Experimental Station. Bull. 7.

Hutchison, M. L., Tester, M. A., Gross, D. C. “Role of biosurfactant and ion channel-forming activities of syringomycin in transmembrane ion flux: a model for the mechanism of action in the plant-pathogen interaction.” Molecular Plant Microbe Interactions. 1995; 8:610–20. [PubMed: 8589416]

Hyre, R. A. 1958. “The development of a method for forecasting downy mildew of lima bean.” Plant Disease Report. Suppl. 257:179-180.

Hyre, R. A., and Cox, R. S. 1952. “Further studies in the physiology of *Phytophthora phaseoli*.” (Abstr.) Phytopathology 42:468

Jun, S. R., et al. “Diversity of *Pseudomonas* genomes, including Populus-associated isolates, as revealed by comparative genome analysis.” Applied Environmental Microbioly. 2015; 82:375–83. [PubMed: 26519390]

Kee, W. E. Glancey, J. L. and Wooten, T. L. 1997. “The lima bean: a vegetable crop for processing.” HortTechnology 7:119-128.

Kee, W. E., Mulrooney, R. P., Caron, D., and Whalen, J. 2006. “Commercial vegetable production recommendations Delaware.” Ext. Bull. 137, University of Delaware, Newark.

Kirk, P. M., Cannon, P. F., David, J. C., and Stapler, J. A. 2001. “Dictionary of the Fungi.” 9th ed. CABI Publishers, Wallingford, Oxon, UK.

Leonian, L. H. 1925. “Physiological studies of the genus *Phytophthora phaseoli*.” American Journal of Botany. 12:444-498.

Mackie, W. W. 1943. “Origin, dispersal and variability of the lima bean, *Phaseolus lunatus*.” Hilgardia 15:1-29

Mhora, T. T., Ernest, E. G., Wisser, R. J., Evans, T. A., Patzoldt, M. E., Gregory, N. F., Westhafer, S. E., Polson, S. W., & Donofrio, N. M. (2016). “Genotyping-by-sequencing to predict resistance to lima bean downy mildew in a diversity panel.” Phytopathology, 106(10), 1152–1158. https://doi.org/10.1094/PHYTO-02-16-0087-FI

Motta-Aldana, J. R., Serrano-Serrano, M. L., Hernández-Torres, J., Castillo-Villamizar, G., Debouck, D. G., & Chacón, M. I. S. (2010). “Multiple origins of Lima bean landraces in the Americas: Evidence from chloroplast and nuclear DNA polymorphisms.” Crop Science, 50(5), 1773–1787. https://doi.org/10.2135/cropsci2009.12.0706

Mulrooney, R. P. 1981. “Fungicide evaluations for control of downy mildew of baby lima beans”, 1980. Fungicide and Nematicide Tests 36:53. The American Phytopathological Society, St. Paul, MN

Mulrooney, R. P. 1983. “Fungicide evaluation for control of downy mildew of baby lima beans”, 1982. Fungicide and Nematicide Tests 38:93. The American Phytopathological Society, St. Paul, MN

Mulrooney, R. P., and Carroll, R. B. 1982. “Fungicide evaluation for control of downy mildew of baby lima beans”, 1981. Fungicide and Nematicide Tests 37:62. The American Phytopathological Society, St. Paul, MN.

Nienhuis, J., Tivang, J., and Skroch, P. 1995. “Genetic relationships among cultivars and landraces of lima bean (*Phaseolus lunatus* L.) as measured by RAPD markers.” Journal of the American Society for Horticultural Science. 120:300-306.

Quat, N. N., 2013. "Conserving Tropical Leguminous food crops. In: Conservation of Tropical Plant Species", Eds, Norman M. N., Chin H. F. and Reed B. M. Pp 213-247. Springer, New York.

Santamaria, L., Ernest, E. G., Gregory, N. F., and Evans, T. A. (2018). "Inheritance of resistance in Lima bean to *Phytophthora Phaseoli*, the causal agent of Downy Mildew of Lima Bean." HortScience, *53*(6), 777-781. doi:10.21273/hortsci12748-18

Smith, C. O. 1904. "Mildew of lima bean." Delelaware Agricultural Experimental Station. Bull. 63:23-24.

Sturgis, W. C. 1897. "The mildew of lima beans." Connecticut Agricultural Experimental Station. Rep. 21:159-166.

Thaxter, R. "A New American *Phytophthora*." Botanical Gazette, vol. 14, no. 11, Nov. 1889, pp. 273–274., doi:10.1086/326464

Thomas, C. A., and Blount, V. L. 1976. "Race D of *Phytophthora phaseoli*." Plant Disease Report. 60:308.29.

Tolomeo, V., Krug, K., Flohr, D., Holm, S., Jeutong, R., Johnson, J., Kugel, B., Letterman, J., McDonnell, J., Olmstead, K., Schwedler, L. and Van Court, J. 2013. California Vegetable Review. United States Department of Agriculture National Agriculture Statistics Service 34.

USDA. Delaware Agricultural Statistics Bulletin 2016-2017. Delaware: USDA, 2017. Web. 15 Aug. 2020

USDA-National Agricultural Statistics Service. 2019. 2017 Census of Agriculture.

Walker, J. C. 1952. "Downy mildew of lima bean." In: Diseases of Vegetable Crops. McGraw-Hill, New York.

Wester, R. E. 1970. "A new race of downy mildew on lima beans." Phytopathology 60:1856.

Wester, R. E., and Jorgensen, H. 1959. "A new race of *Phytophthora phaseoli* from lima beans." Plant Disease Report. 43:184-186.

Wester, R. E. 1968. "New lima beans resistant to "B" strain of *Phytophthora phaseoli*. Plant Disease Report. 52:563-564.

Wester, R. E. and R. C. Cetas. 1959. "Breeding lima beans for resistance to downy mildew." Plant Disease. Report. Suppl. 257:181–182.

Whalen, J., VanGessel, M., Mulrooney, B., & King, S. (2006). “Crop Profile for Beans (Lima) in Delaware.”

Wooten, T. L. 1995. “The effects of heat stress on retention and abscission of lima bean reproductive structures.” M.S. thesis. University of Delaware, Newark.

Chapter 2

EXPLORING EFFECTS OF LEAF SHAPE AND PLANT ARCHITECTURE ON LIMA BEAN DISEASE TOLERANCE AND SUCCULENT SEED QUALITY

Introduction

Lima bean is a cornerstone crop of the Delaware vegetable processing industry providing upwards of 500 jobs and nearly $10 million in annual revenue (USDA-NASS, 2019). Unfortunately, humid conditions during the harvest of lima beans in the mid-Atlantic region of the United States are highly conducive for the oomycete pathogen *Phytophthora phaseoli* (Cox, 1954, Evans et al., 2007). *Phytophthora phaseoli*, the causal agent of lima bean downy mildew, reduces yield annually. In favorable years, Race E has been reported to reduce yield of Delaware's lima bean acreage by over 40% (Evans et al., 2007). Chemical control methods, such as the application of various oomyceticides, provide a respite from the disease, but specific modes of action have potential to select for resistant individuals. In addition, high cost barriers of time and money can limit feasibility of application (https://www.frac.info). The inherent problems of oomyceticides and vertical resistance have pushed the University of Delaware to explore new methods of pathogen avoidance and inhibition that would be affective in avoiding a range of diseases, with less potential for breakdown of disease resistance.

Figure 6 Architecture difference between inbred upright architecture lines (left) and traditional sprawling architecture (right)

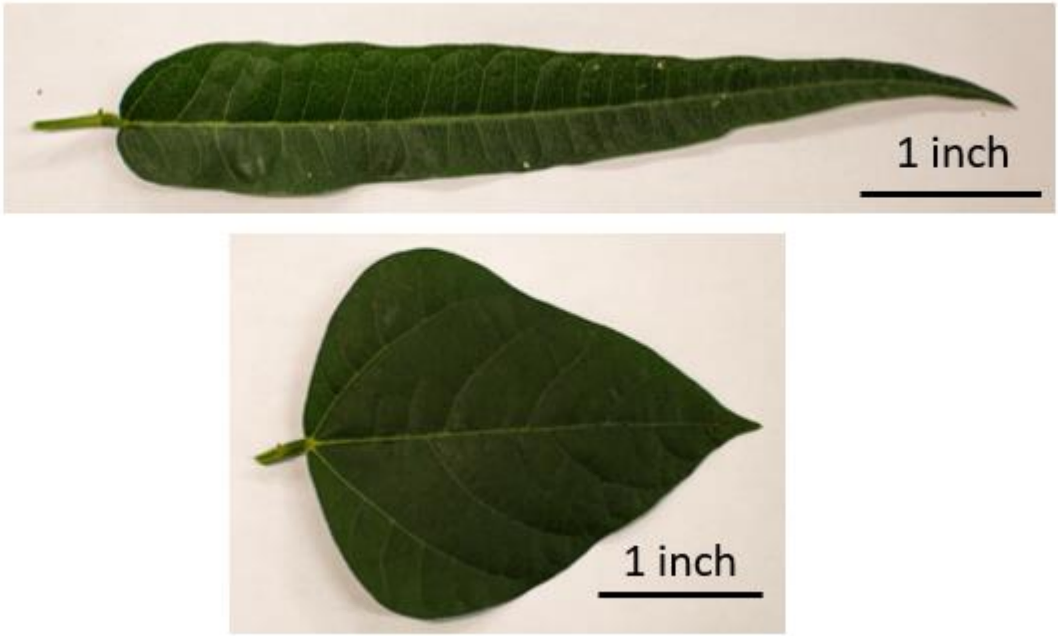

Figure 7 Shape different between lanceolate (top) and traditional ovate (bottom) phenotypes.

It was hypothesized that lanceolate leaf shape will increase airflow through the canopy, thereby reducing the humidity within the lima bean canopy, leading to a decrease in the severity of infection by downy mildew. It was also hypothesized that increased canopy and pod height will lead to less downy mildew infection and severity. The objectives concerning the two parts of this project were to i) plant and compare lanceolate and ovate near isogenic lines for *P. phaseoli* severity and ii) Plant and assess differences in *P. phaseoli* severity in lima bean plants with upright versus sprawling architectures.

Materials and Methods

Leaf shape. NIL pairs were derived from a cross made by Dr. E.G. Ernest between PI 347819 and ‘C-elite Select’. PI 347819 is a vining, white seeded baby lima with lanceolate leaves while ‘C-elite Select’ is a bush green baby lima with reniform leaves. The leaf shape trait is controlled by a single locus and the heterozygous condition produces an intermediate phenotype. Plants may be phenotyped for leaf shape at the primary leaf stage. To create the NIL pairs, bush plants with the heterozygous phenotype were selected from each cross in the F_2 generation. In subsequent generations, individuals with the heterozygous phenotype were selected at the seedling stage and selfed until the F_6 generation. In the F_6 generation, seedlings with lanceolate and reniform leaf shapes were selected from each selfed line and further selfed until the F_8 generation (Figure 7).

Canopy architecture. Lines of lima bean with upright architecture and pods positioned higher in the canopy were developed after lower levels of white mold infection were observed in common bean with a similar structure (Figure 6) (Saindon et al., 1995). Four inbred experimental lines with an upright plant habit (DE1304503, DE1305601C, DE1202203A, and DE1001202E) were chosen to compare with three standard cultivars (C-Elite Select, Cypress, and 184-85) with a sprawling growth habit. The experimental lines were developed by the University of Delaware lima breeding program (Dr. E.G. Ernest) and had been selected for upright plant architecture.

Inoculum preparation. Race F of *Phytophthora phaseoli* inoculum, isolated by Terrence Mhora and Nancy Gregory and henceforth referred to as isolate: PhyP18, was maintained on susceptible Concentrated Fordhook lima beans. On a weekly basis, healthy 6-day old Concentrated Fordhook lima bean hypocotyls were inoculated with a slurry of the previous week's 11-day old inoculum. This slurry was prepared by removing and discarding the cotyledons, leaves, and roots of the infected plant, then slicing the remaining hypocotyl into 2.5-5-centimeter pieces and putting them into a food processor. 100mL of distilled water was added and the lima bean hypocotyls were ground for 45 s, at which point the concentrated slurry was added to another 700mL of distilled water to achieve an approximate sporangial density of 1.16×10^5 sporangia/mL. Approximately 42 hypocotyls were used per inoculum batch. Inoculated plants were stored at 20°C and 87% relative humidity within a digital mist chamber. A 24-hour regiment of florescent light at 5µmol m-2s-1 of photosynthetic active radiation (PAR) was applied in order to stunt plant height.

For field inoculum, approximately 60 hypocotyls infected with PhyP18 (including leaves and cotyledons) were washed in a 3-gallon bucket of water to suspend sporangia. The sporangia solution was passed through two sieves (U.S.A Standard No. 20 (top) and No.100 (bottom)) twice to remove plant debris and sediment. The inoculum was then transferred to a backpack sprayer (SOLO 4 Gallon Backpack Sprayer) and topped off with water. The concentration of field inoculum measured using a hemocytometer and was found to be approximately $9.21*10^{-4}$ sporangia/mL.

Field Trials. In late June of 2019 and 2020, seeds of inbred and NIL's were sown in a research field at the University of Delaware's College of Agriculture and Natural Resource's Newark campus [39°39'55.9"N, 75°44'42.6"W]. Leaf shape and architecture lines were separated within the field and each section was planted using a randomized block design. All lines were replicated three times per season with standard lima bean planting procedures being used. For every line, 40 seeds were planted 7.6 centimeters apart in triplicate per block. Blocks were 3.05 meters long and divided horizontally by 1.5-meter alleys, and vertically by a continuous row of C-elite Select lima beans. Each row was 76.2 centimeters apart. The field was irrigated every-other-day via tripod irrigation for two weeks, at which point 2.5-centimeter blue-stripe trickle tape was deployed running the length of every row. Trickle irrigation was provided for 3-hours between two and five times per week, depending on temperature and rainfall, until inoculations. One week prior to the first inoculation, 5-foot-tall misters were set up at 20-foot intervals throughout the field. After flat pods were observed in late August to early September, inoculations were performed via backpack sprayer during the cooler morning hours. Only the plants in the middle row of each block were inoculated, with two applications being made per inoculation date to cover all surfaces. Inoculations were repeated six days later. At the time of the first inoculation, misters were programmed to spray during the night and early morning hours (4 pm, 6pm, 9pm, 12am, and 4am) for 20 minutes at each time to maintain conducive pathogen conditions. Misters were kept active until the date of first yield harvest. Two weeks after initial inoculations, the number of infected plants and disease severity ratings were taken for each middle row. Disease severity ratings on a scale from 1 (no visible infection) to 4 (widespread, severe infection) were taken twice in 2019 and four times in 2020, with ratings 1 and 2 (2019) and 3 and 4 (2020) coinciding with both years' second field inoculations. At the time of the first rating (2019) and third rating (2020), pods adjacent to those with visible infection were collected for quantitative PCR analysis, with two pods being collected from each replicate of the NILs Thomas, Tessa, Kevin, Kenna, DE1202203A, C-Elite and 184-85 as a positive control. Visual ratings were taken again 5-days after in 2019, and 4, 7, and 12 days later in 2020. Middle rows from three replicates of each line were harvested from late September to early October based on the dryness of their pods and were subsequently analyzed for yield. Disease progress curves were created for all leaf shape pairs in 2020 (Figure 14, 15, 16, 17, 18).

Quantitative PCR analysis. To better understand whether visual ratings were an accurate representation of disease severity among the different lines quantitative PCR analysis was conducted on non-symptomatic pods collected from predetermined plants that were directly adjacent to visibly infected pods. DNA was extracted using the Wizard ® Genomic DNA Purification Kit (Promega Corporation, Madison, WI). DNA was extracted per the included instructions for plant tissues with slight modification of doubling the initial amount of crushed tissue (increased plant tissue from 40mg to 80mg) as well as increasing the amount of "RNAse A Solution" added from 3µL to 5µL. Extracted DNA was stored at 4°C until qPCR analysis could take place. Pure *P. phaseoli* DNA was achieved by growing the pathogen in lima bean broth for 10 days at room temperature on a shaker set to 80rpm. After 10 days, the mycelial ball was cut away from the agar starting plug and dried using a vacuum pump and filter. Tissue was then extracted using the "plant tissues" procedure as mentioned above. A five-fold dilution series (50 ng/uL, 5 ng/uL, 0.5 ng/uL, 0.05 ng/uL, 0.005 ng/uL) was performed using the PhyP18 DNA samples to create a standard curve (Figure 23). Quantitative-PCR (qPCR) was accomplished with a QuantStudio3 PCR machine (Fisher Scientific, Waltham, MA). Using a 2mL 96 well plate to achieve a 20µL qPCR reaction, 10µL of SYBR Green Master Mix (Fisher Scientific, Waltham, MA), 4µL of the forward INF4 primer, 4µL of the reverse INF4 primer, and 2µL of the appropriately diluted *P. phaseoli* DNA was combined in per well replicated in triplicate. The qPCR cycling conditions were as follows: 50°C for 2 minutes; 95°C for 2 minutes; 60°C for 30 seconds (cycled 40 times); 95°C for 15 seconds; 60°C for 1 minute; 95°C for 15 seconds (melting curve). Both the standard curve, 2019 and 2020 qPCR plates were run twice. Standard Curve was plotted using QuantStudio^{TM} Design and Analysis Software (Fisher Scientific, Waltham, MA)

Weather data. To compare climactic conditions within the canopies of leaf shape near isogenic lines four NIL pairs (Thomas/Tessa, Robert/Rebecca, Matt/Morgan, Paul/Phoenix) were planted in a split plot design with four replications at the Carvel Research Station in Georgetown, Delaware [38°38'03.2"N, 75°27'34.4"W]. Plots were three rows wide and temperature/humidity sensors were placed within the lima bean canopy 20 cm from the soil surface for three replications of each of the eight lines. (HOBO Temperature/RH Data Logger).

Results

Visual Disease Severity Ratings:

Leaf Shape. Near-isogenic line data from 2019 showed that for the first rating date, (9/18/19), there was a significant difference between NILs (Figure 8). Performing a pairwise comparison analysis on each pair showed that Kevin and Kenna ($p = 0.028$), along with Robert and Rebecca ($p = 0.028$) had significant differences between them with the ovate line in each pair having the higher severity (Table 1). When comparing all lanceolate NILs to ovate NILs for the first rating date in 2019, lanceolate had significantly less disease severity when compared to their ovate counterparts ($p= 0.024$) (Figure 9). An overview comparison of the two leaf shapes for the second rating date showed no significance.

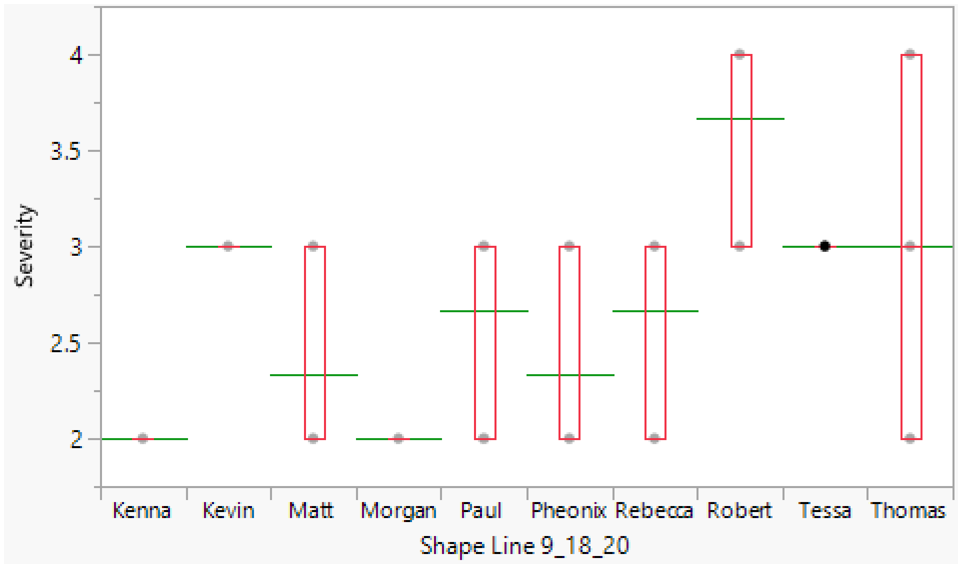

Figure 8 Box plot with distribution of all individual near isogenic lines from the first rating date of 2019.

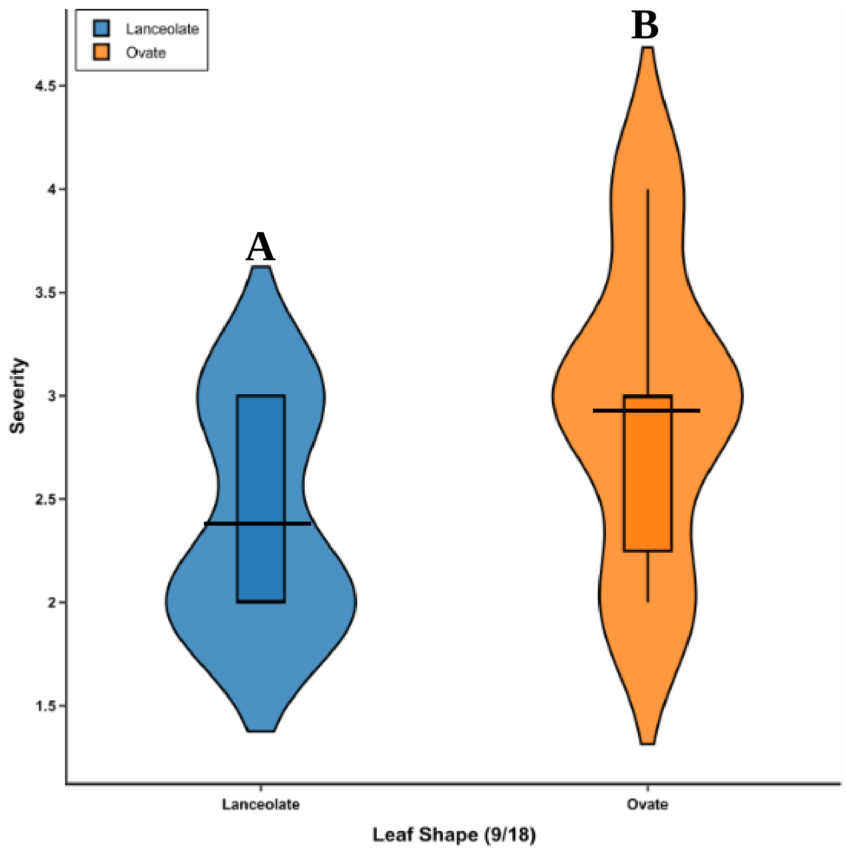

Figure 9 Violin plot depicting distribution of disease severity between all lanceolate and ovate near-isogenic lines from the first rating date of 2019. Significance is denoted by letters above each box.

No significant differences in severity between individual NILs or overall leaf shapes were observed for the first two rating dates of 2020 (9/5/20 and 9/9/20). Rating date 3 (9/12/20) showed significance between NIL pairs as well as overall leaf shapes ($p = 0.006$) (Figure 10, 11). A pairwise comparison showed that Kevin and Kenna ($p = 0.006$), as well as Matt and Morgan ($p = 0.037$) were significantly different with the ovate NILs having the higher severity (Table 1). The final rating date showed no significance between NIL pairs or overall leaf shapes.

Figure 10 Distribution of disease severity among all leaf shape near-isogenic lines from the third rating date of 2020.

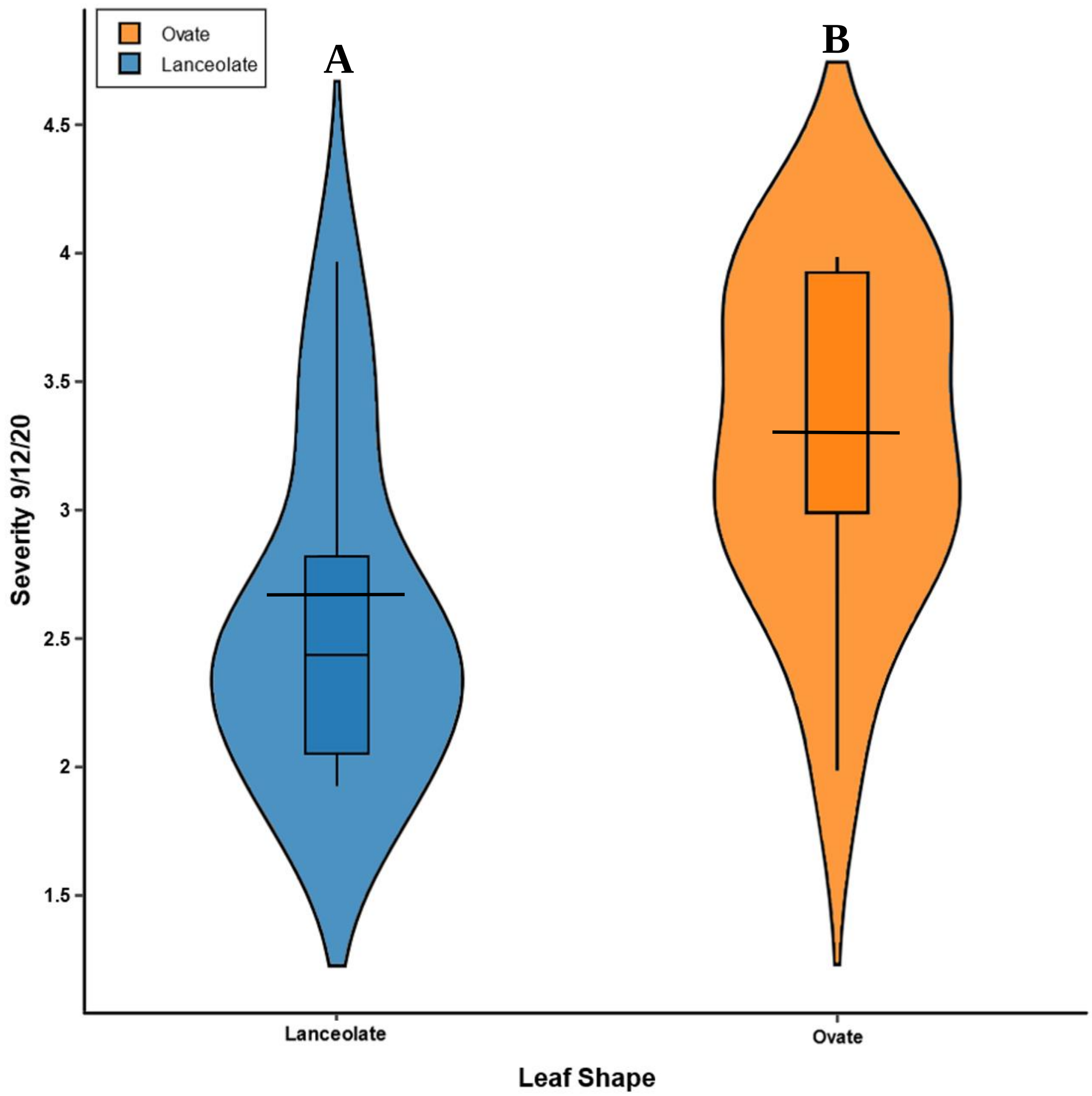

Figure 11 Violin plot depicting distribution of disease severity between all lanceolate and ovate near-isogenic lines from the third rating date of 2020. Significance is denoted by letters above each box.

Architecture. Only one rating date for architecture was taken in 2019 due to the rapid rate at which pods matured and had to be harvested to obtain accurate yield measurement. The first and only rating date in 2019 (9/18/19) for the architecture varieties showed no significant differences between individual lines or upright and sprawling architecture as a whole.

Four ratings were taken in 2020. The first three rating dates (9/5/20, 9/9/20, and 9/12/20) showed no significant differences between individual lines or architecture groups. The fourth rating date (9/17/20) did show significance in both individual pairs ($p = 0.027$) and as groups ($p = 0.029$) (Figure 12). A pairwise comparison showed that DE1305601C had significantly lower severity when compared with Cypress ($p = 0.008$), C-Elite Select ($p = 0.008$), and 184-85 ($p = 0.002$). Upright architecture DE1001202E also had a significantly lower severity than 184-85 ($p = 0.028$).

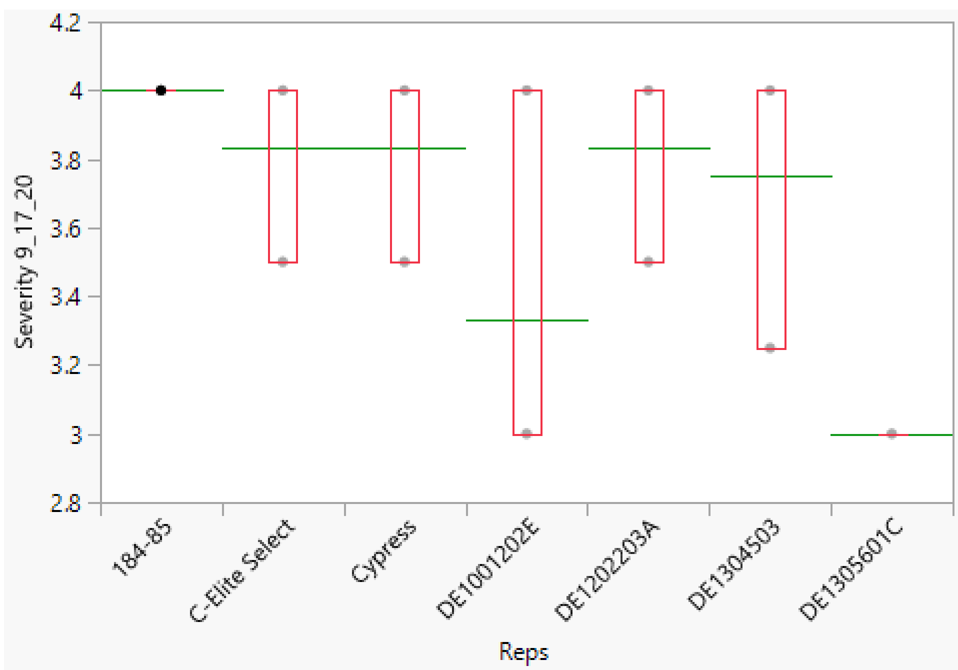

Figure 12 Box plot with distribution of all individual architecture lines from the fourth rating date of 2020.

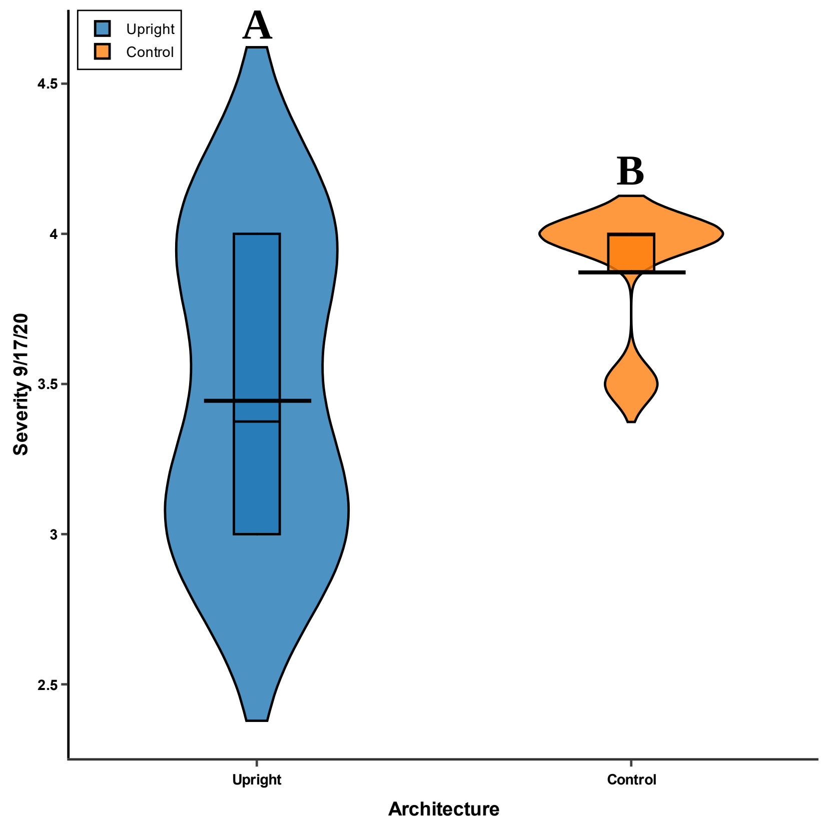

Figure 13 Violin plot depicting distribution of disease severity between all lanceolate and ovate near-isogenic lines from the fourth rating date of 2020. Significance is denoted by letters above each box.

Table 1 Pairwise comparisons of adjoining pairs of leaf shape NILs as well as architectures that showed significant severity differences for both 2019 and 2020 growing seasons.

Severity Contrast				
Year	**Rating Date**	**Line**		**p-Value**
Leaf Shape				
2019	#1	Kevin*	Kenna	0.028
		Robert*	Rebecca	0.028
2020	#1	Kevin*	Kenna	0.006
		Matt*	Morgan	0.037
Architecture				
2020	#2	DE1305601C	Cypress*	0.008
		DE1305601C	C-Elite Select*	0.008
		DE1305601C	184-85*	0.002
		DE1001202E	184-85*	0.028
*Signifies significantly larger value. (p>0.05)				

Disease Progress Curves

Disease progress curves of all near-isogenic line pairs were made to show how the disease progress compared against leaf shapes. Disease progress curves showed significance at the third rating for the Kevin/Kenna and Matt/Morgan NILs as shown by Table 1 and is reflected in Figures 14 and 15 at the September 12^{th} time point. Disease progress curves were only created for the 2020 field season due to the higher frequency of disease ratings.

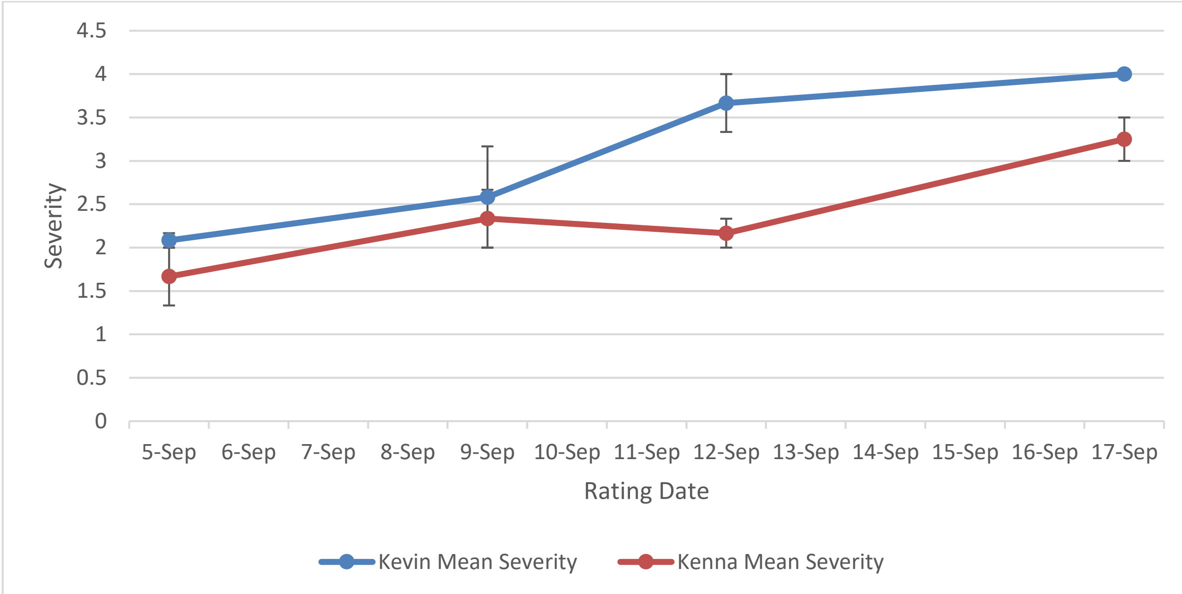

Figure 14 Disease progress curve depicting Kevin and Kenna NILs at all four rating dates. Note: First inoculation (not shown) was applied on 8/26/20 with the second inoculation being applied on 9/5/20.

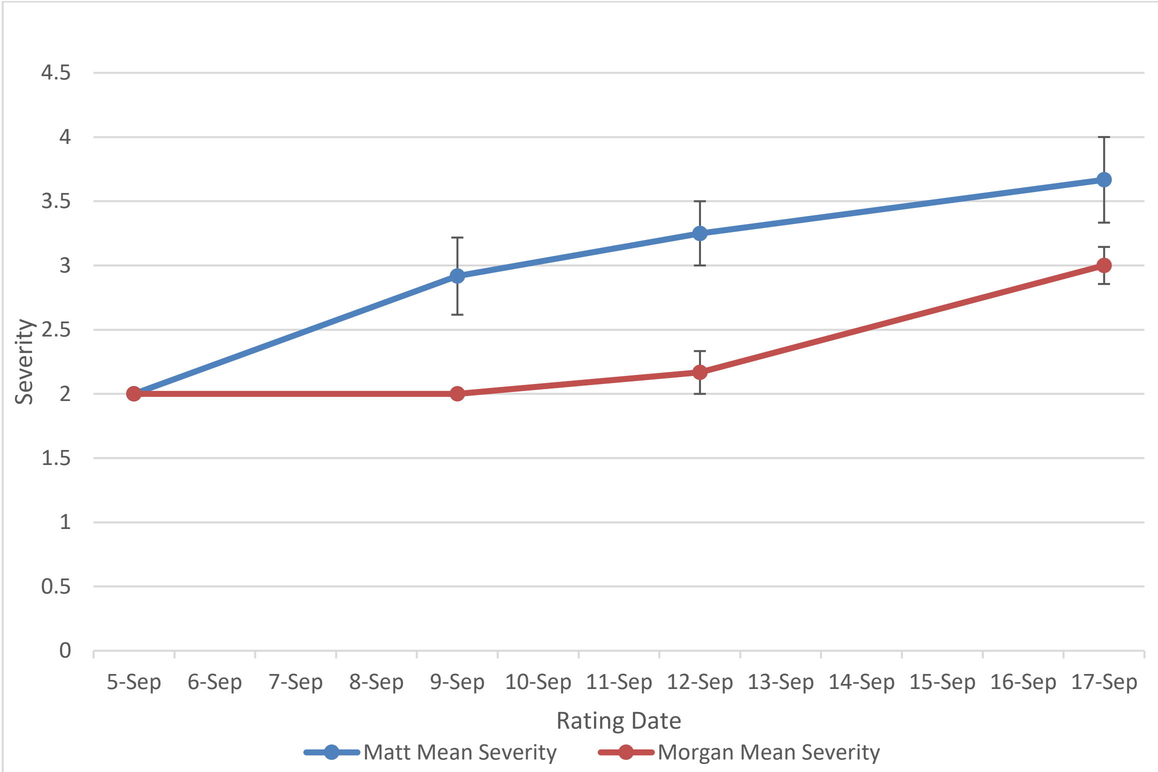

Figure 15 Disease progress curve depicting Matt and Morgan NILs at all four rating dates. Note: First inoculation (not shown) was applied on 8/26/20 with the second inoculation being applied on 9/5/20.

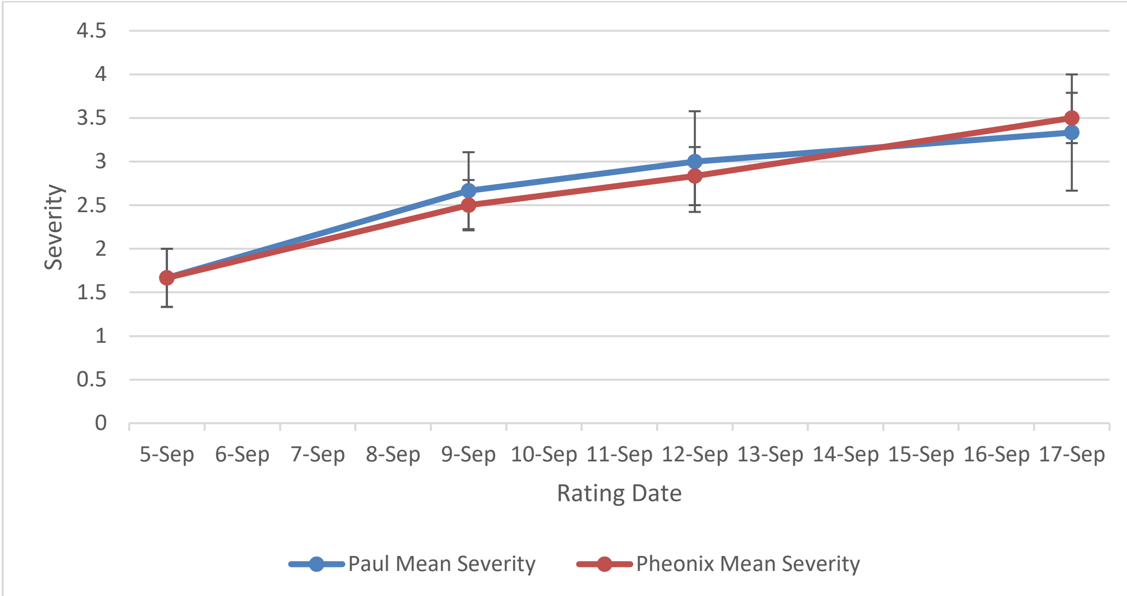

Figure 16 Disease progress curve depicting Paul and Pheonix NILs at all four rating dates. Note: First inoculation (not shown) was applied on 8/26/20 with the second inoculation being applied on 9/5/20.

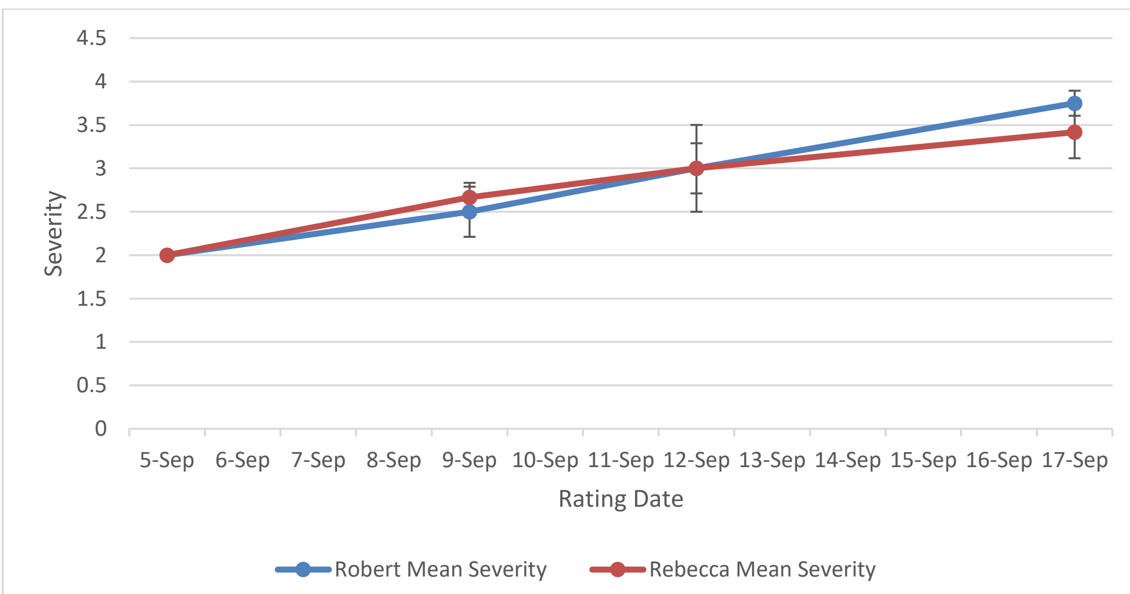

Figure 17 Disease progress curve depicting Robert and Rebecca NILs at all four rating dates. Note: First inoculation (not shown) was applied on 8/26/20 with the second inoculation being applied on 9/5/20.

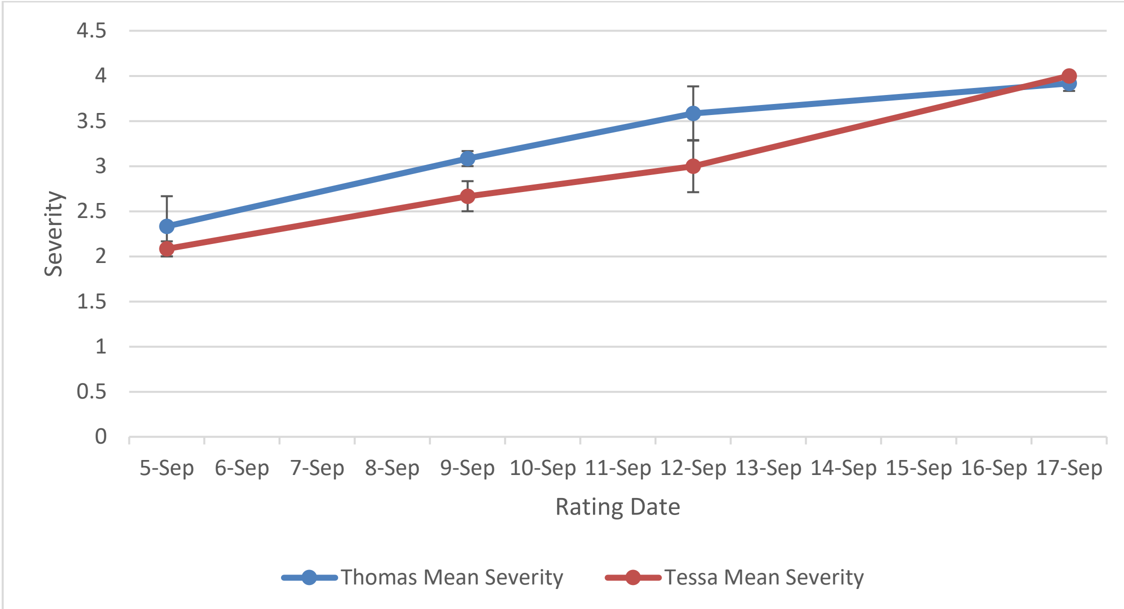

Figure 18 Disease progress curve depicting Thomas and Tessa NILs at all four rating dates. Note: First inoculation (not shown) was applied on 8/26/20 with the second inoculation being applied on 9/5/20.

Yield

Lima bean plants were harvested over the course of several days as they reached approximately 3% dry pods. Architecture varieties with the exception of 184-85 were the first to mature in both 2019 and 2020. The harvesting of 184-85 and lanceolate leaf shapes followed several days after, with ovate leaf shapes being the last set to be harvested in both years. Yield data in 2019 showed upright and control architectures having higher yields than the NILs, though between the architecture varieties as a whole, there were no significant differences (Figure 19). The pairwise comparison, on the other hand, did show significance between DE1202203A and 184-85 ($p = 0.024$), as well as DE1001202E and 184-85 ($p = 0.016$) (Table 2). The upright architectures ("DE" numbers) showed significantly higher yields in both cases. Leaf shape lines showed no significance, but pairwise comparisons uncovered that Matt and Morgan ($p = 0.012$), along with the NILs Robert and Rebecca ($p = 0.024$) had significant differences (Table 2). Matt and Rebecca had significantly higher yields than their counterparts.

Total yield for 2020 from all groups was less than yield recovered in 2019, with upright and control architectures again having a significantly higher yield than the leaf shape NILs (Figure 19). Pairwise comparisons showed that only DE1202203A and C-Elite Select had any significance, with DE1202203A having the higher yield (Table 2).

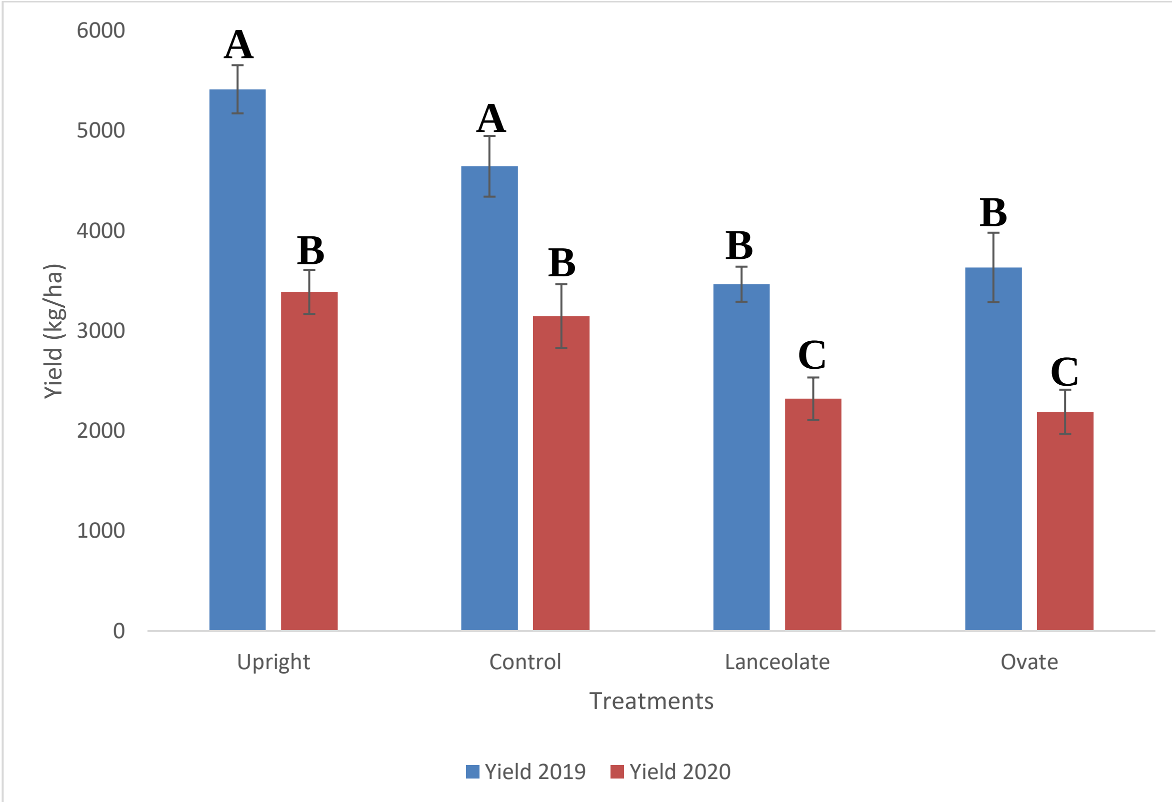

Figure 19 Lima bean yield comparison between all architecture and leaf shape groups for 2019 and 2020. Significance is denoted by letters above bars. Note: values were extrapolated from grams/plot to kilograms/hectare.

Table 2 Pairwise comparisons of adjoining pairs of leaf shape NIL's as well as architectures that showed significance in yield for both the 2019 and 2020 growing seasons.

Yield Contrasts			
Year	**Line**		**p-Value**
Leaf Shape			
2019	Matt*	Morgan	0.012
	Robert	Rebecca*	0.024
Architecture			
2019	DE1202203A*	184-85	0.024
	DE1001202E*	184-85	0.016
2020	DE1202203A*	C-Elite Select	0.042
*Signifies significantly larger value. (p>0.05)			

Weather Data

The relative humidity of the near isogenic line canopies was not significantly different during the nighttime hours, but between the hours of 8 AM and 6 PM, canopy humidity was significantly lower in the lanceolate lines (Figure 20, Table 3).

Relative humidity and leaf wetness were measured over the course of August and September (Figure 21) Leaf wetness hours were considered any hours where the relative humidity was over 95%. No significance was observed between within NIL pairs. It is worth noting that pairs with higher ovate leaf wetness corresponded with a higher disease severity during the 2019 and 2020 field trials.

Averaged canopy temperature readings showed that during nighttime hours (7 PM to 6 AM), lanceolate temperatures were significantly lower than ovate temperatures (Figure 22, Table 4). It was also seen that lanceolate leaf shapes had significantly higher canopy temperatures between the hours of 8-11 AM and 3-5 PM (Figure 22, Table 4).

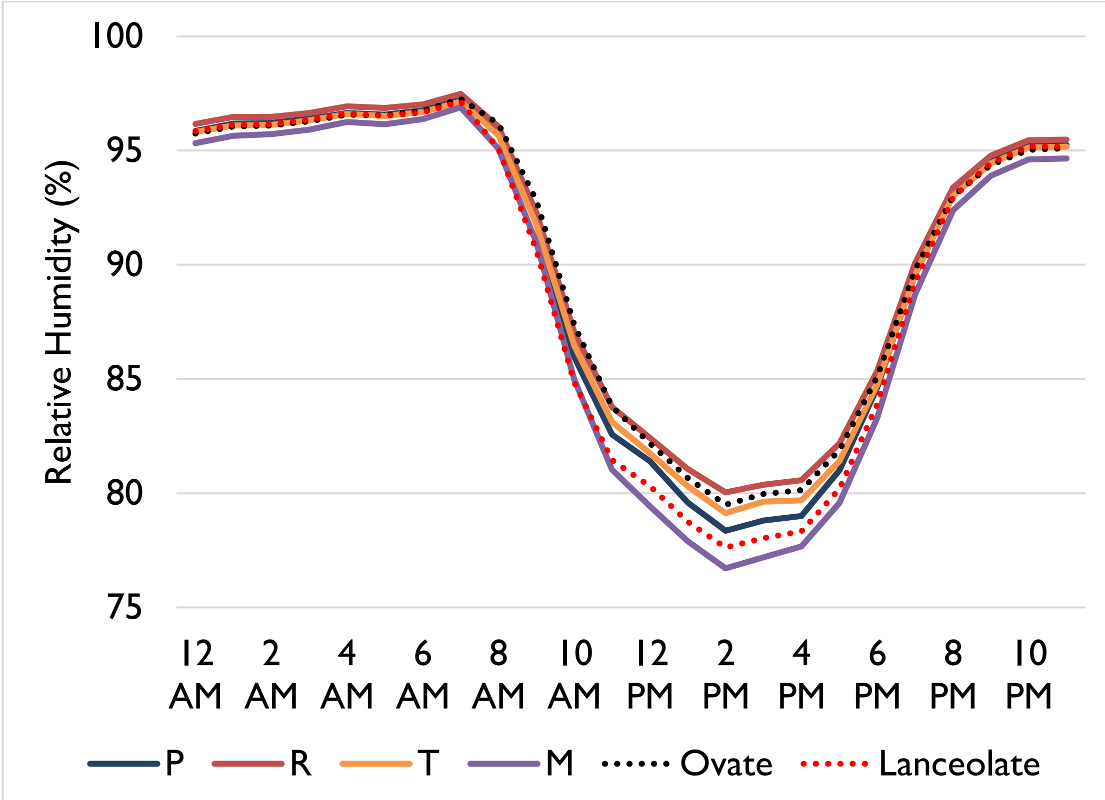

Figure 20 Average canopy relative humidity per hour over the course of the growing season (August 12 – September 20). (Letters in keys represent NIL pairs.)

Table 3 Average canopy humidity p-values at hourly intervals throughout the growing season (August 12 – September 20). Highlighted portions represent significance.

Time	p-value Leaf Shape
12 AM	0.6951
1 AM	0.7334
2 AM	0.7905
3 AM	0.8135
4 AM	0.8195
5 AM	0.9189
6 AM	0.7520

7 AM	0.3780
8 AM	0.0006
9 AM	<0.0001
10 AM	0.0002
11 AM	0.0005
12 PM	0.0030
1 PM	0.0020
2 PM	0.0028
3 PM	0.0011
4 PM	0.0013
5 PM	0.0009
6 PM	0.0051
7 PM	0.1100
8 PM	0.8789
9 PM	0.8034
10 PM	0.5837
11 PM	0.7880

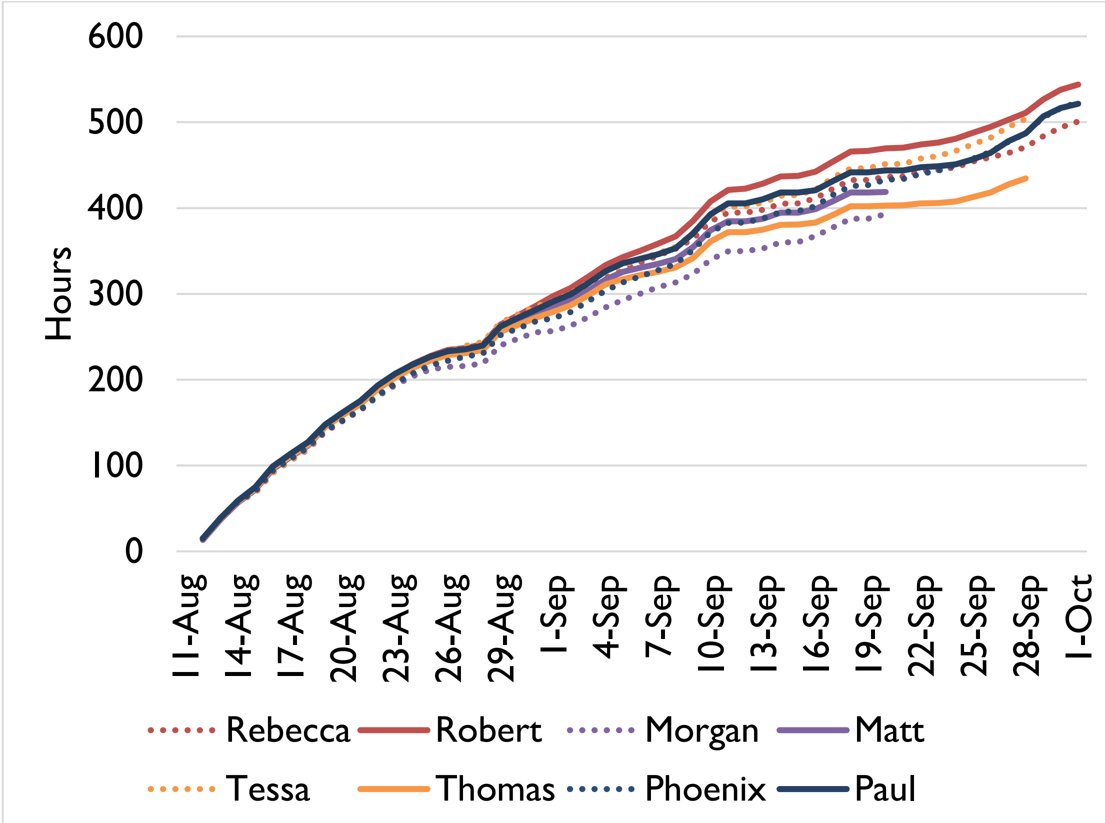

Figure 21 Diagram of total leaf wetness duration in hours for each NIL compiled from sensor readings from August 11th through October 1st.

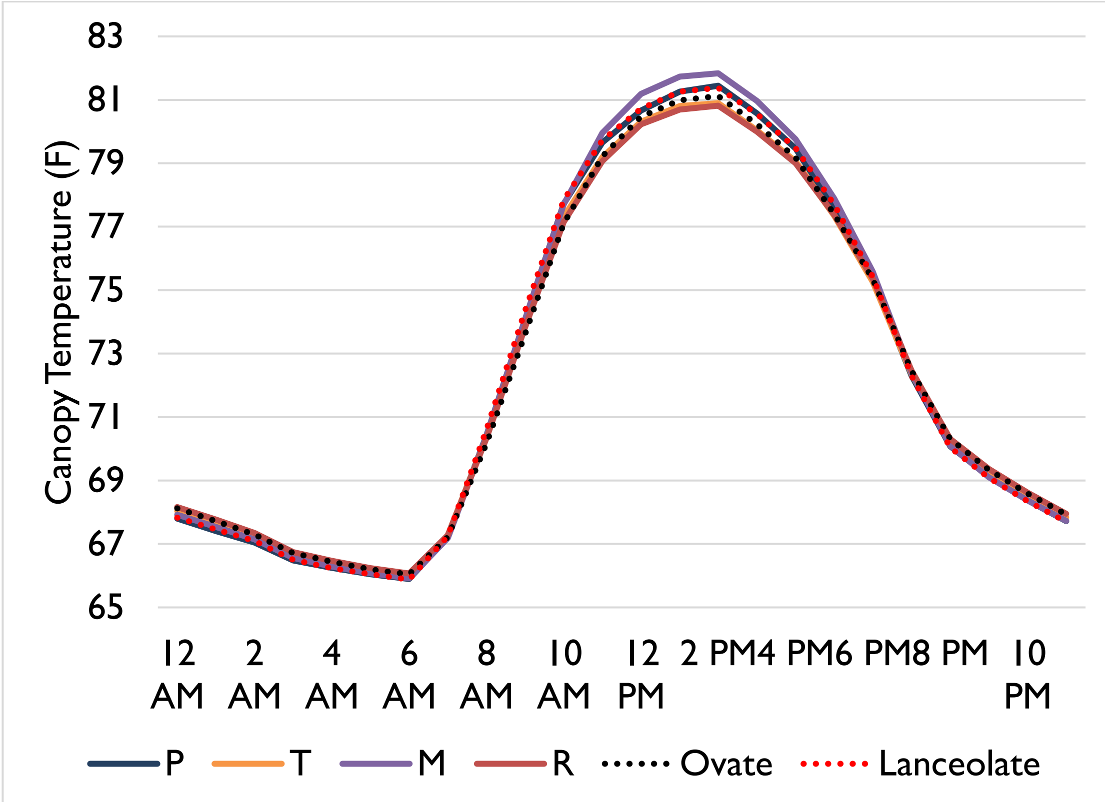

Figure 22 Average canopy temperatures by hour over between August 12th and September 20th.

Table 4 Averaged canopy temperature p-values at hourly intervals throughout the growing season (August 12 – September 20). Highlighted portions represent significance.

Time	p-value Leaf Shape
12 AM	0.0001
1 AM	0.0004
2 AM	0.0018
3 AM	0.0025
4 AM	0.0017
5 AM	0.0051
6 AM	0.0021
7 AM	0.5045

8 AM	0.0001
9 AM	0.0002
10 AM	0.0017
11 AM	0.0160
12 PM	0.1791
1 PM	0.0999
2 PM	0.0846
3 PM	0.0200
4 PM	0.0197
5 PM	0.0054
6 PM	0.1997
7 PM	0.0056
8 PM	0.0002
9 PM	0.0003
10 PM	0.0004
11 PM	0.0009

Quantitative PCR

A standard curve was made using the INF4 primer set and the downy mildew causal agent *Phytophthora phaseoli* (Figure 23). Detectable values ranged from 0.002 to 60 ng/µL. Table 5 shows the concentration of the samples that had greater than 0.002 ng/µL of *P. phaseoli*. Tessa had a pathogen amount above the predefined threshold for both 2019 and 2020 (Figure 24, 25) (Table 5). Tessa-A19 had the highest amount of observed pathogen in 2019, with Kenna-B20 having the highest in 2020. In contrast, the PhyP18 positive control had an average of 36.5437 ng/µL of *P. phaseoli* (Table 5). PhyP18 amounts were not included in Figure’s 24 and 25 to accurately convey the distribution of disease.

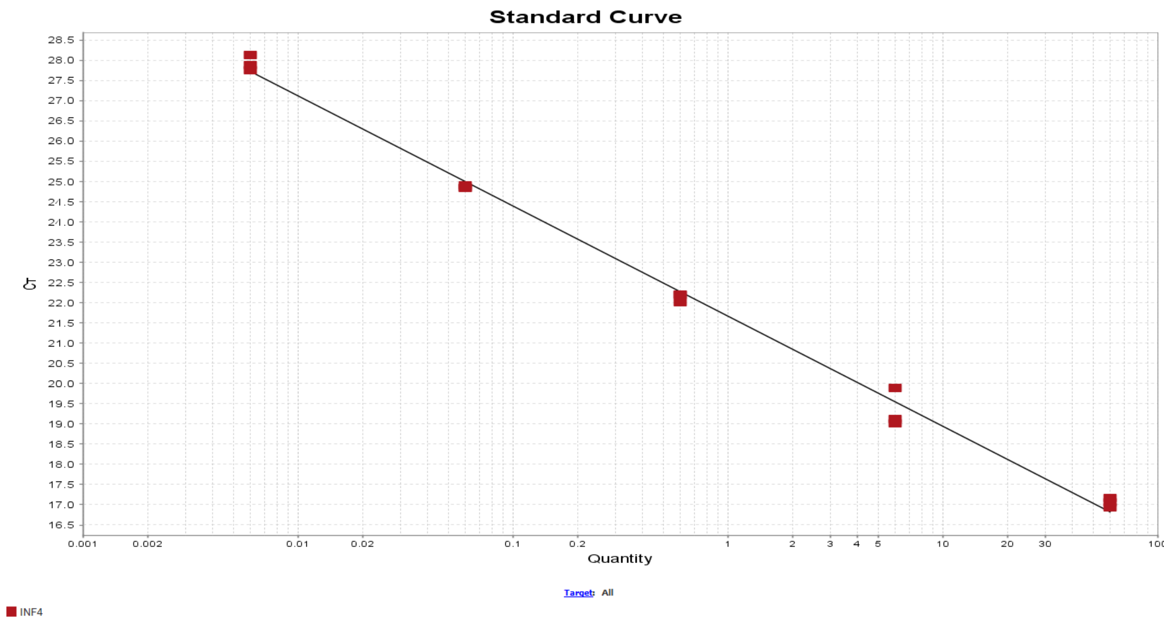

Figure 23 Standard curve of *Phytophthora phaseoli* detection using the INF4 primer set.

Table 5 Detectable values of *Phytophthora phaseoli* that fell within the threshold of significance within the standard curve. The letters following the samples names indicate which replication the samples were collected from.

Detectable values of *P. phaseoli* in lima bean pod tissue by qPCR analysis				
Sample Name	**Leaf Shape or Architecture**	**Year**	**Log [Concentration]**	**Pathogen Amount (ng/μL)**
Tessa-A	Lanceolate	2019	-2.655	0.00221
Kenna-B	Lanceolate	2020	-1.167	0.06809
Kenna-C	Lanceolate	2020	-1.664	0.02167
Tessa-C	Lanceolate	2020	-2.100	0.00795
C-Elite Select-C	Upright	2020	-2.476	0.00334
PhyP18[α]	N/A	N/A	1.5628	36.5437

[α]Standard Curve was based off a five-fold dilution series using DNA from *Phytophthora phaseoli* isolate 'PhyP18'.

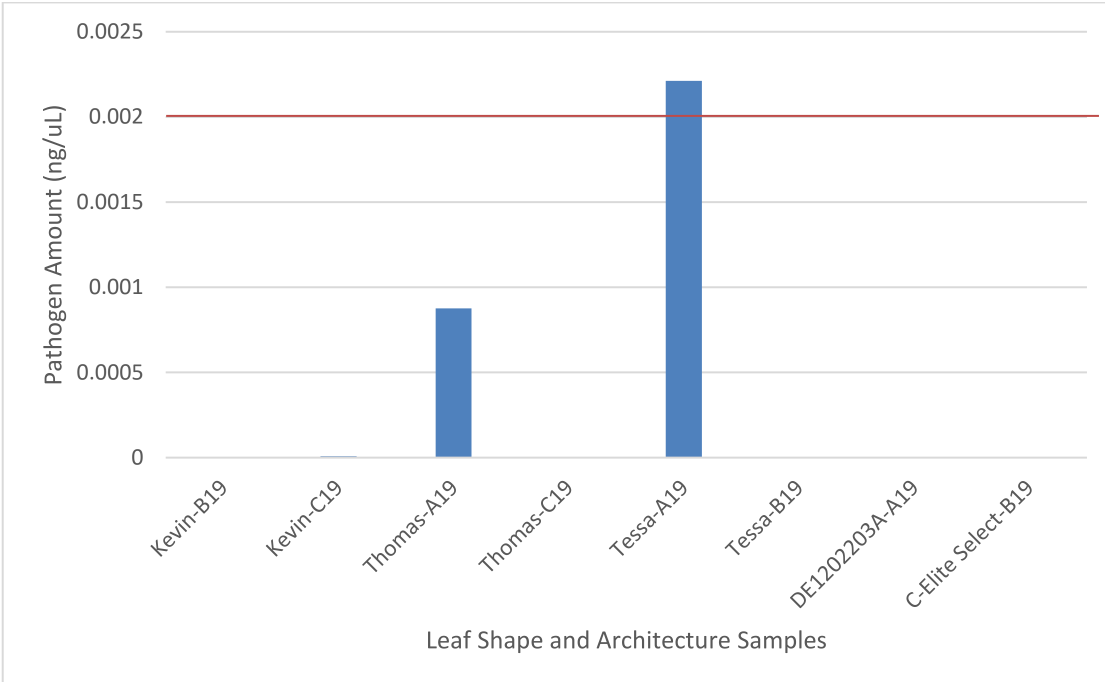

Figure 24 Distribution of detectable *Phytophthora*. *phaseoli* amount per various replications during 2019 field season. Note: All values under 0.002 ng/µL (red line) were deemed insubstantial.

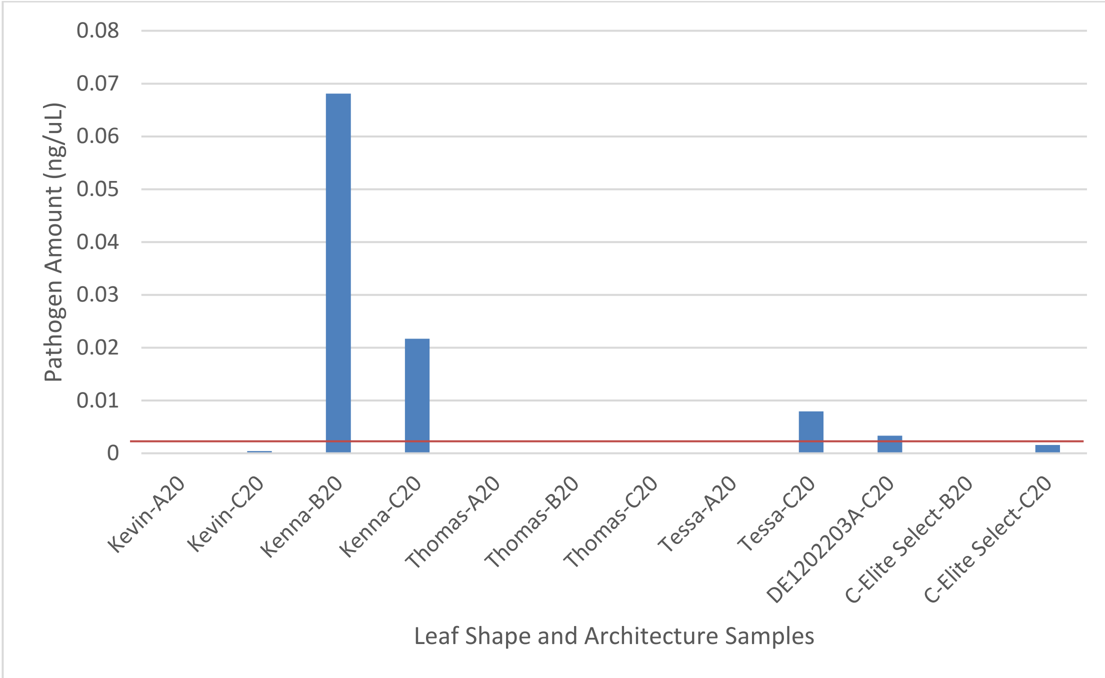

Figure 25 Distribution of detectable *Phytophthora. phaseoli* amount per various replications during 2020 field season. Note: All values under 0.002 ng/µL (red line) were deemed insubstantial.

Discussion

Leaf Shape. Outcomes of the 2019 and 2020 field trials provided three pairs of near-isogenic lines (Table 1) that supported the hypothesis suggesting that a lanceolate (narrower) leaf shape would lead to less disease severity, with one pair (Kevin and Kenna) being present during both trials (Figure 14). This similarity between trials suggests that Kenna, along with the other two lanceolate NILs, could prove to be potential breeding candidates into commercial cultivars. Though, as these are only near isogenic lines, there is reason to believe that there may be other genetic factors at work within these lanceolate lines (Pumphrey et al., 2007). The distribution of disease severity over time among NILs as depicted in Figures 14, 15, 16, 17, and 18 are further proof that there may be underlying genes that are factoring into the tolerance to *P. phaseoli*. The NILs used in this experiment are all progeny from the same parent (C-Elite Select x White Willow PI) and as such, each NIL pair are differing by only the gene controlling for leaf shape, but compared to other pairs of progenies, their genomes are not identical. There are several speculations as to what traits the differing genes may be controlling. First, the genes may have controlled traits relating to plant size. Many of the lanceolate lines were observed having overall smaller sizes when compared to their ovate counterparts. This smaller size may have allowed for a greater amount of airflow resulting in a less humid canopy microclimate. Secondly, the trait(s) effected may have controlled for plant maturation. A more mature plant would be less likely to become infected than a less mature plant. The succulent nature of the younger growth provides *P. phaseoli* with easier entry into the plant tissues (Evans et al., 2007). There is also the possibility of human error or environmental differences within the canopies of the individuals caused the variability in disease tolerance. In terms of human error, it is possible that during the two inoculations, certain lines received a disproportionate amount of inoculum, leading to more widespread infection. Though possible, this theory is rather implausible as precautions were taken to ensure an even application of *P. phaseoli* inoculum during all inoculation periods. Environmental differences could have been caused by unequal exposure to mist from the field misters. Though precautions were taken, and misters checked daily, there were several nozzle failures that could have possibly resulted in some plants having more conducive conditions than others. The possibility of genes controlling resistance or partial resistance to physiological race F of *P. phaseoli* can be dismissed, as both parents were fully susceptible. Further testing will be required to assess the mechanism of the observed tolerance.

Disease progress curves for the two rating dates following the second inoculations of 2019 and 2020 also followed a similar trend with the second rating of 2020 peaking at a rating of 4, just slightly above the 2019 3.75-rating (Figure 26). However, the leaf shape severity ratings from the second rating of 2019 had a much larger range than that of the second 2020 rating (Figure 26). This discrepancy may be attributed to climactic differences between the two years (Figure 28) (Delaware Environmental Observation System). With 2019 having only 1.96 centimeters of rain compared to 2020’s 6.88 centimeters it is possible that the higher rainfall in 2020 led to higher canopy humidity and therefore contributed to a more normalized distribution of disease severities (Figure 28). This low quantity of rain most likely led to less humidity, thus not overpowering the plants disease inhibition via leaf shape, leading to a greater range of disease severity (Cox, 1954).

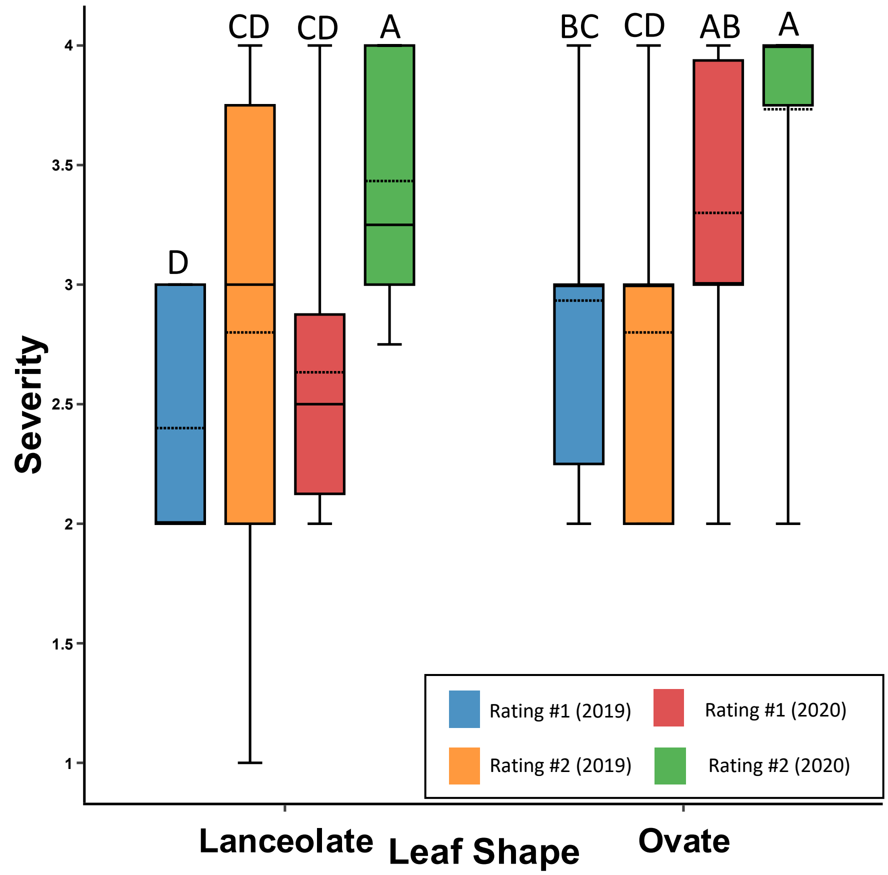

Figure 26 Comparison of leaf shape disease severity between 2019 and 2020 seasons.

The disease progress curves for the Kevin/Kenna and Matt/Morgan NILs continue to support the hypothesis that lanceolate leaf shapes do lead to less disease severity as seen by the significant difference in the September 12th timepoint (Table 1, Figure 22, 23). The lack of significant divergence in disease severity in Figures 24, 25, and 26 suggests that there were other factors, biotic or abiotic, that were interfering with the lanceolate leaf shaped NILs to successfully inhibit *P. phaseoli*'s growth based off of the success of NILs Kenna and Morgan (Figure 22, 23).

This separation of disease severity could prove to be beneficial to growers in terms of oomyceticide applications. According to the model by Hyre, growers need to use their best judgment on when to apply preventative oomyceticides based on weather conditions, as well as when to apply them as a curative measure (Hyre, 1958). This lag in disease progression due to the leaf shape gives growers more time to determine whether a curative application will be needed or if the crop can sustain until harvest.

Lanceolate leaf shape is not meant to eradicate or prevent infection, but is instead meant to slow disease progress and, if bred into cultivars with other resistance traits, provide extra layers of protection from downy mildew.

Architecture. Disease severity in different canopy heights did not have the same level of significance as in the leaf shape trials. This is partially due to the inability to have a second disease rating in 2019 because of the rate at which almost all architecture varieties were maturing. As mentioned previously, these plants had to be harvested immediately after the first rating to ensure accurate yield data could be collected. In 2020, two lines, DE1305601C and DE1001202E were observed to have a lower disease severity at the fourth rating date, with DE1305601C being significantly less infected than all three controls (Figure 27, Table 2).

One of the controls, ‘Cypress’ has a slow mildewing trait that allows for increased tolerance of infection by downy mildew; however, of the course of this experiment, this cultivar was not observed as expressing this trait (Wisser et al., 2020). This partial resistance is believed to have been overwhelmed by the multitude of susceptible architecture lines surrounding the ‘Cypress’ blocks within the field.

The architecture varieties had higher disease severity across almost all lines compared to the distribution of disease among the leaf shape lines (Figure 26, 27). There are several reasons as to why this discrepancy was observed. Firstly, the architecture varieties were bred to have the pods higher in the canopy to prevent splash inoculations from pathogens, namely *P. phaseoli*, that may be on debris in the soil. The issue here is that *P. phaseoli* does not typically infect its host via splash inoculations, nor was that the method of artificial inoculation used in this experiment (Santamaria et al., 2005). All plants were inoculated with a backpack sprayer misting the upper canopy with downy mildew inoculum, fundamentally defeating the purpose of a higher canopy. Secondly, all architecture varieties, including controls had ovate leaf shapes. Data collected from the leaf shape lines support the hypothesis that a thinner leaf shape will allow for more airflow through the canopy, thus decreasing disease severity (Figure 14, 15, 26). The two generous applications of inoculum, along with the ovate leaf shapes and conducive environmental conditions most likely contributed to the high disease severity of the architecture lines between years (Cox, 1954).

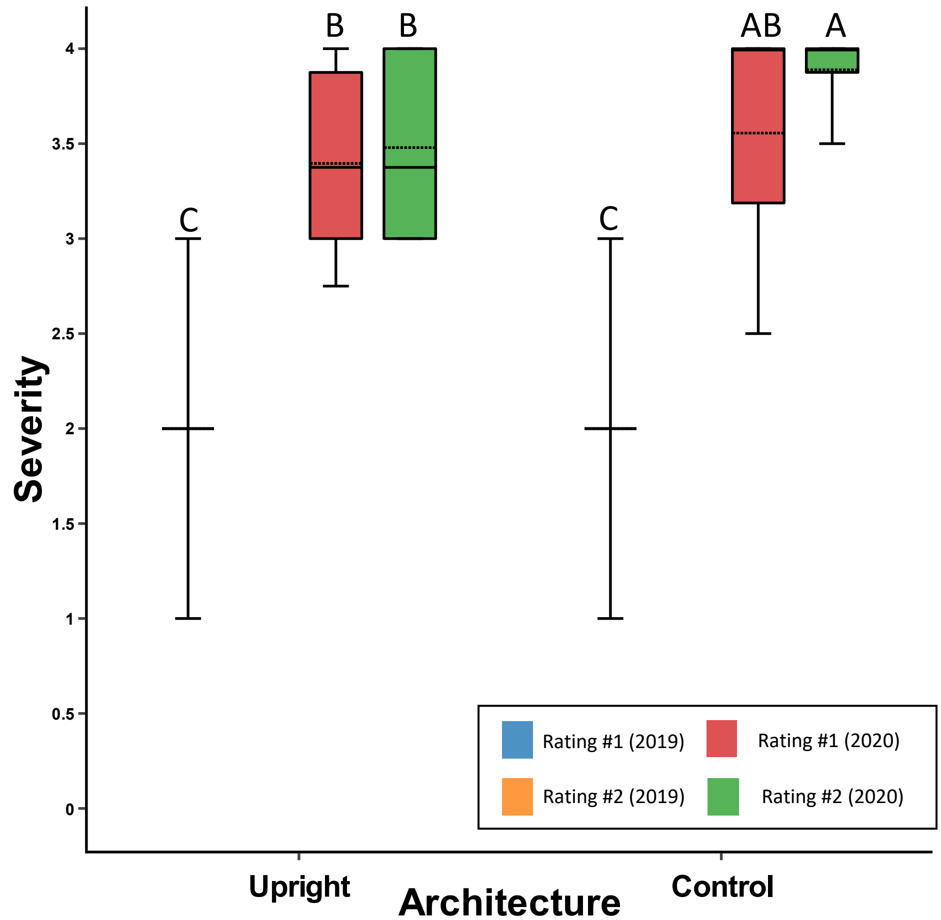

Figure 27 Comparison of plant architecture disease severity between 2019 and 2020 seasons.

Lima bean yield collected for 2019 was significantly higher across all groups when compared to 2020 data. Both upright and sprawling architecture varieties were found to have the higher yields than leaf shape varieties, with upright architectures having more yield than their sprawling counterparts for both 2019 and 2020. Leaf shape NIL's had no significant differences for both years. It is possible that the rate of disease progression is responsible for this discrepancy between years. 2020 had higher rainfall and relative humidity compared to 2019, as well as lower temperatures for the months of September and October (Figure 28) (Delaware Environmental Observation System) (Cox, 1954, Hyre, 1958). The combination of these three conditions provided a more conducive environment for *P. phaseoli* (Kee et al 2007). This in turn may have caused increased disease progress and dispersion among replications leading to the death and subsequent dropping of infected lima bean pods leading to lower yields (Evans et al., 2007)

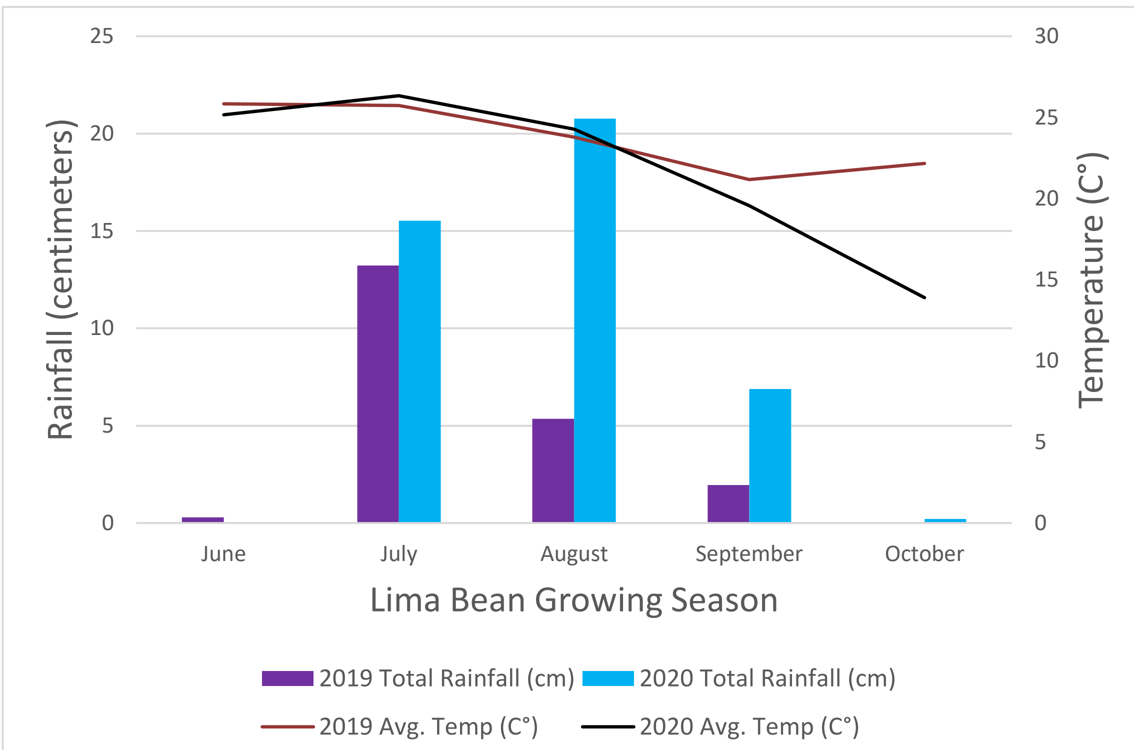

Figure 28 Average Newark, Delaware weather conditions on the University of Delaware Ag Farm (Delaware Environmental Observation System). Note: Weather data for June and October was only included for days when the experiment was running.

Quantitative PCR. In recent years quantitative PCR (qPCR) has been utilized more and more frequently to bypass the human error associated with visual disease severity ratings of oomycetes, fungi, and bacteria, instead providing researchers with quantitative data as to the actual amount of pathogen material per plant (Schena et al., 2003; Böhm et al., 1999; McCarthy et al., 2003; Vandemark and Barker, 2003). Quantitative PCR data was collected from samples taken during the 2019 and 2020 growing seasons with results showed no similarity between seasons, with a large quantity of samples showing no signs of pathogen whatsoever (Figure 24, 25). The detection threshold was determined to be anything greater than 0.002ng/µL according to the standard curve constructed using *P. phaseoli* DNA (Figure 23) (Morrison et al., 1998; Silvar et al., 2005). In addition, all lines that were above the predefined threshold had lanceolate leaf shapes, contradicting my hypothesis (Figure 24, 25). This result is unexpected, as the Kenna varieties had significantly lower visual disease severity than their NIL counterpart during both the 2019 and 2020 growing seasons (Table 2). Tessa and DE1202203A, were not significantly different than their NIL pair or controls, respectively (Table 11). In terms of the NILs only, it is a possibility that Kenna has an unknown underlying tolerance gene to downy mildew, similar to the cultivar, ‘Cypress’ which has a partial resistance to Race F of *P. phaseoli* (Wisser et al., 2020). It is also possible that the increased airflow through the canopy had such a dramatic effect, that the pathogen, though able to infect the plant, was unable to express itself. This theory is in part supported by the fact that Kenna-B, Kenna-C, and Tessa-A were all located at the edges of the plot, allowing for potentially greater airflow and less humid conditions. One reason for this discrepancy could be that it was simply too early in the disease progress curve for pod sampling and or the pathogen may not have been distributed throughout the pod tissue (Rideout et al., 2011).

The fact that the pathogen was not detected in the majority of the samples does not mean that the pathogen was not present. As mentioned above, it is possible that the date of pod collection was too early for detectable amounts of pathogen to have been detected in flat pods. This can be best illustrated by the complete lack of pathogen found in C-Elite Select samples at the time of pod collection. This cultivar is widely known to be susceptible to race F of *Phytophthora phaseoli*, though the barely detectable amount of pathogen suggests that it did not have enough time to infect the plants, especially considering that by the second rating date, the C-Elite Select replication had one of the highest disease severity ratings (Figure 12) (Evans et al., 2007). Future research should focus on replicating the experimental field to make more conclusive assumptions about how pathogen amount is related to overall disease severity and leaf shape/architecture. In a future field trial, following the same planting procedures and random block design, pods would be taken from the predetermined NILs but in greater numbers and at random. The DNA would then be extracted from each pod samples and bulked. Each replication of the NILs would constitute a single 'bulk' and be plotted against a standard curve created from the INF4 primer set and *P. phaseoli* (PhyP18 isolate) DNA. The greater quantity of pod DNA as well as the random nature of its collection would provide researchers with a better understanding of the pathogen amount per pod at that early stage of infection. From this data, it would then be possible to make more conclusive assumptions about how disease severity, leaf shape, architecture, and pathogen amount all relate.

Downy mildew is a devastating disease of lima bean throughout the MAR. With more oomyceticide sensitive isolates appearing it is imperative that alternative methods of pathogen inhibition be found. This experiment provides the framework additional methods of inhibition. With further testing and continued positive results, lanceolate leaf shapes could be candidate for being bred into commercial cultivars and save growers significant amounts of money by reducing oomyceticide costs as well as reducing yield losses.

REFERENCES

Böhm, J., A. Hahn, R. Schubert, G. Bahnweg, N. Adler, J. Nechwatal, R. Oehlmann, and W. Obszwald. "Real-time Quantitative PCR: DNA Determination in Isolated Spores of the Mycorrhizal Fungus Glomus Mosseae and Monitoring of *Phytophthora infestans* and *Phytophthora citricola* in Their Respective Host Plants." Journal of Phytopathology 147.7-8 (1999): 409-16. Print.

Cox, R. S. 1954. "Effects of temperature in the development of downy mildew of lima bean." Phytopathology 44:325-327.

Evans, T. A., Mulrooney, R. P., Gregory, N. F., & Kee, E. (2007). "Lima Bean Downy Mildew: Impact, Etiology, and Management Strategies for Delaware and the Mid-Atlantic Region, U.S." Plant Disease, *91*(2), 128–135. https://doi.org/10.1094/pdis-91-2-0128

Hyre, R. A. 1958. "The development of a method for forecasting downy mildew of lima bean." Plant Disease. Report. Suppl. 257:179-180

Kee, E., Glancey, J. L., and Wooten, T. L. 1997. "The lima bean: A vegetable crop for processing." HortTechnology 7:119-128.

McCartney, A. H., Foster, S. J., Fraaije, B. A., and Ward, E. "Molecular Diagnostics for Fungal Plant Pathogens." Pest Management Science 59.2 (2003): 129-42. Print.

Morrison, T. B. et al. "Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification." BioTechniques vol. 24,6 (1998): 954-8, 960, 962.

Pumphrey, M., Bernardo, R., Anderson, J. A. (2007) "Validating the *Fhb1* QTL for Fusarium head blight resistance in near-isogenic wheat lines developed from breeding populations." Crop Science 200–206.

Rideout, S. L., Hansen M. A., Gregory, N. F., and Evans. T. A. "First Report of Downy Mildew of Lima Bean Caused by *Phytophthora Phaseoli* in Virginia." Plant Disease 95.1 (2011): 71. Print.

Saindon, G., Huang, H. C., and Kozub, G. C. "White-mold Avoidance and Agronomic Attributes of Upright Common Beans Grown at Multiple Planting Densities in Narrow Rows." Journal of the American Society for Horticultural Science 120.5 (1995): 843-47. Print.

Santamaria, L., Mulrooney, R. P., and Evans, T. A. 2005. “Epidemiological studies of downy mildew of lima bean caused by *Phytophthora phaseoli*.” Annu. Rep. Bean Improv. Coop. 49:17-18.

Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. "Real-time Quantitative PCR: A New Technology to Detect and Study Phytopathogenic and Antagonistic Fungi." European Journal of Plant Pathology 110.9 (2004): 893-908. Print.

Silvar, C., Díaz J., and Merino, F. "Real-Time Polymerase Chain Reaction Quantification of Phytophthora Capsici in Different Pepper Genotypes." Phytopathology® 95.12 (2005): 1423-429. Print.

USDA-National Agricultural Statistics Service. 2019. 2017 Census of Agriculture.

Vandemark, G. J., and Barker, B. M. "Quantifying Phytophthora Medicaginis in Susceptible and Resistant Alfalfa with a Real-Time Fluorescent PCR Assay." Journal of Phytopathology 151.11-12 (2003): 577-83. Print.

Wisser, R. J., Oppenheim, S. J., Ernest, E. G., Mhora, T. T., Scanlan, C., Dumas, M. D., Gregory, N. F., Evans, T. A., and Donofrio, N. M. "Genome Assembly of a Mesoamerica-derived *Phaseolus lunatus* Cultivar Enables the Identification and Comparative Genomic Analysis of a Complex Resistance Locus." *BioRxiv* (2020). Print.

Chapter 3

CHARACTERIZATION AND DEPLOYMENT OF NEWLY IDENTIFIED GENES FOR DURABLE RESISTANCE TO DOWNY MILDEW IN LIMA BEAN

Introduction

Physiological races E and F of *Phytophthora phaseoli* are still a threat to the lima bean industry in the MAR and have the potential to cost millions in crop loss (Evans et al., 2007). The damaging effects of these two races are managed using resistant lima bean varieties, in addition to commercial oomyceticides, such as Ridomil Gold (mefenoxam) (Evans et al., 2007). Resistant varieties of lima bean include Bridgeton and 184-85 for Race E, and M-15 and 8-78 for Race F, but currently there are no adapted cultivars with resistance to both Race E and F (Santamaria et al., 2018). Lack of dual resistance is due to the location and characteristics of known resistance genes. The current understanding of dominant resistance in commercial lima bean cultivars, based on genetic mapping show that these single dominant resistance genes are either alleles on the same locus or are so tightly linked that no crossing over is possible during recombination (Ernest et al., 2012; Mhora et al., 2018). The inoperable location of previously identified E and F resistance genes has led researchers to screen diverse germplasm for new sources of resistance.

Recently downy mildew screening at the University of Delaware has uncovered a Costa Rican lima bean genotype, PI 256417, that is resistant to both prominent races of *P. phaseoli* (Mhora, 2018). This discovery has prompted further examination of the PI 256417 genome to discern genes involved in this novel resistance.

The first goal of this project is to learn the genetic architecture underlying E/F resistance in PI 256417. This includes investigating how the resistance trait is inherited, how many genes are involved, and whether the gene(s) involved are recessive or dominant. In addition to this first goal, I aim to discover whether the resistance to E and F are due to the same gene(s). Ideally, accomplishing this goal would provide researchers with genetic markers needed to further investigate the dual-race resistance and utilize this resistance in the development of new cultivars. To accomplish this, the $F_{2:3}$ progeny of a cross between PI 256417 (E and F resistant) and PI 534918 (E and F susceptible) were screened to determine their reaction to both downy mildew races.

It was hypothesized that the resistance genes in PI 256417 will have a dominant inheritance pattern.

Materials and Methods

Germplasm. At the University of Delaware's Georgetown campus PI 256417 (E, F resistant parent) was crossed with PI 534918 (E, F susceptible parent). The F_1 plants were grown out to produce 200 F_2 progeny. Leaf tissue was collected from the F_2 plants for future genotyping. The F_2 generation was selfed and F_3 seeds derived from each F_2 individual were used for the pathogen resistance assay.

Dew Chamber Study. Fifteen $F_{2:3}$ seeds from each line were planted screen for resistance to each race. The screening for each race was completed in three rounds with 5 seeds of each line planted in each round. The 200 lines were randomly divided into three sections, because the full set of 200 lines did not fit into one dew chamber. Sections were randomized in each round to minimize the effect of inconsistencies in dew chamber environment. In each round of screening, the population parents (PI

256417 and PI 534918) were included in one of the three dew chambers. Each dew chamber also contained a resistant and susceptible check to ensure that there was only the specific race of *P. phaseoli* being tested as well as to be sure that the inoculum was virulent. Susceptible checks were DE1001202C (race E) and C-Elite Select (race F), and resistant checks were C-Elite Select (race E) and DE1001202C (race F).

Ten-centimeter pots were filled with Promix BX potting mix and tamped down. The tamped pots were then watered until flowthrough was observed at the bottom of the pots. They were left to drain for approximately 30 minutes at which point five 4cm deep holes were made in the dice pattern using a dibble (Figure 29). Each pot was labeled with its corresponding line number and one seed was placed into each hole and covered lightly with potting mix. The flats were then transferred to 32°C heat mats in the greenhouse. Here they were left to grow for 5 days, at which point the were inoculated and transferred to their respective dew chambers.

Figure 29 Dibble press (right) and resulting hole layout (left) in a 10.2-centimeter pot used for consistent planting depth of resistance assays.

Inoculum. Races E and F (PhyP18) of *Phytophthora phaseoli* inoculum were maintained on susceptible Concentrated Fordhook lima beans. On a weekly basis, healthy 6-day old Concentrated Fordhook lima bean was inoculated with a slurry of the previous week's 11-day old inoculum. This slurry was prepared by removing and discarding the cotyledons and roots of the infected plant, then slicing the remaining hypocotyl into 2.5-5 cm pieces and putting them into a food processor. 100mL of distilled water was added and the lima bean hypocotyls were ground for 45 s, at which point the concentrated slurry was added to another 700mL of distilled water to achieve an approximate sporangial density of 1.16×10^5 sporangia/mL. Approximately 42 hypocotyls were used per inoculum batch. Inoculated plants were stored at 20°C and 87% relative humidity within a digital mist chamber. A 24-hour regimen of florescent light at 5μmol m-2s-1 of photosynthetic active radiation (PAR) was applied in order to stunt plant height.

Inoculation. Approximately 25 infected plants were taken and used to inoculate five trays of experimental lima bean plants. The hypocotyls, cotyledons, and leaves were all washed by hand in 400mL of DI water for one minute, at which point the sporangia solution was filtered twice using a U.S.A Standard No. 10 sieve to remove any large pieces of plant material. The solution was then transferred to a spray bottle and all five trays were misted until runoff on all leaves was observed. Three applications of *P. phaseoli* inoculum were applied three days apart. Average sporangial density was determined to be 4.71 x 10^4 sporangia/mL. Applications of race E and race F inoculum were applied in two separate trials of three rounds each in order to avoid any possible synergism or cross resistance resulting from inoculation with both races.

Incubation. Flats were transferred to their appropriate dew chambers and placed two-per-shelf starting from the top of the chamber. Four shelves in each incubator were placed 43.2 centimeters apart to allow room for plant growth and mist movement. The bottom shelf was occupied by maintained inoculum. Every two days, the experimental flats in each dew chamber were rotated down one shelf to provide consistent conditions. In addition to rotating the flats, all were also misted using a hand mister once a day to ensure conducive conditions were being maintained. Dew chamber temperature and humidity conditions were kept identical to those used to maintain inoculum.

Rating. Two or three ratings were taken with the first being one day after the third inoculation. Each plant was examined for presence of downy mildew. Each plant was rated using designations “R” for resistant (no visible disease), “S” for susceptible (white mycelial growth observed), or “H” for hypersensitive (hypersensitive response on leaf tissue) (Figure 30). In almost all cases, the second rating was considered the final rating, but for any plants that emerged between the second inoculation and first rating, or for those that appeared almost all sensitive, resistant, or hypersensitive, a third rating was taken.

Figure 30 A fully susceptible lima bean plant at approximately 2 weeks (left). A lima bean plant where all leaves are displaying a hypersensitive response (middle). A fully resistant lima bean plant with no visible signs or symptoms of downy mildew infection (right).

Genotyping. DNA was extracted from trifoliate leaves collected from the F_2 generation using DNeasy® Plant Mini Kit 250 (QIAGEN Sciences, Germantown, MD) per the included instructions for plant tissues to genotype using the Genotyping by Sequencing technique (GBS). This analysis will help future researchers ascertain the location of the possible resistance gene(s) within the bulked F_2 progeny.

Analysis. To generate Figures 31 and 32, ratings of races E and F were converted to numerical values (S=1, HR=2, R=3) and plotted against each other using Microsoft Excel, with the correlation significance being calculated using a one-way ANOVA. The correlation coefficient was also computed in Excel. P-values were calculated using the Student's T-test.

Results

Race F. Ratings of "S" or susceptible, "R" or resistant, or "HR" or hypersensitive for each plant were recorded according to their position within their respective pots and then grouped for analysis of all three trials (Appendix A, B). For physiological race F, of the 200 progenies, 31 were all susceptible, 3 were susceptible and hypersensitive, 27 were resistant and hypersensitive, 31 were susceptible and resistant, 91 were susceptible, resistant, and hypersensitive, and 17 were fully resistant (Table 6). Note that for the final rating date of Race F Trial 1, no hypersensitive responses were recorded. This was before it was determined that a hypersensitive response rating should be included.

Race E. The same rating designations were assigned for all three race E trials. Due to insufficient seed sources, five F_2 lines (17, 35, 158, 164, 202) where removed for race E trials. Of the remaining 195 lines, 43 were observed to be all susceptible, 21 were all resistant, 21 were resistant and hypersensitive, 30 were susceptible and resistant, and 80 were susceptible, hypersensitive, and resistant (Table 6).

The susceptible and resistant checks in all rounds of the race E and F trials behaved as expected, with C-Elite Select showing no signs or symptoms of downy mildew when inoculated with race E, though showing massive infection when inoculated with race F, and DE1001202C displaying signs and symptoms when inoculated with race E, but not with race F of *P. phaseoli*. The population parents conformed to their expected resistances in the race F screenings, but during the race E screenings the expected fully resistant parent, PI 256417, was found to have signs and symptoms of downy mildew twice. The checks confirmed that there was no race F present that could explain this anomaly (Appendix A, B).

Table 6 Breakdown of genotypic ratio per disease susceptibility as well as the number of corresponding plants from all three race E and F trials.

Susceptibility	**Genotype**	**Expected Ratio**	**Estimated number of plants**	**Observed number of plants (Race E)**	**Observed number of plants (Race F)[α]**
All Susceptible[ß]	HHRR, HhRR, hhRR	4/16	~50	43	31
All Resistant	hhrr	1/16	~12.5	21	17
Resistant & Hypersensitive	Hhrr	2/16	~25	21	27
Susceptible and Resistant	hhRr	2/16	~25	30	31

Susceptible, Resistant & Hypersensitive	HhRh, HHrr, HHRr.	7/16	~87.5	80	91

[α]Observed values for race F have an X^2 p-value of 0.075 (p>0.05).
[ß]Three progeny were given a disease rating of "Susceptible & Hypersensitive" during the Race F trials, though it is suspected that these were escapes. Parents were not included.

Analysis of races E and F ratings showed a correlation coefficient of 0.891 with a significant p-value of <0.0001 (p>0.05). A line-of-best-fit was also included in Figure 31, with a slope of 0.9171x and an R^2 value of 0.7939. Both the correlation plot and histogram were made using the differences in rating between races E and F to ascertain a general distribution of plant-disease interaction (Figure 32). This showed a normal distribution with no skewing or multiple peaks.

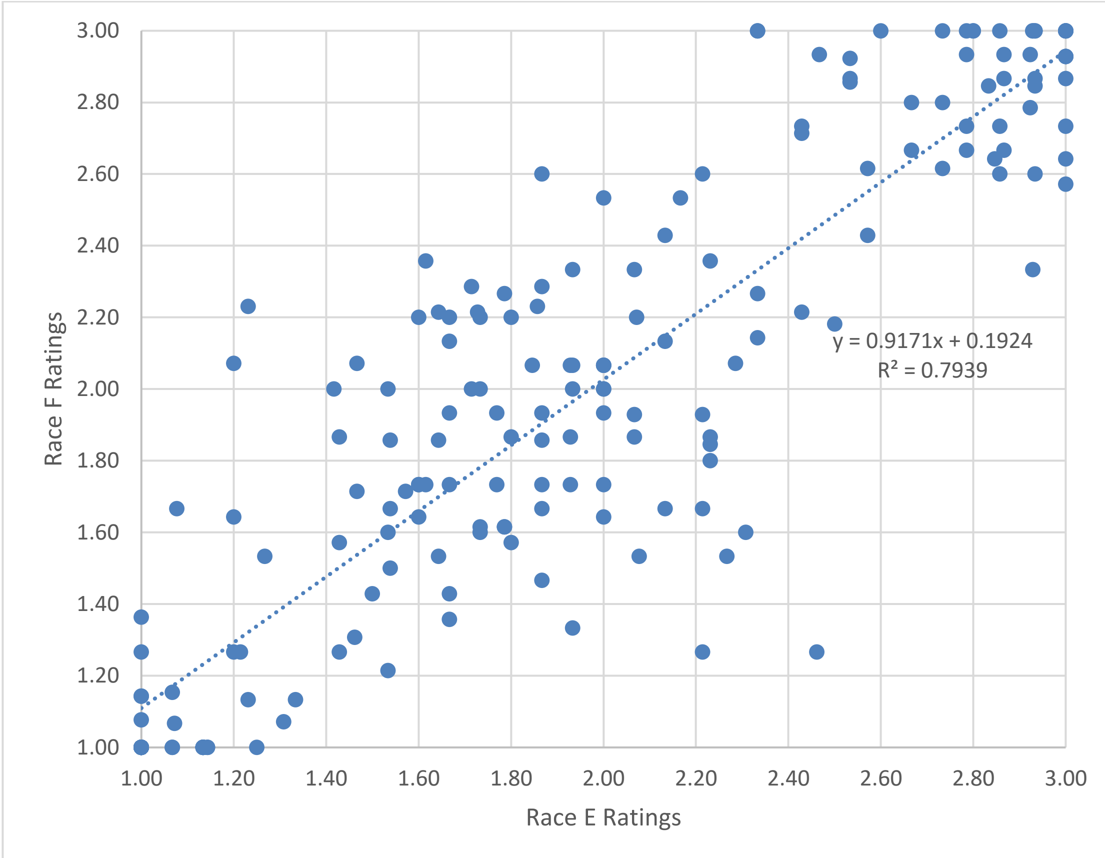

Figure 31 Correlation plot of the race E and F ratings. There is a correlation coefficient of 0.891 with a p-value of <0.0001 (p>0.05).

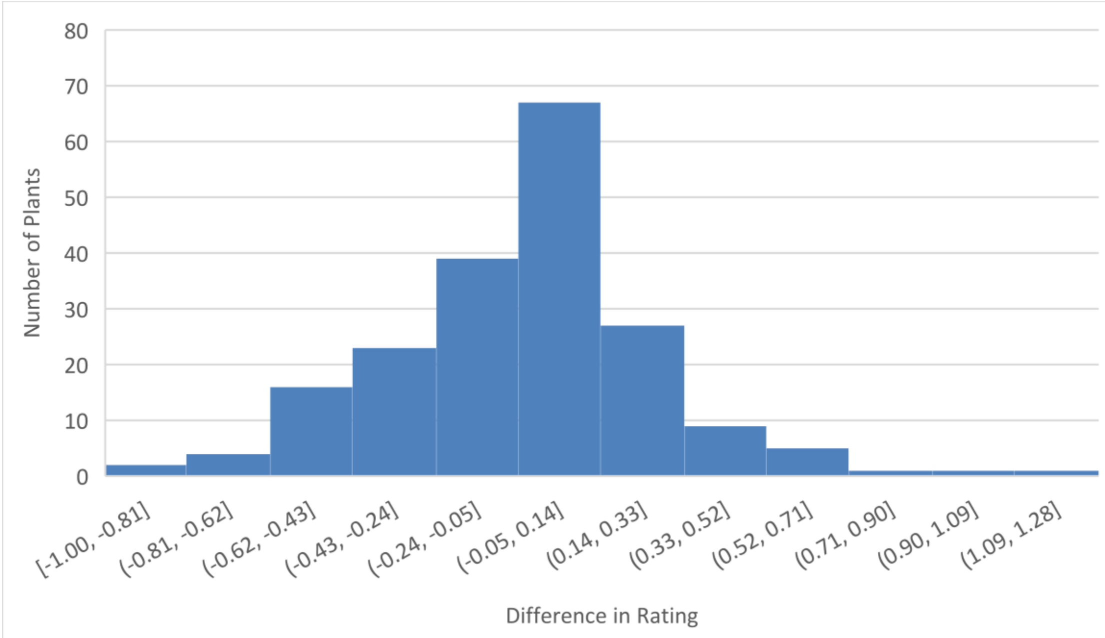

Figure 32 Histogram looking at the differences in assay rating to determine general distribution of plant-disease interactions.

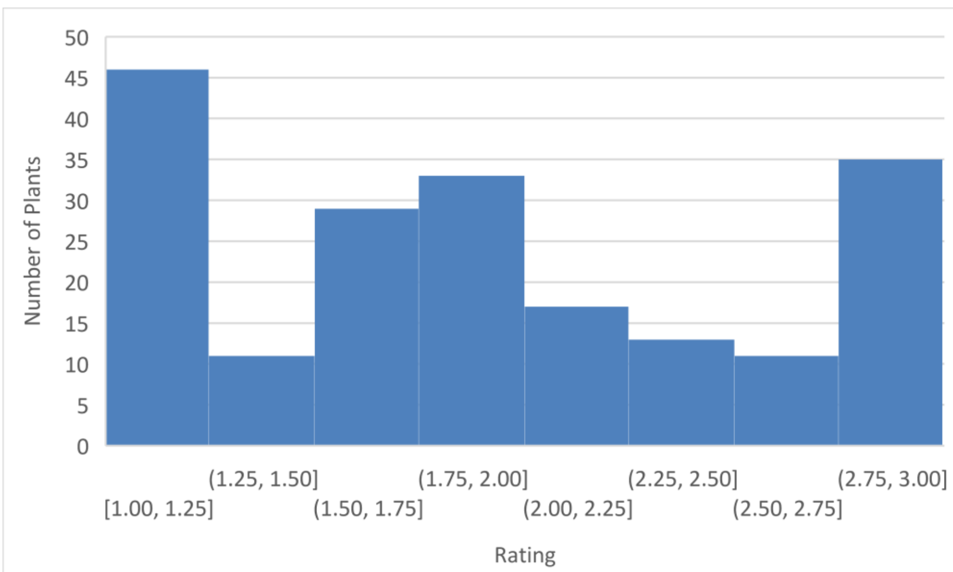

Figure 33 Histogram comparing the average ratings of all race E resistance assay trials.

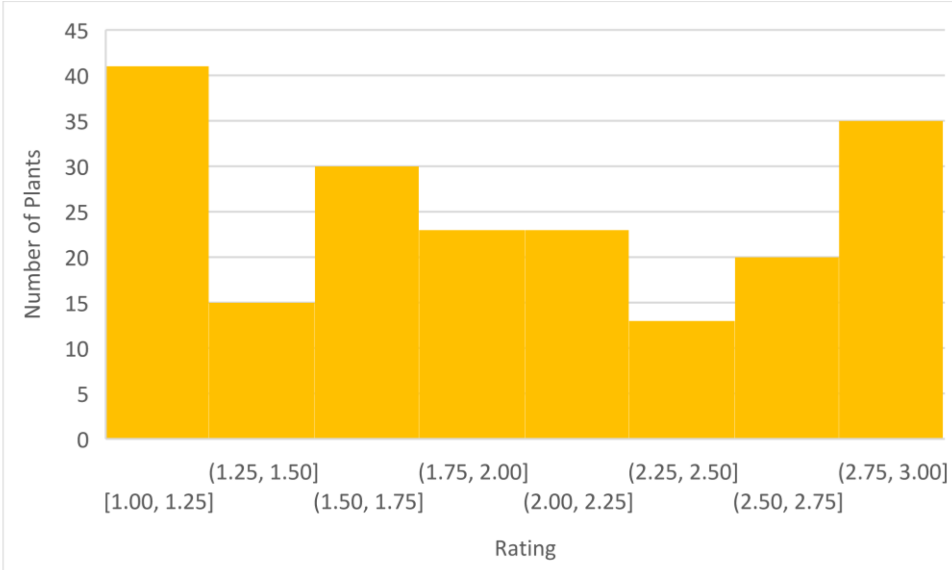

Figure 34 Histogram comparing the average ratings of all race F resistance assay trials.

Discussion

Current lima bean resistance genes are inherited in a dominant fashion (Santamaria et al., 2018). The two histograms constructed using the average ratings for the E and F screenings, respectively, and one histogram displaying the difference between the E and F rating averages provided insight into the type of inheritance occurring in the F2:3 progeny (Figure 32, 33, 34). If these progenies were believed to have a single dominant resistance gene, a higher percentage of the F2:3 progeny would have been resistant (Table 6) (Mhora et al., 2016; Santamaria et al., 2018). Instead, evidence seen in Figures 33 and 34 where the variation between resistance assays shows three peaks (left, middle, and right) corresponding to the three rating categories (Resistant, Hypersensitive, and Susceptible), implies that there is not a single dominant resistant gene present, but multiple recessive resistance genes. This assumption of recessive inheritance is also supported by the normal distribution in the histogram depicting the difference in averages between line in the E and F screens (Figure 32). None of the three histograms have a noticeable skew to the left which would indicate a dominant resistance gene was present from one of the parents. This leads to a conclusion that resistance in the F2:3 progeny is recessively inherited as well as supporting the notions that the mechanism of resistance is similar between parents and that resistance is inherited in a similar manner (Mhora et al., 2016).

After examining the ratios of resistant, susceptible, and hypersensitive response plants it was hypothesized that multiple loci are involved in the resistance present in PI 256417, with possibly two recessive alleles at separate loci based upon the F3 progeny's reaction to the introduction of races E and F of Phytophthora phaseoli over the two race screening trials. The breakdown of genotypes can be found in Figure 35 and Table 6. The abnormality of the observed ratios compared to expected Mendelian ratios could be explained by linkage between the two loci.

Looking further into the resistance genes believed to be present in the F2 progeny, it was observed that there was a significant correlation between the race E and race F ratings ($P<0.0001$) (Figure 31). This correlation suggests that some of the genes that are allowing for resistance to race F of P. phaseoli are also involved in resistance to race E. The distribution of ratings also demonstrates that there are race specific resistance genes that are required in order for the plant to achieve full resistance as displayed in Table 6. In a previous study by Mhora et al., the resistance associated with current race F resistant lima bean cultivars was found to be dominant and located on chromosome 4 (Mhora et al., 2018). Furthermore, Santamaria et al., also found that race E resistance was also located within the same locus (Santamaria et al., 2018). This discovery is the primary reason that there is currently no dual resistance in commercial lima bean cultivars to races E and F of the P. phaseoli, as only one resistance gene can occupy that locus at a time. Based on this information it is inferred that the resistance gene(s) in PI 256417 do not involve the same locus as those described by Santamaria (Santamaria et al., 2018) and Mhora (Mhora et al., 2016). There are several reasons supporting this inference, the first being that several F2:3 lines showed resistance to both E and F in their respective screens (Appendix A, B). This alone suggests that the source(s) of resistance is either in a different location or in several different locations depending on the resistance genes nature.

Secondly, the low number of fully resistant $F_{2:3}$ progeny suggests recessive genes are involved, and this does not follow the pattern of the resistance described at the locus on chromosome 4 (Table 6). And thirdly, the high occurrence of hypersensitive responses within the F2:3 populations implies that there is partial resistance conferred by some of the genes involved, which is, again, different than the resistance described on chromosome 4 (Appendix A, B) (Table 6). If the resistance in PI 256417 is confirmed to be located at loci separate than the previously described chromosome 4 locus found by Mhora et al, it would be beneficial for developing lines with durable resistance to downy mildew. Through a process called pyramiding resistance, the additional resistance genes could be incorporated into cultivars that already contain a dominant resistance gene, thus creating a cultivar that is fully resistant to both races of P. phaseoli, and this resistance would be supported by multiple resistance genes (Pedersen and Leath, 1988).

Only clear resistant and susceptible reactions were expected in the F2:3 progeny, however, in the first trial, an intermediate reaction was observed, which appeared to be a hypersensitive response (Figure 30). The hypersensitive plants had crumpled, non-expanded leaves with brown spots. It was also observed that some, while not healthy when compared to fully resistant plants, also did not become visibly infected by *P. phaseoli*. This reaction matches observations made by Santamaria et al. 2018 during previous resistance testing where a resistant and susceptible cross was made, and progeny were inoculated with race F of *P. phaseoli*. This partial resistance response to *P. phaseoli is* indicative of other minor genes playing a role in the plants defense as well as a weak resistance (R)-avirulance (Avr) gene interaction, similar to the slow mildewing properties observed in the commercial Cypress cultivar (Flor, 1971; Vleeshouwers et al., 2000; Wisser et al., 2020).

Ultimately, the data collected during this experiment does not support the hypothesis that the E and F resistance in PI 256417 is controlled by dominant resistance gene(s). Rather, the data suggest that the resistance is conferred by recessive alleles at multiple loci.

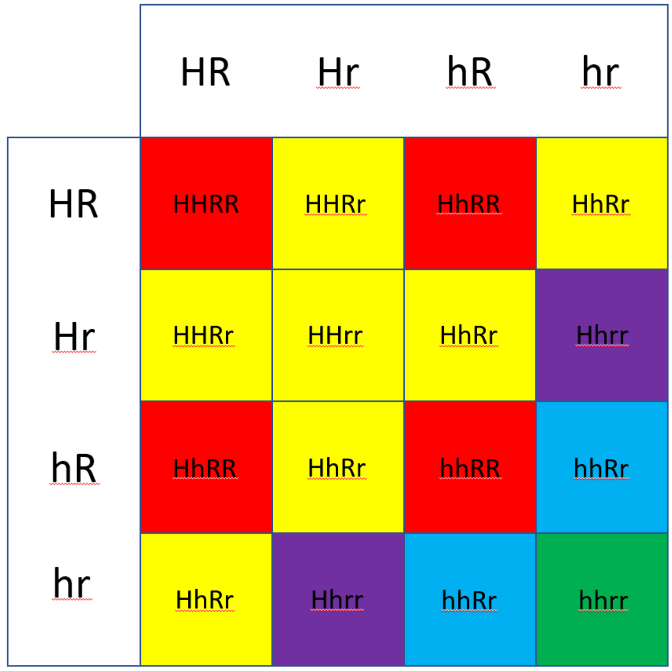

Figure 35 Punnett square depicting infection type per genotype. (Red) All susceptible; (Yellow) Susceptible, Resistant and Hypersensitive; (Purple) Resistant and Hypersensitive; (Blue) Susceptible and Resistant; (Green) All Resistant.

The data presented in this chapter only covers data for the first goal of the experiment. The second goal of this experiment was to use bulk segregant analysis to map the resistance loci in PI 256417 and determine if these genes were located near the resistance locus identified by Mhora et al (2016) for single-dominant resistance genes for physiological race F (Mhora et al., 2016). The complex, multigenic nature of the resistance present in PI 256417 means that sequencing bulked DNA from the F_2 plants is unlikely to achieve the goal of mapping resistance loci. The final interpretation of genotypes as constructed via resistance assay data was non-conforming to the standard Mendelian ratio suggesting that if ‘bulks’ were to be constructed using the extracted DNA, the resolution to conclusively identify genetic markers or produce accurate location of resistance genes would not be adequate (Table 6, Figure 35). The current plan is to genotype each individual F2 using genotyping-by-sequencing (GBS). and us GWAS to identify QTLs associated with resistance. The ultimate goal is to develop markers linked to the resistance alleles in PI 256417 (Mhora et al., 2016). The development of these markers would increase efficiency of selecting for desirable alleles and combining multiple resistance genes to achieve more durable resistance. (Kumar et al., 2011; Schneider et al., 1997).

The information attained via the resistance assay and BSA work will provide the groundwork needed to effectively incorporate the novel E and F resistance in PI 256417 into adapted cultivars that can be produced in the mid-Atlantic region. If further tests and analysis continue to prove fruitful, these new sources of resistance may be potential candidates for being bred into commercial cultivars, finally providing growers with lima beans that are resistant to both race E and race F of *P. phaseoli*, thereby removing the gamble of whether or not their lima bean crop will fall victim to one race or the other of downy mildew.

REFERENCES

Ernest, E. G., Evans, T. A., Gregory, N. F., and Wisser, R. J. "Annual Report of the Bean Improvement Cooperative." Annual Report of the Bean Improvement Cooperative. Vol. 55. USDA, 2012. 27-28. Print.

Evans, T. A., Mulrooney, R. P., Gregory, N. F., & Kee, E. (2007). “Lima Bean Downy Mildew: Impact, Etiology, and Management Strategies for Delaware and the Mid-Atlantic Region, U.S.” Plant Disease, *91*(2), 128–135. https://doi.org/10.1094/pdis-91-2-0128

Flor, H. H. "Current Status of the Gene-For-Gene Concept." Annual Review of Phytopathology 9.1 (1971): 275-96. Print.

Kumar, J., Choudhary, A. K., Solanki, R. K., and Pratap, A. "Towards Marker-assisted Selection in Pulses: A Review." Plant Breeding 130.3 (2011): 297-313. Print.

Mhora, T. T. “A Genomics Based Approach to Managing Downy Mildew of Lima Bean, University of Delaware”, Ann Arbor, 2018. *ProQuest*, https://login.udel.idm.oclc.org/login?url=https://www.proquest.com/dissertations-theses/genomics-based-approach-managing-downy-mildew/docview/2193365531/se-2?accountid=10457.

Mhora, T. T., Ernest, E. G., Wisser, R. J., Evans, T. A., Patzoldt, M. E., Gregory, N. F., Westhafer, S. E., Polson, S. W., and. Donofrio, N. M. "Genotyping-by-Sequencing to Predict Resistance to Lima Bean Downy Mildew in a Diversity Panel." Phytopathology® 106.10 (2016): 1152-158. Print.

Pedersen, W L, and S Leath. “Pyramiding Major Genes for Resistance to Maintain Residual Effects.” Annual Review of Phytopathology, vol. 26, no. 1, 1988, pp. 369–378., doi:10.1146/annurev.py.26.090188.002101.

Santamaria, L., Ernest, E. G., Gregory, N. F., and Evans, T. A. "Inheritance of Resistance in Lima Bean to *Phytophthora phaseoli*, the Causal Agent of Downy Mildew of Lima Bean." HortScience 53.6 (2018): 777-81. Print.

Schneider, K. A., Brothers, M. E., and Kelly, J. D. "Marker-Assisted Selection to Improve Drought Resistance in Common Bean." Crop Science 37.1 (1997): 51-60. Print.

Vleeshouwers, V. G., van Dooijeweert, W., Govers, F., Kamoun, S., and Colon, L. T. "The Hypersensitive Response Is Associated with Host and Nonhost Resistance to *Phytophthora infestans*." Planta 210.6 (2000): 853-64. Print.

Wisser, R. J., Oppenheim, S. J., Ernest, E. G., Mhora, T. T., Scanlan, C., Dumas, M. D., Gregory, N. F., Evans, T. A., and Donofrio, N. M. "Genome Assembly of a Mesoamerica-derived *Phaseolus lunatus* Cultivar Enables the Identification and Comparative Genomic Analysis of a Complex Resistance Locus." BioRxiv (2020). Print.

Chapter 4

EFFECTS OF RED LIGHT ON SPORANGIAL GROWTH AND DEVOLOPMENT OF DOWNY MILDEW AND *PHYTOPHTHORA CAPSICI* ON LIMA BEAN

Introduction

Since the discovery of *Phytophthora phaseoli* by Thaxter in 1889, there have been six recorded physiological races labeled Race A-F, with F being the prominent in today's fields (Evans et al., 2007). Control measures that growers typically employ include using resistant cultivars or applying regiments of oomyceticide, though, these methods of avoiding downy mildew are not always effective (Evans et al., 2007). Currently, there are no commercial cultivars that are resistant to physiological races E and F of *P. phaseoli* and it is unknown whether E is still present in today's fields (Santamaria et al., 2018). This poses a risk to growers, in that if they plant an E-susceptible variety and race E is still present in the field, they could suffer larger yield losses. Oomyceticides, while also effective, come at the cost of selecting for resistant individuals (https://www.frac.info). In addition, they also have harmful impacts on non-target plants, animals, and insects (https://www.frac.info). In Delaware, instances of newly resistant *P. phaseoli* due to over or under application of mefenoxam have been found as recently as 2017 (Mhora et al., unpublished; T. A. Evans, *personal communications*). This risk of oomyceticide-induced resistance as well as the current culture of "organically grown" fruits and vegetables have caused growers and researchers to look at alternatives for downy mildew control, with one promising method being the application of red light (Patel et al., 2016).

Plants are exposed to visible and invisible wavelengths of light every day with differing wavelengths being utilized by different photoreceptors (Islam et al., 2002).

Certain wavelengths have been associated with an increase in plant defensive materials, such as phytoalexins (Bridge and Klarman, 1973). Red light with a wavelength between 600-700nm's has shown promising results in the inhibition of downy mildew caused by *P. capcisi* on eggplant, pepper, and watermelon, as well as downy mildew on basil (Cohen et al., 2013; Patel et al., 2016). The method of inhibition is unknown, but it is reported these wavelengths of light also correspond with increased levels of phytoalexins within plant tissue (Islam et al., 2002). Phytochrome B, a photoreceptor sensitive to red and far-red wavelengths of light, has been shown to cause physiological changes in *Arabidopsis* plants, thereby increasing the plants defense against pathogens via the salicylic acid (SA) pathway and hypersensitive response (HR). (Reed et al. 1993; Nagendran and Lee, 2014). Phytochrome B is also found in various *Phytophthora* species, giving researchers a starting point to better understand the connections between red light, fungal inhibition, and plant defense (GenBank Accession [POM76165.1] and [XP_008902503.1]).

In most plants, phytochrome play a significant role in effecting plant development and metabolism (Smith, 1975). Red lights can affect plants via induction of flowering, stem growth, leaf growth, and germination timing (Smith, 1975; Park and Runkle, 2017; Kurepin et al., 2007). Interestingly, in a study performed by Borthwick et al., red and far-red wavelengths of light had opposite effects on plant morphogenesis. Within lettuce seed germination, seeds treated with a far-red wavelength of light had significantly lower germination rates than those treated with normal red light (Borthwwich et al., 1952). This difference in plant response to two closely related wavelengths of light promotes questions on how pathogens may respond to young and adult plants treated with differing wavelengths of red light.

Phytochromes are found within all parts of most plants, ranging from the roots to the fruits, though the highest concentrations are most often found within meristematic tissues responsible for photosynthetic activities (Smith, 1975). There are five categories of phytochromes, named A-E, with all five absorbing both red light (~600-700nm) as well as far-red light (700+nm) (Smith, 1975). Normal red light is absorbed by the biologically inactive form of phytochrome, known as 'Pr'. When exposed to red wavelengths of light, the Pr isoform will convert to its biologically active form, 'Pfr', which ideally absorbs far-red wavelengths of light (Smith, 1975). Due to the photoreversible nature of these two isoforms, Pfr will eventually be converted back into Pr when enough far-red light is absorbed (Smith, 1975). Both of these forms play a role in photosynthesis, with photosystem I absorbing the highest amount of far-red light and photosystem II absorbing the highest amount of red light (Smith, 1975).

There has been extensive research on red light as it relates to photosynthesis. The effects red light has on photosynthesis may shed light as to how *P. phaseoli* and *P. capsica* are inhibited on lima bean tissue. In photosynthesis, different wavelengths of light are absorbed by corresponding photoreceptors in the plant leaves (Taiz and Zeigler, 2010). Phytochrome, along with chlorophyl, are able to absorb the highest amounts of red light (Muneer et al., 2014).

I hypothesize that application of red wavelengths of light to *in planta* and *in vitro* isolates of *P. phaseoli* will inhibit growth and sporulation of the pathogen. Most applications of red light have been conducted in greenhouse settings, with very few being conducted in actual field settings (Islam et al., 2002; Patel et al., 2016). The objective of this study is to measure the effects of nocturnal application of red light on sporulation and pathogen growth.

Materials and Methods

Isolate acquisition. A previous study on downy mildew of lima bean collected 110 *P. phaseoli* and 16 *P. capsici* isolates from infected lima bean crop throughout the state of Delaware and surrounding area. Oomyceticide sensitivity assays were conducted on all isolates to determine whether any resistance to mefenoxam (Ridomil Gold Copper) was present. Isolates were chosen based on sensitivity to mefenoxam and race.

Isolate maintenance. *P. phaseoli* isolates were maintained on a rotating schedule of 2% lima bean agar and ¼ strength potato dextrose agar. All isolates were directly cultured from lima bean agar in each experiment. The *P. capsici* isolate was maintained on a rotating schedule of V-8 Agar and ¼ strength potato dextrose agar and cultured directly from V-8 agar to insure active sporangia in each experiment. All cultures were maintained in a dark incubator at 20°C with transfers being made every two weeks.

Race F of *Phytophthora phaseoli* inoculum was maintained on susceptible Concentrated Fordhook lima beans. The specific isolate used was designated as “PhyP18”. This isolate is a fully genotyped race F isolate of *P. phaseoli* and has been extensively used in various field and greenhouse trials throughout the past several years at the University of Delaware. On a weekly basis, healthy 6-day old Concentrated Fordhook lima beans were inoculated with a slurry of the previous week’s 11-day old inoculum. This slurry was prepared by removing and discarding the cotyledons, leaves, and roots of the infected plant, then slicing the remaining hypocotyl into 2.5-3-centimeter pieces and putting them into a food processor. 100mL of distilled water was added and the lima bean hypocotyls were ground for 45 seconds, at which point the concentrated slurry was added to another 700mL of distilled water to achieve an approximate sporangial density of 1.16 x 10^5 sporangia/mL. Approximately 42 hypocotyls were used per inoculum batch. Inoculated plants were stored at 20°C and 87% relative humidity within a digital mist chamber. A 24-hour regiment of florescent light at 5µmol m-2s-1 of photosynthetic active radiation (PAR) was applied in order to stunt plant height.

In vitro Study. Both pathogens were grown on their preferred media, lima bean agar for *P. phaseoli* and V-8 agar for *P. capsici* (Calvert et al., 1960, Guo and Ko, 1993). A 7mm-diameter punch was used to transfer three plugs of *P. phaseoli* on 2% lima bean agar to plates of 2% lima bean agar with 6 grams of dextrose. Dextrose was added to increase growth rate. Single 7mm-diameter plugs of *P. capsici* were transferred to plates of V-8 agar. 6 plates of each isolate were made and three were designated as ‘controls’ with the other three being ‘experimental’. *P. capsici* and *P. phaseoli* isolates were stored in a digital incubator at 20°C for 20 and 21 days, respectively. Control isolates were separated from experimental isolates by a tinfoil-lined piece of cardboard to assure no light contamination between samples. A red LED grow light (HQRP 630nm LED Plant Grow Panel Lamp System) was suspended 30.48cm above the experimental isolates, with an intensity of 22µmol s^{-1} m^{-2} (Figure 36) Control plates were subjected to 24 hours of total darkness, while experimental plates experienced a regiment of 12-hours of red light, and 12-hours of darkness. At 5 (*P. capsici)* and 7 (*P. phaseoli*) day increments post inoculation, the diameter of growth for each plug was measured and 7mm plugs were taken from the edge of the mycelial growth for sporangial evaluation (Figure 37). Sample plugs were washed 7 times with 100µL of distilled water via a 1000µL pipette. A 10µL aliquot of the suspension was transferred to a hemocytometer where sporangia were counted. After final measurements, 7mm plugs were taken from all plates and transferred to their preferred media and placed in a separate dark incubator at 20°C to assess whether the isolates were still alive.

Figure 36 Image of the *in vitro* setup of red-light apparatus. Not shown: control isolates were placed directly on top of the upper tinfoil-lined cardboard in an identical layout as experimental isolates.

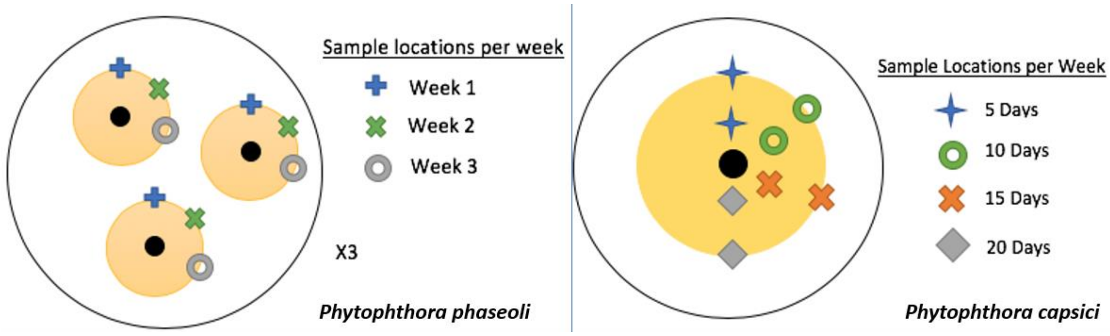

Figure 37 Diagram of plug removal for *Phytophthora phaseoli* (left) and *Phytophthora capsici* (right). Symbols correspond to location of plug removal on the various rating dates. Due to the rapid growth of *P. capsici*, plugs were taken from the edge and center of the mycelial mat for sporangial analysis.

In planta. Two identical mist chambers were constructed from PVC pipe, black trash bags, and clear trash bags inside a walk-in growth chamber (Figure 38). Each mist chamber measured 121.92cm x 60.96cm x 50.8cm, with 48.26cm x 25.4cm flaps cut into the front side of the enclosures, and an additional 7.62cm x 5.08cm hole cut 30.48cm up on the left side of each. Humidifiers (Walgreens Cool Mist Humidifier) were placed outside of each encloser with the mist exhaust opening lined up with the left-facing hole. A red LED growth light was suspended above the clear plastic top of one enclosure at a height of 43.18cm. Misters were filled with distilled water, set to “Low”, and placed on a timer set for 30 minute on-off intervals. Misters were refilled every 48 hours. Three separate time trials were conducted, 13-hours white/11-hours red, 13-hours white/5.5-hours dark/5.5-hours red (alternating each hour), and 13-hours red/11-hours dark (with no inoculum). The LED light was adjusted appropriately for each light regiment. Two trials were conducted per light regiment, with the LED being moved to the opposite between trials to rule out environmental factors. Light intensity was measured using a Li-COR Photometer (LI-195B). White light intensity was $50 \mu mol\ s^{-1}\ m^{-2}$, while red light intensity was $22 \mu mol\ s^{-1}\ m^{-2}$.

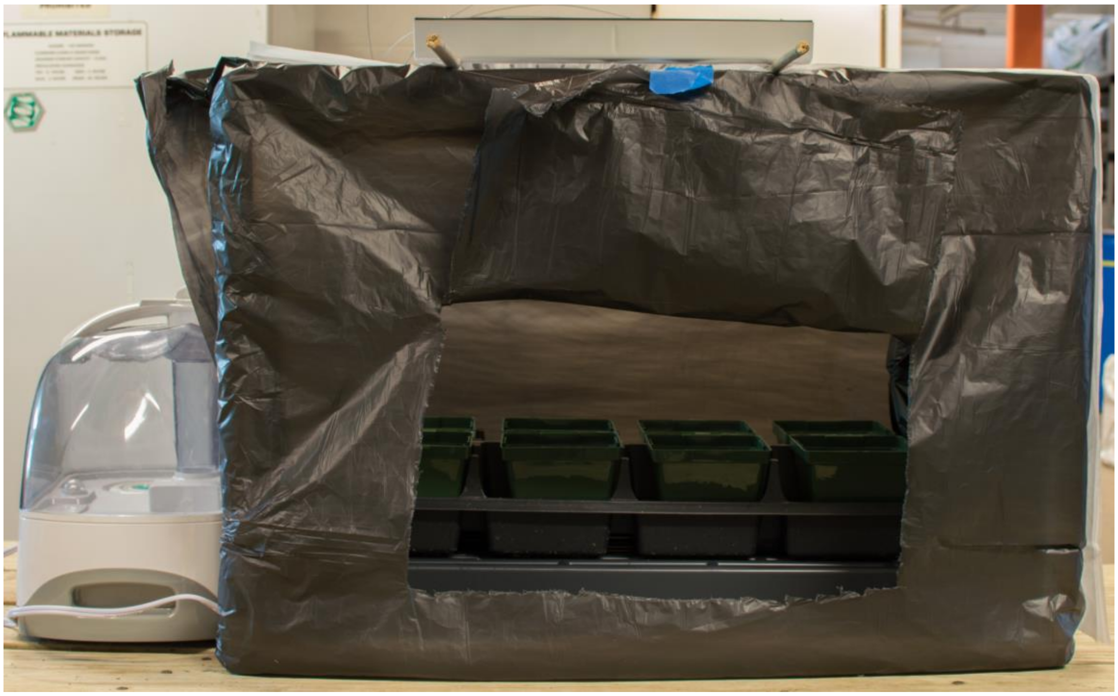

Figure 38 Set up of homemade dew chambers used for *in planta* trails.

Five Concentrated Fordhook lima bean seeds were arranged in a "5-dice" shape in each pot for 16 total pots, all containing "ProMix Bx" planting substrate. Six days after planting, 5mL of inoculum was dispended via broad-nose pipette on the exposed hypocotyls. Heights of every plant were taken along with pictures to ensure non-sprouted plants would not be counted. Eight pots were transferred to each mist chamber at 7am on the day of inoculation. Five days post inoculation, plants were removed from the mist chambers and plant height and disease severity were measured. Disease severity was measured on a scale of 1-4 (1 = >25% disease coverage, 2 = 25-

49% disease coverage, 3 = 50-74% disease coverage, and 4 = 75-100% disease coverage). Pictures were taken of individual pots along with entire flats.

Analysis. Graphs were generated in JMP Pro 15® and Microsoft Excel. Significance was analyzed using one-way ANOVA's also using Microsoft Excel and JMP Pro 15 ®. P-values were calculated using the Student's T-test. *In vitro m*easurements of the control and experimental plants were taken at the time of inoculation and five days post inoculation during the severity rating. The change in height was recorded, purged of outliers, and analyzed via one-way ANOVA for significance. Correlation significance was calculated using one-way ANOVA, and the correlation coefficient and graph was accomplished using Excel.

Results

In vitro Study. Diameters of all *Phytophthora phaseoli* Control isolates for ratings 1, 2, and 3, hereby denoted by a "XXXX_C" in future figures, were all significantly larger than their red-light treatment counterparts, denoted by "XXXX_R" (Figure 39). All red-light treatments, apart from isolate PhyP 26.3, showed no growth during all three weeks of the experiment. Of the Control isolates, PhyP62A had the largest diameter in all three rating dates, with PhyP12C having the smallest diameter (Figure 39). When rating 3 diameters were compared to the percent growth of their controls, all isolates showed 0% of control growth except for PhyP 26.3, which only showed an average of 30.2% growth compared to its control (Figure 41).

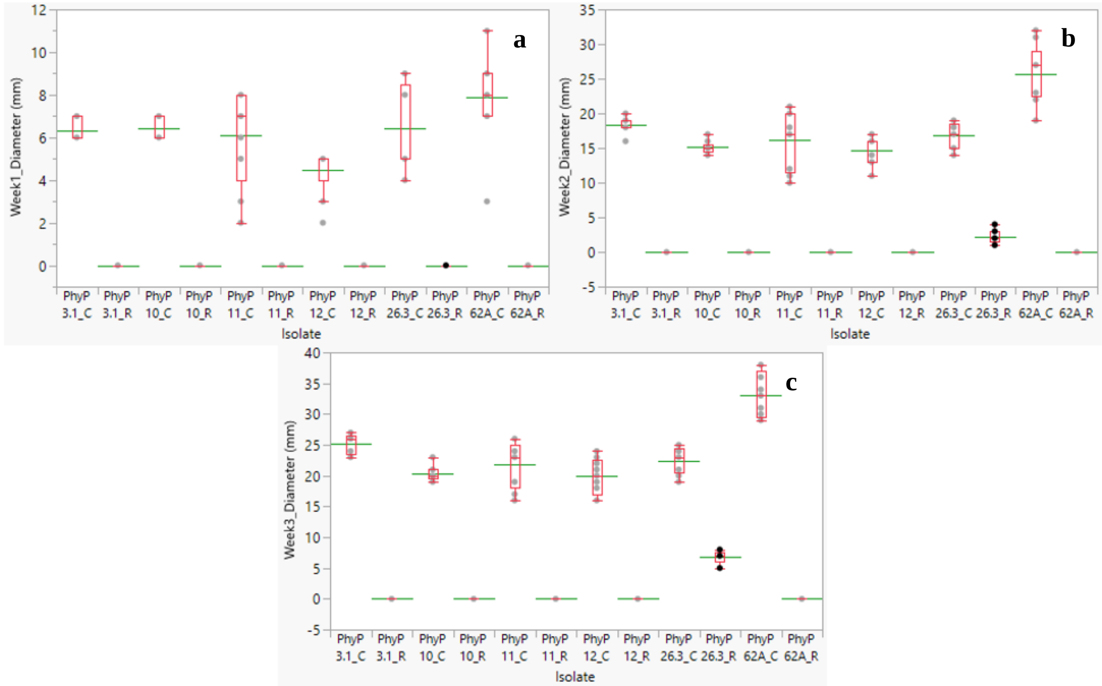

Figure 39 Box plots showing the growth of the *Phytophthora phaseoli* isolates on ratings 1 (a), 2 (b), and 3 (c).

Phytophthora capsica isolate 93 showed faster and wider growth under red light than *P. phaseoli*, though still followed a similar pattern in growth. By day 10, control variants of PC93 had already grown the diameter of the plate (83mm), while experimental treatments had only grown to a diameter of 46.6mm at the same time point (Figure 40b). At Day 20, the average of the experimental group had only reached a diameter of 66.2mm, with no individuals growing the full diameter of the plate (Figure 40d). When compared against their controls, the diameter of the *P. capsici* isolates were 20.3% less (Figure 41).

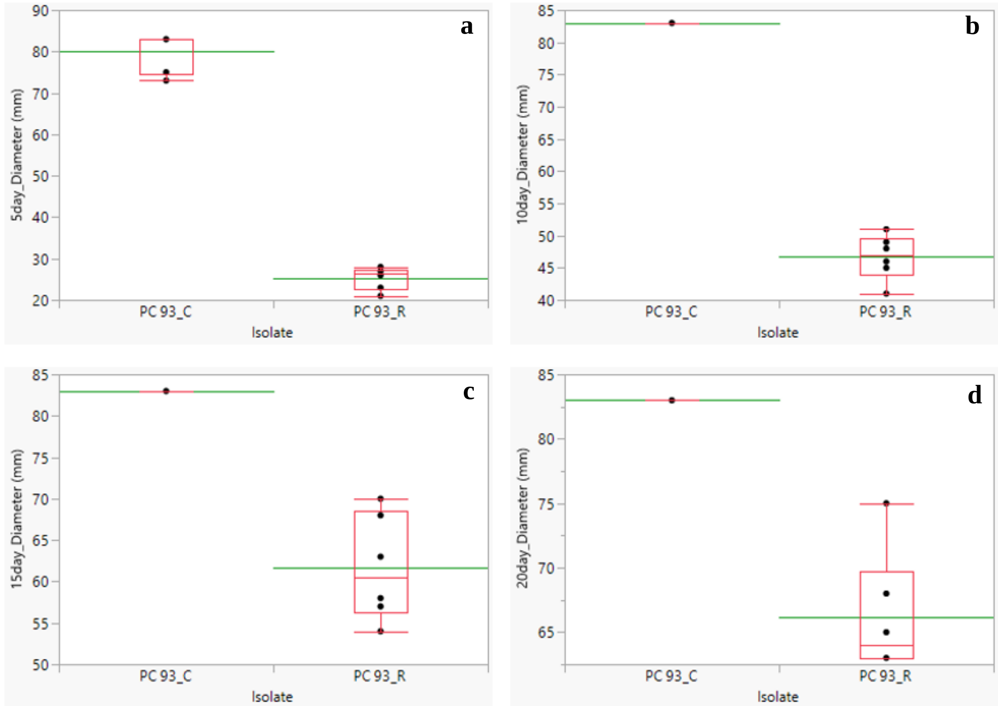

Figure 40 Box plots showing the growth of the *Phytophthora capsici* isolates on days 5 (a), 10 (b), 15 (c), and 20 (d) post inoculation.

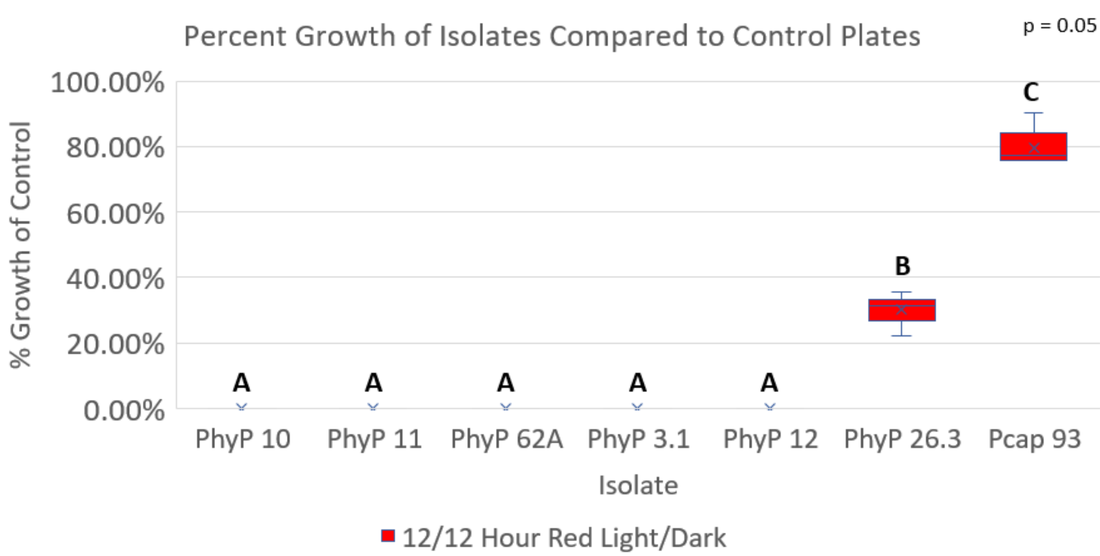

Figure 41 Comparison of red light treatment to control growth of *Phytophthora phaseoli* and *P. capsici* isolates.

In planta. Two light regiments, 13-hour-light 11-hour-dark/red timing, as well as a 13-hour-light, 5.5-hour-dark/red-alternating timing, were tested in a plant trial, a using *Phytophthora phaseoli* isolate "PhyP18". In the 13-hour-light 11-hour-dark/red treatments, the control group had a significantly higher (p=0.0002) severity five days post inoculation with an average rating of approximately 3.5, compared to the red-light treatment with an average rating of 2.5 (Figure 42).

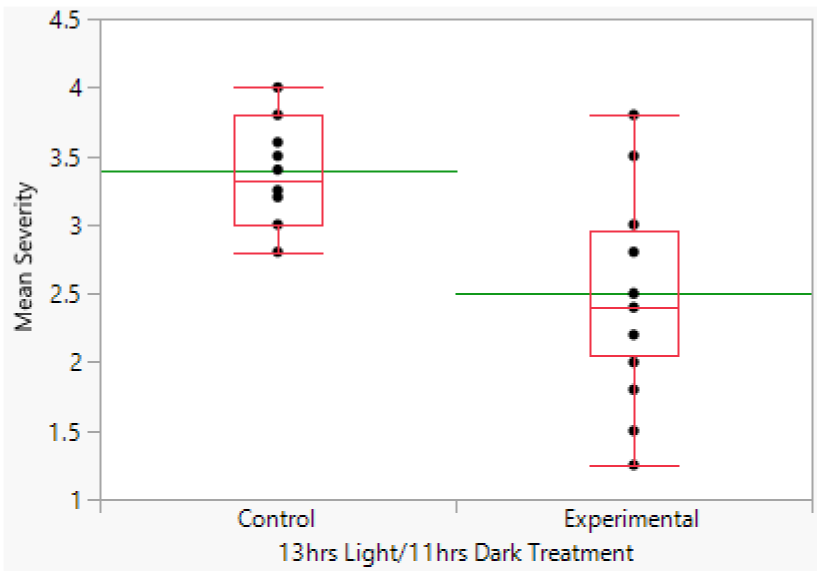

Figure 42 Comparison of severities in the 13-hour-light, 11-hour-dark/red trials.

The 13-hour-light, 5.5-hour-dark/red-alternating treatment showed no significant difference (p=0.934) between the control and experimental groups. Both mean severities were approximately 2.8 (Figure 43). Both mean severities are lower than 13-hour-light 11-hour-dark/red control treatments, and slightly higher than their experimental.

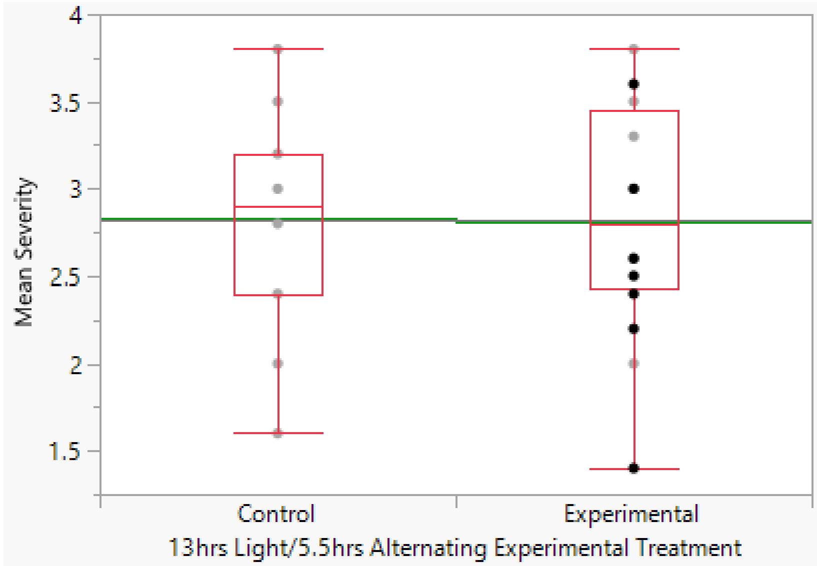

Figure 43 Comparison of severities in the 13-hour-light, 5.5-hour-dark/red alternating trials.

Control and experimental chambers were switched after each two-week replication to ensure even treatments. The difference in rating between chambers was found to be insignificant (p=0.169).

The change in height of control plants and experimental plants was insignificant during all three-time regiments as well as between chamber trials (Figure 44, 45, 46). Disease severity ratings were plotted against change in height measurements. No correlation was observed between the two variables in the 13-hour light/5.5 hour alternating trials, but a positive correlation was observed in the 13-hour light/11-hour dark/red trials (p=0.005) (Figure 47, 48). A higher severity was associated with a larger change in plant height.

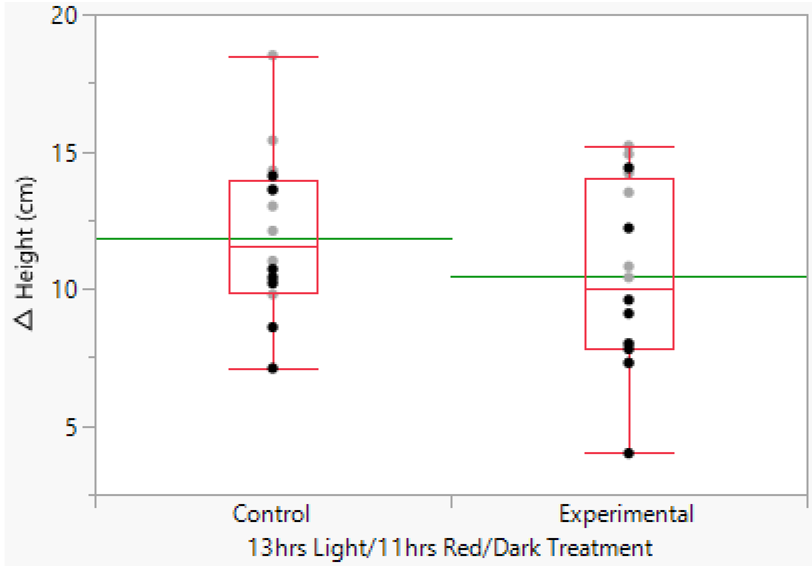

Figure 44 Comparison of change in height between 11-hour darkness and 11-hour red light treatments (p=0.2194).

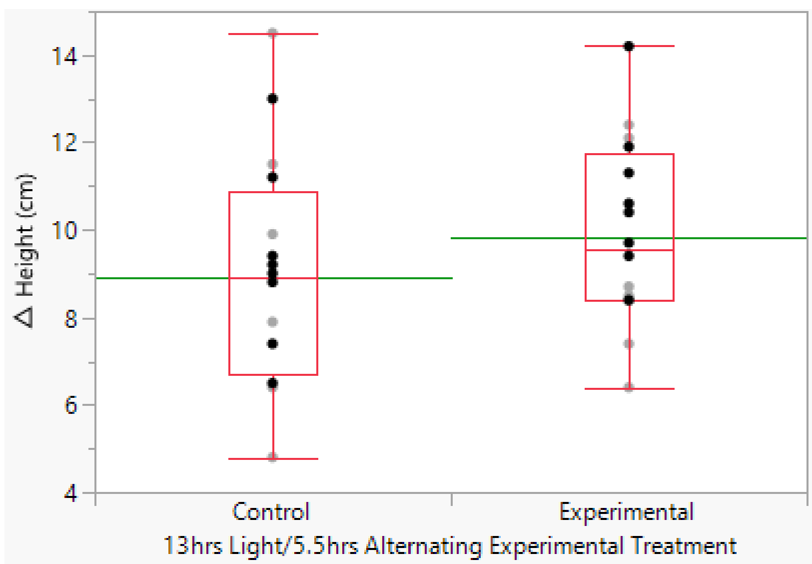

Figure 45 Comparison of change in height between 11-hour darkness and 5.5-hour red light/darkness alternating treatments (p=0.2884).

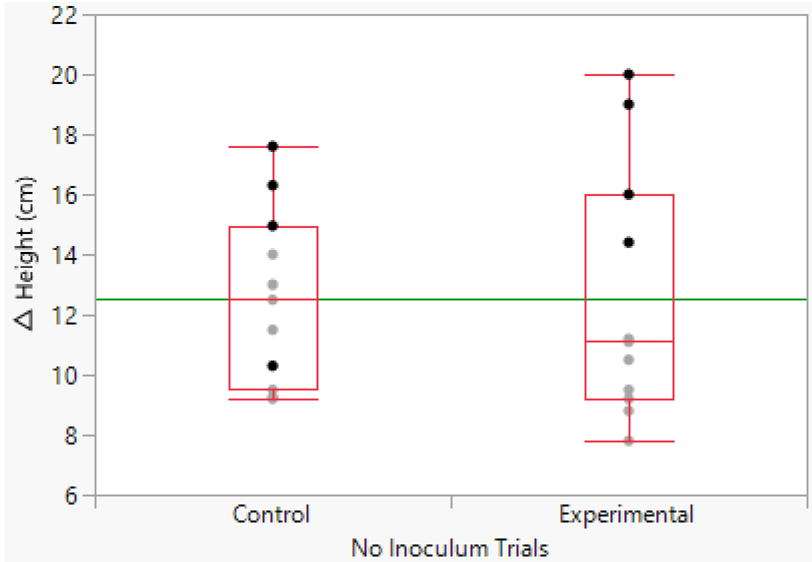

Figure 46 Comparison of change in height between uninoculated 11-hour darkness and 11-hour red light treatments (p=0.9722).

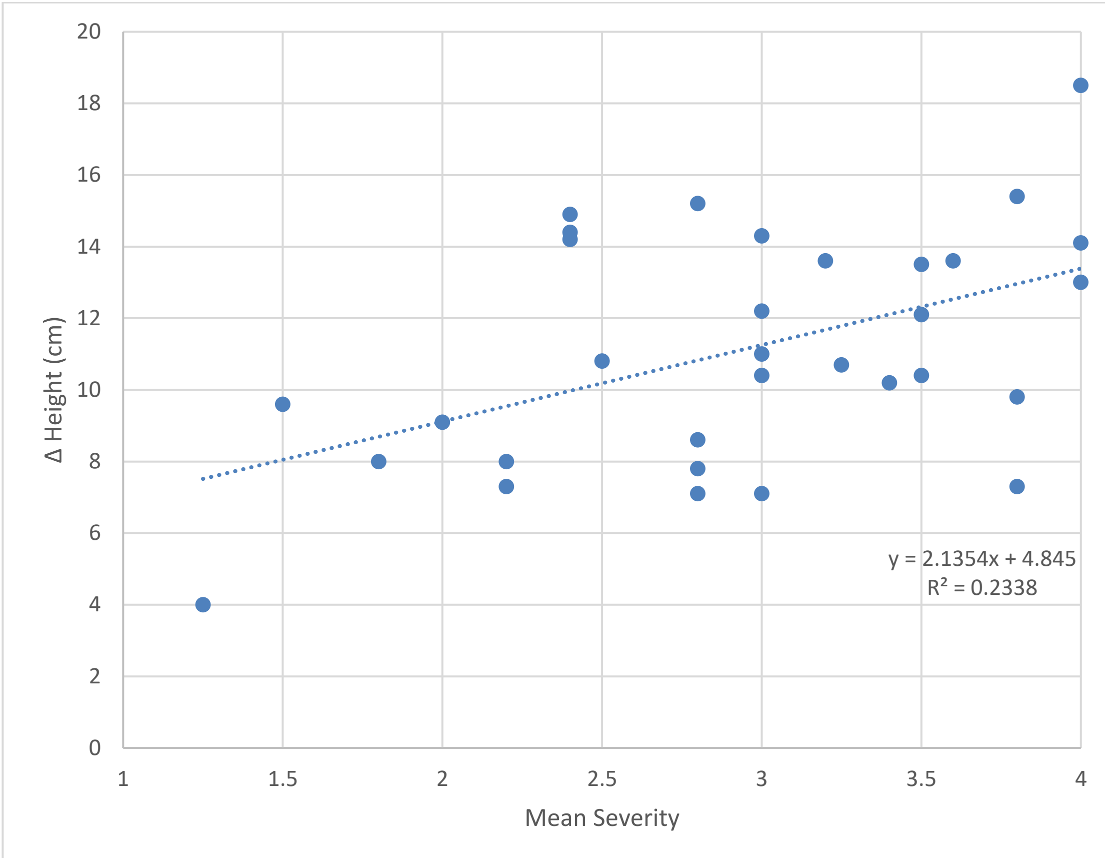

Figure 47 Correlation between the mean severity and change in height of the 13-hours light/11-hours darkness/red light treatments (p=0.0005).

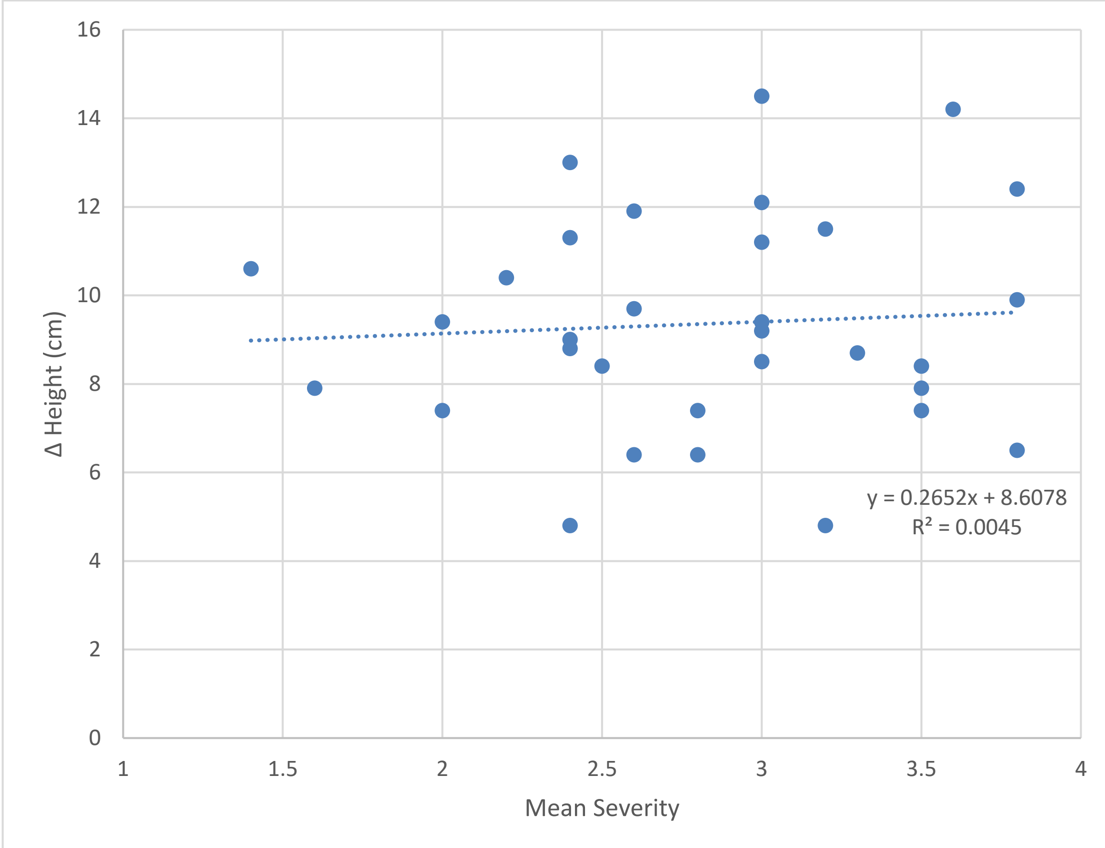

Figure 48 Correlation between the mean severity and change in height of the 13-hour light and 5.5-hour red light/darkness alternating treatments (p=0.7158).

Discussion

This experiment served as a proof-of-concept to determine whether the results found on other *Phytophthora* species by Patel et al. would translate in a similar manner to *Phytophthora phaseoli* (Patel et al., 2016).

In vitro. We confirmed that red light did significantly inhibit the growth of both pathogens on both of their preferred medias (Figure 41). While *P. phaseoli* was not tested by Islam et al., the results concerning the effects of red light on *P. capsici* is consistent with his findings (Islam et al., 2001). *Phytophthora capcisi* is a faster growing pathogen compared to *P. phaseoli*, being able to cover a 100mm petri plate in 4-5 days at 20°C. During the *in vitro* tests, the red-light treated *P. capcisi* was observed to only cover 79.7% of the plate compared to its controls even after 20 days. Similarly, the experimental *P. phaseoli* isolates also showed significantly hindered growth, with only one of the five isolates tested showing any growth whatsoever. It was suggested that the right light treatment had killed the four *P. phaseoli* isolates, but upon agar plug transfer to fresh plates of the pathogens preferred under control conditions, all isolates grew at the same rate and to the same size as historically seen. From this test, we were able to conclude that the red light was the point source on inhibition, supporting the hypothesis.

The *P. phaseoli* isolate, PhyP 26.3, grew significantly more than all other 'PhyP' isolates, reaching approximately 31% of its control counterpart. Currently, there are no theories as to why this isolate was significantly different than the others. All abiotic variables such as lighting and temperature were controlled and the lima bean agar for all plates came from the same batch. In addition, the growth of the 'PhyP 26.3'controls was not significantly different than other isolates, regardless of race of mefenoxam sensitivity used in the *in vitro* trials continuing to suggest that a genotypic trait or other biotic factor unique to this specific isolate allowed for some partial resistance against inhibition by red wavelengths of light. (Table 7).

Table 7 Description of the various *Phytophthora* species used for the trials, along with their sensitivity to the current prominent oomyceticide as well as physiological race where applicable.

Pathogen Designation	**Pathogen**	**Physiological Race**	**Reaction to Mefenoxam**[α]
PhyP 3.1	*Phytophthora phaseoli*	F	Partially Resistant
PhyP 10	*Phytophthora phaseoli*	F	Sensitive
PhyP 11	*Phytophthora phaseoli*	E	Partially Resistant
PhyP 12	*Phytophthora phaseoli*	E	Resistant
PhyP 26.3	*Phytophthora phaseoli*	F	Sensitive
PhyP 62A	*Phytophthora phaseoli*	F	Sensitive
Pcap 93	*Phytophthora capsici*	N/A	Sensitive

[α]Sensitivity designations were obtained by Mhora, et al., unpublished.

Due to low spore counts, collection and subsequent analysis of sporangia from control and experimental lima bean plants was unable to be accomplished. Sporangia formation of all plates was either inconsistent or non-existent. Similar issues have been observed in fungi that have remained on media other than their host plant for too long, which is the case for all isolates used in this experiment (Toussoun, 1976).
The addition of dextrose allows for *P. phaseoli* to grow rapidly without undue stress, therefore, decreasing the need for the pathogen to produce reproductive bodies. In both cases, it may be surmised that the absence of any wavelengths of light other than red light can be attributed to the lack of any sporangia formation. If this experiment were to be repeated, the lima bean agar would not contain dextrose and the default 12-hour dark cycle would be substituted with a 12-hour white light cycle in an attempt to induce sporangial formation.

In planta. During plant trials, it was confirmed that red light did significantly inhibit *P. phaseoli* growth during the 13-hour white/11-hour red-light trial, while the 13-hours white/5.5-hours dark/5.5-hours red alternating did not show any significant differences in disease severity, thereby supporting the hypothesis. The positive results from the longer treatment of red light prompted further research questions, specifically, would increasing the intensity of the red light allow for a shorter treatment time with just as effective disease inhibition? During these trials, the red light was suspended 43.18cm above the pots, in future tests, it would be prudent to half the distance between the pots and the same red-light source and subject the inoculated lima bean plants to the same time regiments as previously described. Furthermore, the differences between the 13-hour white/11-hour red-light and 13-hours white/5.5-hours dark/5.5-hours red-light alternating trials raise questions about the type of red light being applied, as studies have shown differences between red and far-red wavelengths have differing effects on plants growth characteristics (Chen et al., 2005; Huber and Wiggerman, 1997; Finlayson, 2007; Delvin, 1998). These various wavelengths in conjunction with varying intensities and treatment timings could prove useful in determining an ideal treatment regimen to meaningfully inhibit downy mildew on susceptible lima bean crop. *Phytophthora phaseoli* isolate 'PhyP 18' was the only isolate tested during this section of the experiment.

A no-inoculum trial was conducted using the 13-hour light/11-hours dark or red-light lighting regiment to determine whether increased exposure to red wavelengths of light over a short time scale affected the growth rate of the Fordhook lima bean plants. Previous studies have demonstrated that elevated level of red wavelengths of light can cause a litany of shade avoidance syndrome (SAS) responses in many plant species including but not limited to accelerated flowering rates, abnormal stem elongation, and reduced branching (Huber and Wiggerman, 1997; Finlayson, 2007; Delvin, 1998). From these no-inoculum trials, it was determined that elevated exposure to red wavelengths of light did not change the rate of plant growth in any significant capacity ($p=0.9722$), at least over the short term (Figure 46). This discovery may be translated into treating field lima bean with short-term high-intensity regiments of red light without fear of adversely affecting the crops morphology and growth rate.

The change in plant height was compared against the severity of disease among the two different light regiment trials to deduce whether these two variables were correlated. There was a positive correlation between growth rate and disease severity ($p=0.0005$) for the 13-hour light/11-hour dark/red light regiments, where a larger increase in plant height was associated with a higher severity (Figure 47). While not a causation, there are some possible explanations as to why this correlation may exist. The first possibility is that the faster growing lima bean plants had certain defense pathways inhibited which allowed for easier disease infection and spread in these individuals, though no current data supports this theory.

Another possibility would be that the lima bean plants are attempting to out-grow the pathogen, leading to higher severity in taller plants (Kay et al., 2007). It is well known that there is a fitness cost associated with plant defense responses. Most responses cause dwarfism and other setbacks in plant development, though other cause hypertrophy and hyperplasia (Seilaniantz et al., 2011; Kay et al., 2007). It possible that the red light in combination with this specific pathogen triggers a similar growth hormone and its pathway.

Further testing is required to identify the optimal conditions for pathogen inhibition in terms of the intensity and wavelength of red light, the exposure time needed to have an effect, and at what point or points in the plant's life cycle the light treatment would need to be applied to see meaningful inhibition. If continued research yields positive results, red light treatments could one day be added to the disease toolbox utilized by growers to combat downy mildew on lima bean and minimize the need for expensive oomyceticides.

REFERENCES

Borthwich, H. A., Hendricks, S. B., Parker, M. W., Toole, E. H. and Toole, V. K. (1952) Proceedings of the National Academy of Science, U. S. A., 38, 662.

Bridge, M.A. and Klarman W.L. 1973. “Soybean phytoalexin, hydroxyphaseollin, induced by ultraviolet irradiation.” Phytopathology 63:606– 608.

Calvert, O. H., Williams, L. F. and Whitehead, M. D. 1960. “Frozen lima bean agar for culture and storage of *Phytophthora sojae*.” Phytopathology 50:136-137.

Chen, M., Tao, Y., Lim, J., Shaw, A., Chory, J., 2005. “Regulation of phytochrome B nuclear ocalization through light-dependent unmasking of nuclear-localization signals.” Current Biology. 15, 637–642. https://doi.org/10.1016/j.cub.2005.02.028.

Delvin, P.F., 1998. “Phytochrome E influences internode elongation and flowering time in Arabidopsis.” Plant Cell Online 10, 1479–1488. https://doi.org/10.1105/tpc.10.9. 1479

Finlayson, S. A., 2007. “Arabidopsis TEOSINTE BRANCHED1-LIKE 1 regulates axillary bud outgrowth and is homologous to monocot TEOSINTE BRANCHED1.” Plant Cell Physiology. 48, 667–677. https://doi.org/10.1093/pcp/pcm044.

Guo, L. Y., and Ko W. H. "Two Widely Accessible Media for Growth and Reproduction of *Phytophthora* and *Pythium* Species †." Applied and Environmental Microbiology 59.7 (1993): 2323-325. Print.

Huber, H., Wiggerman, L., 1997. “Shade avoidance in the clonal herb *Trifolium fragiferum*: a field study with experimentally manipulated vegetation height.” Plant Ecology. 130, 53–62. https://doi.org/10.1023/A:1009702611270.

Islam, S. Z., Babadoost, M., and Honda, Y. (2002). “Effect of Red Light Treatment of Seedlings of Pepper, Pumpkin, and Tomato on the Occurrence of.” Islam Zeitschrift Für Geschichte Und Kultur Des Islamischen Orients, 37(July 2001), 678–681.

Kay, S., Hahn, S., Marois, E., Hause, G., and Bonas, U. "A Bacterial Effector Acts as a Plant Transcription Factor and Induces a Cell Size Regulator." Science 318.5850 (2007): 648-51. Print.

Kurepin L.V., Walton L.J., Reid D.M. “Interaction of red to far red light ratio and ethylene in regulating stem elongation of *Helianthus annuus*.” Plant Growth Regulations. 2007;51:53–61. doi: 10.1007/s10725-006-9147-x.

Park Y., Runkle E.S. “Far-red radiation promotes growth of seedlings by increasing leaf expansion and whole-plant net assimilation.” Environtal and Experimental Botany. 2017;136:41–49. doi: 10.1016/j.envexpbot.2016.12.013.

Patel, J. S., Zhang, S., and McGrath, M. T. (2016). “Red Light Increases Suppression of Downy Mildew in Basil by Chemical and Organic Products.” Journal of Phytopathology, 164(11–12), 1022–1029. https://doi.org/10.1111/jph.12523

Reed. J. W., Nagpal, P., Poole, D. S., Furuya, M., and Chory, J. (1993) “Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development.” Plant Cell 5:147–157.

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. "Hormone Crosstalk in Plant Disease and Defense: More Than Just JASMONATE-SALICYLATE Antagonism." Annual Review of Phytopathology 49.1 (2011): 317-43. Print.

Santamaria, L., Ernest, E. G., Gregory, N. F., and Evans, T. A. (2018). “Inheritance of resistance in Lima bean to *Phytophthora Phaseoli*, the causal agent of Downy MILDEW of Lima Bean.” HortScience, *53*(6), 777-781. doi:10.21273/hortsci12748-18

Smith, H., “Phytochrome and Photomorphogenesis.” London: McGrall Hill Book, 1975. Print.

Taiz, L., and Zeiger, E. Plant Physiology. Fourth Edition ed. USA: Sinauer Associates, 2010. Print.

Toussoun, T. A. "Fusarium: A Pictoral Guide to the Identification of Fusarium Species According to the Taxonomic System of Snyder and Hansen." Fusarium: A Pictoral Guide to the Identification of Fusarium Species According to the Taxonomic System of Snyder and Hansen. University Park - Pa. & London: Pennsylvania State UP, 1976. 43. Print.

Appendix A

FINAL RATINGS FOR RACE E NOVEL RESISTANCE ASSAY

Line #	Rating 2 Round 1					Rating 2 Round 2					Rating 2 Round 3				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	H	S	R	--	S	S	S	S	S	S	S	--	S	S	S
2	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	S	S	S	H	S	S	S	R	S	S
5	--	S	R	R	S	S	S	S	S	S	H	S	H	R	H
6	S	--	R	S	R	R	S	S	R	S	S	R	S	H	S
7	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8	S	R	S	H	S	S	S	S	S	S	R	H	R	R	R
9	H	H	--	S	H	--	S	S	S	--	S	S	S	S	S
10	--	S	H	--	S	H	H	H	H	S	S	S	S	S	R
11	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
12	S	S	S	S	S	S	S	S	S	S	R	H	S	S	S
13	R	R	R	R	R	R	R	R	R	--	--	R	R	H	H
14	H	R	H	R	R	S	S	S	H	H	H	H	H	H	R
15	--	R	R	R	R	R	H	R	R	R	R	R	R	R	R
17	MISSING					MISSING					MISSING				
18	S	RR	S	R	R	H	--	S	R	R	S	S		R	R
19	R	R	H	R	R	R	R	R	R	R	--	H	R	R	R
20	H	S	--	S	H	S	S	H	H	S	H	S	R	S	S
21	S	R	R	R	R	H	S	H	S	H	S	R	S	S	S

22	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
23	R	R	R	R	R	R	H	H	R	R	R	R	R	R	R
24	S	H	H	S	H	S	S	S	S	S	R	R	R	S	H
25	R	S	S	R	R	H	H	S	S	R	S	S	S	S	S
26	--	S	R	R	H	H	R	S	S	S	H	H	S	H	H
27	R	S	S	--	R	S	S	S	S	S	S	R	RR	S	S
28	R	R	R	R	R	H	R	S	S	R	S	--	H	R	R
29	S	R	H	S	H	H	S	S	H	S	H	S	R	S	S
30	S	R	H	S	R	H	--	R	R	--	S	H	--	H	R
31	S	H	S	S	H	S	R	H	R	R	S	S	R	H	S
32	R	H	H	H	H	R	S	--	H	H	S	--	R	R	R
33	R	R	R	R	R	H	H	R	R	R	R	R	R	R	R
34	--	R	R	R	R	R	R	R	R	R	R	R	R	R	R
35	MISSING					MISSING					MISSING				
36	S	R	H	--	R	R	R	--	R	S	R	R	S	H	S
37	S	--	R	S	R	H	R	H	R	R	S	R	S	R	--
38	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
40	R	S	S	S	S	S	--	S	H	H	--	R	S	S	H
41	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
42	H	S	R	R	R	S	R	H	S	H	R	R	S	S	R
43	R	S	S	S	R	H	R	S	S	S	R	S	R	--	R
44	S	S	--	S	S	S	S	S	S	S	S	S	S	S	S
45	S	R	R	R	R	S	S	H	S	S	S	S	S	S	H
46	S	S	S	R	S	S	S	S	--	S	S	S	S	S	S
47	H	R	RR	R	R	H	R	H	R	R	R	R		R	R

48	S	S	R	S	S	S	H	S	S	H	H	S	R	H	S
49	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S
50	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
51	R	S	--	--	S	R	R	R	R	R	S	H	R	R	R
52	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
53	R	S	H	R	R	S	S	S	S	R	S	S	R	R	S
54	R	H	--	R	R	H	H	R	R	R	R	R	R	R	R
55	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
56	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
57	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
58	--	S	S	H	S	R	R	S	S	--	H	S	R	R	S
59	H	R	--	S	S	S	S	S	S	H	H	R	R	H	S
60	S	H	H	H	R	H	S	S	S	S	R	R	R	H	S
61	S	S	S	S	S	S	H	S	S	S	S	S	S	R	S
62	H	H	H	H	H	H	H	H	H	H	R	R	R	R	R
63	H	R	R	R	R	R	R	R	R	R	R	R	R	R	R
64	S	H	S	H	S	R	R	S	S	H	R	R	R	R	S
65	S	H	S	H	H	S	S	--	S	S	H	R	S	S	--
66	R	R	R	R	R	R	R	H	R	--	R	R	R	R	R
67	R	S	H	H	S	H	R	S	R	S	S	S	R	R	R
68	S	S	S	S	S	S	S	S	S	S	--	S	S	H	S
69	S	--	H	--	S	S	S	H	H	H	S	R	R	S	S
70	R	R	R	R	--	R	H	R	R	R	R	R	R	R	R
71	R	R	S	S	H	H	H	S	H	S	S	S	H	S	S
72	S	R	R	S	S	S	S	S	S	R	S	S	S	R	R

73	S	H	S	--	H	R	S	S	S	R	S	S	S	R	S
74	S	S	S	S	S	S	S	S	S	S	S	H	S	S	S
75	R	--	H	R	S	R	S	S	R	S	H	H	S	S	S
76	S	S	S	R	S	S	R	S	S	S	--	H	S	H	S
77	H	H	H	H	R	R	R	H	H	H	R	R	R	R	R
78	H	H	S	S	H	S	S	S	S	S	S	S	R	S	R
79	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
80	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
81	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
82	S	S	S	S	S	S	S	S	S	S	S	--	S	S	S
83	R	R	R	--	R	S	S	S	S	R	S	R	R	H	R
84	S	H	S	S	S	S	R	S	R	S	R	R	R	S	S
85	R	R	R	--	R	--	--	H	R	H	R	R	R	R	R
86	S	H	R	S	S	S	S	S	R	H	S	S	S	R	S
87	R	R	S	H	R	S	R	R	R	R	S	R?	S	S	--
88	R	H	H	H	H	R	R	R	R	R	R	R	R	R	R
89	--	--	--	H	S	S	--	H	R	S	H	S	H	H	H
90	S	R	R	R	R	R	S	H	S	R	R	H	H	R	H
91	H	S	S	S	R	R	S	S	S	R	S	S	S	H	S
92	S	R	S	R	S	S	S	S	S	S	H	S	S	R	R
93	R	S	H	H	S	R	H	H	R	H	H	R	H	--	S
94	S	S	H	R	H	H	R	S	R	R	H	H	R	H	H
95	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
96	S	S	--	S	S	S	S	S	S	S	S	S	S	S	S
97	R	R	R	R	R	R	R	R	R	R	R	R	H	R	R

98	R	S	S	H	S	S	S	S	S	S	R	R	S	R	R
99	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
100	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
101	R	R	R	R	R	R	R	R	--	R	R	R		R	R
102	R	H	S	S	S	S	R	S	R	H	S	S	R	H	R
103	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
105	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
106	--	R	R	R	--	H	H	H	H	H	R	H	--	R	R
107	R	R	R	R	R	R	R	R	R	R	R	R	R	H	R
108	R	R	R	H	H	R	R	H	H	H	R	R	R	R	R
109	R	S	R	S	S	S	S	S	S	S	H	R	S	R	S
110	S	H	H	S	R	S	S	S	--	S	--	S	S	S	S
111	H	S	S	S	H	S	S	S	H	H	S	H	--	--	--
112	S	R	R	S	R	R	R	R	R	R	R	R	R	R	R
113	H	H	H	H	H	S	R	H	R	S	R	H	S	S	H
114	--	R	S	H	R	S	S	S	R	S	S	S	H	--	S
115	R	R	R	--	R	R	R	H	H	H	H	H	H	H	H
116	S	S	S	S	S	S	S	S	S	S	H	H	S	S	H
117	R	R	H	R	H	H	H	H	H	H	R	R	R	R	R
118	S	S	S	S	S	S	S	S	S	S	S	S	H	S	S
119	H	R	R	R	H	R	R	R	R	R	R	R	--	R	R
120	S	S	--	S	S	S	S	S	S	S	R	R	S	S	R
121	S	S	R	H	S	--	S	S	S	R	S	S	S	R	R
122	S	S	H	R	S	S	S	S	R	S	S	S	H	S	?

123	--	S	H	R	R	R	R	S	S	S	R	S	S	R	H
124	S	S	H	S	S	H	S	S	S	S	R	--	R	H	R
125	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
126	R	H	H	R	R	S	--	R	S	R	R	R	R	R	R
127	R	H	R	H	R	R	R	R	R	R	R	R	H	--	R
128	--	S	S	--	S	S	S	S	S	S	S	S	S	H	S
129	R	--	R	R	R	H	R	R	R	R	R	R	R	--	R
130	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
131	H	R	R	R	R	R	S	R	S	R	R	R	R	R	R
132	R	R	H	S	R	S	S	S	S	R	R	H	R	S	R
133	R	R	R	R	R	H	H	H	H	R	R	R	R	R	R
134	S	H	H	S	S	S	S	S	S	S	S	H	S	S	H
135	R	R	R	R	--	R	R	R	R	R	H	H	R	R	H
136	--	S	R	H	R	R	H	S	S	S	S	S	S	H	H
137	S	S	S	R	R	S	R	S	R	S	S	S	H	S	R
138	R	R	R	R	R	R	R	H	H	H	R	H	H	H	H
139	R	--	R	--	R	R	H	R	R	R	R	R	R	R	R
140	R	H	S	H	R	H	S	S	S	H	S	--	R	R	R
141	R	--	S	S	S	S	S	S	S	S	S	S	S	S	S
142	R	R	R	R	R	R	R	--	R	R	R	R	R	R	R
143	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
144	R	R	S	R	S	S	R	R	R	S	H	S	S	H	S
145	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
146	H	R	S	S	S	S	R	R	S	R	H	H	H	H	R
147	R	R	R	S	S	S	H	S	R	H	H	S	S	S	H

148	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
149	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
150	R	R	R	R	R	--	S	R	S	S	H	H	H	S	R
151	S	S	S	S	S	S	S	S	S	S	S	S	S	--	S
152	R	S	R	S	R	S	S	S	S	S	R	R	S	S	S
153	R	R	S	H	S	R	S	S	S	S	S	R	R	R	H
154	R	R	R	R	R	S	S	S	R	S	R	S	S	S	H
155	R	H	R	R	R	S	S	S	S	S	S	S	H	R	H
156	--	--	S	--	S	S	S	S	S	S	S	S	S	S	S
157	R	R	R	R	R	S	S	H	S	R	S	H	S	S	S
158	MISSING					MISSING					MISSING				
159	S	R	S	S	R	S	S	S	S	S	H	S	S	R	S
160	S	S	--	S	R	S	S	S	S	S	S	S	H	S	R?
161	H	R	H	S	S	R	R	R	S	R	S	H	S	--	S
162	--	R	R	R	R	R	S	R	R	R	R	R	R	R	R
163	R	R	R	R	R	S	S	R	S	R	S	S	S	H	R
164	MISSING					MISSING					MISSING				
165	H	R	R	R	R	R	R	R	R	R	H	H	H	R	S
166	S	S	--	--	S	S	S	S	S	S	S	S	S	S	S
167	R	R	R	R	--	R	S	S	R	S	H	R	H	?	S
168	R	R	R	R	R	H	H	H	H	S	R	S	R	H	H
169	R	S	R	S	H	S	--	R	H	S	--	S	S	R	S
170	R	S	S	S	S	S	S	R	R	S	S	H	R	R	S
171	R	S	R	--	H	R	S	S	S	S	S	R	S	S	R
173	R	R	--	R	R	H	S	H	H	H	R	R	S	R	S

174	H	R	R	S	S	S	S	S	S	S	H	R	R	R	H
175	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
176	S	S	S	S	S	S	S	H	S	S	S	S	S	S	S
177	R	R	R	R	R	H	H	R	R	R	R	R	R	H	R
178	R	S	R	H	S	S	S	S	S	S	S	S	S	S	S
179	R	R	R	R	R	R	R	S	R	R	R	H	H	H	H
180	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
181	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
182	R	H	R	R	R	R	R	R	R	R	R	R	R	R	R
183	S	S	H	S	R	S	S	S	H	H	R	R	R	R	R
184	R	R	R	R	R	S	S	S	H	H	S	R	R	R	R
185	R	R	R	R	R	H	R	R	R	R	R	R	R	R	R
186	S	S	S	--	S	S	S	--	H	S	H	H	R	S	R
187	R	--	--	R	R	R	R	R	R	R	R	R	R	R	R
188	S	R	H	R	S	S	S	S	R	H	R	R	R	R	S
189	H	R	R	R	R	R	R	H	H	S	R	H	H	R	R
190	S	H	S	R	--	--	H	H	R	S	R	S	S	S	R
191	H	R	R	R	R	H	H	H	H	H	S	--	R	R	R
192	R	R	R	--	R	H	S	R	S	R	S	--	H	S	S
193	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
194	S	S	--	S	R	S	S	S	R	R	H	R	R	S	R
195	S	R	--	S	H	R	H	R	--	R	H	R	H	H	R
196	R	R	S	H	R	R	H	H	S	S	R	R	R	S	R
197	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
198	H	H	H	R	S	--	R	S	H	S	R	R	R	S	S

199	R	H	H	R	R	H	--	R	R	R	R	R	R	R	R
200	R	R	R	R	R	R	R	R	R	R	R	R	--	R	R
201	S	S	S	S	S	S	--	S	S	S	H	S	S	S	R
202	MISSING					MISSING					MISSING				
203	R	R	R	R	S	S	S	R	H	S	S	R	R	S	S
PI 534918	S	S	S	S	S	S	S	S	S	S	S	S	--	S	S
PI 256417	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R

Appendix B

FINAL RATINGS FOR RACE F NOVEL RESISTANCE ASSAY

Line #	Rating 2 Round 1					Rating 2 Round 2					Rating 2 Round 3				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
2	S	S	S	S	--	S	S	S	R	S	S	S	S	S	S
3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	S	R	R	S	S	H	S	S	S	S	S	S	R	S	S
5	R	S	S	R	S	S	S	S	H	H	R	S	S	S	S
6	R	R	R	R	R	S	S	R	R	S	S	R	S	H	R
7	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8	R	R	S	S	R	S	S	H	S	S	R	S	S	R	R
9	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10	R	--	R	R	S	H	S	S	H	S	S	R	S	R	S
11	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
12	S	R*	S	S	R	S	S	S	S	S	S	S	S	S	S
13	R	R	R	R	R	R	R	R	--	R	H	H	H	H	H
14	R	S	R	R	S	H	S	S	S	R	S	H	S	R	H
15	--	R	R	R	R	R	R	R	R	R	R	R	R	R	R
17	S	--	S	R	R*	S	S	S	R	S	S	S	S	S	S
18	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S
19	R	R	R	R	R	H	R	R?	R	H	R	R	H	R	H
20	R	R	--	S	S	S	S	S	S	S	H	S	H	S	S
21	S	S	S	S	R?	S	R	S	R	R	R	S	R	R	S
22	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

23	R	R	R	R	R	R	R	R	H	H	R	R	R	R	R
24	R?	R?	S	S	S	R	R	R	S	R	R	S	H	R	S
25	R	R	R	S	R	S	S	S	S	S	R	S	R	R	S
26	R	S	--	S	R	H	R	S	--	R	H	S	R	R	R
27	S	R	S	S	S	R	S	S	S	R	--	S	S	S	S
28	R	R	--	S	R	S	R	S	H	H	H	H	H	R	R
29	R	R	R	R	S	S	S	S	S	S	S	S	?	H	S
30	R	R	R	R	R*	R	S	R	R	R	H	H	H	H	H
31	S	R	R	S	R	R	R	R	R	R	H	S	S	H	S
32	R	R	R	R	R	H	H	S	S	H	S	S	S	S	S
33	R	R	R	R	R	H	H	R	H	H	R	H	R	R	R
34	R	R	R	R	R	H	H	H	H	R	R	R	R	R	R
35	--	R	--	--	--	R	R	R	S	R	R	--	R	S	H
36	R	R	--	--	R	S	S	S	S	S	H	S	R	R	S
37	R	S	S	R	S	S	S	R	H	H	H	S	S	R	H
38	S	S	S	S	S	S	S	S	S	S	S	S	S	--	S
40	R	R	S	R	R	S	S	R	S	S	S	S	S	S	S
41	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
42	S	S	R	R	?	R	S	R	R	R	R	S	R	R	R
43	S	S	S	R	R	H	R	S	R	R	S	S	S	S	S
44	--	?	S	S	S	S	S	S	S	S	S	S	S	H	S
45	S	S	S	R	S	S	R	R	R	S	H	S	S	H	H
46	S	S	--	S	S	S	S	R	S	S	S	S	S	S	S
47	R	R	R	--	R	R	R	R	R	R	--	R	R	R	R
48	R	S	S	R	R	H	S	S	S	R	S	S	S	S	S
49	S	--	S	S	S	S	S	S	S	S	S	S	S	S	S

50	R	R	R	R	R	R	H	R	R	R	R	R	R	R	R
51	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S
52	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
53	R	--	S	R	R	S	R	S	H	H	S	S	S	R	S
54	R	R	R	R	R	R	R	R	R	R	H	H	H	H	R
55	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
56	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
57	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
58	S	R	R	S	S	S	S	R	H	H	S	R	S	H	S
59	S	S	R	R?	R	H	S	R	R	S	R	H	H	S	S
60	R	S	S	S	R	H	S	R	H	S	S	H	S	S	R
61	S	S	R*	R*	?	H	R*	S	S	S	S	S	S	R	S
62	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
63	R	R	R	R	?	R	R	R	R	R	R	R	R	R	R
64	S	S	S	R	R	S	S	R	S	R	R	H	R	H	R
65	S	R	S	R	S	S	S	S	S	--	S	--	S	S	S
66	R	R	R	R	R	H	H	H	H	H	H	H	H	H	H
67	S	R	R	S	S	R*	S	S	R	S	R	R	R	R	S
68	S	S	S	S	S	S	S	S	S	S	H	S	S	S	S
69	S	R	R	R	S	R	H	S	R	R	R	R	--	R	S
70	R	R	R	R	R	R	R	R	R	R	R	R	R	R	--
71	S	S	S	S	S	S	S	S	R	S	R	R	H	R	R
72	R	S	S	R	R	S	S	R	R	S	R	S	R	R	R
73	R	S	--	R	S	S	R	S	H	S	S	S	R	S	H
74	S	S	S	S	S	S	S	S	S	S	S	S	S	--	S
75	S	R	R	S	R	S	S	S	S	S	--	R	S	S	--

76	S	R	S	S	S	H	S	S	S	H	H	S	H	R	--
77	R	R	R	R	R	R	H	R	R	H	R	R	R	R	R
78	S	S	S	S	S	R	R	S	R	S	R	S	--	R	S
79	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
80	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
81	S	S	S	S	S	R	H	--	--	--	S	H	--	S	S
82	S	S	--	S	S	--	S	S	S	S	S	S	S	S	S
83	R	R	R	R	R	R	H	R	R	R	R	R	S	S	H
84	R	R	S	R	S	--	S	S	S	--	R	R	R	S	H
85	R	R	R	R	R	R	R	R	R	R	S	--	--	R	R
86	S	S	S	S	S	S	S	--	S	S	R	S	S	S	H
87	R	S	S	R	S	R	R	S	S	R	H	S	R	--	S
88	R	--	R	R	R	H	H	H	H	H	R	R	--	R	R
89	R	R	S	S	R	R	R	--	S	R	H	H	H	H	H
90	R	R	R	R	S	S	S	S	R	R	H	--	R	H	S
91	R	S	S	S	R	S	H	S	R	H	S	--	R	R	R
92	R	S	S	S	S	R	H	S	R	S	S	R	S	S	R
93	R	R	S	R	R	R	R	R	S	R	S	R	S	S	S
94	S	R	R	R	R	R	H	S	H	H	S	H	H	H	H
95	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
96	S	S	S	S	--	S	S	S	S	S	S	--	S	S	S
97	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
98	R	R	S	S	R	H	H	S	R	S	R	S	R	R	R
99	R	R	R	R	R	R	R	H	R	R	R	R	R	R	--
100	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
101	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

102	R	R	R	R	?	S	H	R	S	S	R	H	R	R	S
103	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
105	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
106	R	--	--	R	R	H	H	H	H	H	S	H	--	--	H
107	R	R	R	R	R	R	R	S	R	R	H	S	R	H	R
108	R	R	R	R	R	S	S	R	R	R	R	--	--	--	R
109	R	S	S	R	R	R	H	S	S	R	R	H	R	R	S
110	S	S	?	S	S	H	S	S	S	S	S	S	S	S	S
111	R	R	S	R	--	S	R	H	H	S	H	H	S	H	H
112	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
113	S	S	S	R	S	S	S	S	S	S	S	S	R	S	H
114	R	S	R	R	S	S	H	S	H	H	S	S	S	R	S
115	R	R	R	R	R	H	H	H	R	R	R	R	H	R	R
116	R	S	S	R	R	H	H	R	S	H	R	--	H	S	H
117	R	R	R	--	R	R	R	R	--	R	H	R	R	R	R
118	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
119	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
120	S	S	S	S	S	S	S	R	S	S	R	S	S	S	S
121	R	R	R	R	R	H	S	H	R	H	--	S	R	S	S
122	S	S	R	S	S	R	S	S	H	R	R	R	S	S	R
123	S	S	R	R	S	S	S	H	H	H	R	S	S	S	R
124	S	S	R	R	R	H	S	H	H	--	H	R	S	S	S
125	R	R	R	R	R	R	R	R	H	--	R	R	R	R	R
126	S	R	S	R	R	R	R	H	R	R	R	S	H	--	R
127	R	R	R	R	S	S	H	R	R	R	R	R	R	R	R

128	S	R	R	S	S	S	S	H	S	H	R	S	S	R	S
129	R	R	R	R	R	R	R	R	H	R	R	R	R	R	R
130	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
131	R	R	R	R	R	H	H	H	R	R	R	R	R	R	R
132	S	R	S	S	R	R	R	R	R	R	R	S	H	R	H
133	R	R	R	R	R	R	H	R	H	H	R	R	R	R	R
134	S	S	S	S	S	S	R	S	S	S	S	S	R	R	R
135	R	R	R	R	R	H	R	R	R	R	R	R	R	R	R
136	R	R	S	R	R	R	R	R	H	S	S	R	H	S	--
137	S	S	S	R	R	S	H	R	S	H	S	S	S	--	--
138	R	R	R	R	R	--	R	R	R	R	H	H	R	R	R
139	R	R	R	R	R	R	R	R	H	R	R	R	H	H	--
140	R	R	R	R	R	S	H	H	S	H	H	S	H	S	S
141	S	S	S	S	S	S	S	S	S	S	S	S	S	--	S
142	--	R	R	R	R	R	R	H	H	H	H	H	R	R	H
143	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
144	R	R	R	R	R	H	H	S	R	R	S	H	H	H	H
145	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S
146	S	S	S	S	S	R	S	R	R	S	R	R	R	R	H
147	S	--	R	S	S	H	R	R?	S	S	S	S	S	S	H
148	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
149	R	R	R	R	R	R	R	R	R	R	R	R	H	H	R
150	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R
151	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
152	S	R	S	S	R	S	S	--	S	S	S	S	S	S	H
153	R	R	R	S	R	S	R	R	H	H	H	S	S	H	S

154	R	S	S	R	S	R	S	R	R	H	S	H	R	S	R
155	R	S	S	S	S	S	S	R	R	H	S	S	S	R	H
156	S	--	S	S	S	S	S	S	S	S	S	S	S	S	S
157	S	S	R	R	R	S	R	H	H	S	H	--	H	H	H
158	R	R	R	R	R	S	H	R	H	H	R	R	[redacted]	H	R
159	R	S	S	R	--	R	H	R	S	S	S	S	R	R	R
160	R	R	R	S	R	S	R	H	S	H	R	S	R	--	--
161	R	R	S	S	R	H	S	S	R	S	R	H	S	S	H
162	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R
163	R	R	R	S	R	S	S	S	H	H	S	S	S	S	S
164	R	R	R	--	R	S	S	R	R	R*	--	S	S	--	R
165	R	R	R	R	R	R	R	R	R	R	R	R	R	H	R
166	S	S	S	S	--	S	S	S	S	S	--	S	S	--	--
167	R	R	R	R	R	H	H	S	R	H	S	--	R	H	H
168	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
169	R	R	S	R	S	H	H	S	S	H	H	S	H	H	R
170	S	S	S	R	R	S	R	S	S	S	S	S	H	R	S
171	R	S	S	S	R	S	S	S	H	S	S	R	--	H	--
173	R	R	S	R	R	S	S	S	R	R	--	H	S	H	H
174	R	R	S	R	R	R	H	R	R	H	R	R	R	S	R
175	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
176	S	S	S	S	--	S	S	S	S	S	S	R	S	--	S
177	R	R	R	R	R	R	R	R	R	R	R	R	R	--	R
178	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S
179	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
180	S	S	S	S	S	S	S	S	S	--	S	S	S	S	S

181	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
182	R	R	--	R	--	R	H	H	R	R	R	R	R	R	R
183	S	R	S	--	S	S	S	R	S	R	S	R	S	S	H
184	R	R	S	R	R	R	R	S	S	R	R	R	H	S	S
185	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
186	S	S	R	R	S	S	S	S	S	S	--	R	H	S	S
187	R	R	R	--	R	R	R	H	R	R	R	H	H	H	H
188	R	S	R	R	R	S	--	S	S	H	S	H	H	S	R
189	--	R	R	R	R	R	R	--	R	H	R	H	H	H	H
190	R?	S	S	S	S	R	R	H	R	H	H	H	R	R	S
191	R	--	S	R	R	R	R	R	R	R	R	R	R	H	H
192	S	S	S	R	S	R	H	S	S	H	S	R	S	S	S
193	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
194	R	R?	R	R	R	S	S	R	S	R	S	H	S	S	H
195	S	S	R	R	S	S	R	S	S	S	H	S	S	H	H
196	S	S	R	S	R	R	S	S	S	H	H	S	S	S	S
197	R	R	R	R	R	R	R	H	R	R	R	--	R	R	R
198	R	R	R	R	R	S	R	H	R	S	S	R	R	R	R
199	R	R	R	R	R	R	R	R	R	R	R	R	--	R	R
200	R	R	R	R	R?	R	R	R	R	R	R	R	R	R	R
201	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S
202	R?	R	R	R	R	R	R	R	R	R	R	R	R	R	--
203	S	R	R	S	S	R	R	S	S	S	R	S	R	H	H
PI 534918	S	S	S	S	S	S	S	S	S	S	S	S	S	S	--
PI 256417	R	R	R	--	R	R	R	R	R	R	R	R	R	R	R

Appendix C

CAUSAL AGENT OF BACTERIAL LEAF SPOT OF LIMA BEAN

Introduction

History of bacterial brown spot. One particular pathovar of the common bacterial pathogen *Pseudomonas syringae, pv. syringae*, hereby referred to as PSS, is the causal agent for a myriad of plant diseases, including what is commonly referred to as bacterial brown spot in bean (Muedi et al., 2015). Bacterial brown spot was first reported by Walter Burkholder in 1930 on common beans (Harveson, 2013). Initially thought to be a rare disease, brown spot is a major pathogen of several legume species throughout the United States (Jung et al., 2003). This bacterial pathogen has been observed in dry bean (*Phaseolus vulgaris* L.), snap bean (*Phaseolus vulgaris* 'Derby'), pea (*Pisum sativum* 'Bolero'), soybean (*Glycine max*), and alfalfa (*Medicago sativa*) (Fogg et al., 2004; Muedi et al., 2015). Bacterial brown spot is a problem in many places throughout the world where beans are grown, including the United States, South Africa, and South and Central America (Muedi et al., 2015). In South Africa, this pathogen was responsible for 55% yield loss of dry bean in 2002, along with up to 25% estimated yield loss in the United States (Muedi et al., 2015; Singh & Schwartz, 2010). The potential yield loss makes brown spot one of the more destructive disease of bean crops in the United States (Jung et al., 2003).

Life cycle. In the first phase of *Pseudomonas syringae*'s life the pathogen lives as an epiphyte on the surface of plant leaves and tissues (Hirano et al., 2000; Rouse et al., 1985). The pathogen must passively gain entry into the plant which it can accomplish through a variety of ways, the first being entry through a stomate. The second method involves accessing the plant apoplast via injury to the plant (Hirano et al., 2000). *P. syringae* is unique in that it contains a gene, *INA*, that encodes for an ice-nucleation protein (Hirano et al., 2000). This protein causes water to form ice crystals at temperatures above freezing, leading to damage throughout the affected plant tissues, which can, in turn, provide access for various pathogen including *P. syringae* (Hirano et al., 2000; Lindow et al., 1982). Once inside the plant, *P. syringae* begins the endophytic half of its life (Xin et al., 2017). This pathogen is both biotrophic and necrotrophic with an end goal of multiplying within its hosts apoplastic tissue (Xin et al., 2017). In order to bypass the hosts detection of pathogen-associated molecular patterns (PAMPs), *P. syringae*'s T3SS produces two distinct phytotoxins, coronatine and syringomycin (Xin et al., 2017). Coronatine effectors bind to jasmonic acid receptors, locking stomata in the open position, thus allowing for further infection by *Pseudomonas* (Melotto et al., 2008; Katsir et al., 2008). Syringomycin, on the other hand, improves conditions of the host to provide a more conducive environment to *P. syringae*. Examples include the induction of pore formation as well as increasing leaf wetness on the outer portion of leaf tissue (Hutchison et al., 1995). Humid conditions are an abiotic factor that favors *Pseudomonas* growth leading to higher virulence and aggregation on plant tissues (Hirano et al., 2000; Monier and Lindow, 2004; Dechesne and Smets, 2012).

Signs and Symptoms and identification. Bacterial brown spot presents itself as water-soaked lesions along the margins of infected leaves (Figure 49) (Fogg et al., 2004). These spots will, within several days, become chlorotic and then necrotic and spread throughout the leaf, eventually leading to leaf death (Fogg et al., 2004). Lesion samples can be cut and grown on nutrient agar at 21-23°C to isolate the bacteria. The isolated bacteria can be identified as PSS if it fluoresces on King's B agar after 24-48 hours (Fogg et al., 2004). Fatty Acid analysis as well as sequencing of the extracted genetic material is another, more exact method of identification (Kunitzky et al., 2005).

With the MAR having some of the highest production of lima beans within the United States (USDA-NASS, 2019), the discovery of this pathogen is worrying and requires careful attention in order to minimize its spread.

Materials and Methods

Inoculum acquisition, isolation, and storage. Symptomatic leaf tissue from the lima bean variety ‘C-Elite’ was obtained by the University of Delaware Plant Diagnostic Clinic in August 2020 from a commercial grower in Sussex Country, Delaware. The diseased leaf tissue displayed necrotic lesions with yellow ‘halos’ along the leaf’s margin (Figure 49) Symptomatic leaves were surface disinfested in 0.9% NaOCl for 90 s and rinsed in sterile water before abscising a necrotic lesion and macerating it within a 2mL microcentrifuge tube containing 1mL of sterile deionized (DI) water. The macerated leaf solution was triple streaked onto King’s B agar plates and grown for two days at room temperature. Fluorescent colonies were identified using Spectroline^{TM} UV Darkroom Cabinet (Fisher Scientific) with a wavelength of 364nm. These colonies were triple streaked onto additional King’s B agar plates and allowed to grow for an additional two days, again at room temperature. This process was repeated until a pure culture was believed to have been achieved. Pure cultures were maintained in a digital incubator at 20°C and transferred via single streak every 14 days.

Identification. In addition to screening for fluorescent colonies, pure samples were sequenced using fatty acid methyl esters (FAME) analysis at Microbial ID, Inc.

(Kunitsky et al., 2005). Results proved that the isolated colony was indeed *Pseudomonas syringae* pv. *syringae* (PsS) with a similarity index (SIM) of 0.941 (Microbial ID, Inc). This identification technique was again used to confirm a successful resiolation of PsS after completion of Koch's postulates.

Inoculation. Koch's postulates were completed using this newly isolated PsS strain. Two plates of nutrient agar with carpeted PsS were maintained in in a digital incubator at 20°C. Simultaneously, four 15.2-centimeter pots, two for control and two for inoculum, containing 4 seeds of the lima bean cultivar Fordhook 242 were grown in a greenhouse set at 85°C to the unifoliate stage. Upon observation of unifoliate leaves, the PsS from the two previously mentioned nutrient agar plates were suspended in 50mL of sterile DI water to achieve an approximate concentration of $1x10^6$ bacteria/mL. The bacterial suspension was then transferred to a clean mister bottle. An identical mister bottle with sterile DI water was also filled. Before applying the bacterial inoculum, all unifoliate leaves were surface sterilized using 70% ethanol and allowed to dry. Control plants were misted with DI water until runoff was observed, while the other two pots were sprayed with the bacterial solution. All plants were transferred to a transparent plastic tub on a 26°C heatmat within the greenhouse. Upon observation of yellow halos and necrotic lesions along the margins of the inoculated unifoliates, the symptomatic leaves were harvested.

Re-isolation. Three lesions were cut from the infected leaves and allowed to stream into 1mL of sterile DI water for 30 minutes (Figure 50). After, 200µL of the bacterial suspension was transferred to three King's B agar plates and spread using a glass rod. Plates were then placed in a 20°C incubator for two days at which point fluorescent colonies were identified under the above mentioned 364nm UV light and transferred via triple streak to fresh King's B agar plates. This process was repeated until pure colonies were observed. Pure colonies were kept at maintenance conditions on nutrient agar until FAME analysis could be conducted. Maintained isolates were single streaked onto King's B agar and stored at maintenance temperatures for 4 days. Each day, isolates were put under UV light along with a *Pectinobacteria carotovora* isolate also on King's B agar to serve as a negative control whole assessing for fluorescence.

Figure 49 Bacterial leaf spot on lima bean trifoliate.

Figure 50 *Pseudomonas syringae* pv. *syringae* streaming out of a section of infected lima bean leaf tissue. Note: 'fuzzy' area within the red circle are a cloud of the bacteria that had previously been streaming out. (Courtesy of Jill Pollok).

Results and Discussion

Several methods of reisolating the bacteria were tried, though their effectiveness for the desired bacteria was questionable. The first methods involved plating a single 5mm x 5mm square of leaf tissue with 50% containing the yellow halo and/or the necrotic lesion and the other 50% consisting of healthy leaf tissue after cleaning sterilizing the leaf tissue in a 10% bleach solution for 90 seconds. This method did not produce ideal results, with multiple unique bacterial colonies growing within extremely close proximity to each other. The second method involved macerating the same type of leaf tissue as described above in a microcentrifuge tube with 1mL DI water, then triple streaking this bacterial suspension onto King's B agar. This method produced better results and cleaner colonies. Samples were sent for FAME analysis on multiple occasions, but results consistently showed that the isolated bacteria were, *Pseudomonas putida*. This saprophytic bacterium is most often found within the soil, leading us to theorize that as the seed grew, the leaves were coming in contact it, thus leading to contamination during reisolation attempts (Albert and Anderson, 1987). A series of tests with the *P. putida* and *P. syringae* showed that the former grew larger colonies at a faster rate than PsS, leading to this pathogen potentially covering the sought after PsS. In order to mitigate this contamination, all leaves were surface sterilized with 70% ethanol before the inoculum was administered. In addition, the sterilization of leaves, the plants were also stored in a large clear plastic tub to ensure that the leaves were not in contact with each other, or the sides of the container. Previously, humid conditions were maintained by placing a clear plastic bag over the individual plants, leading to leaves being plastered against the plastic. No tests were conducted to determine if this was a source of contamination, but the factor was removed for the final, successful test.

As mentioned above, FAME analysis showed that the first isolation was indeed *Pseudomonas syringae* pv. *syringae* (PsS) with a similarity index (SIM) of 0.941 (Microbial ID, Inc). Analysis of the reisolation confirmed that Koch's postulates was successful and that *Pseudomonas syringae* pv. *syringae* was successfully isolated with a similarity index of 0.916 to 0.954 over three separate isolations. From these results, we can confidently conclude that *Pseudomonas syringae* pv. *syringae* is the causal agent of bacterial leaf spot of lima beans in Delaware.

REFERENCES

Berge O, et al. “A user’s guide to a data base of the diversity of *Pseudomonas syringae* and its application to classifying strains in this phylogenetic complex.” PLoS One. 2014; 9:e105547.

Clarke, C. R., Cai, R., Studholme, D. J., Guttman, D. S., Vinatzer, B. A. “*Pseudomonas syringae* strains naturally lacking the classical *P. syringae* hrp/hrc Locus are common leaf colonizers equipped with an atypical type III secretion system.” Molecular Plant Microbe Interactions. 2010; 23:198–210. [PubMed: 20064063]

Dechesne, A., Smets, B. F. “Pseudomonad swarming motility is restricted to a narrow range of high matric water potentials.” Applied Environtal Microbiology. 2012; 78:2936–40. [PubMed: 22327576]

Evans, T. A., Mulrooney, R. P., Gregory, N. F., and Kee, E. (2007). “Lima Bean Downy Mildew: Impact, Etiology, and Management Strategies for Delaware and the Mid-Atlantic Region, U.S.” Plant Disease, *91*(2), 128–135. https://doi.org/10.1094/pdis-91-2-0128

Fogg, M. L., Reedy, R. M., Kobayashi, D. Y., Johnston, S. A., and Kline, W. L. (2004). “Occurrence of bacterial blight of fenugreek caused by *Pseudomonas syringae* pv. *syringae* and isolation of the pathogen from infested seed.” Canadian Journal of Plant Pathology, 26(2), 129–133. https://doi.org/10.1080/07060660409507123

Frederick, A. and Anderson, A. J. "The Effect of *Pseudomonas putida* Colonization on Root Surface Peroxidase." Plant Physiology 85.2 (1987): 537-41. Print.

Gutiérrez-Barranquero, J. A., Cazorla, F. M., and de Vicente, A. (2019). “*Pseudomonas syringae* pv. *syringae* associated with mango trees, a particular pathogen within the “hodgepodge” of the *Pseudomonas syringae* complex.” Frontiers in Plant Science, 10(May), 1–20. https://doi.org/10.3389/fpls.2019.00570

Hirano, S. S., Upper, C. D. “Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*-a pathogen, ice nucleus, and epiphyte.” Microbiology Molecular Biology Review. 2000; 64:624–53. [PubMed: 10974129]

Jung, G., Ariyarathne, H. M., Coyne, D. P., and Nienhuis, J. (2003). “Mapping QTL for bacterial brown spot resistance under natural infection in field and seedling stem inoculation in growth chamber in common bean.” Crop Science, 43(1), 350–357. https://doi.org/10.2135/cropsci2003.3500

Katsir, L., Schilmiller, A. L., Staswick, P. E., He, S. Y., Howe, G. A. "COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine." Proceedings of the National Academy of Science, U S A. 2008; 105:7100–5. [PubMed: 18458331]

Kunitsky, C., Osterhout, G., and Sasser, M. 2005. "Identification of microorganisms using fatty acid methyl ester (FAME) analysis and the MIDI Sherlock Microbial Identification System." Encyclopedia of Rapid Microbiological Methods. MIDI, Inc., Newark, DE.

Lindow, S. E., Arny, D. C., "Upper, C. D. Bacterial ice nucleation: a factor in frost injury to plants." Plant Physiology. 1982; 70:1084–9. [PubMed: 16662618]

Melotto, M. et al. "A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein." Plant Journal. 2008; 55:979–88. [PubMed: 18547396]

Monier, J. M., Lindow, S. E. "Frequency, size, and localization of bacterial aggregates on bean leaf surfaces." Applied Environmental Microbiology. 2004; 70:346–55. [PubMed: 14711662]

Muedi, H. T. H., McLaren, N. W., and Fourie, D. (2015). "Reaction of selected common bean germplasm accessions to bacterial brown spot in South Africa." Crop Protection, 77, 87–93. https://doi.org/10.1016/j.cropro.2015.07.011

Rouse, D. I., Nordheim, E. V., Hirano, S. S., Upper, C. D. "A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens." Phytopathology. 1985; 75:505–509.

Singh, S. P., and Schwartz, H. F. (2010). "Breeding common bean for resistance to diseases: A review." Crop Science, 50(6), 2199–2223. https://doi.org/10.2135/cropsci2009.03.0163

USDA-National Agricultural Statistics Service. 2019. 2017 Census of Agriculture.

Xin, X., Kvitko, B., Yang He, S. (2017). "*Pseudomonas syringae*: what it takes to be a pathogen." Physiology & Behavior, 176(12), 139–148. https://doi.org/10.1038/nrmicro.2018.17.

Appendix D

CAUSAL AGENT OF BROWN BEAN OF LIMA BEAN IN DELAWARE

Introduction

Rhizoctonia solani is a plant pathogenic basidiomycete. It was first recorded in 1858 by Julius Kühn and has since been seen throughout the world (Parmeter, 1965). In legumes, *R. solani* often causes pod, root, and hypocotyl rot (Galindo, 1983a; Warren et al., 1972). On bean pods, the signs and symptoms of this fungal pathogen typically manifest themselves as depressed brown lesions, while the infected beans themselves are brown versus their normal pale green (Figure 51). (Hedgcock, 1904). *R. solani* has does not create any spores of its own but has been observed overwintering in plant debris (Agrios, 2005; Galindo, 1983b). With the recent sighting of this potentially destructive pathogen, it is imperative that growers and extension services keep an eye on weather conditions and new reports to minimize its spread and to warn growers of potential epidemics.

Figure 51 50 Infected lima bean within the pod (left), lima beans at varying stages of infection (right).

Materials and Methods

Inoculum acquisition, isolation, and storage. In November of 2019, symptomatic lima bean pods of the ‘Cypress’ variety were brought to the University of Delaware’s Georgetown campus. These pods contained beans with a brown discoloration that were only observable when the pods themselves were open. Approximately 1-5% of beans were found to have this discoloration. Symptomatic beans were collected, and surface disinfested in 0.85% sodium hypochlorite for 30 seconds and rinsed in sterile deionized water for 30 seconds. They were then sectioned into four pieces and plated onto potato dextrose agar (PDA) amended with 50µg/ml penicillin G and streptomycin sulfate. Petri dishes were incubated at 23ºC and observed for colony morphology. Pure cultures were obtained and tan colonies had mycelia with right angle branching and septations near the branch, consistent with the description of *Rhizoctonia solani* Kuhn (Sneh et al. 1991).

Identification. A representative isolate was selected for DNA extraction and pathogen identification was confirmed by sequencing of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA using primers ITS4/ITS5 (White et al. 1990). The isolate was identified as *Rhizoctonia solani* AG 4 (99.9% sequence identity with GenBank Accession [MN106359.1].) The sequence was deposited in GenBank as accession number MW560551.

Inoculation and Re-isolation. To confirm Koch's postulates, 10 asymptomatic 'C-Elite Select' lima bean pods at the succulent seed stage were harvested from a university field and surface sterilized in 75% ethanol for 60 seconds, then rinsed in Milli-Q water. The 10 pods were then evenly divided into two 150 mm Petri dishes containing a single 150 mm filter paper. Prior to this, the newly identified *R. solani* AG 4 was grown out on three-quarter-strength PDA for approximately 14 days. Agar plugs one mm^2 were cut and placed hyphae-side down and 0.5 cm apart along the top of every pod, resulting in 5-8 plugs added per pod. The filter paper was then saturated with 5 mL of Milli-Q water with excess water being poured off and the plates sealed with Parafilm. Control pods were kept in identical conditions excluding the agar plugs. Petri dishes were kept in a biosafety cabinet at room temperature for one week until symptoms began to manifest on the pod surfaces, at which point the beans from infected pods were removed and placed on PDA, three to a plate. The plates were stored in identical conditions and monitored for 5 days until tan colonies were observed. Colonies were sub-cultured once and placed in an incubator at 20°C for 1 week. Culture morphology was consistent with the original isolate. No symptoms were observed on control pods. Reisolated cultures were again proven to be *Rhizoctonia solani* AG 4 via sequencing of the ITS region.

Results and Discussion

As mentioned above, sequencing of the ITS 4/5 region confirmed that the pathogen in question for both the initial and reisolation was *Rhizoctonia solani* AG 4. *R. solani* is most commonly associated with root rot, though in recent years, the no root symptoms have been found in relation to the “brown bean” symptoms observed during harvest (Sharma-Poudyal et al., 2015). From these results, we can conclude that *Rhizoctonia solani* AG 4 is the causal agent of brown bean of lima bean in Delaware.

REFERENCES

Agrios, G. N. “Diseases Caused by Fungal-Like Organisms.” Plant Pathology, 5th ed., Elsevier, 2005, p. 409-414.

Galindo, J. J. "Effect of Mulching on Web Blight of Beans in Costa Rica." Phytopathology 73.4 (1983): 610. Print.

Galindo, J. J. "Source of Inoculum and Development of Bean Web Blight in Costa Rica." Plant Disease 67.9 (1983): 1016. Print.

Hedgcock, G. G. "A NOTE ON RHIZOCTONIA." Science 19.476 (1904): 268. Print.

Parmeter, J. R. “Rhizoctonia Solani, Biology and Pathology: Based on an American Phytopathological Society Symposium on Rhizoctonia Solani, Held at the Miami Meeting of the Society, October, 1965.” Berkeley: U of California, 1970. Print.

Sharma-Poudyal, D., et al. 2015. Plant Disease, 99(5), 604-613. American Phytopathological Society, St. Paul, MN. doi:10.1094/pdis-08-14-0803-re

Sneh, B., et al. 1991**. “**Identification of *Rhizoctonia* Species**.”** APS Press, St. Paul, MN. https://scholar.google.com/scholar_lookup?hl=en&publication_year=1991&author=B.+Sneh&author=L.+Burpee&author=A.+Ogoshi&title=Identification+of+Rhizoctonia+Species

Warren, H. L., Helfrich, R. M., and Blount, V. L. 1972. “Evaluation of lima bean varieties and selections for resistance to *Rhizoctonia solani*.” Plant Disease Report. 56:286-270.

White, T. J., et al. 1990. Page 315 in**:** PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.