Investigating the biology, epidemiology, and management of the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* from Wisconsin

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Phytophthora infestans (Mont.) deBary causes late blight, one of the most important diseases of potato and tomato worldwide. Multiple aspects of the biology and epidemiology of three recently identified clonal lineages of *P. infestans*, US-22, US-23, and US-24 were investigated to further an understanding of changing *P. infestans* populations and enhance management of late blight. Collection and characterization of *P. infestans* isolates from Wisconsin from 2009 to 2012 revealed three new clonal lineages, US-22 (A2 mating type, mefenoxam sensitive), US-23 (A1, intermediately sensitive), and US-24 (A1, intermediately sensitive). In an investigation of host range, it was discovered that the US-22, US-23, and US-24 lineages all produced lesions and sporulated well on potato and also on hairy nightshade, a common solanaceous weed. Sporulation on tomato was limited for US-24, but not for US-22 or US-23. Through testing of low temperature survival on tomato seeds, survival by all three lineages was seen for 14 days at -3°C and for 3 months at 0°C, indicating the potential for overwintering of *P. infestans* in tomatoes buried in fields or in protected areas such as compost piles. Resistance in tomato cultivars was explored and cultivars with *Ph* late blight resistance genes had variable efficacy against US-22, US-23, and US-24. Surprisingly, effective broad-spectrum resistance was found in some heirloom cultivars, including 'Wapsipinicon Peach' and

'Matt's Wild Cherry'. These cultivars can be immediately used by growers and further explored by breeders. Several organic-approved and biorational fungicides, particularly EF400 and Zonix, showed efficacy against the new lineages, indicating the potential for more environmentally friendly management of tomato late blight through non-conventional fungicides. The pathogen characteristics of lesion growth and sporulation rate were experimentally determined and simulated epidemics generated for each lineage using the LATEBLIGHT simulation model, resulting in conclusions that the US-23 lineage may have the potential to initiate the largest, fastest epidemics and be the most likely to persist in the pathogen population. Together, these results will further an understanding of the biology and epidemiology of *P. infestans* and lead to enhanced control of the devastating disease of late blight.

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CHAPTER 1:

Introduction

Introduction to the Pathogen

Late blight, caused by *Phytophthora infestans* (Mont.) deBary, is one of the most limiting diseases of potato production worldwide. Originating in the Peruvian Andes (33) or the highlands of central Mexico (35, 43), *P. infestans* spread to the U.S. in the 1800s before arriving in Ireland in the mid 1800s. There it contributed to the Irish Potato Famine, an event that led to widespread starvation and migration of the Irish to the U.S. More than 160 years later, this disease is still a problem, and many clonal lineages of the pathogen infect both potato and tomato. In the U.S., it has been estimated that this disease costs growers $507 per acre per year in lost revenue and fungcide costs, adding up to $287.8 million annually (45). Economic data do not exist for tomato, but in cool, wet years, losses can be high. Worldwide, *P. infestans* has been estimated to cause $6.7 billion dollars in losses in potato production each year (48).

P. infestans is an oomycete belonging to the kingdom Chromista. It is heterothallic, requiring two mating types, designated A1 and A2 for sexual oospores to form (30). Oospores are thick-walled and can survive desiccation and freezing temperatures (24). This is in contrast to the asexual sporangia, which are short lived and quickly die if exposed to adverse conditions. Sporangia are the primary means of dispersal within a growing season and can infect directly or, at lower temperatures, differentiate to produce 8-10 biflagelate swimming zoospores (22). The organism itself is considered a hemibiotroph, as it initially infects the plant as a biotroph, but then changes to a necrotrophic phase (57). Therefore, in the absence of living plant material and sexual oospores, *P. infestans* dies and must be reintroduced from other areas. This is often the case in areas with harsh winters, however, potato tubers protected from freezing, such as those buried in fields or in cull piles can initiate epidemics in northern areas (7). Additionally, the

pathogen may be harbored in infected seed tubers and thereby be reintroduced to an area (52, 76).

Although the earliest accounts of *P. infestans* were on potato, many strains of the pathogen, or clonal lineages, infect both potato and tomato. Foliar symptoms on these hosts are similar and include dark brown circular lesions not confined within veins, often with a halo of fuzzy white sporangial growth visible under humid conditions. Stems may become similarly infected. Potato tuber and tomato fruit infection are both characterized by sunken, firm, darkened lesions. Internal infected tuber tissue has a reddish granular appearance without distinct margins. Tuber infection can be particularly devastating, as the disease can spread in storage and destroy the harvested crop. Late blight infection may also cause an entry point for other pathogens, such as soft rotting bacteria (*Pectobacterium carotovorum*), which further destroy the crop (28, 76)

Review of the Literature

P. infestans has been intensively studied for more than 150 years, and yet still remains an important pathogen of potato and tomato wherever these crops are grown. In the U.S. where sexual recombination is not known to occur, distinct clonal lineages have been identified, usually having been introduced from Mexico, which then reproduce asexually to cause large-scale epidemics (13, 27, 35, 38, 42, 49, 56, 63, 70, 71). These asexual descendants of a single genotype are often referred to as clonal lineages (29), which are named sequentially as they are identified in the U.S. with 'US' followed by a dash and a number (i.e. US-1). These designations that can be associated with several genotypic characteristics such as the RG57 restriction

fragment length polymorphism fingerprint (36) and allozyme fingerprint (37) and several practical phenotypic characteristics important for management such as mating type and sensitivity to the fungicide active ingredient mefenoxam (14, 56, 71, 72). Many clonal lineages are represented by single isolates or by geographically and temporally limited findings, and just a few lineages are responsible for most of the destruction caused by this disease (38). The US-1 lineage was historically the predominant clonal lineage worldwide, but in the early 1990s, the US-8 lineage appeared and almost entirely displaced the US-1 lineage and a few others, including US-6 and US-7 (35, 38). Several other clonal lineages have since been identified, with only a few being widespread or prevalent (US-11, US-17), but US-8 had predominated until recently when US-22 made a widespread appearance in 2009 (38, 69).

New clonal lineages are usually introduced to the U.S. from Mexico, as genetic recombination is prevalent in that region (34, 44). Variations in pathogen populations have been distinguished in several different ways. In early *P. infestans* studies, the primary means of assessing variability was by race designation (67). In the 1980s, variation at the *glucose-6-phosphate isomerase* and the *peptidase* allozyme loci, identified using starch gel electrophoresis, were found to be unique among different isolates, and the combination of these enzyme characteristics distinguished numerous clonal lineages (77). The use of cellulose acetate electrophoresis further streamlined this testing, making genotype designation fast and simple (37). However, new pathogen strains began to appear that had different phenotypic characteristics but were not distinguished with the two allozyme loci. Therefore, a restriction fragment length polymorphism (RFLP) assay was developed to evaluate the RG57 locus (36). Numerous other molecular markers have been used to determine genetic differences among

isolates including AFLPs, SSRs, mitochondrial DNA haplotype analysis, SNPs, and sequence analysis (1, 8, 10, 83) but variation detected using the allozyme assay and the RG57 RFLP assay are nearly always determined for each new isolate and are key to assigning a clonal lineage designation.

Host range is a trait that can vary between clonal lineages. Potato is considered the original host of *P. infestans* but lineages have emerged with reported specificity on tomato (27, 38, 49, 61, 70). In particular, so-called "tomato isolates" are characterized by the ability to initiate infection and sporulate while causing little or no leaf necrosis, while "non-tomato" isolates will quickly cause necrosis when inoculated onto tomato (80). The infection of other potential hosts has also been explored. Eggplant, green pepper, and tobacco are usually considered non-hosts (66), while petunia can become infected (3-5). In Mexico, there are numerous wild solanaceous plants that host the pathogen, but there are many fewer in the US. Erwin and Ribeiro (15) list pages of hosts, but few can become infected under anything other than ideal lab conditions, and potato and tomato remain the most common hosts from which the pathogen is recovered in the field. However, *P. infestans* clonal lineages US-1, US-8, US-11 and several unknown lineages have occasionally been collected from hairy nightshade (*S. physalifolium*) in the field (2, 16, 18, 39, 41, 65, 68, 78). Isolates have been collected from black nightshade (*S. nigrum* or *S. ptycanthum*) in the field in Wales (19) and the Netherlands (25), but black nightshade is usually considered a non-host (12, 66). Infection of bittersweet nightshade (*S. dulcamara*) has been observed only occasionally in the field (11, 25) and overwintering on this perennial host is unlikely (32). Host range can vary between clonal lineages, so new lineages cannot be assumed to have the same weed host preferences as previous lineages.

Recently, the genome of *P. infestans* was sequenced and this revealed a large repetitive, polymorphic region that is thought to play a role in the unique ability of this pathogen to adapt and cause disease in spite of the best efforts control efforts (47). The discovery of late blight resistance genes seemed to promise a solution to this devastating disease, but perhaps due to that highly variable region of the genome, these have had limited success, with some genes lasting only one to two years (28, 47). Nevertheless, the search is ongoing for durable resistance genes and/or horizontal resistance (26, 28, 64). There are currently five tomato late blight resistance genes, designated *Ph-1* through *Ph-5,* that have been found in the wild tomato species *S. pimpinellifolium* and bred into commercial tomato lines (26, 31, 58). Resistance genes have similarly been found and used in potato, mostly from the wild species *S. demissum* and more recently from *S. bulbocastanum,* and several others (62, 73). It is not unusual for isolates within a single clonal lineage to have different races, (6, 21, 40) and even a more thorough genotypic analysis with SSR markers and mtDNA haplotyping many not detect the genotypic diversity that is observed phenotypically (6, 46). Clonal lineages that have been present the longest often exhibit the greatest amount of genetic diversity, but usually the greatest diversity is observed between lineage (40, 51).

Mefenoxam, a phenylamide fungicide, was the first systemic fungicide effective against this pathogen, and in some cases remains the best (9). Mefenoxam inhibits ribosomal RNA synthesis by blocking the incorporation of uridine (23). However, clonal lineages have arisen with intermediate or no sensitivity to this fungicide (17, 63, 82). The genetic basis for this is still unclear (23, 53-55). Regardless, the response to mefenoxam is often used to characterize isolates and has direct applications for late blight management.

Calendar-based fungicide applications historically were the primary means of late blight control, but the introduction of disease forecasting systems in some regions has changed this. BLITECAST was developed in 1975 at Pennsylvania State University to incorporate both the Hyre (50) and Wallin (81) models for late blight prediction (60). BLITECAST uses temperature and relative humidity data to determine severity values, which accumulate to predict when the pathogen is most likely to be actively causing disease. This program was modified for use in Wisconsin and released for private use in 1983 (74, 75). This system is still used in Wisconsin and surrounding states and has been reported to save up to two fungicide sprays per season, which lessens the environmental impact of potato production and saves growers money without sacrificing yield (74).

Most forecasting systems used in northern areas assume that the pathogen does not survive freezing temperatures, even though it does survive in potato cull piles (7). Survival in tomato plant parts has, to date, been understudied. Vartanian and Endo (79) showed some survival and subsequent seedling infection from buried tomato fruits, but these were not exposed to temperatures below freezing. Kirk (59) showed survival of some lineages for a short time at –3°C, but this was on media and not on plant tissue. DeBruyn (20) showed survival in several substrates at temperatures from –12 to –25°C, with manure as the best substrate and clay soil the poorest, indicating that the medium may influence survival of the pathogen.

Three clonal lineages of *P. infestans* have been recently identified in Wisconsin and the U.S. In the present work, isolates were collected from Wisconsin to determine the prevalence and distribution of these new lineages. Numerous aspects of the biology, ecology, and epidemiology of the US-22, US-23, and US-24 clonal lineages were studied in depth to achieve a

more complete understanding of these lineages and of the organism *P. infestans* as a whole, with the goal of enhancing management of this pathogen. The host range of isolates of US-22, US-23, and US-24 was determined on several nightshade weeds and cultivated solanaceous plants. The survival of *P. infestans* on tomato seeds at temperatures near and below freezing was explored to estimate the overwintering potential of this pathogen on tomato. Heirloom and hybrid tomato cultivars were investigated for potential resistance to the US-22, US-23, and US-24 lineages. Conventional and biorational fungicides were also tested for efficacy against these new lineages. To further investigate epidemiology, the biological characteristics of sporangial size, sporulation rate, and lesion growth rate were determined and the epidemiology investigated using simulation modeling. Together, these objectives will lead to a deeper understanding of the current *P. infestans* population. Additionally, this increased knowledge will contribute to better-informed management decisions resulting in fewer losses to the devastating disease of late blight.

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CHAPTER 2:

Characterization and distribution of three new clonal lineages of *Phytophthora infestans* causing late blight in Wisconsin from 2009 to 2012

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Abstract

Phytophthora infestans causes late blight of potato and tomato, a disease that has been estimated to cost U.S. potato growers $287.8 million annually. We collected isolates of *P. infestans* from Wisconsin from 2009 to 2012 and determined distribution of clonal lineages and mating types and sensitivity to the systemic fungicide mefenoxam. We also sought to evaluate the current utility of an analysis of the *Glucose-6-phosphate isomerase* (*Gpi*) allozyme locus for predicting mefenoxam sensitivity with the aim of delivering timely information to growers. Overall, 143 isolates were collected from 52 locations in 20 Wisconsin counties from 2009 to 2012. Three clonal lineages, US-22, US-23, and US-24, were identified and were novel to Wisconsin and the U.S. US-22 is of the A2 mating type and sensitive to mefenoxam, with *Gpi* 100/122. US-23 and US-24 are of the A1 mating type and primarily intermediately sensitive to mefenoxam, with *Gpi* 100/100 and 100/100/111, respectively. Because of this close correlation and the unique *Gpi* patterns for each lineage present, we were able to predict mefenoxam sensitivity directly from samples using the allozyme assay and quickly deliver management information to growers. Both mating types were present in Wisconsin in 2009 and 2010 but were spatially separated and no evidence of sexual recombination or soil persistence was detected. The presence of new clonal lineages of *P. infestans* in Wisconsin indicates a need for continued close monitoring of late blight to facilitate generation of timely information for enhanced short-term and long-term late blight management.

Introduction

Late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) deBary, is one of the most important diseases of potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) worldwide. In potato in the U.S., late blight is estimated to cost growers $507 per acre per year, equaling $287.8 million annually (Guenthner et al. 2001). Worldwide it can cause losses of $6.7 billion annually (Haverkort et al. 2008). In Wisconsin in 2011, potatoes were grown on 25,500 hectares, for a total production of 1.1 million metric tons and an average production value of $262.5 million (National Agricultural Statistics Service 2012). Tomato production covers a smaller area in Wisconsin, at 1,600 hectares, but is still important due to the broad geographic and grower profile of the crop. *P. infestans* causes late blight on nearly all tomato and potato plant parts: leaves, petioles, stems, and tomato fruit and potato tubers (Erwin and Ribeiro 1996). Tuber infection can be particularly devastating, as the harvested crop can be destroyed by late blight in storage and infected seed tubers can serve as a source of the disease for the subsequent production season.

P. infestans is found recurrently in the potato and vegetable producing regions of the U.S., but this pathogen is native to either the Peruvian highlands (Gomez-Alpizar et al. 2007) or the central valley of Mexico (Goodwin et al. 1994). Sexual recombination occurs freely in these latter two regions, as the two mating types, termed A1 and A2 are present in nearly equal proportions. The oospores formed when both mating types are in contact results in a highly diverse *P. infestans* population in these regions. This is in contrast to the population of *P. infestans* in northern North America. Both mating types are usually not in proximity and only limited evidence of sexual recombination in the U.S. and Canada has been reported (Goodwin et

al. 1998, Goodwin et al. 1995b, Kalischuk et al. 2012). Instead, reproduction, spread and persistence is almost entirely via asexual sporangia. These ephemeral sporangia are produced in abundance on late blight lesions during the growing season and can be wind-dispersed to infect potato and tomato in other regions (Erwin and Ribeiro 1996). These sporangia cannot survive desiccation or freezing temperatures, so *P. infestans* is normally eliminated from regions with harsh winters between growing seasons (Stevenson et al. 2001). However, oospores have the potential to survive desiccation and freezing temperatures in the soil (Stevenson et al. 2001, Fay and Fry 1997), so the presence of these in the northern U.S. would potentially greatly change the way late blight is currently managed in these regions, both because of increased winter survival, and because of increased genetic diversity.

Late blight is typically managed with numerous fungicide applications throughout the growing season (Gevens et al. 2011). There are several protectant fungicides that are effective and commonly used, but these must be applied before initial late blight infection to be effective. Ridomil was introduced in the 1970s, and its active ingredient, metalaxyl (enantiomer mefenoxam), has excellent systemic activity against *P. infestans* and many other *Phytophthora* species (Cohen et al. 1979). Ridomil could be applied after infection and still provide good control of the disease. However, in the late 1980s, the US-8 clonal lineage became widespread and was insensitive to mefenoxam (Goodwin et al. 1998, Marshall-Farrar et al. 1998). Perhaps because of continued use of mefenoxam, eliminating sensitive lineages, the US-8 clonal lineage quickly predominated the pathogen population. In Wisconsin, US-1 was almost completely displaced by US-8 in just two years (Marshall-Farrar et al. 1998). Since then, other lineages

have again emerged that have sensitivity to mefenoxam, so it is important to test each new lineage to determine if applications of mefenoxam may be an effective control measure.

Due to the economic importance of the potato industry in Wisconsin, late blight is a yearly and well-recognized disease risk in the state. However, after 2002 and until 2009, there were no detections of late blight in Wisconsin. Then in 2009, late blight was identified widely in the state, on both potato and tomato. To enhance understanding of the epidemic and the ecology of the current pathogen population, we collected isolates of *P. infestans* from Wisconsin production fields and gardens from 2009 to 2012 and determined clonal lineage, mating type, and mefenoxam sensitivity. In an effort to provide growers with timely management information we also sought to validate the utility of the relatively fast allozyme assay for distinguishing isolates in the current *P. infestans* population and quickly predicting mating type and mefenoxam sensitivity. Together this research will contribute to both short-term and long-term management of late blight in Wisconsin.

Materials and Methods

Collection and isolation of P. infestans. Isolates of *P. infestans* were obtained from diseased plant samples submitted to the Plant Disease Diagnostic Clinic or the Potato and Vegetable Pathology Lab at the University of Wisconsin by growers, professional crop consultants, county extension agents, state specialists, home gardeners, and regulatory agencies throughout Wisconsin during 2009, 2010, 2011, and 2012. Potato and tomato leaves expressing symptoms consistent with late blight were placed on a thin layer of water agar in a 9-cm-diameter petri dish for 48 hours to induce sporulation. With the aid of a dissecting microscope, a

1- to 2-mm^2 block of agar was contacted with several sporangia causing them to abscise onto the agar. The small block was placed on rye A agar (Caten and Jinks 1968) amended with rifampicin and ampicillin (rye A+rif+amp) and incubated at 18°C in darkness to generate pure cultures. Excisions from tomato fruit and potato tuber lesions were processed either as described above for leaf lesions or were plated directly on rye A+rif+amp and incubated at 18°C. In 2010, one isolate was obtained from each sample received. In 2009, 2011, and 2012, 1 to 13 isolates were obtained per sample, depending on sample quality. All cultures were maintained on rye A agar and single zoospore-derived cultures generated according to the method of Erwin and Ribeiro (1996) using sterilized water from Lake Mendota (Madison, Wisconsin). For long-term storage, two 8-mm-diameter agar plugs containing mycelia and sporangia from 2- to 4-week old actively growing cultures and three autoclaved hemp seeds were placed in a sterile 1.5 ml microcentrifuge tube with 1 ml sterile distilled water. Five tubes were made for each isolate, incubated at 18°C for 4 weeks to encourage colonization of the hemp seeds by the pathogen, and then stored at 12 to 18°C for long-term maintenance (modified from Lamour and Hausbeck (2001)).

Allozyme assay. For each isolate, an analysis of the *Gpi* locus using cellulose acetate electrophoresis was performed according to the method of Goodwin et al. (1995a). If sporulation was evident upon receipt of the sample, the assay was performed as outlined below. If sporulation was not evident, entire leaves or excisions from lesions of tomato fruit or potato tubers were placed directly on a thin layer of water agar in petri dishes and incubated at 18°C in complete darkness. After sporulation was visible (24 to 48 hours), sporulating tissue was excised and placed in a 1.5 ml microcentrifuge tube with 1 ml of sterile distilled water and agitated with

a vortex mixer to dislodge sporangia. After removing the leaf tissue, the tube was centrifuged at 13,000 rpm for 2 minutes to pellet the sporangia, and all but 50 to 75 μl of the supernatant was removed. The remaining supernatant and pellet were used to perform the assay according to Goodwin et al. (1995a) using the agar overlay. Migration distance of the *Gpi* proteins was determined by comparing bands from unknown isolates with known reference isolates and information on migration distances from previously published results was used to assign clonal lineage (Hu et al. 2012, Kalischuk et al. 2012).

Analysis using restriction fragment length polymorphisms (RFLP). Analysis of RFLP using the RG57 probe was performed on a subset (n=7) of *P. infestans* isolates by the laboratory of Dr. William E. Fry at Cornell University according to the method of Goodwin et al. (1992). Reference cultures from each year from each clonal lineage, as identified by the allozyme analysis, were submitted to corroborate the association between *Gpi* genotype and clonal lineage designation.

Mating type determination. All isolates collected during 2009 and 2010 were tested for mating type on rye A media. Plugs of mycelia and sporangia from 2- to 3-week-old cultures of new *P. infestans* isolates grown on rye A agar media were placed 4 to 5 cm apart from plugs of known A1 (US-1) and A2 (US-7) mating types on rye A agar media in separate 9-cm-diameter petri dishes. After 3 to 4 weeks of incubation at 18°C in complete darkness, portions of the culture at the interface of the two colonies were excised and examined microscopically. The presence of oospores indicated that the new isolate was of the mating type opposite to the known reference culture. Opposite mating type reference isolates were inoculated 4 to 5 cm apart from each other on rye A media as a positive control; isolates of the same mating type were similarly

inoculated on a single rye A plate as a negative control. A chi-square test was performed using SigmaPlot (Systat Software, San Jose, CA) to establish correlation between mating type and clonal lineage. Mating type was not tested for the 2011 and 2012 isolates but was inferred from the allozyme genotype.

Mefenoxam sensitivity. The colony diameter on rye A media was compared to growth on rye A amended with 100 ppm mefenoxam (Ridomil Gold SL, Syngenta Crop Protection, Greensboro, NC) after 10 to 12 days of incubation at 18°C in complete darkness. For each isolate, 8-mm-diameter plugs from actively growing *P. infestans* cultures were used. The average colony diameter of two non-amended and two fungicide-amended plates was measured, the diameter of the original plug subtracted, and the insensitivity value expressed as a percentage of growth of the isolate on fungicide-amended media to non-amended media: (average colony diameter on amended media)/(average colony diameter on non-amended media)*100%. Isolates with values of 0 to <10% were considered sensitive, ≥10 to ≤60% intermediately sensitive, and >60% insensitive (Daayf et al. 2000, Deahl et al. 1995, Marshall-Farrar et al. 1998, Shattock et al. 1988). The assay was performed twice on at least one isolate from each sample received in 2009, 2010, and 2011, and mean values calculated from the two repetitions. No difference in sensitivity was detected between isolates from individual samples, so one sensitivity designation was assigned for each sample received. A chi-square test was performed using SigmaPlot (Systat Software, San Jose, CA) to establish correlation between mefenoxam sensitivity and clonal lineage. Mefenoxam sensitivity was not tested for the 2012 isolates but was inferred from the allozyme genotype.

Results

Overall, 143 isolates of *P. infestans* were collected from field samples from 52 locations in 20 counties in Wisconsin during 2009, 2010, 2011, and 2012 (Table 1, Fig. 1). Three allozyme banding patterns, 100/122, 100/100 and 100/100/111, were distinguished (Table 1). The subset of isolates analyzed with RFLP using the RG57 probe corroborated that 100/122, 100/100, and 100/100/111 corresponded to the US-22, US-23, and US-24 clonal lineages, respectively.

Mefenoxam sensitivity correlated well with allozyme genotype and clonal lineage ($P<0.001$, $X^2=30.55$, df=4). All US-22 isolates tested (n=16) were sensitive to mefenoxam, with a mean insensitivity value of 4.1% (Table 1, Fig. 2); the range was narrow, at 1.2 to 7.8. US-23 isolates from all but one sample tested intermediately sensitive to mefenoxam; the exception was sensitive (Fig. 2). Overall the mean for US-23 isolates tested (n=6) was 11.7% and the range was 8.0 to 17.0. US-24 isolates from all but one sample also tested intermediately sensitive to mefenoxam, but unlike US-23, the exception was insensitive (Fig. 2). The mean for all US-24 isolates tested (n=11) was also much larger, at 34.58%, and the range was also large, spanning from 13.9 to 72.8.

Mating type also correlated well with clonal lineage ($P<0.001$, $X^2=28.0$, df=2). All US-22 isolates formed oospores with the A1 tester isolate, but not with the A2 tester isolate, indicating the A2 mating type. All US-23 and US-24 isolates formed oospores with the A2 tester isolate but not with the A1 tester isolate, indicating the A1 mating type. Both mating types were found concurrently in Wisconsin during 2009 and 2010, with A1:A2 mating type ratios in Wisconsin of 1:7 in 2009 and 11:9 in 2010 (Fig. 3B). Only the A1 mating type was found in

2011 and 2012 (Fig. 3B). Isolates of opposite mating types were separated by location and/or growing season over the 4-year span, limiting the potential for sexual recombination and oospore formation (Fig. 1).

Isolates of just one of the clonal lineages (US-22, -23, and -24) of *P. infestans* were detected from individual counties in Wisconsin in each year of this study, with the exception of Adams County in 2011, where both US-23 and US-24 were recovered from separate potato fields (Fig. 1). Though together in the same county, these two lineages were never found in the same field, even though isolates were obtained from up to 13 lesions in some fields in the area (Table 1). Throughout the 4-year period, US-22 was recovered from 16 samples, US-23 from 25, and US-24 from 11 samples. US-22 was the predominant isolate in 2009, recovered from 88% of all late blight samples received (Fig. 3A). In 2010, US-22 was detected in just 45% of samples, and in 2011 and 2012 US-22 was not detected in Wisconsin (Fig. 3A). US-23 was found in all four years, recovered from 13% of late blight samples in 2009, 15% in 2010, 40% in 2011, and 100% in 2012 (Fig. 3A). The report of US-23 in Vernon County in 2009 was the first incidence of this lineage in the Midwestern U.S.; all other detections of US-23 were from the eastern U.S. that year (Fry et al. 2012, Gevens and Seidl 2013a, Hu et al. 2012). US-24 was identified only in 2010 and 2011, comprising 40 and 60% of late blight samples received in those years, respectively (Fig. 3A). It was not found in 2009 or 2012 in Wisconsin (Fig. 3A).

The three clonal lineages appeared to demonstrate differential host and tissue preference during 2009, 2010, 2011, and 2012 impacting the crop profiles of the epidemics. In 2009, tomato was affected by late blight more than potato, with tomato comprising most (63%) of the late blight cases diagnosed (Fig. 3C). Conversely, in 2010, 2011, and 2012, late blight was

recovered primarily from potato (60, 80, and 63% of all samples received, respectively) (Fig. 3C). US-22 was recovered more often from tomato (63%) than from potato, but US-24 was recovered exclusively from potato. Conversely, US-23 was recovered in near equal proportions from the two crops, with tomato being the host in 46% of the samples received and potato only slightly more, at 54% of samples received. Of all the potato samples received, most (77%) included blighted foliage, with only 23% infected tubers (Table 1). All three lineages of *P. infestans* were recovered from both potato foliage and potato tubers over the course of the study. Of all the tomato late blight samples received during 2009, 2010, 2011, and 2012, 71% contained diseased fruit and 43% contained both diseased leaf and fruit tissue. US-22 and US-23 were isolated in near equal percentages from fruit samples (n=14), with US-22 from 47% of samples received and US-23 from 53% of fruit samples received.

Discussion

The late blight epidemics in Wisconsin during 2009, 2010, 2011, and 2012 were notable, initiated by three new clonal lineages of *P. infestans*, US-22, US-23, and US-24, which led to occurrence of late blight in a state that had not seen the disease for the previous six years. The US-8 and US-1 clonal lineages, which had previously been common in Wisconsin (Stevenson 2001, Stevenson and James 1997, Stevenson and James 1998, Marshall-Farrar et al. 1998) were not found.

The correlation between allozyme genotype and mefenoxam sensitivity and mating type was key to enabling us to deliver timely management information to growers. Such a correlation has been observed in the past, but allozyme genotypes can be redundant among clonal lineages;

the most extreme example is the 100/100 *Gpi* genotype which corresponds to the US-4, US-5, US-6, US-9, US-13, US-15, US-18, and US-23 clonal lineages, all with varying levels of sensitivity to mefenoxam. However, historically, populations of *P. infestans* in regions of the United States have been clonal or nearly clonal, and our results indicate that this trend has been repeated in Wisconsin in recent years. Because of this, and our data showing a correlation of mating type and mefenoxam sensitivity to allozyme genotype, we were able to quickly predict the mefenoxam sensitivity of subsequent isolates from field samples after the first few had been thoroughly tested. Testing mefenoxam sensitivity of an isolate can take several weeks, as an axenic culture must first be generated from a sample. However, we could complete the allozyme analysis within 2 hours from receipt of a sporulating sample, allowing timely delivery of management information to growers. This assay is straightforward and easy to perform, and if populations remain nearly clonal as demonstrated in our results, we would recommend this assay, particularly for extension or diagnostic labs that may not be equipped to perform commonly used but more sophisticated molecular diagnostics such as analysis of restriction fragment length polymorphisms (RFLP) (Goodwin et al. 1992) or analyses of simple sequence repeats (SSRs) (Lees et al. 2006, Widmark et al. 2011).

This study represents a survey of late blight in Wisconsin from 2009 to 2012 and was initiated in response to a rapidly expanding epidemic. As such, immediate information was needed, and a systematic sampling of potato and tomato fields was not established in the initial year of the study. Instead, growers were strongly encouraged, through multiple communication venues, to submit potential late blight samples to the Potato and Vegetable Pathology Lab, with the understanding that they would receive phenotypic information on mefenoxam sensitivity

within a day or two. Therefore, although systemic systematic sampling was not performed, the data collected likely still represent an accurate picture of the late blight situation in Wisconsin from 2009 to 2012. Admittedly, many samples came from just a few counties, particularly in 2010 and 2011. However, potato production in Wisconsin is primarily concentrated in the center of the state in an area known as the Central Sands, so receiving many samples from this area is likely representative of what is happening in the state.

Mefenoxam is potentially one of the best fungicide active ingredients available against *P. infestans*, as it has systemic curative activity against sensitive isolates (Cohen et al. 1979). However some clonal lineages, such as US-8, are insensitive, rendering the fungicide ineffective. Our data show that mefenoxam sensitivity is still well correlated to clonal lineage, so a genotypic test, such as the relatively fast allozyme assay used in this study, can be used to estimate the phenotypic character of mefenoxam sensitivity, which would otherwise take much longer to determine. Isolates of US-24 showed the most variability in their sensitivity, ranging from means of 13.8 to 72.8% (Fig. 2), but nevertheless, all but one isolate was in the intermediately sensitive range. It is uncertain why so much variability is seen with US-24, but further laboratory experiments may shed light on the impact of this. US-23 had a much smaller range between isolates, from 8.0 to 17.0, also with all but one isolate falling in the intermediately sensitive category. The US-23 clonal lineage in Wisconsin is borderline sensitive, so applications of mefenoxam may control late blight in the field, but the fungicide may not be as effective as it could be. US-22 had the tightest range, from 1.2 to 7.8, and all isolates were categorized as sensitive, indicating that applications of mefenoxam in a field infected with this lineage would be effective. Mefenoxam sensitivity appears to have no correlation to mating

type, as the A2 US-22 isolates we collected were entirely sensitive, but previously tested isolates of the A2 US-8 clonal lineage were insensitive (Groves 2002, Kalischuk et al. 2012, Marshall-Farrar et al. 1998).

Knowledge of the mating types present in a *P. infestans* population is important for immediate and long-term management of late blight. By knowing the distribution of mating types, future changes in the population due to increased sexual recombination can be anticipated and potential problem fields monitored. Additionally, a soil persistent oospore phase of *P. infestans* would drastically change current management practices. Prior to the late 1980s in Wisconsin, the A1 mating type was the only mating type detected in Wisconsin (Marshall-Farrar et al. 1998). Then between 1993 and 1994, with the coming of the A2 US-8 clonal lineage, the distribution of mating types was completely reversed, and by 1995 the A2 mating type was found nearly exclusively (Marshall-Farrar et al 1998). In 2009 in Wisconsin the distribution of mating types was similar to what had been seen in 1995 and through 2002: nearly exclusive presence of the A2 mating type (Fig. 3B) (Stevenson et al. 2001, Stevenson and James 1998). Then in 2010 that changed, and both the A1 and A2 mating types were present in near equal proportions (Fig. 3B). Furthermore, by 2011, the mating type distribution exhibited a complete reversal back to the A1 mating type exclusively, returning Wisconsin to the mating type distribution seen before the late 1980s.

The nearly equal distribution of mating types identified in 2010 raises concern over the potential for oospore formation. However, opposite mating types were largely never found in the same Wisconsin counties. One exception is a US-22 (A2) isolate that was recovered from Waushara County in central Wisconsin, a county that had otherwise only seen the A1 mating

type (US-24). However, this was a late season tuber sample, from tubers grown in another region and shipped to Waushara County for storage. Based on our data, no other county ever had both mating types together in the same year, so the two mating types were likely still separated spatially and temporally. Additional late blight infected fields not represented in our collection may have been present in the state, but the majority of Wisconsin potato and tomato producers proactively and aggressively manage field infections, by careful scouting and often by destroying diseased plants and healthy bordering rows within fields, limiting the potential for contact between opposite mating types. Additionally, we have seen no evidence in Wisconsin of the increased pathogen diversity that would be associated with sexual recombination. On the contrary, the *P. infestans* population was entirely clonal in 2012, with US-23 being the only clonal lineage found in Wisconsin (Fig. 3A).

The hosts and plant organs from which each lineage was collected indicate the natural preferred hosts of each clonal lineage. US-22 and US-23 were recovered often from both potato and tomato foliage, tomato fruit, and potato tubers, indicating that they are good natural pathogens of these hosts and plant parts. Conversely, the US-24 clonal lineage was never recovered from tomato foliage or fruit. Due to the non-systematic method of isolate collection that was used for this study, this could simply be a coincidence, but further laboratory tests have suggested that US-24 is a poor tomato pathogen (Seidl et al. 2012). Overall, incidence of tuber infection was rare, comprising only 13% of all samples received. This, however, may be more indicative of grower management practices than any inability of the US-22, US-23, or US-24 to infect tubers, but further laboratory experimentation would be needed to confirm this.

The appearance and dissemination of the US-22 clonal lineage in Wisconsin may have been impacted by movement of infected transplants and by potential overwintering. US-22 was a novel clonal lineage in Wisconsin and much of the eastern U.S. in 2009 and was able to quickly infect tomatoes, and to a lesser extent, potatoes, over a large geographic area (Fry et al 2012, Gevens and Seidl 2013b). This widespread distribution and detection of US-22 was likely due to broad geographic movement of infected tomato transplants, which were purchased and transplanted by growers and home gardeners (Hu et al. 2012, Ristaino et al. 2010). This may explain the scattered distribution of isolates collected in Wisconsin in 2009 and the high percentage of isolates collected from tomato (Figs. 1 and 3C). In addition to widespread dissemination of the pathogen, the weather was particularly conducive for late blight development in 2009. *P. infestans* requires moderate temperatures (8 to 24°C) and free water to infect, (Stevenson et al. 2001) and the summer of 2009 was abnormally cool with average temperatures in July of 3.2°C below normal (Enloe 2012). The winter weather following the 2009 season may partly explain the persistence of US-22 into 2010. *P. infestans* purportedly dies during Wisconsin winters due to the cold temperatures and absence of living plant material, but the winter of 2009 was initiated throughout the state by an early heavy snowfall providing an insulating layer. At a 5-cm depth, soil temperature from southwestern WI (Spring Green) dropped to just -1.5°C for only 3 days during the entire 2009 to 2010 winter (UW Weather 2010). In protected areas in cities and/or compost piles, where temperatures can be slightly higher, mycelia or sporangia in fallen or composted plant tissues might not have encountered killing freezing temperatures and may have survived the winter. This presumption is supported by the continued scattered distribution of US-22 on tomatoes around the state in 2010 (Fig. 1).

In 2011, the absence of US-22 suggests that the average winter conditions the preceding winter were sufficient to freeze plant material and pathogen propagules and that this lineage was not introduced to Wisconsin for the 2011 season.

The appearance of US-23 and US-24 in Wisconsin was likely a result of multiple factors coinciding. The sources of introduction of US-23 and US-24 in 2009, 2010, 2011, and 2012 are unknown, although US-23, like US-22, may have been introduced on tomato transplants in 2009. This source of introduction of US-23 was documented in Canada that year (Kawchuk et al. 2011), but was not widely documented in the U.S. in 2009. In fact, US-23 was reported at only a few locations in the eastern U.S. that year (Hu et al. 2012), and our report of US-23 in Vernon County in Wisconsin is the first report of US-23 in the Midwestern U.S. (Gevens and Seidl 2013a). US-23 was not isolated from Vernon County in subsequent years, indicating the likelihood of separate subsequent introductions into the state. In 2010, persistence of each lineage after initial introduction may be attributed to the abnormally wet weather in Wisconsin. In July alone, Wisconsin received nearly double the precipitation typically received (Enloe 2012), making ideal conditions for late blight. Sporangia of US-24 may have been aerially transported into Wisconsin from surrounding states in 2010, but introduction on seed potatoes is also likely, as this lineage was recovered from only two Central Wisconsin counties (Fig. 1) and was reported as the predominant lineage affecting potatoes in several other U.S. potato-producing states (Fry et al. 2012, Gevens and Seidl 2013c). Spread of US-24 to other areas of the state was limited, perhaps due in part to aggressive management of late blight by the producers in this region. In 2011, temperature and precipitation were average for Wisconsin,

resulting in conditions less favorable for late blight, which is reflected in the fewer number of late blight samples received in 2011 (Table 1, Fig. 1).

The late blight epidemics in Wisconsin during 2009, 2010, 2011, and 2012 were noteworthy, initiated by three new clonal lineages of *P. infestans* introduced into the state during seasons of conducive weather, which led to occurrence of late blight in a state that had not seen the disease for the previous six years. Among the four years of late blight in Wisconsin, each year has had a unique profile with respect to crops and regions of the state affected, as well as clonal lineages present. Regardless, we were able to successfully use the allozyme assay to quickly predict the mefenoxam sensitivity profile of isolates from fields and inform grower fungicide choices. Continued monitoring of genotypic and phenotypic characteristics of the *P. infestans* population will contribute to both short-term and long-term management of late blight in Wisconsin and surrounding states.

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Table 1. Genotypes and characteristics of isolates of *Phytophthora infestans* collected from Wisconsin from 2009 to 2012.

Year	County	Isolates[a]	Host	Plant Tissue	Gpi[b]	Clonal Lineage	MT[c]	Mefenoxam[d]
2009	Vernon	3	Potato	Leaf	100/100	US-23	A1	S
2009	Columbia	2	Tomato	Fruit	100/122	US 22	A2	S
2009	Columbia	1	Tomato	Fruit	100/122	US-22	A2	S
2009	Columbia	2	Potato	Leaf	100/122	US 22	A2	S
2009	Dane	1	Tomato	Fruit	100/122	US 22	A2	S
2009	Dane	3	Tomato	Leaf	100/122	US 22	A2	S
2009	Portage	1	Tomato	Fruit	100/122	US 22	A2	S
2009	Langlade	1	Potato	Tuber	100/122	US 22	A2	S
2010	Marquette	1	Potato	Leaf	100/122	US 22	A2	S
2010	Waukesha	1	Tomato	Fruit	100/100	US-23	A1	I
2010	Waukesha	1	Tomato	Leaf	100/100	US-23	A1	I
2010	Portage	1	Potato	Leaf	100/100/111	US-24	A1	I
2010	Portage	1	Potato	Leaf	100/100/111	US-24	A1	I
2010	Portage	1	Potato	Leaf	100/100/111	US-24	A1	I
2010	Portage	1	Potato	Leaf	100/100/111	US-24	A1	I
2010	Portage	1	Potato	Leaf	100/100/111	US-24	A1	I
2010	Langlade	1	Potato	Leaf	100/122	US-22	A2	S
2010	Waushara	1	Potato	Leaf	100/100/111	US-24	A1	I
2010	Waushara	1	Potato	Leaf	100/100/111	US-24	A1	R
2010	Vernon	1	Tomato	Leaf	100/122	US-22	A2	S
2010	Brown	1	Tomato	Leaf and Fruit	100/122	US-22	A2	S
2010	Brown	1	Tomato	Leaf	100/122	US-22	A2	S

2010	Kewanee	1	Tomato	Fruit	100/122	US-22	A2	S
2010	Manitowoc	1	Tomato	Leaf and Fruit	100/122	US-22	A2	S
2010	Ozaukee	1	Tomato	Fruit, Leaf, Stem	100/100	US-23	A1	I
2010	Columbia	1	Potato	Leaf	100/122	US-22	A2	S
2010	Waushara	1	Potato	Tuber	100/122	US-22	A2	S
2010	Portage	1	Potato	Tuber	100/100/111	US-24	A1	I
2011	Waukesha	2	Tomato	Leaf	100/100	US-23	A1	I
2011	Adams	6	Potato	Leaf	100/100	US-23	A1	I
2011	Adams	6	Potato	Leaf	100/100/111	US-24	A1	I
2011	Adams	13	Potato	Leaf	100/100/111	US-24	A1	I
2011	Waushara	2	Potato	Tuber	100/100/111	US-24	A1	I
2012	Barron	4	Potato	Leaf, Stem	100/100	US-23	A1	I
2012	Adams	2	Potato	Leaf	100/100	US-23	A1	I
2012	Adams	3	Potato	Leaf	100/100	US-23	A1	I
2012	Portage	5	Potato	Leaf	100/100	US-23	A1	I
2012	Waushara	6	Potato	Leaf	100/100	US-23	A1	I
2012	Waushara	4	Potato	Leaf	100/100	US-23	A1	I
2012	Oneida	5	Potato	Leaf	100/100	US-23	A1	I
2012	Oneida	3	Potato	Leaf	100/100	US-23	A1	I
2012	Adams	6	Tomato	Leaf	100/100	US-23	A1	I
2012	Waushara	5	Tomato	Leaf, Fruit	100/100	US-23	A1	I
2012	Marathon	4	Potato	Leaf	100/100	US-23	A1	I
2012	Waushara	6	Tomato	Leaf, Stem, Fruit	100/100	US-23	A1	I
2012	Sheboygan	2	Tomato	Fruit	100/100	US-23	A1	I
2012	Rusk	3	Tomato	Fruit	100/100	US-23	A1	I
2012	Sauk	3	Tomato	Leaf	100/100	US-23	A1	I

2012	Eau Claire	4	Tomato	Leaf, Stem, Fruit	100/100	US-23	A1	I
2012	Barron	5	Potato	Tuber	100/100	US-23	A1	I
2012	Waushara	4	Potato	Tuber	100/100	US-23	A1	I
2012	Waushara	4	Potato	Tuber	100/100	US-23	A1	I

[a] The number of isolates collected from a given field in the given county.

[b] *Glucose-6-phosphate isomerase* locus determined using cellulose acetate electrophoresis (Goodwin et al. 1995a).

[c] Mating type, determined as outlined in the main text.

[d] S = sensitive, I = intermediately sensitive, and R = resistant to the fungicide mefenoxam, determined as outlined in the main text.

Fig. 1. Distribution of Wisconsin counties from which late blight samples were collected from 2009 to 2012. Different patterns represent the three clonal lineages, US-22, US-23, and US-24.

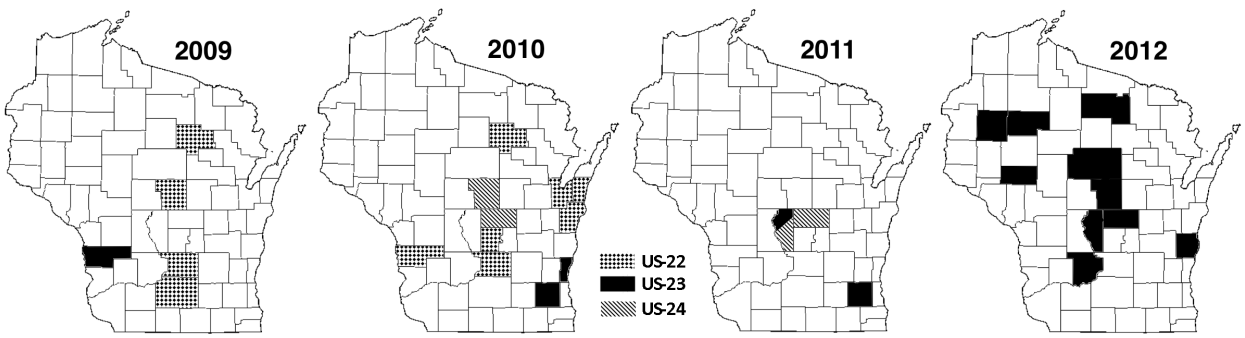


Fig. 2. Mefenoxam sensitivity profile of *Phytophthora infestans* isolates collected from Wisconsin during 2009, 2010, and 2011. Bars represent the mean sensitivity values (as percent culture growth of amended to non-amended media) of one isolate from each sample received, determined as outlined in the text. Error bars represent the standard deviation between replicates of the assay. The horizontal lines at 10 and 60% indicate thresholds for determining categories of sensitivity to mefenoxam. Isolates with mean values <10% were considered sensitive, ≥10% to ≤60% were considered intermediately sensitive, and >60% were considered insensitive to mefenoxam (Daayf et al. 2000, Deahl et al. 1995, Marshall-Farrar et al. 1998, Shattock et al. 1988).

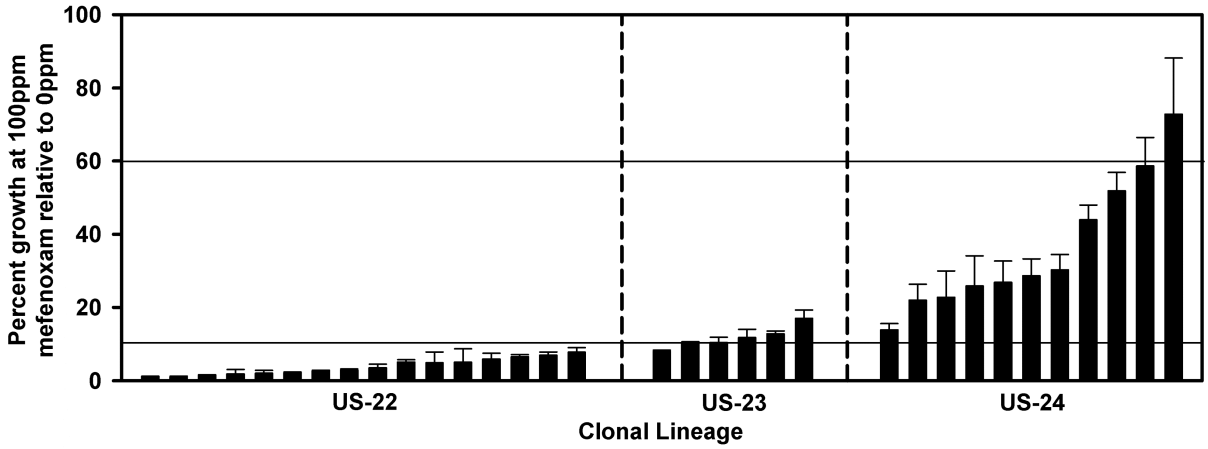
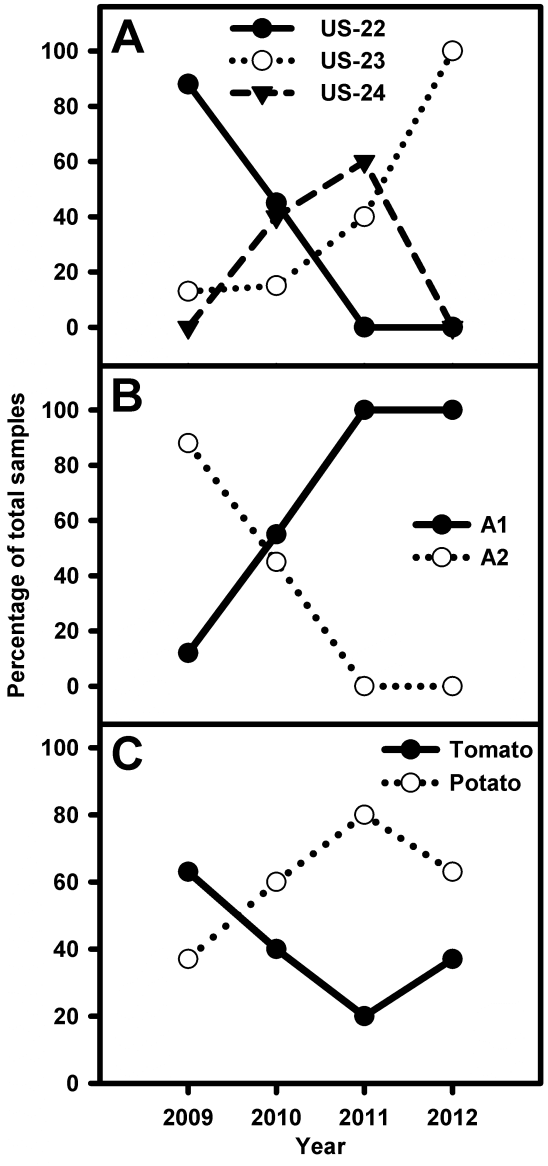


Fig. 3. Genotypic and phenotypic characteristics of *Phytophthora infestans* isolates collected from late blight samples from Wisconsin from 2009 to 2012. (A) Percentage of the sampled population identified as clonal lineage US-22, -23, or -24 in each year. (B) Percentage of the sampled population identified as either A1 or A2 mating type in each year. (C) Percentage of the sampled population present on potato and tomato.



CHAPTER 3:

Investigating the host range of the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* on cultivated solanaceous plants and common solanaceous weeds

Abstract

Phytophthora infestans causes late blight, one of the most important diseases of potato and tomato worldwide. Recently in the U.S., three newly identified clonal lineages, US-22, US-23, and US-24, have become widespread. While potato and tomato are the most commonly infected solanaceous hosts for *P. infestans*, new lineages may have a broader or different host range. We determined the host range of US-22, US-23, and US-24 on cultivated solanaceous plants and solanaceous weeds common to the upper midwestern production region. None of the clonal lineages produced late blight symptoms or pathogen signs on foliage of selected cultivars of eggplant, pepper, tomatillo, or ground cherry. Symptoms and signs were evident on the potato and tomato cultivars tested, though with US-24, infection on tomato was limited. None of the lineages sporulated on the common weed black nightshade, but sporulation and disease was observed with all lineages on bittersweet nightshade. Hairy nightshade hosted abundant pathogen signs and symptoms, and sporangial production was not significantly different than that on tomato for each of the three lineages, indicating the potential for this weed to produce inoculum and contribute to late blight epidemics. Interestingly, black nightshade had the highest incidence of sporulation on berries, but the lowest on leaves, suggesting the importance of testing multiple plant organs when determining susceptibility of a species. Our results update knowledge of the host range of an ever-changing *P. infestans* population and will help to inform management strategies for enhanced efficacy of late blight control.

Introduction

Phytophthora infestans (Mont.) deBary, one of the most important pathogens worldwide, causes late blight on potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.). Many clonal lineages, which are defined as the asexual descendants of a single genotype (22), are considered to be pathogenic primarily on potato, which is thought to be the original host of *P. infestans* (42). Other clonal lineages infect both potato and tomato (20, 31, 42, 52), and still others appear to be pathogenic primarily on tomato and will not infect potato (38). So called "tomato isolates" are members of clonal lineages that are characterized by the ability to cause abundant leaf sporulation on tomato with little or no initial necrosis (56). In the U.S. and Canada, *P. infestans* populations are mostly clonal, as the two mating types required for sexual recombination, designated A1 and A2, have historically not been in proximity. However, in South and Central America, and more recently, in The Netherlands, both mating types have been present in near equal proportions, and sexual recombination is commonplace (15, 35). Because of this, greater diversity has been observed in genotypic and phenotypic characteristics, including the ability to infect diverse hosts (18).

Potato and tomato are the most common hosts from which *P. infestans* is isolated, but many other species can become infected. In their review of the topic, Erwin and Ribeiro (17) listed 90 *P. infestans* host species, not all of which are solanaceous. Some non-solanaceous hosts listed include maple (*Acer* sp.), morning glory (*Ipomoea hederacea*), and fleabane (*Erigeron multicaulis*). Other common solanaceous vegetable and ornamental species that can become infected with *P. infestans* have been reported and include eggplant (*Solanum melongena*) (36), tomatillo (*Physalis ixocarpa*) (23), ground cherry (*Physalis angulata*) (47), and petunia (*Petunia*

hybrida) (3, 6, 47). In most cases, these species became infected only when artificially inoculated under ideal environmental conditions.

In central Mexico, the center of origin of tomato and many other solanaceous species and a potential center of origin for *P. infestans* (30), there are many wild solanaceous species that naturally become infected with *P. infestans* (17, 18). Throughout the U.S. and Europe, there are fewer wild solanaceous species than in Mexico, and only some of these have ever been shown to be hosts to *P. infestans*. Black nightshade (*Solanum ptychanthum* Dunal, syn. *Solanum nigrum* auct. non. L.) is usually not a host to *P. infestans* (7, 48), though some artificial inoculations have led to the production of sporangia (47). *P. infestans*-infected black nightshade (reported as *S. nigrum*) has been found in fields in Wales (14) and The Netherlands (19), but it is unclear in the Wales case whether the plant was a genetically unique variant of black nightshade; the morphological description of the host is not entirely consistent with descriptions of black nightshade in the U.S. (27), and plasticity and hybridization among the solanaceous weeds is common (16). Artificial inoculation of bittersweet nightshade (*Solanum dulcamara* L.) with diverse clonal lineages of *P. infestans* has often resulted in some disease and sporangial production (29, 50). Additionally, natural infections of bittersweet nightshade have been observed, though rarely, and sporulation has been seen in some cases (5, 6, 13, 19). Among the solanaceous weeds, hairy nightshade (*Solanum physalifolium* Rusby syn. *Solanum sarrachoides* auct. non Sendtn.) is most often reported infected with *P. infestans* in the U.S. (1, 10-12, 31, 32, 34, 46, 49, 55), however, these reports are still rare compared to reports of late blight on potato and tomato. Additionally, while the susceptibility of nightshade weeds has been investigated,

studies have focused on the foliage; infection of berries from nightshade weeds has been evaluated only on wounded berries (7).

Hairy and black nightshade are important and widespread weeds of cultivated crops (2). Bittersweet nightshade is also a common weed, although it is usually found in woodlands, windbreaks, or near waterways instead of directly in agricultural fields. Black or hairy nightshade plants growing without competition can produce 1000 to 2000 berries, leading to 20,000 to 50,000 seeds produced per plant per year (41). The seeds have a long dormancy, which makes eradicating black or hairy nightshade from an area nearly impossible (D. Heider, *personal communication*). Additionally, very few herbicides are effective against black and hairy nightshade, and many function only to eliminate competition from other weeds (D. Heider, *personal communication*). Both nightshades have seeds that germinate only in warm soil, so seeds often do not germinate in time to be destroyed by pre-plant tillage. Additionally, the seeds will germinate throughout the summer (41), producing a crop of seedlings that can quickly take advantage of increased light as potato vines senesce or are vine-killed in late summer. This period may often be long enough for the plants to bloom and produce berries and mature seeds (41). Because of all of these factors, black and hairy nightshade will likely continue to be troublesome weeds. As such, knowledge of their susceptibility to late blight will be important for current and future management of this disease on potato and tomato.

Broad plant pathogen host ranges can challenge disease management for producers, particularly if a new strain of a pathogen has an uncharacterized host range. Unrecognized *P. infestans* host plants may serve as unmonitored sources of inoculum that may contribute to disease on potato or tomato. The overwintering of *P. infestans* on perennial solanaceous weeds,

even in colder climatic regions has been speculated to occur, although this has, to date, been found to be unlikely (29). In milder climatic regions, infected weeds may serve as a bridge between successive seasons of potatoes or tomatoes, providing a living host even when the main crop is no longer present. Further, it has been suggested that late blight infection on hairy nightshade may select for a more aggressive population of *P. infestans* (34). This is because hairy nightshade, unlike the potato crop, reproduces sexually, and so can evolve with a sexually reproducing *P. infestans* population, applying selection pressure on the pathogen to maintain a high level of virulence (34).

In the U.S., despite a lack of evidence for sexual recombination, new clonal lineages of *P. infestans* have been identified over the years. US-1 had been the most dominant lineage up until the late 1980s, when the US-8 clonal lineage effectively displaced it in just a few years (31, 43). Other successively named lineages have been identified, but their occurrence has typically been ephemeral and their presence often limited to a particular geographic region. However, in 2009 the US-22 clonal lineage quickly spread across much of the eastern and Midwestern U.S. In the years following, US-23 and US-24 were also identified (8, 24-26, 38). These three new lineages have caused much crop loss and added expense of fungicide applications across the U.S. and in Wisconsin from 2009 to the present.

It is important to understand the host range of new clonal lineages of *P. infestans* in Wisconsin and throughout the U.S. to best prescribe integrated late blight management through appropriate crop selection and rotation, fungicide timing and selection, and weed control in and around production fields. To determine host range, we evaluated pathogen signs and disease symptoms of isolates of US-22, US-23, and US-24 collected from Wisconsin inoculated onto

detached leaves of eggplant, ground cherry, pepper, petunia, tomatillo, and tomato, and the three common solanaceous weeds hairy nightshade, bittersweet nightshade, and black nightshade. We also compared sporangial production of each lineage inoculated onto leaves of hairy nightshade to that on potato and tomato to establish the relative potential role of this weed in late blight epidemics. Because the nightshade weeds produce such a large number of persistent berries, we also determined sporulation incidence on both wounded and non-wounded berries of the three solanaceous weeds by each of the three clonal lineages.

Materials and Methods

P. infestans *isolates*. The US-22 (Pi7-09a) US-23 (Pi16-10a) and US-24 (Pi21-10a) isolates used in this study were collected from Wisconsin and have been previously characterized (53). Pi7-09a (A2, mefenoxam sensitive) was isolated from tomato fruit, Pi16-10 (A1, mefenoxam sensitive) from tomato foliage, and Pi21-10a (A1, mefenoxam intermediately sensitive) from potato foliage. Each isolate originated from a single zoospore. Isolates are maintained in the Vegetable Pathology pathogen collection of Dr. Amanda Gevens at the University of Wisconsin-Madison in the Plant Pathology Department. Isolates were transferred minimally in culture and all experiments were performed within 18 months of collection of the original isolate to ensure that the original virulence was not lost.

Plant species and growing conditions. The solanaceous plants selected for inoculation were the cultivated plants eggplant (*S. melongena* L.) 'Florida Highbush', ground cherry (*Physalis peruviana* L.), petunia (*Petunia* x *hybrida* Vilm), potato (*S. tuberosum* L.) 'Katahdin', pepper (*Capsicum annuum* L.) 'Goliath', tomatillo (*P. ixocarpa* Lam.) 'Toma Verde', and

tomato (*Solanum lycopersicum*) 'Brandywine Red', and the solanaceous weeds bittersweet nightshade (*S. dulcamara*), black nightshade (*S. ptycanthum*, also known as eastern black nightshade or west Indian nightshade), and hairy nightshade (*S. physalifolium*). All plants except potato were grown from seed in a growth chamber with a constant temperature of 28°C and a 14-hour photoperiod. Potatoes were grown from cuttings in a greenhouse with a daytime temperature of 18 to 21°C and a nighttime temperature of 21 to 24°C in an 18-hour photoperiod. All seeds were sown directly into potting mix except for hairy nightshade and black nightshade, which were soaked in 1500 ppm gibberellic acid for 12-14 hours before sowing to break dormancy. Seeds for hairy and black nightshade were collected from wild plants that were growing at the University of Wisconsin – Hancock Agricultural Research Station, Hancock, WI. Bittersweet nightshade seeds were collected from Madison, WI. All vegetable seeds were purchased from Reimer Seed (Saint Leonard, MD).

Leaf inoculations. A detached leaf method was used for all leaf inoculations. Fully expanded leaves from near the top of 4- to 6-week-old plants were removed with a sharp razor blade and immediately placed with the adaxial surface up in a petri dish on a thin layer of 10% water agar, with three leaves per plate and two plates per host/isolate combination. Isolates were grown for 2 to 4 weeks on rye A media (4) before they were flooded with sterile distilled water and brushed gently with a glass rod to dislodge sporangia. The resulting suspension was quantified with a hemacytometer and adjusted to 50,000 sporangia/ml. For each host/isolate combination, each of five leaves (in 2 plates) was inoculated with one 10-μl droplet of the sporangial suspension applied at the midvein. The sixth leaf was mock inoculated with a 10-μl droplet of sterile distilled water as a control. All petri dishes were incubated at 18°C in darkness.

The experiment was conducted two times. Percent mycelial cover and percent symptomatic tissue on a 1.6-cm-diameter circle centered on the inoculation drop was estimated at 0, 5, 7, 9, 12, and 14 days after inoculation using a modified Horsfall-Barratt scale (37). Mycelial cover was defined as any visible pathogen growth including mycelia, sporangiophores, and/or sporangia, and symptomatic tissue was defined as chlorotic and/or necrotic plant cells, as visible with a dissecting microscope (16 to 40× magnification).

Nightshade berry inoculations. Mature berries of hairy and black nightshade were harvested from plants grown in a growth chamber. Due to the difficulty in producing bittersweet nightshade berries in the growth chamber, these berries were harvested from plants growing wild on the UW-Madison campus. To avoid wounding, the calyx was not removed from the berries. For each isolate, 20 berries were placed in each of two petri dishes containing a thin layer of 10% water agar with small indentations carved to prevent movement of the berries. The berries in one petri dish were wounded by piercing the skin with a sharp, sterile dissecting probe immediately prior to inoculation. Inoculum was prepared as previously described for the leaf inoculations and each of 18 berries from each plate was inoculated with a 10-μl droplet of the sporangial suspension. The remaining two berries were mock-inoculated with a 10-μl droplet of sterile distilled water for control. Berries were incubated at 20°C in complete darkness and evaluated after 14 days for presence or absence of sporangia using a dissecting microscope. The experiment was performed two times.

Quantification of sporangia. Fully expanded leaves were detached from the upper half of 4- to 6-week-old plants of 'Katahdin' potato, 'Brandywine Red' tomato, and hairy nightshade. Three 15-mm-diameter leaf disks were cut from each leaf with a cork borer and placed with the

adaxial surface up in three separate 9-cm-diameter petri dishes containing a thin layer of water agar. This was repeated seven more times for a total of eight leaf disks per plate, with one plate for each plant species/clonal lineage combination. Individual petri dishes were inoculated with the US-22, US-23, and US-24 isolates as previously described for leaf inoculations. An additional leaf disk in the center of each plate was mock-inoculated with a 10 μl droplet of sterile distilled water as a control. Inoculated plates were sealed with Parafilm and incubated in a single layer at 20°C with a 12-hour photoperiod. After 9 days, four sporulating leaf disks were selected from the eight inoculated disks in each plate and were individually mixed with a vortexer in 2 ml of sterile distilled water in a 25-ml plastic centrifuge tube to which 1 drop (12 to 18 μl) of Tween 20 (Agdia Inc. Elkhart, IN) had been added. The addition of Tween 20 was essential to obtain uniform and accurate sprorangial counts from each of the three plant species and clonal lineages (54). Sporangial suspensions were quantified within 20 minutes using a hemacytometer. The number of sporangia in suspension was then calculated and divided by the area of the leaf disk, π*0.75cm^2, to determine the number of sporangia produced per square centimeter of leaf area. The experiment was performed twice.

Statistical analysis. For all of the experiments, the mock-inoculated leaves, berries, and leaf disks that were included in each plate never showed signs or symptoms of late blight, so it was determined that each leaf, berry and leaf disk was independent of petri dish location for statistical analysis. In all cases, the two repetitions of each experiment gave statistically similar results and were pooled for analysis (P=0.696, P=0.102, P=0.206, and P=0.663 for the mycelial cover, symptomatic tissue, sporangia quantification, and berry inoculation experiments, respectively). For the leaf inoculations, Horsfall-Barratt scale numbers were converted to their

midpoint percentages prior to statistical analysis. From those percentages, the area under the disease progress curve (AUDPC) was calculated for each host/clonal lineage combination using the Agricolae package in R (9). A two-way ANOVA was performed on the AUDPC values using SigmaPlot (Systat Software, San Jose, CA). Tukey's test (P=0.05) was used to determine significant differences among hosts and lineages. For the berry inoculation, percent incidence for each clonal lineage/species combination was determined for each repetition of the experiment and significant differences determined using Tukey's test (P=0.05). For the sporangial quantification, the data were square root transformed to ensure homogeneity of variance prior to performing a 2-way ANOVA. Significant differences were determined using Tukey's test at P=0.05.

Results

Leaf inoculations. No mycelial coverage was observed on detached leaves of eggplant, ground cherry, pepper, or tomatillo inoculated with US-22, US-23, or US-24 (Table 1). Additionally, no symptomatic tissue or hypersensitive response was observed on these four species for any of the clonal lineage inoculations (Table 1). On petunia, mycelial cover and symptomatic tissue were observed with inoculation by each of the three clonal lineages (Figs. 1 and 2). While both metrics for each lineage were not significantly different than zero statistically (Table 1), the presence of sporulation indicated that infection is possible on petunia for each of the new lineages. While inoculations with US-23 and US-24 demonstrated a trend for greater mycelia cover and symptomatic tissue on petunia than US-22, there were no statistically significant differences among lineages (Figs. 1 and 2). On tomato, inoculations with all three

lineages produced abundant mycelial cover and symptomatic tissue, although US-24 produced significantly less mycelial cover than either US-22 ($P<0.001$) or US-23 ($P<0.001$) (Figs. 1 and 2).

With inoculations of US-24 on tomato leaflets, streaks of dead cells were observed in several instances radiating from the inoculation point prior to the development of pathogen signs. Inoculations with US-22 and US-23, however, produced sporulation up to 3 days before any plant cell death became apparent. This is reflected in the significantly higher symptomatic tissue AUDPC values for US-24 compared to US-22 ($P<0.001$) and US-23 ($P=0.011$), and in the significantly lower mycelial cover values for US-24 compared to US-22 ($P<0.001$) and US-23 ($P<0.001$) (Figs. 1 and 2).

Among the nightshade weeds tested, black nightshade was the poorest foliar host for the US-22, US-23, and US-24 lineages of *P. infestans*. Both mycelial cover and symptomatic tissue ratings were not significantly different than zero for all three lineages (Figs. 1 and 2, Table 1). However, unlike the responses of eggplant, ground cherry, pepper, and tomatillo to inoculations with the lineages, a few dead cells were observed at the inoculation point for US-22 and US-23. On inoculated bittersweet nightshade leaves, the mean AUDPC for mycelial cover was 15.3, 129.5, and 152.9 for US-22, US-23 and US-24, respectively, and atypical branching patterns of necrotic tissue were often observed instead of a continuously expanding lesion (Figs. 1 and 3, Table 1). Hairy nightshade was the best nightshade host for each lineage tested, with mean mycelial cover AUDPC values of 643.6, 784.4, and 741.3 and mean symptomatic tissue AUDPC values of 468.1, 656.2, and 622.5 for US-22, US-23, and US-24, respectively (Figs. 1 and 2, Table 1). For each lineage, these mycelial cover and symptomatic tissue values were

significantly greater than both mycelial cover and symptomatic tissue values on tomato, except in the case of US-22 mycelia, where there was no significant difference between the two hosts (Table 1).

Nightshade berry inoculations. The US-23 clonal lineage of *P. infestans* sporulated exclusively on wounded berries; no sporulation by US-23 was observed on non-wounded inoculated berries (Fig. 4). US-23 also failed to sporulate on bittersweet nightshade berries, but both US-22 and US-24 sporulated on all three species inoculated (Fig. 4). Wounded black nightshade berries inoculated with US-22 exhibited the greatest sporulation incidence among all the lineage/host combinations tested, with an average of 39.3% (range 30.0 to 44.4) between both repetitions of the experiment (Fig. 4). For all three lineages inoculated onto hairy nightshade, the attached calyx of the berry often hosted abundant sporulation, but sporulation on the berry itself was limited.

Sporangial production on leaf tissue. For each of the clonal lineages, significantly more sporangia were produced on inoculated potato than on inoculated hairy nightshade ($P=<0.001$, <0.001, and 0.003 for US-22, US-23, and US-24, respectively) or tomato ($P=<0.001$ for all lineages) (Fig. 5). Sporangial production was not significantly different between tomato and hairy nightshade for each of the US-22, US-23, or US-24 inoculations ($P=0.802$, 0.965, 0.243, respectively) (Table 1). Across hosts, the US-23 lineage produced significantly more sporangia than US-22 or US-24 ($P<0.001$) (Fig. 5). Considering all three lineages together, an average of 72,731 sporangia/cm^2 were produced on hairy nightshade. While this was significantly less than the average sporangia production across lineages on potato, ($P<0.001$) it was not significantly different than average sporangial production on tomato ($P=0.098$). Inoculations with the US-22

lineage produced significantly more sporangia than US-24 on tomato (P=0.036) but the two lineages were not significantly different on hairy nightshade or potato (P=0.941 and 0.142, respectively) (Fig. 5).

Discussion

We have shown that isolates of US-22, US-23, and US-24, three new clonal lineages of *P. infestans* common to Wisconsin and the U.S., can readily infect and sporulate on foliage of tomato, potato, and hairy nightshade. There is a differential host preference between fruits and leaves of nightshade weeds, a finding that has not previously been reported for *P. infestans*. Foliage of eggplant, ground cherry, pepper, and tomatillo did not become infected in our study, a result that has been corroborated by other researchers of *P. infestans*. However, with new lineages of *P. infestans*, it is important to continue to evaluate host range on solanaceous plants to monitor potential changes in the pathogen population. Further, we have demonstrated significant differences in sporangial production among each of the three clonal lineages on hairy nightshade, potato, and tomato. This knowledge aids in our further understanding of the pathogen, resulting in effective and informed late blight management recommendations.

Investigation of a single representative isolate of each *P. infestans* clonal lineage was appropriate for this study, as virulence variation in non-sexually recombining populations of *P. infestans* has been shown to be low. Goodwin et al. (33) concluded that only 37% of the variation in virulence that they found among a *P. infestans* population was from within lineages, but the majority was from between lineages. This variation is most prevalent in clonal lineages that have persisted for many years, as it is presumed that mutations can accumulate, giving rise

to variable virulence patterns (33, 42). The US-22, US-23, and US-24 lineages were only recently identified (38, 40, 53), and Danies et al. (8) showed that variation in several biological characteristics among isolates of US-22, US-23, and US-24 is low. As such, testing of single representative isolates from each clonal lineage is likely adequate for profiling the current host range status of the population.

The absence of pathogen growth and hypersensitive response by US-22, US-23, and US-24 on inoculated detached leaves of eggplant, ground cherry, pepper, and tomatillo indicates that these species may be considered non-hosts. These agricultural crops may not need to be aggressively managed for late blight through their vegetative phases of development. Additionally, the lack of sporulation on foliage suggests that these solanaceous plants are not likely to contribute to epidemics through secondary inoculum production. Further studies investigating the susceptibility of fruit tissues of pepper, eggplant, tomatillo, and ground cherry may further define a need for management later in the crop cycle. Our finding is consistent with previous determinations of host range for isolates of the US-1, US-8, and US-11 clonal lineages collected in North America tested on eggplant and pepper (48). However, for tomatillo and ground cherry, very little historic information and no recent studies on *P. infestans* infection can be found in the published literature (23, 47). As such, our results are novel and particularly useful for filling these knowledge gaps and informing late blight management decisions on these minor crops.

Late blight infection on petunia leaves was limited for all three *P. infestans* clonal lineages, with mycelial cover and symptomatic tissue ratings significantly lower than tomato. However, infection was evident, and in a nursery or transplant production greenhouse where

there is a concentration of petunia plants and humid, consistently favorable temperature conditions, late blight management may be required. However, due to the low level of infection by US-22, US-23, and US-24 observed in this study, petunia late blight management may not be necessary in botanical or home gardens where plants are not as concentrated and environmental conditions may be less favorable.

Phylogenetic relationships of the plant species tested may offer some insight into the susceptibility of the species to *P. infestans*. Pepper, tomatillo, and ground cherry are in the family *Solanaceae* but not in the genus *Solanum*, so one might expect these to not be hosts. However, petunia is not a *Solanum* species and yet it hosted sporulation. Conversely, eggplant and black nightshade are *Solanum* species and the leaves of neither of these became infected. The *Solanum* species tested were more likely to become infected than the non-*Solanum* species, suggests that further studies of host range should focus on the genus *Solanum*.

Different disease responses were observed among the three clonal lineages of *P. infestans* across solanaceous plants, particularly on tomato. Clonal lineages US-22 and US-23 produced abundant mycelial cover and high numbers of sporangia per square centimeter when inoculated onto tomato leaf tissue. This was in contrast to US-24, which produced significantly less mycelial cover and sporangia per square centimeter (Figs. 1 and 5). US-24 has never been isolated from tomato in Wisconsin, but has been isolated from numerous potato samples (53). A similar situation has been seen around the U.S., with very few US-24 isolates collected from tomato (8, 38). The mechanisms causing the streaks of dead cells observed on tomato leaves upon inoculation with US-24 remain unknown, but it is evident that this resulting cell death limits the early biotrophic phase of infection characteristic of a successful late blight infection.

Because of this, tomato may be able to inhibit US-24 before it can produce a full sporulating lesion, particularly under environmental conditions that are less than ideal for the pathogen. US-24 may still require management on tomato if initial infection is successful, but our data on pathogen growth and sporangial production suggest that spread within the crop may be limited when compared to that with US-22 or US-23.

The disease response observed on tomato leaves inoculated with US-24 was unique among the lineages, but a similar, though more pronounced, response occurred on bittersweet nightshade leaves inoculated with each of the three lineages. Rather than the typical hemibiotrophic response of *P. infestans*, which is characterized by pathogen sporulation followed by cell death (21, 39), dead cells were often observed several days before sporulation was visible. Additionally, instead of the typical uniformly expanding lesion, the dead cells often appeared in a branching pattern radiating from the point of inoculation (Fig. 3). Sporulation, if it occurred, typically formed in a ring at the edge of the branches of dead cells and not throughout the lesion. The dead cells that were observed surrounding the advancing pathogen hyphae may have been a resistance response initiated by the plant to limit pathogen spread (28). However, under the ideal disease conditions in the detached leaf assay, the pathogen often eventually overcame this attempted resistance response and produced sporangia. Under less ideal conditions, such as what may be encountered under natural conditions, infection may be effectively limited. Our data on pathogen sporulation on bittersweet nightshade suggest that this weed may contribute to a late blight epidemic when infected with US-22, US-23, and US-24 if cool, wet, humid weather conditions persist.

Our results on the infection of nightshade weed berries by US-22, US-23, and US-24 was largely in contrast to what has previously been reported for *P. infestans*. Dandurand et al. (7) determined that the US-1 and US-8 lineages were most likely to infect wounded berries of hairy nightshade, while wounded black and bittersweet nightshade berries showed a very low incidence of infection when inoculated with US-1 and US-8. In our work with US-22, US-23, and US-24, black nightshade berries exhibited the greatest incidence of sporulation but sporulation incidence was much lower on hairy nightshade (Fig. 4). Such differing results stress the importance of continuing to monitor changes in host range of a changing *P. infestans* population.

Sporulation incidence was low on all nightshade berries, but even this low incidence may be epidemiologically significant. Black and hairy nightshade are common weeds, and the authors have observed heavy infestations of hairy or black nightshade in Wisconsin potato fields. A healthy individual plant can produce 1000 to 2000 berries (41), so even a low incidence of infection can result in a large number of infected berries. If late blight is a threat late in the production season, potato growers in the upper Midwestern U.S. are advised to wait at least two weeks between complete chemical vine kill and harvest. This practice is intended to ensure that potato vines are no longer viable and able to harbor *P. infestans* that could potentially infect tubers at harvest. However, the authors have observed that hairy and black nightshade berries can persist on the plants beyond two weeks after vine kill, and this has been further corroborated (45). If infected and sporulating, nightshade berries could serve as a source of inoculum to the tubers both before and after harvest. It is therefore important to manage these weeds throughout the entire season for effective late blight control.

The dissimilar disease and pathogen sporulation responses between leaf and fruit tissue of the nightshade weed species indicates the importance of testing multiple plant organs for late blight susceptibility. Hairy nightshade leaves produced abundant mycelial cover and high numbers of sporangia, but the berries exhibited a low incidence of sporulation when inoculated with the three lineages (Figs. 1, 4, and 5). In contrast, black nightshade leaves hosted no mycelial cover when inoculated with the US-22, US-23, or US-24 clonal lineages, but the berries yielded the highest sporulation incidence of all nightshade berries tested. By including an evaluation of late blight sporulation on fruit, we can conclude that black nightshade is a host for *P. infestans*, which would have been overlooked if only the leaves had been tested.

Control of hairy nightshade may be important in limiting inoculum sources that can contribute to late blight epidemics. Inoculation of hairy nightshade leaves with the US-22, US-23, and US-24 clonal lineages of *P. infestans* resulted in sporangial production not significantly different than sporangial production on inoculated tomato leaflets (Fig. 5, Table 1). Late blight infected tomato plants can contribute to potato late blight epidemics, as demonstrated on a large scale across the eastern and Midwestern U.S. in the 2009-growing season (8, 38, 51). It follows, then, that hairy nightshade should also be managed to reduce inoculum sources and limit spread of late blight from weeds to potatoes and tomatoes. If hairy nightshade is in a potato or tomato field, it should receive the same fungicide program as the main crop, limiting late blight infection, but field edges or non-potato/tomato fields would not receive this treatment. If these areas are infested with hairy nightshade they may be sources of inoculum and should be monitored to avoid inoculum production and late blight spread to susceptible potato and tomato crops.

Our work has updated knowledge of the host range of *P. infestans* on cultivated plants and nightshade weeds common to the U.S. While some of our results corroborate that of other researchers who have investigated the host range of previously prominent lineages, the body of host range knowledge outside of potato and tomato is generally limited. Additionally, some of our results are novel, reflecting a changing *P. infestans* population. Therefore, the *P. infestans* host range should continue to be monitored, as new clonal lineages will likely continue to be identified in the U.S. as they have for the past 20 to 25 years (31). Results of this work on *P. infestans* will further inform late blight management recommendations and will lead to more efficient and effective control of this potentially devastating disease.

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Table 1. Mycelial cover, symptomatic tissue, and sporulation measurements indicating significant differences among plant species within each clonal lineage.

Metric[w], host[x]	**Rating Value[y]**		
	US-22	**US-23**	**US-24**
Mycelial cover			
HNS	643.6 a[z]	784.4 a	741.3 a
Tomato	573.0 a	553.6 b	258.7 b
BitNS	15.3 b	129.5 c	152.9 bc
Petunia	13.2 b	56.4 c	51.1 c
BkNS	3.5 b	0.0 c	0.8 c
Eggplant	0.0 b	0.0 c	0.0 c
GrCh	0.0 b	0.0 c	0.0 c
Pepper	0.0 b	0.0 c	0.0 c
Tomatillo	0.0 b	0.0 c	0.0 c
Symptomatic tissue			
HNS	468.1 a	656.3 a	622.5 a
Tomato	193.1 b	249.8 c	397.2 b
BitNS	115.0 bc	459.2 b	481.3 ab
Petunia	10.7 c	63.5 d	83.4 c
BkNS	2.7 c	24.0 d	0.0 c
Eggplant	0.0 c	0.0 d	0.0 c
GrCh	0.0 c	0.0 d	0.0 c

Pepper	0.0 c	0.0 d	0.0 c
Tomatillo	0.0 c	0.0 d	0.0 c
Sporangial production			
Potato	134,450 a	242,099 a	102,840 a
HNS	57,309 b	109,496 b	51,388 b
Tomato	45,664 b	114,162 b	21,613 b

[w]The mycelial cover and symptomatic tissue metrics are data taken from the same sets of leaves, and sporangial production was quantified on leaf disks in a separate experiment.

[x]HNS = hairy nightshade, BitNS = bittersweet nightshade, BkNS = black nightshade, and GrCh = ground cherry.

[y]For the Mycelial Cover and Symptomatic Tissue experiments, the rating value is the area under the disease progress curve calculated from ratings taken at 0, 5, 7, 9, 12, and 14 days after inoculation. For the sporangial production experiment, the rating value is the number of sporangia produced per square centimeter of leaf area.

[z]Numbers in each column within each metric followed by the same letters are not significantly different by the Tukey test (P=0.05)

Figure 1. *Phytophthora infestans* mycelial cover on solanaceous hosts inoculated with the US-22, US-23, and US-24 clonal lineages. No mycelial cover was observed on eggplant, ground cherry, pepper, or tomatillo, so these were omitted from the figure. Percent mycelial cover was determined at 0, 5, 7, 9, 12, and 14 days after inoculation and the area under the disease progress curve (AUDPC) calculated for each host/lineage combination. Error bars are standard deviations. Different letters over the bars indicate significant differences among clonal lineages within each host (Tukey test, P=0.05). HNS=hairy nightshade, BitNS=bittersweet nightshade, and BkNS=black nightshade.

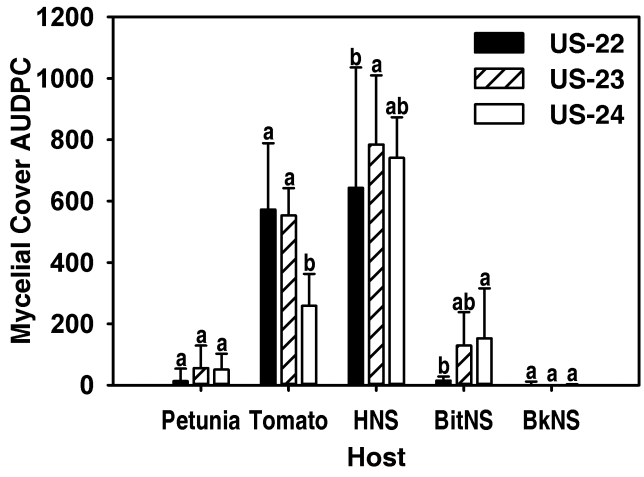


Figure 2. Cumulative late blight symptom expression on foliage of solanaceous hosts inoculated with the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans*. No disease was observed on ground cherry eggplant, pepper, or tomatillo, so these plants were omitted from the figure. Percent disease was determined at 0, 5, 7, 9, 12 and 14 days after inoculation and the area under the disease progress curve (AUDPC) calculated for each host/lineage combination. Error bars are standard deviations of the combined date from two repetitions of the experiment. Different letters over the bars indicate significant differences among clonal lineages within each host (Tukey test, P=0.05). HNS=hairy nightshade, BitNS=bittersweet nightshade, and BkNS=black nightshade.

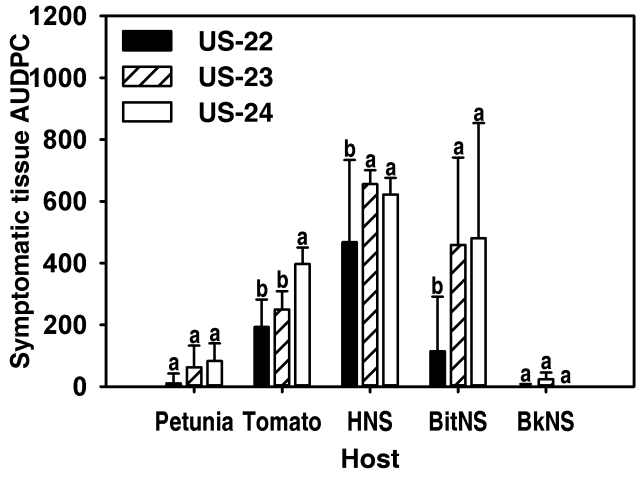


Figure 3. Unique inoculation response often observed on bittersweet nightshade with the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans*. The leaf pictured was inoculated with the US-23 clonal lineage and the image was taken 6 days post inoculation.

Figure 4. Incidence of *Phytophthora infestans* sporulation on berries of solanaceous weeds inoculated with the US-22, US-23, and US-24 clonal lineages. Bars represent the mean sporulation incidence of two repetitions of the experiment. Error bars represent the standard deviation between the two repetitions. There was a statistically higher incidence of sporulation with inoculation of US-22 than US-23 on wounded black nightshade berries, but otherwise there were no statistically significant differences among lineages (Tukey test, P=0.05).

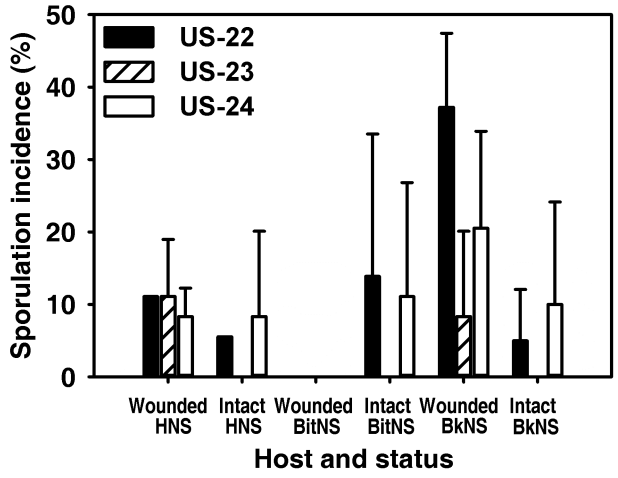
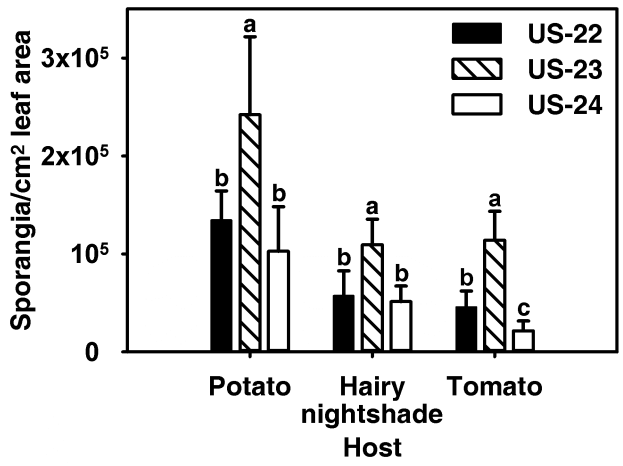


Figure 5. *Phytophthora infestans* sporangial production on hairy nightshade, tomato, and potato leaf disks inoculated with the US-22, US-23, and US-24 clonal lineages. Bars are the mean number of sporangia from eight leaf disks (four per experiment) and error bars are standard deviations. Different letters over the bars indicate significant differences between clonal lineages within each host (Tukey test, P=0.05).



CHAPTER 4:

Asexual survival of three clonal lineages of *P. infestans* on tomato seeds under simulated Upper Midwestern overwintering conditions

Abstract

Asexual survival of *Phytophthora infestans*, the cause of potato and tomato late blight, is generally limited under freezing conditions due to the inability of this phase of the pathogen to survive in the absence of living plant tissues. Potato tubers insulated in cull piles can harbor the asexual stages of this pathogen over winter, but overwintering in tomato is thought to be negligible and has been little researched. We tested survival on wet tomato seeds for up to 112 days at temperatures of 18, 4, 0, -5, and -3°C using isolates of three recently identified clonal lineages of *P. infestans,* US-22, US-23, and US-24. To facilitate the exploration of four applied examples, we compared the data to recent winter soil temperatures in four USDA hardiness zones in the upper Midwestern United States. All lineages survived for 112 days at 18 and 4°C and for 84 days at 0°C. The US-23 lineage showed the greatest cold tolerance, surviving for 21 days at -3°C and 7 days at -5°C on wet tomato seeds. Comparing the survival data from each lineage to 5-cm winter soil temperatures suggests that overwintering of each of the three lineages at each of the four example locations may be possible, but is more likely in the Zones 5a and 5b locations than in Zones 4a and 4b locations. Together, these data suggest that *P. infestans* has the potential to overwinter asexually in tomato production systems in northern regions. Seedlings arising from buried infected fruit should be monitored and managed to prevent the initiation of tomato and potato late blight epidemics.

Introduction

Phytophthora infestans (Mont.) deBary, the causal agent of late blight, is one of the most destructive pathogens of potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.)

production worldwide. Asexual survival of *P. infestans* at freezing temperatures is generally limited because *P. infestans* is essentially an obligate parasite in its asexual state (8, 22). Sexual reproduction and development of resilient oospores is not common in northern North America (9, 13, 16, 21), and this pathogen is therefore usually eliminated from northern regions each winter when temperatures below 0°C destroy all annual plants.

Asexual overwintering of *P. infestans* in some regions has been investigated. In potato production systems, overwintering in potato cull piles has been demonstrated (3). Even in regions were winter air temperatures fall well below 0°C, tubers in the center of the pile may be insulated enough to remain above freezing and may give rise to infected sprouts the following season (3, 15). Additionally, tubers buried in fields after harvest may be protected from freezing conditions and harbor the late blight pathogen through the winter (3, 15). In tomato production systems in northern regions of the U.S., survival in tomato is assumed to be limited due to freezing conditions, but overwintering of the pathogen in compost piles has been suggested (12). In California, where temperatures do not fall below freezing, infected seedlings can emerge from buried tomato fruit after the winter season (24). Further research indicated that wet tomato seeds infested with *P. infestans* could give rise to infected plants, but the seeds were exposed to temperatures only as low as 10°C (23).

Few studies have explored the possibility for *P. infestans* to survive freezing temperatures in its asexual state. In 1926 deBruyn tested survival of *P. infestans* in various substrates and concluded that the pathogen could survive in manure for 5 days with temperatures as low as -25°, but the possibility that sexual oospores were formed in the cultures could not be eliminated (7). More recently, Kirk (17) tested survival of mycelia of four clonal lineages of *P.*

infestans in artificial media, and found survival for up to 3 days at -3°C, but survival for no longer than 24 hours at -5°C. Dandurand et al. (5) found successful low temperature survival on natural substrates when berries of nightshade weeds infected with *P. infestans* were frozen for 48 hours at -15°C. However, when compared to the length of winters in northern regions, these experiments have all been limited in duration. Additionally, other natural substrates, such as tomato seeds, have not been investigated.

The US-22 clonal lineage of *P. infestans* was newly identified in the U.S. in 2009 when it caused widespread late blight, mostly on tomato, across the eastern and Midwestern U.S. (6, 14, 20, 21). In Wisconsin, tomato late blight caused by the US-22 lineage was widespread in 2009, and was again scattered and widespread in 2010 (21), raising questions about its ability to overwinter in tomato production systems. In the following years, two other new clonal lineages, US-23 and US-24 also became widespread across the U.S. (10, 11). We investigated the viability of US-22, US-23, and US-24 on wet tomato seeds subjected to long term freezing conditions. Additionally, to facilitate the evaluation of four applied examples, soil temperature data were collected from one location in each of four northern hardiness zones (25), zones 4b, 4a, 5b, and 5a, and compared to our survival data. These results will contribute to an understanding of *P. infestans* biology and improve management of late blight.

Materials and Methods

All *P. infestans* isolates were collected from Wisconsin in 2009 or 2010 and were previously characterized (21). One isolate from each clonal lineage was used. The US-22 isolate (Pi7-09a) is of the A2 mating type and was originally collected from tomato fruit. The

US-23 isolate (Pi16-10a) is of the A1 mating type and was isolated from tomato leaves. The US-24 isolate (Pi21-10a) is of the A2 mating type and was isolated from potato leaves. All cultures were derived from single zoospore isolations, and oospore production in single-isolate cultures was never observed. Isolates used for the experiment were transferred minimally in culture after isolation to maintain the original characteristics of the isolates. Isolates are maintained in hemp seed long term storage vials (18) in the Potato and Vegetable Pathology Laboratory of Dr. Amanda Gevens at the University of Wisconsin-Madison.

Three commercially-processed, non-fungicide treated tomato seeds (cv. Brandywine Red, Reimers Seeds, Saint Leonard, MD) were placed in each of 110 1.5 ml microcentrifuge tubes and sterilized by autoclave. To each tube, 0.75 ml of sterile distilled water and one 7-mm-diameter plug of mycelia/sporangia from a 2- to 4-week-old actively growing rye A culture (4) of a single isolate was added. All tubes were closed and incubated at 18°C for 4 weeks to allow colonization of the tomato seeds. After incubation, 22 tubes were randomly placed in each of five 80-well plastic microcentrifuge tube racks. The entire procedure was repeated for each of the three *P. infestans* isolates, so that each microcentrifuge tube rack contained 66 tubes (22 for each isolate). One rack each was placed at -5, -3, 0, 4, and 18°C.

Incubator temperatures were measured with Watch Dog B Series temperature buttons (Spectrum Technologies Inc., Aurora, IL, margin of error: ±1.1°C) located near the microcentrifuge tube racks of tomato seeds. Prior to beginning the experiment, five buttons were placed in one incubator and the collected temperature data checked for consistency between the buttons. Buttons were then moved to their respective incubators. Through the course of the experiment temperatures fluctuated no more than 0.5°C from the desired temperatures.

Survival of each isolate of *P. infestans* was tested at 1, 2, 3, 5, 7, 14, 21, 28, 56, 84, and 112 days after exposure to each temperature treatment. For the time points at or before 28 days, the sampling was performed at exactly the time specified, ±5 minutes. Later time points were exact ±2 hours. For each of the 11 time points, two tubes of each isolate at each temperature were randomly selected and removed from the incubators. Within two hours of removal, the six seeds from the two tubes were placed on rye A medium in petri plates and incubated at 20°C. Individual seeds plated on medium were evaluated at 7, 14, and 21 days after placing the seeds on the medium by examining each by eye or with a dissecting microscope, as needed. The amount of *P. infestans* growth from each seed was quantified using a survivability index of 0 to 4, where 0 = no *P. infestans* growth, 1 = less than 10 short mycelial strands, 2 = more than 10 longer mycelial strands but not a wide-spreading colony, 3 = a wide-spreading colony, but less than the maximum expected, and 4 = a thick profuse colony of mycelia/sporangia, the maximum expected. Varying degrees of vigor are characteristic among the isolates in culture, so the scale was calibrated by observing several (>20) cultures of each isolate that had not been exposed to any temperature treatment to visually determine the maximum expected growth of each isolate.

The three *P. infestans* survivability index ratings (taken at 7, 14, and 21 days after placing the seeds on medium) were summed individually for each of the 11 exposure times for each isolate/temperature combination to achieve one survivability index value for each combination, with the maximum value being 12. Sampling for a given isolate/temperature combination was ended before 112 days of exposure if all the seeds in two consecutive samplings showed no *P. infestans* growth 21 days after plating the seeds on medium. The experiment was performed three times. Due to minimal (±0.5°C) differences in incubator temperatures between and within

repetitions of the experiments, the data from the three repetitions were pooled and analyzed together. A three-way ANOVA was performed on the three executions of the experiment and significant differences at P=0.05 determined using Tukey's test.

To facilitate the exploration of four applied examples, soil temperature data were obtained from single locations in four different USDA hardiness zones, 4b, 4a, 5b, and 5a. Four years of minimum soil temperature data at 5 cm below the surface were aggregated from 15 November through 15 April from public databases beginning with the 2009 to 2010 winter. Soil temperature data for the Zone 5b location (Charlotte, Michigan, in south-central Michigan) were downloaded from the Michigan State University Enviro-Weather database (http://www.agweather.geo.msu.edu/mawn/station.asp?id=clt&rt=24). Soil temperature data for the Zone 5a location (Shagbark Hills, Iowa, in northwest Iowa) and the Zone 4a location (Crescent Lake, Minnesota, in central Minnesota) were downloaded from the Natural Resources Conservation Service National Water and Climate Center (http://www.wcc.nrcs.usda.gov/scan/). Soil temperature data for the Zone 4a location (Hancock, Wisconsin, in central Wisconsin) were downloaded from the University of Wisconsin Soil Science website (http://www.soils.wisc.edu/uwex_agwx/awon/daily_soil). Data were summarized to reflect the number of days at or below 0, -3, and -5°C for each winter at each location and survivability criteria of temperature and duration applied categorically.

Results

All three clonal lineages had high survivability indexes on tomato seeds at 18°C for the duration of the 112-day experiment (Fig. 1, Table 1) alhough there were some statistical

differences between lineages at this temperature, particularly early in the experiment (Table 2). Recovery of *P. infestans* at 4°C was also high, and usually not significantly different than survival at 18°C (Table 1). For the US-22 lineage, the survivability values at 4°C dropped off sharply after 56 days, but prior to that had been steadily near maximum and not significantly different than the 18°C treatment (Fig. 1a, Table 1). For all the lineages at 0°C, survival was observed up until 84 days after freezing, but none of the lineages survived for 112 days at 0°C (Fig. 1, Table 3). Survival at -3°C was variable between the three lineages, although statistically significant only at 1 and 2 days of exposure (Table 2). For the US-22 clonal lineage, survival was seen after 5 days, but not after 7 days, but the survival seen at 14 days indicated that the pathogen was still alive during that period (Fig. 1a). The US-24 clonal lineage showed similar results at -3°C (Table 3). For the US-23 clonal lineage, however, survival values higher than zero were consistently seen up until 21 days after freezing at -3°C (Fig. 1b), even though these values were not statistically greater than zero (Table 1). At -5°C, survival was further reduced compared to the higher temperatures, but it was still observed. US-24 survived the shortest time, failing to show survival between 3 and 5 days after freezing (Fig. 1c, Table 3). The US-22 and US-23 clonal lineages both survived for 7 days, but died before the 14-day sampling (Fig. 1a and 1b, Table 3).

The zone 5b location had the most moderate 5-cm soil temperatures of locations analyzed (Table 4). All lineages of *P. infestans* could survive for 84 days at 0°C (Table 3) and temperatures in the zone 5b location were never below 0°C for longer than 85 days in the past four winters (Table 4). Additionally, temperatures never dropped below 3°C in this location in the past four winters.

In the zone 5a location, 5-cm soil temperatures at or below 0°C were usually in duration of less than 85 days, except in the 2012-2013 winter, where there were 91 days below 0°C (Table 4). Extrapolating data between the 85 and 112 sampling points suggests that the US-22, US-23, and US-24 lineages would likely still be alive 6 days after the 84-day sampling point, and so would survive for 91 days (Fig. 1). Additionally at this location, the temperature dropped below -3°C for just a few days in two of the winters, and only once dropped down to -5.4°C (Table 4). Survival of the US-22, US-23, and US-24 clonal lineages of *P. infestans* was longer than each of these durations at both temperatures (Table 3).

In the zone 4b location, more days had soil temperatures below 0°C than in the other states, with the 2010 to 2011 winter having the most, at 130 days. None of the lineages survived to the 112-day sampling point at 0°C, so 130 days would have been lethal (Table 3). The 2011 to 2012 and 2012 to 2013 winters had 97 and 99 days, respectively, of soil temperatures below 0°C (Table 4), and extrapolating the survivability results between the 85 and 112 sampling points indicates that *P. infestans* may have survived when 0°C data alone are considered (Fig. 1). However, the 2012 to 2013 winter soil temperatures were very cold, dropping down to -11.45 and having 7 days below -5°C (Table 4). The 2011-2012 winter was not as cold, with minimum temperatures reaching just -5.68°C and having only 3 days at or below -5°C (Table 4), which is within the survival ranges of the US-22, US-23, and US-24 lineages (Table 3). However, 17 days at or near below -3°C but greater than -5°C may have been long enough to eliminate all except the US-23 clonal lineage. US-23 was able to survive for 21 days at -3°C on tomato seeds, while the US-22 and US-24 lineages had only a very low survivability index at 14 days (Fig. 1).

The 2009 to 2010 winter had the warmest soil temperatures of the four years, with 80 days below 0°C and just 3 days at or below -3°C (Table 4), so survival would have been likely in this case.

In the zone 4a location, the 2011 to 2012 winter was the coldest winter between 2009 and 2013, dropping down to a minimum of -7.3°C and having 4 days below -5 (Table 4). However, in the winter of 2010 to 2011, even though the 5-cm soil temperature never dropped below -3°C, 111 days were spent below 0°C (Table 4), which is longer than *P. infestans* can survive at 0°C; all lineages failed to grow out of the tomato seeds after 112 days at 0°C (Table 3). The winter of 2012 to 2013 had 90 days at or below 0°C, and 11 of those were at or near below -3°C, which are greater than than the lethal temperatures and less than the lethal durations for each lineage (Table 3). The 2009 to 2010 winter soil temperatures were the warmest, with only 64 days below 0°C and a minimum soil temperature of only -0.5°C (Table 4), which is within the range of survival of each lineage (Table 3).

Discussion

Asexual survival of *P. infestans* at low temperatures has been explored on artificial media (17) and nightshade berries (5), but never on tomato seeds and never for extended durations. We have demonstrated that three clonal lineages of *P. infestans* recently identified in the U.S., US-22, US-23, and US-24, are able to survive on wet tomato seeds for extended periods at temperatures near and below freezing. In tomato fields or home gardens, tomato fruits may likely be buried or plowed under at the end of the season and thereby be protected from the harsh extreme winter air temperatures. The possibility that new clonal lineages of *P. infestans* may be

able to overwinter in northern regions on tomato seeds has important implications for tomato late blight management in these areas.

The evaluation of four example locations in conjunction with the laboratory data suggests that there is a possibility that *P. infestans* can persist long enough in wet tomato seeds at temperatures near and below freezing to survive the winter in northern regions. Zone 5b was the warmest hardiness zone considered and overwintering was most likely in this location in our analysis. Soil temperatures at 5 cm of depth never dropped below -3°C over the four years analyzed and far fewer than 85 days were ever below 0°C in each of the last four winters. Soil temperatures were colder in the location evaluated in zone 5a, but still warm enough that the US-22, US-23, and US-24 lineages that we tested would likely survive each winter. The potential for survival of *P. infestans* on wet buried tomato seeds in the zone 4b and 4a locations was less, but still likely in some of the winters from 2009 to 2012. All of the soil temperature data were the minimum temperature for each day, and this temperature may have been reached for just a short time each day, so survivability may be higher than predicted. Additionally, actual soil temperatures may fluctuate and our experimental parameters were kept constant. Field studies over many years and in various regions would validate the survivability of *P. infestans* over winter, but our data suggest that *P. infestans* may survive on buried tomato seeds in some winters and locations in the northern U.S.

Extrapolating results from the four example locations to other locations in each hardiness zone may be appropriate, but should be done with caution. Hardiness zones are delimitated based on air temperatures, so these temperatures should be consistent within each hardiness zone (25). However, different soil types and/or moisture contents can affect how cold a given soil will

become at a given air temperature (1). Additionally, many microclimates may exist within the hardiness zones. Backyard garden plots in cities are often more sheltered and warmer than rural areas, and compost piles may be kept warmer due to microbial activity (19), both of which would increase the likelihood of survival of *P. infestans*. Additionally, more abundant snowfall or drifts in certain years or areas may insulate the ground and prevent lower temperatures. The affect of soil microclimate is evident even in our four examples. Zone 4b is technically warmer than zone 4a, but soil temperatures were actually more moderate in the zone 4a location than the zone 4b location over the four years analyzed. Generalizing results from a single location to the rest of the hardiness zone in these locations may contribute to general knowledge of overwintering potential, but may lead to inappropriate conclusions on the risk of tomato late blight overwintering in certain microclimates.

Instead of extrapolating from our examples and generalizing about *P. infestans* survival, winter soil temperatures from anywhere and at any depth can be compared directly to our laboratory results in a similar way as we have compared the four example locations. As we demonstrated with the zone 4a location, even if the temperature does not fall very low in a given winter, *P. infestans* cannot survive for 112 days at 0°C, so if soil temperatures remained at or near 0°C for that long, the likelihood of survival is low. Alternatively, a short cold snap may not eliminate *P. infestans*, as it can survive for several days at or near -5°C in tomato seeds. For some regions or production systems, higher or lower soil depths may be of interest, and our laboratory data can easily be applied in any of these circumstances. Our survivability data, therefore, have advantages over field data from a few locations over a few seasons because it can

be easily applied to numerous situations to individually evaluate the potential for survival of *P. infestans* on wet tomato seeds at freezing temperatures.

Differences between biological and statistical significance of the data should be noted. There were several instances when survivability values were numerically greater than zero but not statistically different than treatments that demonstrated no growth. Biologically, it is significant that some treatments grew, even if only a little, before the final growth assessment at 21 days. The statistics indicating no significant difference from treatments with no growth suggest that there is a low likelihood of survival at some of these temperatures and durations. However, under conditions conducive to pathogen growth and disease development, even the smallest surviving pathogen propagule may initiate an epidemic. Therefore, any survivability values numerically greater than zero should be considered as biologically significant.

The ability of *P. infestans* to cause late blight on tomato seedlings from infected/infested seeds has been demonstrated (23). Vartanian and Endo (23) showed a 34% rate of disease on tomato seedlings arising from infected seeds on sterile soil. Even on non-sterile field soil, 18% of seedlings arising from late-blight infected seeds showed signs and symptoms of late blight. Additionally, in natural tomato field settings, seedlings infected at the crown with late blight and growing in clumps have been observed, suggesting that these seedlings arose from a single buried infected tomato fruit instead of from aerial inoculum, which would have caused disease on the top of the plant (24). Our data indicate that *P. infestans* may be able to survive the winter in buried wet tomato seeds in northern regions. Along with the knowledge that infected seedlings can arise from infested/infected seeds containing viable *P. infestans*, we can conclude

that volunteer tomato seedlings may be a source of inoculum for initiating late blight epidemics, even in regions with freezing winters.

This overwintering potential has important implications for late blight management, not only in tomato, but also in potato. Potato cull piles are carefully managed and even regulated because of their documented ability to initiate late blight, even in northern regions with freezing winters (3, 26). Tomatoes are assumed to freeze over winter, destroying the living plant material needed for the hemibiotrophic *P. infestans* to survive, and so are not currently regulated, even if infected with late blight. Our results indicate that tomato growers should be aware of the potential for late blight to overwinter on tomato seeds in buried infected tomato fruits and manage volunteer tomatoes the subsequent spring. These tomatoes could host sporangial production that could spread late blight to both tomato and potato production fields.

As the global climate changes (2), some areas may become warmer and increase the likelihood of survival of *P. infestans* over winter. Regions that may have normally had soil temperatures cold enough to eliminate *P. infestans* may become warmer in the coming years. Monitoring soil temperatures over time and comparing these data to our results on survival can elucidate a need to monitor volunteer tomatoes for reduction of initial inoculum for tomato and potato late blight.

In many cases, a delayed initiation of *P. infestans* growth from the tomato seeds after the cold treatment was observed. Occasionally, some tomato seeds showed no *P. infestans* growth until the 3-week evaluation point. This apparent recovery period may explain why late blight is typically a mid- to late season disease. Our grow-out conditions were on sterile medium, but if the same growth rate was seen on tomato seedlings, it would likely be well over 1 month before

enough lesions would be produced for the disease to become evident on a field scale. The initial delay in growth from the tomato seeds in many cases may partly explain the phenology of late blight.

Many clonal lineages of *P. infestans* have been identified over the past 30 years in the U.S. and these new lineages usually replace the old ones, suggesting changes in the biology that impart enhanced fitness to new lineages. In our work, we tested isolates from three recently emerged clonal lineages of *P. infestans* that have been the most widespread lineages in the U.S. in the past several years (6, 14, 16, 21). Some differences were seen among the lineages, particularly at the lower temperatures, with US-24 surviving for the shortest duration at -5°C and US-23 for the longest of the lineages at -3°C. However, all three lineages that were tested on tomato seeds survived low temperatures for longer than any previous tests on *P. infestan*s (5, 7, 17). This may be due to differences in the medium tested, as we used tomato seeds, which had not been previously tested, but it may also indicate that new lineages are better able to survive temperatures at or below freezing than previously common lineages.

We have demonstrated that *P. infestans* can survive for extended periods near or below freezing on the natural medium of tomato seeds. Previous research has indicated that *P. infestans* can cause late blight on volunteer tomatoes arising from buried infected fruit. Our data suggest that *P. infestans* can survive on fruits buried in the soil over winter in northern regions. These results suggest that tomato production systems should be monitored to mitigate the potential overwintering of *P. infestans* on tomato seedlings in northern regions and thereby reduce the initial inoculum available to infect the tomato or potato crop.

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Table 1. Mean survivability index values and statistical differences between temperatures for each exposure time/clonal lineage combination for *Phytophthora infestans*.

	US-22					US-23					US-24				
Exposure time[y]	18°C	4°C	0°C	-3°C	-5°C	18°C	4°C	0°C	-3°C	-5°C	18°C	4°C	0°C	-3°C	-5°C
1	12.0a[z]	11.9a	11.9a	7.7b	7.0c	10.6a	10.8a	10.4a	6.2b	4.3c	11.2a	10.8a	10.3a	8.4b	6.3c
2	11.7a	11.8a	10.6ab	7.9c	8.4bc	8.5a	10.3a	8.7a	4.3b	5.0b	9.9a	8.6ab	6.4b	3.9c	2.7c
3	11.7a	11.6ab	9.4b	4.7c	1.7d	8.4a	8.7a	6.5a	2.8b	0.3c	9.1a	8.3a	8.2a	3.2b	1.8b
5	11.9a	11.4ab	9.6b	4.0c	0.0d	8.3ab	10.0a	7.0b	2.7c	1.1c	10.8a	9.9a	8.6a	2.1b	0.0b
7	11.8a	11.7a	10.3a	0.0b	0.4b	10.1a	9.2ab	7.6b	1.1c	0.5c	9.8a	8.6a	8.4a	0.0b	0.0b
14	11.7a	11.9a	9.7a	0.5b	0.0b	11.6a	11.8a	8.6b	0.3c	0.0c	11.1a	9.1a	5.5b	0.2c	0.0c
21	11.3a	12.0a	9.0b	0.0c	0.0c	11.3a	11.0a	5.3b	1.2c	0.0c	10.5a	9.6a	4.4b	0.0c	0.0c
28	11.8a	11.7a	5.6b	0.0c	0.0c	10.8a	11.2a	6.8b	0.0c	0.0c	10.8a	7.6a	5.8b	0.0c	0.0c
56	11.4a	8.9a	1.8b	0.0b	0.1b	7.8a	9.1a	0.6b	0.0b	0.0b	9.6a	6.0b	1.1c	0.0c	0.0c
84	11.9a	6.8b	0.9c	0.0c	0.0c	9.3a	9.3a	1.1b	0.0b	0.0b	11.3a	6.8b	0.6c	0.0c	0.0c
112	10.1a	1.0b	0.0b	0.0b	0.0b	11.3a	8.8a	0.0b	0.0b	0.0b	11.4a	3.4b	0.0c	0.0c	0.0c

[y] Exposure time (days) of each lineage to each temperature treatment.

[z] Values are means between the three executions of the experiment. Means within each exposure time and clonal lineage followed by the same letters are not significantly different (Tukey test, P=0.05).

Table 2. Significant differences between survival of each clonal lineage of *Phytophthora infestans* at each temperature and exposure time.

	Exposure temperature														
	18°C			4°C			0°C			-3°C			-5°C		
Exposure Time[y]	US-22[z]	US-23	US-24	US-22	US-23	US-24	US-22	US-23	US-24	US-22	US-23	US-24	US-22	US-23	US-24
1	a	b	a	a	b	a	a	b	a	a	b	a	a	b	a
2	a	b	ab	a	ab	b	a	a	b	a	b	b	a	b	c
3	a	b	b	a	b	b	a	a	a	a	a	a	a	a	a
5	a	b	a	a	a	a	a	a	a	a	a	a	a	a	a
7	a	a	a	a	b	b	a	a	a	a	a	a	a	a	a
14	a	a	a	a	a	b	a	a	b	a	a	a	a	a	a
21	a	a	a	a	a	a	a	b	b	a	a	a	a	a	a
28	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
56	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
84	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
112	a	a	a	a	b	c	a	a	a	a	a	a	a	a	a

[y] Exposure time (days) of each lineage to each temperature treatment.

[z] Different letters indicate significant differences between clonal lineages at each temperature and exposure combination (Tukey test, P=0.05).

Table 3. Summary of the length of survival (days) of the three clonal lineages of *Phytophthora infestans* at each tested temperature.

Clonal lineage	18°C	4°C	0°C	-3°C	-5°C
US-22	112	112	84	14	7
US-23	112	112	84	21	7
US-24	112	112	84	14	3

Table 4. Summary of daily minimum winter soil temperatures from 5 cm below the surface from 2009 through 2013 from single locations in four USDA hardiness zones and a qualitative assessment of survival of each clonal lineage of *Phytophthora infestans*.

		Number of days at or below each temperature			Clonal lineage survival		
HZ[a], year[b]	Minimum[c]	0°C	-3°C	-5°C	US-22	US-23	US-24
Zone 5b							
2009-2010	-2	24	0	0	Y[d]	Y	Y
2010-2011	-1	45	0	0	Y	Y	Y
2011-2012	-0.1	4	0	0	Y	Y	Y
2012-2013	-1.9	72	0	0	Y	Y	Y
Zone 5a							
2009-2010	0.1	0	0	0	Y	Y	Y
2010-2011	-2.4	76	0	0	Y	Y	Y
2011-2012	-4.4	62	5	0	Y	Y	Y
2012-2013	-5.4	91	6	1	Y/N	Y/N	Y/N
Zone 4b							
2009-2010	-4.2	80	3	0	Y	Y	Y
2010-2011	-3.4	130	2	0	N	N	N
2011-2012	-5.7	99	20	3	N	Y/N	N
2012-2013	-11.5	97	12	7	Y/N	Y/N	N
Zone 4a							
2009-2010	-0.5	64	0	0	Y	Y	Y
2010-2011	-1.2	111	0	0	N	N	N
2011-2012	-7.3	75	14	4	Y/N	Y/N	Y/N
2012-2013	-4.6	90	11	0	Y	Y	Y

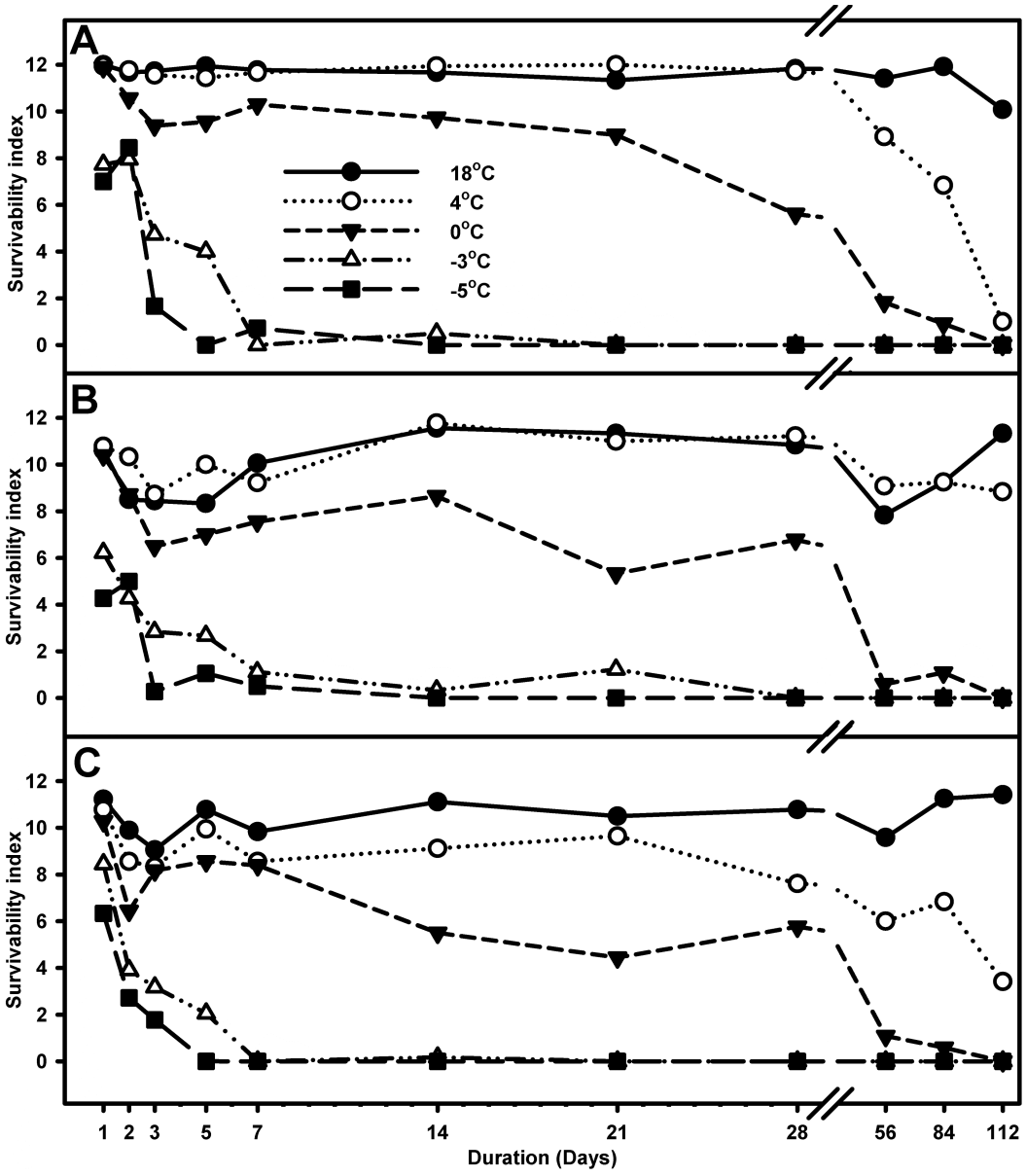
[a] USDA Plant Hardiness Zone designation (25).

[b] Data was aggregated from 15 November to 15 April for each winter period.

[c] Minimum temperature (°C) recorded each winter period.

[d] The designation Y (yes) indicates that survival would have been likely with the given set of temperature conditions in each row. The designation N (no) indicates that survival would not have been likely. The designation Y/N indicates that the winter temperature/duration ranges were outside of those ranges experimentally determined.

Fig. 1. Survival of the (A) US-22, (B) US-23, and (C) US-24 clonal lineages of *Phytophthora infestans* on tomato seeds at five different temperatures for up to 112 days. Survivability was evaluated for each lineage on a 0 to 4 scale at 7, 14, and 21 days after plating out seeds from each temperature/duration treatment and those values summed for a maximum possible survivability index of 12 for each seed. Each symbol represents the mean of 18 seeds (6 seeds per execution, three executions of the experiment).



CHAPTER 5:

Heirloom and hybrid tomato cultivar resistance to late blight caused by the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans*

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Abstract

Late blight, caused by the oomycete *Phytophthora infestans,* causes serious losses in tomato production worldwide. Application of fungicides throughout the growing season is a primary means of control in many regions, but cultivar resistance, primarily through *Ph* resistance genes from *Solanum pimpinellifolium*, can provide a cost-effective and environmentally friendly foundation to an overall disease management program. Due to highly adaptable pathogen populations, cultivar resistance against late blight is often short-lived. We evaluated 11 tomato cultivars against three clonal lineages (US-22, US-23 and US-24) of *P. infestans* novel to the U.S. to determine the efficacy of currently deployed *Ph* genes in hybrid cultivars and the validity of claims of late blight resistance in heirloom cultivars. The US-24 clonal lineage showed a low level of virulence on all the cultivars tested, but US-22 and US-23 were highly virulent on tomato. 'Plum Regal' (*Ph-3*) and 'Legend' (*Ph-2*) demonstrated some resistance against US-23 but both were susceptible to US-22. Interestingly, when the *Ph-2* and *Ph-3* resistance genes are combined in 'Mountain Magic', resistance was effective against all three lineages, including US-22, which may be explained by the different genetic backgrounds in the cultivars, or a possible residual effect of broken resistance genes. Surprisingly, three heirloom cultivars, 'Wapsipinicon Peach', 'Matt's Wild Cherry', and 'Pruden's Purple' showed resistance to all three clonal lineages, suggesting broad-spectrum resistance unlike that conferred by the currently deployed *Ph* genes. Wapsipinicon Peach may have *S. pimpinellifolium* in its background and Matt's Wild Cherry may have been subjected to selection pressure in Mexico, but the resistance in Pruden's Purple is currently without explanation. The heirloom cultivars may be useful for future tomato late blight breeding efforts. All of the cultivars investigated in

this work are currently commercially available to growers and home gardeners to limit losses to tomato late blight and reduce reliance on fungicides. Additionally, resistant cultivars limit the production of inoculum, reducing overall late blight risk in tomato and potato.

Introduction

Phytophthora infestans (Mont.) deBary causes late blight, a serious disease in tomato (*Solanum lycopersium* L.*)* production worldwide. In the U.S., late blight is considered the eighth most important disease of tomato production based on potential for economic loss (10). Tomato late blight can also spread to potato production, and potato late blight losses are estimated at $6.7 billion annually around the globe (30). Lesions produced on leaves and stems can cause a plant to defoliate and collapse; lesions are also produced on ripe or unripe fruit making them unmarketable and contributing directly to economic loss (35). *P. infestans* is a heterothallic oomycete pathogen with a short generation time of four to five days, and under cool, wet, humid conditions, the asexual sporangia can quickly spread throughout a field and into surrounding fields, rapidly devastating susceptible and unprotected crops (35).

Since the time of its discovery as the biological cause of the Irish potato famine in the late 1840s, *P. infestans* had largely been considered a potato pathogen (26). However, isolates have been identified that infect both potato and tomato (27). In the U.S., the late blight population has limited diversity due to lack of sexual recombination and has been categorized into clonal lineages, or asexual descendants of a single genotype (17). In the U.S., US-8 was the first widespread clonal lineage that required vigilant management on both potato and tomato (27). Since then, several other lineages have been identified that readily infect tomato and potato

(27). Additionally, some lineages have been identified as primarily pathogenic on tomato and will not cause disease on potato under field conditions (31). It is therefore important to understand which lineages are present for optimized tomato late blight control.

Management of late blight on tomato requires an integrated approach of cultural and chemical strategies. In the U.S., asexual reproduction of *P. infestans* by ephemeral sporangia is the primary means of reproduction, as the A1 and A2 mating types of this heterothallic pathogen have historically not been in proximity to establish a soil persistent oospore population (27, 35, 50). Therefore, in northern regions with freezing winters, the pathogen can, in theory, be eradicated between growing seasons unless viable infected plant tissues are harbored in warm compost piles, greenhouses, or potato cull piles (3). Removing these overwintering environments can reduce pathogen overwintering potential and reduce late blight risk in a region the following season. However, the pathogen can be present in seed potatoes and/or tomato transplants, and sporangia can be aerially transported from other regions, so late blight management is often still necessary (34, 48). During the growing season, fungicides are commonly used to manage late blight, often in conjunction with disease forecasting models that optimize timing of applications based on environmental conditions favorable to *P. infestans* (38, 53). Tomato late blight fungicides are available with contact, protectant, translaminar systemic, and truly systemic activity (8), but all fungicides are most effective when applied before late blight infection has occurred. Additionally, clonal lineages have been identified that are insensitive to the systemic active ingredient mefenoxam, rendering it useless against certain pathogen populations (27, 51). The use of fungicides adds chemical and labor costs to

production and some may be environmentally unfriendly. Additionally, choices for effective fungicides are limited in organic production.

To enhance integrated late blight management within the growing season and reduce reliance on chemical control, host resistance has been investigated for tomato late blight management. Modern tomato late blight resistance breeding began in 1946 with screening of close relatives of tomato for resistance genes (47). Since that time, several resistance genes, designated *Ph-1*, *Ph-2* and *Ph-3* have been discovered in *S. pimpinellifolium* and mapped to regions of the tomato chromosomes (2, 7, 15, 19, 33, 41, 43). Most recently, further screening of this species has resulted in the discovery of genes tentatively named *Ph-5-1* and *Ph-5-2* (43). The first two *Ph* genes have been traditionally bred into commercial tomato cultivars and proved effective for a few years, but have since been overcome by races of *P. infestans* (15, 28, 43). More recently, new tomato cultivars containing *Ph-3* have been released, and while this resistance was hoped to be superior to *Ph-1* and *Ph-2* resistance, isolates of *P. infestans* have been found that overcome the resistance (6, 15, 21, 37, 43).

Throughout its history, *P. infestans* has been infamous for its ability to overcome resistance genes (16). The history of resistance breeding in potato is longer than that in tomato, and *P. infestans* isolates have thus far overcome all 11 potato resistance genes deployed (4, 28). In both potato and tomato, there is potential that broad-spectrum resistance or QTL-based field resistance may have targets of reduced specificity and thus may be less easily overcome than single, dominant genes (1, 15, 43). However, the durability of these kinds of late blight resistance has yet to be rigorously tested. Mutations in the asexual *P. infestans* population have largely been responsible for overcoming resistance genes deployed thus far; isolates within a

single clonal lineage can vary in virulence (28). With the introduction of several clonal lineages to an area, the potential for overcoming multiple resistance genes is increased.

In the U.S., genetic diversity in the *P. infestans* population was very limited up until the late 1980s, with US-1 as the only clonal lineage found in northern North America (27). Since that time, many new clonal lineages have been discovered and assigned a US-# designation (27, 50). In the absence of sexual recombination in the U.S., it is hypothesized that these lineages have been introduced from Mexico, where sexual recombination is common (25, 29). With the arrival of the US-8 clonal lineage, the US-1 and other lineages were quickly completely displaced and US-8 remained the most common lineage for many years (27). In 2009, the US-22 clonal lineage quickly became widespread throughout the eastern and Midwestern U.S., due largely to the distribution of late blight-infected tomato transplants (18, 31, 48). In subsequent years, US-23 and US-24 were also identified, and the previously predominant US-8 clonal lineage was almost completely displaced (9, 36, 50).

The arrival of new lineages that are highly virulent on tomato in the field brought into question the effectiveness of currently available late blight resistant tomato cultivars. Additionally, in commercial field surveillance in 2009, some cultivars with no known resistance genes appeared to have less severe symptoms under the high natural disease pressure than others. To further investigate, we used isolates from three new clonal lineage of *P. infestans*, US-22, US-23, and US-24, and evaluated the late blight disease response on several commercially available tomato cultivars containing *Ph* resistance and on several hybrid and heirloom cultivars with putative late blight resistance based on field observations and previous experimentation.

Materials and Methods

The US-22 (Pi7-09a), US-23 (Pi16-10a), and US-24 (Pi21-10a) isolates used in this study were collected from symptomatic plant tissues from Wisconsin production fields and gardens and have been previously characterized (50). Pi7-09a (A2, mefenoxam sensitive) was isolated from tomato fruit, Pi16-10 (A1, mefenoxam sensitive) from tomato foliage, and Pi21-10a (A1, mefenoxam intermediately sensitive) from potato foliage. Isolates are maintained in the Potato and Vegetable Pathology Laboratory of Dr. Amanda Gevens at the University of Wisconsin-Madison.

Ten tomato cultivars with putative resistance to late blight and one susceptible control were chosen for the experiment. The cultivars were chosen based on one or more of several criteria: (a) documented presence of resistance genes, (b) previous field studies indicating resistance with other *P. infestans* lineages (12, 32), (c) claims by seed companies of late blight resistance, or (d) personal observations of reduced late blight severity in some cultivars in commercial field surveillance in 2009 (Table 1). Seed was purchased from Johnny's Selected Seeds ('Pruden's Purple'), Totally Tomatoes ('Legend', 'Roma', 'Green Zebra', 'Wapsipinicon Peach', 'Juliet',), Seedway LLC ('Mountain Magic', 'Plum Regal'), and Reimer Seeds ('Matt's Wild Cherry', 'Brandywine Red', 'Slava') and all tomato plants were grown in a growth chamber at 28°C with a 14-hour photoperiod. For each cultivar, six fully expanded leaflets from 4-to 5-week-old tomato plants were detached using a sharp razor blade and immediately placed adaxial surface up in three 9-cm-diameter petri dishes directly on a thin layer of water agar, two leaves per plate. To prepare inoculum, 2- to 4-week-old actively growing cultures of each of the three clonal lineages of *P. infestans* were flooded with sterile distilled water and brushed gently

to dislodge sporangia. The resulting suspensions were transferred to sterile plastic 50-ml centrifuge tubes and the concentrations quantified using a hemacytometer. Each suspension was adjusted to 50,000 sporangia/ml by adding sterile distilled water. Five leaflets in each six-leaflet cultivar set were inoculated with 10-μl droplets of a suspension of a single clonal lineage. The sixth leaflet was mock-inoculated with a 10-μl droplet of sterile distilled water as a control. Plates were placed in plastic boxes with non-sealing lids and incubated at 20°C in the dark. Lesions were defined as either visible sporulation or necrotic/chlorotic diseased tissue and the lesion length was measured along the midvein with a ruler at 0, 5, 7, and 9 days post inoculation (dpi). Aerial pathogen growth (mycelia, sporangia, sporangiophores) was estimated and assigned to the categories of 0, 25%, 50%, 75%, or 100% coverage of the leaflet surface at each of the same time points. The experiment was performed twice.

Results from the two executions of the experiment were pooled for analysis. Area under the disease progress curve (AUDPC) was calculated for both lesion length and pathogen growth for each cultivar/clonal lineage combination using the AUDPC function in the Agricolae package for R (11). A two-way ANOVA was performed on the resulting values using SigmaPlot (Systat Software, San Jose, CA) and significant differences between cultivars and clonal lineages determined using Tukey tests (P=0.05).

Results

Most of the cultivars were resistant when challenged with the US-24 clonal lineage. Overall, US-24 produced the smallest cumulative lesion length values across all of the tomato cultivars inoculated (mean=20.6) compared to US-22 (mean=50.2, P<0.001) and US-23

(mean=49.3, P<0.001). Similarly, overall pathogen growth values were less when inoculated with US-24 (mean=86.6) than with US-22 (mean=207.3, P<0.001) or US-23 (mean=199.1, P<0.001). Individual cultivars inoculated with US-24 had cumulative lesion length and cumulative pathogen growth values significantly less than cultivars inoculated with US-22 and US-23 on almost all of the tomato cultivars tested (Figs. 1 and 2).

When inoculated with US-22 and US-23, the cultivars Mountain Magic, Matt's Wild Cherry, Wapsipinicon Peach, and Pruden's Purple demonstrated the greatest resistance with both cumulative lesion length and cumulative pathogen growth values significantly less than other cultivars (Table 1). Additionally, Plum Regal and Legend showed low cumulative lesion growth values when inoculated with US-23, however, pathogen growth values were higher and not significantly different than susceptible cultivars (Table 1). Juliet, Roma, Slava, Green Zebra, and Brandywine Red all showed high cumulative lesion length and pathogen growth values (Table 1, Figs. 1 and 2).

The cultivars containing *Ph* resistance genes demonstrated varying levels of resistance when inoculated with each lineage. Plum Regal (*Ph-3*) had significantly larger lesions when inoculated with US-22 compared to US-23 (P=0.001) and US-24 (P<0.001) (Fig. 1). For US-22, the cumulative lesion growth on Plum Regal was not significantly different than the most susceptible cultivar (P=0.277), however, cumulative pathogen growth was significantly less than the cultivar with the greatest pathogen growth (P<0.001), although still greater than the cultivar with the least pathogen growth (P=1.0) (Table 1). For US-23 inoculated onto Plum Regal, cumulative lesion length was statistically similar to the most resistant cultivar (P=0.416) and cumulative pathogen growth was statistically less than the most susceptible (P<0.001) (Table 1).

On Legend (*Ph-2*), US-22 and US-23 exhibited the same level of virulence, with both cumulative lesion length and cumulative pathogen growth not significantly different between lineages (P= 0.197 and P=0.140, respectively) (Figs. 1 and 2). However, Mountain Magic (*Ph-2+Ph-3*) showed a high level of resistance when inoculated with each of US-22, US-23, and US-24; cumulative lesion length and pathogen growth values were not significantly different among lineages on this cultivar (Figs. 1 and 2).

Discussion

We have demonstrated that commercially available tomato cultivars have effective resistance against late blight caused by the recently identified clonal lineages of *P. infestans*, US-22, US-23, and US-24. In addition to cultivars that have been bred to contain *Ph* resistance genes, we identified three heirloom cultivars with no known resistance genes that were resistant to the new lineages. While the US-24 lineage caused minimal disease signs and symptoms on all of the tomato cultivars tested, US-22 and US-23 were highly virulent on tomato, indicating the need for further management of these lineages on this crop. Knowledge of the effectiveness of resistance genes currently deployed in readily available tomato cultivars can help growers and home gardeners to manage late blight with less reliance on fungicides and reduce the overall late blight inoculum load. Additionally, the currently uncharacterized resistance in Matt's Wild Cherry, Wapsipinicon Peach, and Pruden's Purple may prove useful in future breeding efforts.

Surprisingly, the heirloom cultivar Wapsipinicon Peach was resistant to all three clonal lineages of *P. infestans* tested. The history of this cultivar has not been carefully documented, but it may have *S. pimpinellifolium*, the species from which the *Ph* resistance genes have been

found, in its background (14). Many Wapsipinicon Peach seed sources indicate that the seed they sell originated with Dennis Schlicht, who named the tomato after the Wapsipinicon River in northern Iowa (Seed Savers, Decorah IA; Baker Creek Heirloom Seed Co., MO). Some seed sources further indicate that Dennis Schlicht was not the originator of this cultivar, but that instead it was bred by Elbert S. Carman and released in 1890 as White Peach (49). No detailed records of Carman's tomato breeding efforts can be found, but it has been documented that he made crosses with *S. pimpinellifolium* (14). We cannot be certain that White Peach was the result of those crosses, or, further, that Wapsipinicon Peach has *S. pimpinellifolium* in its background. However, we have demonstrated that Wapsipinicon Peach is resistant to the US-22, US-23, and US-24 lineages of *P. infestans* and the historical evidence suggests that *S. pimpinellifolium* may be the source of this late blight resistance.

Further work is needed to determine if the resistance in Wapsipinicon Peach is from one of the known *Ph* genes or other currently uncharacterized genes. However, our results suggest that this resistance is likely not from *Ph-2* or *Ph-3* alone. These genes in Legend and Plum Regal, respectively, did not impart resistance against US-22, but Wapsipinicon Peach was highly resistant when inoculated with US-22. Pyramiding both resistance genes offers better resistance, as was seen in Mountain Magic, but this is unlikely the case for Wapsipinicon Peach, as there is no record of Carman breeding and selecting specifically for late blight resistance. Instead, the resistance in Wapsipinicon Peach may be novel to modern late blight resistance breeding.

Matt's Wild Cherry is another heirloom cultivar that demonstrated late blight resistance when challenged with the US-22, US-23, and US-24 clonal lineages. This cultivar is a variety of tomato known as *S. lycopersium* var. *cerasiforme*. There is disagreement among taxonomists on

the degree of relatedness of this variety to *S. piminellifolium* or the common garden tomato *S. lycopersicum*, with some taxonomists presenting evidence that it is simply a feral garden tomato (45, 46). The seed for the cultivar Matt's Wild Cherry was originally collected by Teresa Arellanos de Mena from her family's home near Hildalgo, Mexico before being given to Dr. Matt Leibmen, who subsequently gave it to Johnny's Selected Seeds (Winslow, Maine). Hildalgo, Mexico is only about 150 km from the Toluca Valley, a center of diversity for *P. infestans*. As such, the wild population from which this cultivar originated likely had been subjected to selection pressure for late blight resistance, possibly explaining the resistance response that was observed in our work. However, the underlying genetic cause for this resistance is unknown. To our knowledge, no late blight resistance from *S. pimpinellifolium* has ever been deliberately bred into this cultivar. Therefore, this cultivar may carry a source of resistance that is novel to current late blight resistance breeding and could be further explored for utility and durability.

Pruden's Purple demonstrated some resistance to the US-22, US-23, and US-24 lineages. Little is known about the history of this heirloom cultivar and no associations to wild tomatoes can be found in its background. Consistently, Pruden's Purple had numerically higher lesion length and pathogen growth ratings than Wapsipinicon Peach, Matt's Wild Cherry, and Mountain Magic, but these values were statistically similar, indicating that it may offer comparable resistance. The fruit of Pruden's Purple is larger than that of the aforementioned cultivars, so this cultivar may be useful to producers or gardeners desiring late blight resistance and larger fruit size. Like Wapsipinicon Peach and Matt's Wild Cherry, the resistance of Pruden's Purple was consistently observed between the clonal lineages tested, indicating

potential broad-spectrum resistance. This resistance may be highly useful in further late blight resistance breeding, but this cultivar can also be immediately useful to home gardeners and commercial tomato growers.

The genetic backgrounds of the cultivars containing resistance genes may explain the unexpected results obtained when these were inoculated with US-22. Legend, an older cultivar released in 2001 containing *Ph-2,* and Plum Regal, a newer cultivar released in 2010 containing *Ph-3* were both not significantly more resistant than the most susceptible cultivar when challenged with the US-22 clonal lineage. However, when *Ph-2* and *Ph-3* were combined in the cultivar Mountain Magic, a high level of resistance was achieved. In Legend, *Ph-2* resistance is derived from an accession of *S. pimpinellifolium* known as West Virginia 700 (5), but the source of *Ph-2* in Mountain Magic is from Richter's Wild Tomato (20, 42). However, the *Ph-2* resistance in Richter's Wild Tomato has been determined to be allelic to that in Legend, indicating that these are likely the same *Ph-2* (5). Another explanation may be the nature of the *Ph-3* resistance. The *Ph-3* resistance in both Mountain Magic and Plum Regal originates from the same accession of *S. pimpinellifolium*, L3707 (21, 42). However, studies on this resistance have concluded that the late blight resistance in this accession should be attributed to two genes, one partially dominant gene, and a second independent dominant gene that is epistatic to the first and necessary for expression of resistance (33). One or the other of these genes may not be expressed as strongly in Plum Regal as in Mountain Magic, simply as an artifact of the breeding process. This may explain why two apparently ineffective resistance genes appear to become effective when combined.

An alternative explanation for the resistance demonstrated by Mountain Magic when inoculated with US-22 may be a residual effect of broken resistance genes. This effect has been shown in potato (55) and many other crops (13, 40, 44). It is speculated that the defeated genes still somehow contribute a low level of resistance, and when these genes are combined in cultivars, a more complete resistance will be seen (1, 55). Neither Plum Regal nor Legend showed a very high level of resistance when challenged with US-22, indicating that the *Ph-2* and *Ph-3* resistance genes had been defeated by this clonal lineage, but Mountain Magic was quite resistant. This supports the hypothesis of a residual effect of broken resistance genes in Mountain Magic.

The different resistance responses of the clonal lineages underscore the importance of testing new clonal lineages of *P. infestans* for virulence. That US-24 is not virulent on any of the tomato cultivars tested indicates that tomato growers may not need to be as rigorous in applying protective fungicides if it is known that US-24 is the only prevalent lineage in a region. This reduced virulence of US-24 on tomato has been previously suggested based on tests of single cultivars (9, 50); our results on 11 different tomato cultivars add support to this conclusion. US-22 and US-23 both appear to have similar virulence on the range of tomato cultivars tested. An exception was Plum Regal, which was susceptible to US-22 but resistant when inoculated with US-23. Knowledge that Plum Regal offers resistance against prominent clonal lineages of *P. infestans* may be immediately useful to potato growers. Continued testing of new clonal lineages of *P. infestans* as they are identified will keep our knowledge of the virulence of the population current and will be useful to breeders and growers.

Generalizing results from a single isolate of each clonal lineage to make conclusions about the lineage as a whole was appropriate in our work. Within a clonal lineage, virulence patterns can vary, but the majority of the variation in *P. infestans* populations in the U.S. is between clonal lineages (28, 39). This is particularly true in newer clonal lineages that have not yet been subjected to years of selection pressure; there is very little virulence variation observed in these lineages (28). The US-22, US-23, and US-24 clonal lineages in the U.S. have only recently been discovered (9, 22-24, 31), so our testing of a single isolate from each new clonal lineage is likely representative of the US-22, US-23, and US-24 pathogen populations in the U.S.

Commercial and private growers of both tomato and potato can benefit from our work. Late blight is a "community disease" easily spread from the thousands of sporangia produced within each lesion and carried on air currents (35, 54). Fungicide applications are not usually recommended or feasible for home gardeners, yet these small plots can serve as a late blight inoculum source for larger scale tomato or potato production (48), resulting in significant increases in fungicide usage. The use of resistant cultivars should contribute to an overall reduced level of inoculum in a region, lessening the risk of late blight for both tomato and potato. In addition, cultivar resistance to late blight, even if only partial, can contribute to integrated late blight management in both organic and conventional tomato production. Resistant cultivars require reduced fungicide applications, which is better for the environment and can reduce input costs (52). These cultivars may be particularly useful in organic production where options for effective fungicides are often limited. We have demonstrated that several tomato cultivars are resistant to currently common clonal lineages of *P. infestans*, and these cultivars are currently readily available. Therefore, our results demonstrating effective late blight resistance in tomato

cultivars may be highly useful to both the tomato and potato industry and be economically important for growers.

We tested heirloom and hybrid tomato cultivars for resistance to US-22, US-23, and US-24, three recently identified clonal lineages of *P. infestans,* and discovered potentially new sources of resistance in four heirloom cultivars. We also showed the usefulness of *Ph* resistance in three hybrid cultivars against these new lineages. The potentially new sources of resistance in the heirlooms may be useful in future breeding efforts, but both the heirloom and hybrid cultivars that tested resistant against the US-22, US-23, and US-24 lineages are immediately useful for limiting tomato late blight. The use of these cultivars can also limit the overall *P. infestans* inoculum in a region, benefiting tomato and potato growers alike.

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Table 1. Tomato cultivars tested for late blight resistance against the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* and their resistance/susceptibility response.

Cultivar[v]	Selection Parameter[w]	AUDPC Lesion Length[x]			AUDPC Pathogen Growth[y]		
		US-22	US-23	US-24	US-22	US-23	US-24
Wapsipinicon	Field observation	80.5a[z]	116.2a	12.6a	72.5a	155.0bcd	0.0a
Mountain Magic	*Ph-2* and *Ph-3*	96.1ab	67.1a	49.8ab	73.8a	22.5a	27.5ab
Matt's Wild Cherry	Seed company	87.6ab	128.7ab	22.2ab	70.0a	118.8ab	10.0a
Pruden's Purple	Other research	177.0abc	153.0ab	48.4ab	180.0ab	136.3abc	20.0ab
Legend	*Ph-2*	213.6cd	161.5abc	79.2abc	271.3bc	201bcde	96.3abcd
Plum Regal	*Ph-3*	243.9cde	137.5ab	110.6bc	206.3b	147.5bcd	106.3abcd
Juliet	Seed company	180.0bc	214.0bcd	108.2abc	228.8b	246.3cdef	150.0cd
Roma	Field observation	257.3cde	251.8cd	81.7abc	272.5bc	261.3def	48.8abc
Slava	Other research	249.3cde	277.1d	100.5abc	271.3bc	293.8ef	136.3bcd
Green Zebra	Other research	321.0e	295.5d	114.9bc	365.0c	322.5f	152.5cd
Brandywine Red	Susceptible control	293.7de	304.1d	160.9c	268.8bc	285.0ef	205.0d

[v] Mountain Magic, Plum Regal, and Juliet are hybrids. Legend was bred to contain *Ph-2* resistance, but is open pollinated, so is considered an heirloom by some. The remaining cultivars are heirlooms.

[w] Cultivars were selected based on anecdotal field observations from 2009, claims by seed companies of late blight resistance, field trials using other *P. infestans* clonal lineages (12, 32) or the presence of resistance genes *Ph-2* and/or *Ph-3*.

[x] Lesion length was measured at 0, 5, 7, and 9 days after inoculation and area under the disease progress curve (AUDPC) was calculated.

[y] Percent leaf coverage of pathogen growth was determined at 0, 5, 7, and 9 days after inoculation and area under the disease progress curve (AUDPC) was calculated.

[z] Values in each column followed by the same letter are not significantly different (Tukey test, P=0.05).

Fig. 1. Variation in late blight lesion lengths on the different cultivars inoculated with the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans*. Lesion length was measured at 0, 5, 7, and 9 days post inoculation (dpi) and area under the disease progress curve (AUDPC) calculated. Bars with the same letters indicate no significant difference between the lineages on a given cultivar (Tukey test, P=0.05).

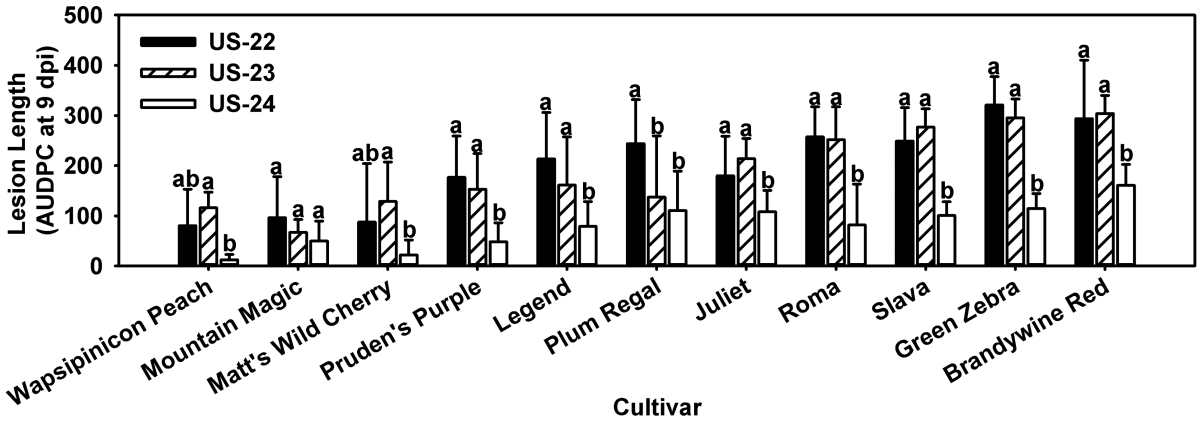
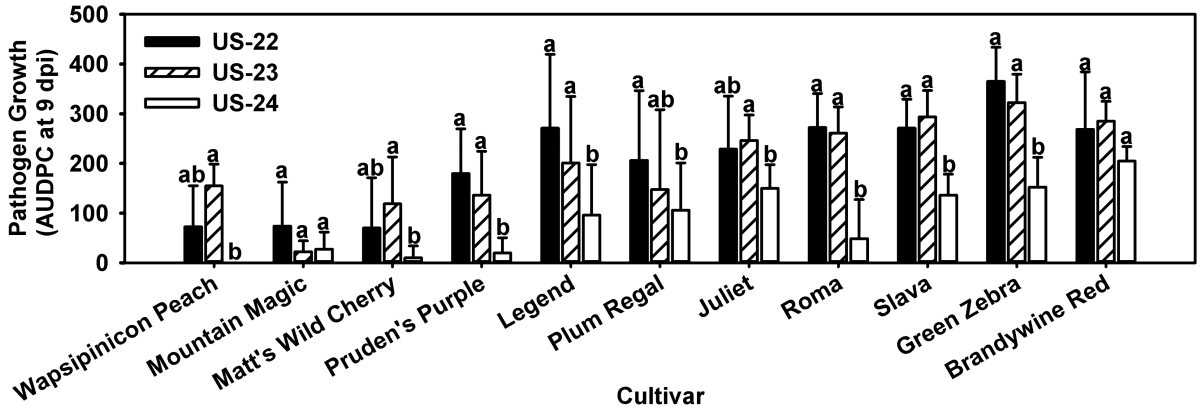


Fig. 2 Variation in percent leaf coverage of pathogen growth on the different cultivars inoculated with US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans*. Percent coverage of pathogen growth was measured at 0, 5, 7, and 9 days post inoculation (dpi) and area under the disease progress curve (AUDPC) calculated. Bars with the same letters indicate no significant difference between the lineages on a given cultivar (Tukey test, P=0.05).



CHAPTER 6:

Efficacy of biopesticides for control of tomato late blight caused by the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans*

Abstract

Late blight, caused by *Phytophthora infestans,* is one of the most economically important diseases of potato and tomato worldwide. Repeated preventative application of fungicides is the primary means of control on susceptible crops. For organic production, fungicide choices are limited, and little efficacy data are available from which to base control recommendations. Twelve fungicides, including organic and conventional standards, were evaluated for control of three recently identified *P. infestans* clonal lineages, US-22, US-23, and US-24, using a detached tomato leaf assay. A subset of the most effective fungicides was also tested against US-23 on potted tomato plants under laboratory conditions. Fungicide applications made 2 days after inoculation failed to control late blight on detached leaves. Compared to US-22 and US-23, US-24 demonstrated a low level of virulence on tomato leaves, and all fungicides applied preventatively effectively limited disease. The biopesticides EF400 and Zonix, and Phostrol applied prior to inoculation with US-22 or US-23 resulted in significantly less disease than the copper hydroxide treatments. All conventional fungicide treatments demonstrated a high degree of efficacy when applied two days before inoculation. Similar trends with all fungicides were seen with control of US-23 in potted tomato plant assays. These results indicate that currently available conventional and organic fungicides can effectively control late blight caused by new clonal lineages of *P. infestans*.

Introduction

Phytophthora infestans (Mont.) deBary causes late blight, one of the most important diseases of potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) production

worldwide. In the U.S. it is estimated that $77.1 million per year is spent on fungicides to control potato late blight (27). In spite of this, losses can still be high and late blight has been estimated to cost growers $507 per hectare per year, or $287.8 million annually (27). Worldwide, the disease has been estimated to cost growers $6.7 billion annually (28).

The biology of *P. infestans* in the U.S. influences management of late blight. *P. infestans* is a heterothallic oomycete that reproduces primarily asexually in the U.S. (13, 15). The A1 and A2 mating types have not historically been in proximity and the possibility for sexual reproduction of resilient oospores has not been widely documented (18, 22, 23, 40). As such, the pathogen requires living plant material to survive, limiting survival in northern winters, and genetic variation within the population is limited (8, 19, 43). Instead, reproduction is primarily via asexual sporangia. These short-lived reproductive structures are produced in large numbers on lesions and are easily wind-blown to quickly spread late blight to surrounding plants and fields, often quickly devastating an unprotected susceptible crop (52). These asexual descendants of a single genotype are often referred to as clonal lineages (16), a designation that can be associated with several genotypic characteristics such as the RG57 restriction fragment length polymorphism fingerprint (20) and allozyme fingerprint (21) and several practical phenotypic characteristics important for management such as mating type and sensitivity to the fungicide active ingredient mefenoxam (9, 32, 43, 46). Several clonal lineages of *P. infestans* have been discovered throughout the years in the U.S. (22). Some have been limited in distribution, being found only in limited geographic regions or for single seasons (22, 25, 32), but others, such as the US-1 and US-8 clonal lineages have been widespread spacially and temporally (19, 22). However, new lineages usually quickly displace previously predominant

lineages (22, 33, 43) making it important to continue to study new lineages of *P. infestans* to optimize management.

There are several integrated control strategies to manage late blight. In northern regions where freezing temperatures prevail in the winter months, living plant material containing *P. infestans* is usually destroyed. However, infected tubers could be protected from freezing in potato cull piles (4) or buried in production fields after harvest so it is important to destroy cull piles and manage volunteers to eliminate potential inoculum sources for the following season. Planting late blight-free seed tubers is also very important to reduce initial inoculum (30, 52). Resistance genes have been deployed in both potato (24, 36, 47, 53) and tomato (2, 34, 38) and these can aid in an overall integrated management plan (50). However, *P. infestans* has historically demonstrated an exceptional ability to overcome plant resistance, sometimes carrying genes to overcome resistance genes that have not yet been deployed (15, 24). Complete reliance on genetic resistance for late blight control, therefore, has been impossible. Instead, fungicides are usually necessary and can greatly decrease yield losses due to late blight (17, 51).

Fungicides are usually applied in conjunction with a disease forecasting system to optimize timing of application to achieve the highest levels of late blight control with the minimum amount of fungicide (35, 51). Disease forecasting systems usually use knowledge of pathogen biology in conjunction with temperature and humidity conditions to predict when the pathogen is most likely to be active and when fungicide applications will be the most effective (35, 51). Most available fungicides are protectants and must be applied before disease onset to be effective (5). Other types of fungicides include antisporulants, which can reduce further late blight spread from an infected field, translaminar fungicides which have limited ability to halt

established infections, and truly systemic fungicides, such as the active ingredient mefenoxam (5, 7, 17). Mefenoxam can stop established infections, provided the disease-causing lineage is sensitive (7). Several clonal lineages, such as the very prominent US-8 lineage, were completely insensitive to mefenoxam, rendering this normally superior fungicide ineffective (10, 22, 25, 26, 31, 37)

In organic potato and tomato production systems, fungicides are often necessary to control late blight just as in conventional systems. Many years of research have established that formulations containing copper are the most effective organic-approved fungicide options (12, 48, 49). However, there are concerns over the extensive use of copper in organic production. Copper kills a broad range of microorganisms, and with repeated applications, there is concern with the accumulation of copper in soil and detrimental effects on soil microorganisms (3, 11, 14). Additionally, copper residues on plant tissues can cause dermatitis (41), so field workers may need appropriate personal protective equipment when working in recently treated fields. Residues on harvested fruit may also be undesirable to consumers. Some organic non-copper containing fungicides have been developed, but the efficacy of these products is often limited or not substantiated by unbiased, third-party research (12).

With new clonal lineages of *P. infestans* displacing the US-8 clonal lineage in recent years, and a growing interest in the production of organic approved fungicides as the market for organic potatoes and tomatoes expands (39), testing the efficacy of fungicides against new lineages of *P. infestans* is needed. We evaluated 12 fungicides, including organic, biorational and conventional formulations, against three recently identified clonal lineages of *P. infestans*, US-22, US-23, and US-24, using a detached tomato leaf assay in the laboratory. We also

evaluated a subset of the most effective fungicides against the US-23 lineage, which has become the most common lineage (1, 43), on potted tomato plants under controlled conditions. Results will better inform fungicide recommendations for new, prominent clonal lineages of *P. infestans* in organic, conventional, and integrated systems leading to decreased losses to late blight.

Materials and Methods

P. infestans *isolates*. The US-22 (Pi7-09a), US-23 (Pi16-10a), and US-24 (Pi21-10a) isolates used in this study were collected from symptomatic plant tissues from Wisconsin production fields and gardens and have been previously characterized (43). Pi7-09a (A2, mefenoxam sensitive) was isolated from tomato fruit, Pi16-10 (A1, mefenoxam sensitive) from tomato foliage, and Pi21-10a (A1, mefenoxam intermediately sensitive) from potato foliage. Isolates are maintained in hemp seed long-term storage vials (Lamour 2001) in the Potato and Vegetable Pathology Laboratory of Dr. Amanda Gevens at the University of Wisconsin-Madison.

Inoculum preparation. Two- to four-week-old actively growing cultures of *P. infestans* grown on rye A media (6) at 18°C in darkness were flooded with sterile distilled water and brushed gently with a sterile glass rod to dislodge sporangia. The resulting suspension was transferred to a sterile, plastic 50-ml centrifuge tube and the concentration quantified using a hemacytometer. The concentration was adjusted as needed for each experiment by adding sterile distilled water.

Fungicide evaluation on detached leaves. Tomato plants (cv. Brandywine Red, Reimer Seeds, Saint Leonard, MD) were grown in a growth chamber at 28°C with a 14-hour

photoperiod. Fully expanded leaflets from 4- to 5-wk old plants were detached using a sharp razor blade and were placed directly on a thin layer of water agar in 9-cm-diameter petri plates agar. Two leaflets were placed in each plate. Fungicide treatments were mixed in 100 ml of tap water directly in the canister of a 1 L Solo sprayer (Solo, Newport News, VA) according to the rates outlined in Table 1. The sprayer was pumped 35 times to establish pressure in the tank and ensure a small droplet size. The canister was agitated thoroughly prior to application of each fungicide to ensure a homogeneous suspension. Eight leaves from four petri plates were placed together and sprayed to runoff with each fungicide treatment. For the preventative application experiment, treated leaves were returned to the petri plates, two leaves per plate, and the lids were replaced after the fungicides dried. One full spray from an atomizer (average 75 µl) containing 50,000 sporangia/ml of *P. infestans* was applied to each leaf within 2 hours of the fungicide application. The post-inoculation experiment was set up the same way, except leaves were inoculated two days before the fungicide applications. All leaves were incubated in their petri plates (100% relative humidity) at room temperature conditions (21-23°C, ambient lighting) for the duration of the experiment. Seven days after inoculation, disease was assessed on individual leaves using a 0-5 scale: 0=no disease, 1=1 to 11% disease (midpoint 6%), 2=12 to 38% disease (midpoint 25%), 3=39 to 61% disease (midpoint 50%), 4=62 to 88% disease (midpoint 75%), and 5=89 to 100% disease (midpoint 95%).

Fungicide evaluation on potted plants. All plants were grown in 9.5-cm-diameter pots in a growth chamber at 24°C with a 14-hour photoperiod and inoculated 4-5 weeks from planting. Plants were removed from the growth chamber for fungicide treatment and inoculation. The fungicide treatments were prepared as outlined for the detached leaf experiments. Four plants

for each treatment were arranged together and sprayed as a unit, just until runoff. The plants were then moved and randomly situated over an approximately 1-2.5m^2 area to dry. Inoculum for the US-23 clonal lineage of *P. infestans* was produced as outlined above and adjusted to 10,000 sporangia/ml. The block of randomly arranged plants was uniformly sprayed with 400 ml of inoculum until the volume was exhausted. Plants were immediately individually covered with clear plastic bags for 24 hours and arranged in a random design on a lighted shelf maintained at laboratory temperature conditions (21-23°C) with a 14-hour photoperiod. The lighted shelf was loosely covered with clear plastic to promote humid conditions for disease development. The plants were misted daily with distilled water using the Solo sprayer and watered as needed. Disease ratings were assigned using the same scale as for the detached leaves at 7 days after inoculation.

Data analysis. The detached leaf experiments were performed two times for each of the three *P. infestans* lineages. The potted plant experiment was performed two times on the US-23 lineage only. The 0 to 5 disease ratings were converted to their corresponding midpoint percentages and the data analyzed using analysis of variance (ANOVA). Tukey tests (P=0.05) were used to determine significant differences between treatments and between clonal lineages within each experiment.

Results

When the US-22 and US-23 clonal lineages were inoculated onto detached leaves, prior to fungicide treatments, all treatments had disease ratings significantly greater than the non-inoculated control (Table 2). Additionally, all treatments except Phostrol for US-22 and Bravo

Ultrex for US-23 were not significantly different than the inoculated control (P=0.003 and P<0.001, respectively). When fungicides were applied prior to inoculation with US-22 and US-23, many treatments showed significantly less disease than the inoculated controls (Table 2). Additionally, for the US-22 lineage, Zonix was not significantly different than the non-inoculated treatment (P=0.828), and was statistically similar to the three conventional fungicides (Table 2). For the US-23 lineage, the low rate of EF400 demonstrated disease not significantly different than the non-inoculated control (P=0.114) and statistically similar to the three conventional fungicides (Table 2). For both the US-22 and US-23 lineages, Zonix, EF400, and Phostrol treatments had the lowest disease ratings, apart from the conventional standards (Table 2). These disease ratings were also significantly less than those treatments containing copper hydroxide for both lineages (Table 2).

For the US-24 lineage, numerically similar levels of disease control were achieved when the fungicide treatments were applied either before or after inoculation. Overall, the US-24 clonal lineage caused low levels of disease, with mean disease ratings for the inoculated control leaves of only 24.7% and 15.3% for both experiments (Table 2, Fig. 1). For the experiment where fungicides were applied prior to inoculation with US-24, most treatments were effective, and only three treatments, Regalia, Serenade Soil, and the high rate of Mycostat, showed disease values significantly greater than the non-inoculated control (Table 2). However, many of the treatments inoculated with US-24 that were not significantly different than the non-inoculated control were also not significantly different than the inoculated control (Table 2).

In the fungicide efficacy tests on potted plants with the US-23 lineage, none of the treatments except the conventional fungicides resulted in disease significantly similar to the non-

inoculated control (Fig. 2). All other the treatments had significantly more disease than the conventional standards. However, the high rate of EF400 had statistically less disease than the inoculated control (Fig. 2, Table 2).

Comparing among lineages, the US-24 lineage always had lower disease ratings than the US-22 and US-23 lineages, except with the conventional standard treatments (Bravo Ultrex, Ridomil Gold SL, and Revus) applied before inoculation; all lineages had a statistically similar low level of disease (Fig. 1a). When the fungicides were applied prior to inoculation, inoculations with US-23 resulted in significantly less disease than inoculations with the US-22 clonal lineage for the EF400, Phostrol, Mycostat, and the high rate of Champ Formula 2 treatments (Fig. 1a). Otherwise, the two lineages had statistically similar levels of disease with all other treatments. When the fungicides were applied two days after inoculation, there were statistically similar high levels of disease between the US-22 and US-23 lineages for all of the treatments except the Bravo, Zonix, Phostrol, and Oxidate treatments (Fig. 1b); however, disease ratings were still high in all cases.

Discussion

Application of fungicides is one of the primary means of controlling late blight on potato and tomato during the production season (5). Effective fungicide choices are limited in organic production systems and little unbiased, replicated efficacy data are available for many fungicides. New clonal lineages of *P. infestans* continue to be identified in the U.S. and it is important to determine efficacy of fungicides against each current genetically distinct population. We have demonstrated, through detached leaf assays and potted plant trials, that effective non-

copper based fungicides are available to contribute to late blight control in organic or transitional organic systems, or in conventional systems desiring to use more environmentally friendly chemistries. Additionally, we have validated that several conventional fungicides remain effective against new lineages of *P. infestans* when applied preventatively.

Apart from the conventional fungicides, EF400 and Zonix provided the best control of each lineage evaluated. The active ingredients for EF400 are a mixture of clove, peppermint, and rosemary oil. The mode of action of these essential oils is unclear, but they appear to provide good control of new clonal lineages of *P. infestans* on both detached leaves and potted plants. Trials in Michigan with the US-22 lineage suggest that EF400 is effective against late blight in a field setting with polycyclic late blight pressure (44). Zonix is a rhamnolipid biosurfactant that alters the osmotic potential of zoospore membranes, resulting in rupture and death (54). In laboratory microscope evaluations, a drop of Zonix applied to a zoospore suspension immediately eliminated all swimming zoospores (data not shown). Zoospores are released by *P. infestans* at lower temperatures (8-18°C) when free water exits, but at higher temperatures sporangia germinate directly (52). Our inoculations and incubations were performed at 21-23°C, higher than the optimal temperature for indirect germination of sporangia into zoospores, but Zonix was still effective. This would suggest that the isolates of *P. infestans* that we tested have a low rate of effective direct sporangial germination. Indeed, a low rate of direct germination at 20 and 25°C has been observed for other isolates of the US-22, US-23, and US-24 lineages (Danies 2013). Further greenhouse or field trials are needed to further confirm the efficacy of EF400 and Zonix, but our results suggest that they may be useful in a late blight fungicide program.

It is uncertain why the treatments containing copper performed so poorly against the US-22 and US-23 clonal lineages. Fungicide formulations containing copper have a long history of being the most effective organic fungicides available (12, 48, 49). Our experiments were replicated with statistically similar results for the copper-containing treatments, and each time the copper formulation performed statistically worse than many other fungicides. The potential for copper tolerance in new clonal lineages of *P. infestans* requires further investigation.

All of the fungicides tested provided good control of late blight caused by the US-24 clonal lineage. However, when the untreated inoculated controls from each experiment were compared, the US-24 lineage demonstrates much less virulence on tomato. US-24 has been infrequently isolated from tomato since it was first identified in the U.S. (8, 29, 43). Additionally, numerous laboratory experiments have shown that infection and disease progression (42) and sporulation (8, 45) are limited with the US-24 clonal lineage on tomato. This reduced virulence suggests that a broader range of fungicides, including those that only partially control more virulent lineages, may be used to effectively control tomato late blight caused by the US-24 lineage of *P. infestans*.

Many of the fungicides tested provided little to no control of late blight caused by the US-22 and US-23 lineages, even when applied prior to inoculation. Mycostat, like, EF400, is an oil blend, but it was ineffective. Baking soda and OxiDate also provided ineffective control, but because of their ability to kill sporangia, may have a part in an overall late blight management program by reducing inoculum and, subsequently, the spread of disease. The active ingredient in Regalia is an extract from giant knotweed (*Reynoutria sachalinensis*) that is reputed to enhance plant defenses. This fungicide was ineffective in our tests, but further tests on cultivars with

partial resistance may demonstrate its merit. Serenade Soil, containing the *Bacillus subtilis* QST 713 bacterium, was ineffective against late blight in our trials. Information on the ineffectiveness of several common fungicides against currently common lineage of *P. infestans* can inform also management recommendations.

The effectiveness of the conventional fungicides tested indicates that there is currently no resistance in the *P. infestans* population against these fungicides. In *in vitro* tests, isolates of the US-24 lineage often test partially insensitive to mefenoxam (43), but the Ridomil treatment was effective against US-24 on tomato leaves. Phostrol contains salts of phosphorus acids and is generally considered less toxic than some of the other available conventional fungicides. This characteristic, combined with demonstrated efficacy, may make this a preferred choice among conventional and/or integrated systems.

Our experiments were performed on tomato leaves, but the fungicides tested are also approved for use on potato late blight and similar efficacies can be extrapolated. The exception would be the results from the US-24 clonal lineage, as this lineage has low virulence on tomato but is virulent on potato (29, 43). Further field trials would likely confirm results for potato, but our work has demonstrated the potential of several non-conventional fungicides to provide good late blight control.

The contrast between the efficacy of any of the fungicides applied before inoculation compared to after inoculation was striking. Two days after inoculation, when the fungicides were applied, no sign or symptom of disease was observed. However, the leaves were latently infected with *P. infestans* in its biotrophic phase (13). The established infection was not effectively controlled with any of the fungicides. This is important to consider for management,

as our results indicate that all of the fungicides tested must be applied prior to the onset of disease to be effective, and infected leaves may show no sign of disease for several days. Disease forecasting models can predict when environmental conditions are most conducive to late blight infection and recommend the application of fungicides (35, 51). Our results underscore the utility of these forecasting systems for adequately controlling late blight.

Late blight can be a challenging disease to manage, particularly in an organic production system. We have demonstrated that several non-copper fungicide formulations are effective in controlling this potentially devastating disease when applied prophylactically. With concerns over copper build up in soil and unsightly copper residues on organic produce, our results showing efficacy of non-copper formulations may be particularly timely. Our results provide much-needed fungicide efficacy data that support effective recommendations for fungicide selection to achieve the best management of late blight on potato and tomato in conventional, integrated, or organic production systems.

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Table 1. Fungicide treatments, manufacturers, active ingredients, and label rates tested for efficacy against the US-22, US-23, and US-24 clonal lineages of *P. infestans* on tomato.

Treatment	Manufacturer	Active ingredient	Label Rates[a]	Rate for 100ml water[b]
Bravo Ultrex	Syngenta Crop Protection, Greensboro, NC	chlorothalonil	1.8 lb/acre	0.019 g
Ridomil Gold SL	Syngenta Crop Protection, Greensboro, NC	mefenoxam	1 pt/acre	0.011 ml
Revus	Syngenta Crop Protection, Greensboro, NC	mandipropamid	0.5 pt/acre	0.006 ml
Zonix	Jeneil Biosurfactant Co., Saukville, WI	rhamnolipid biosurfactant	100 ppm	1 ml in 99 ml water
EF400	USAgritech, Inc. Paso Robles, CA	Herbal extract mixture	4 pt/100 gal water	0.49 ml
EF400	USAgritech, Inc. Paso Robles, CA	Herbal extract mixture	6 pt/100 gal water	0.75 ml
Phostrol	NuFarm Americas Inc., Burr Ridge, IL	phosphorous acids	10 pt/acre	0.11 ml
Mycostat	Diacon Technologies Ltd., Richmond BC	oil extract	0.5 pt/acre	0.006 ml
Champ Formula 2	Nufarm Americas Inc., Burr Ridge, IL	copper hydroxide	2 pt/acre	0.022 ml
Baking soda	Arm & Hammer, Princeton, NJ	sodium bicarbonate	1 Tbs/gal water	0.5 tsp. in 630 ml water
Champ Formula 2	Nufarm Americas Inc., Burr Ridge, IL	copper hydroxide	1.33 pt/acre	0.014 ml
Regalia	Marrone Bio Innovations, Davis, CA	*Reynoutria sachalinensis* extract	2 pt/acre	0.022 ml
Serenade Soil	AgraQuest, Inc. Davis, CA	*Bacillus subtilis* QST 713	12.5 pt/acre	0.136 ml
OxiDate	BioSafe Systems LLC, East Hartford, CT	hydrogen dioxide	1 gal/100 gal water	1 ml in 99 ml water
Mycostat	Diacon Technologies Ltd., Richmond BC	oil extract	1 pt/acre	0.011 ml

[a] The rates chosen are common recommended rates and units of measurements on each label. Both a high and a low recommended rate were used in some cases. The abbreviation ppm=parts per million, pt=pint, Tbs=tablespoon, tsp=teaspoon and gal=gallon.

[b] Recommended label rates were converted to the amount needed in 100ml of water and applied until runoff.

Table 2. Mean disease ratings of fungicide treatments applied against the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* on tomato.

	Mean disease severity						
	Detached Leaves						Potted plants[y]
	Preventative[w]			Non-preventative[x]			Preventative
Treatment[v]	US-22	US-23	US-24	US-22	US-23	US-24	US-23
Inoculated control	93.8c[z]	100e	24.7cde	84.4cd	100c	15.3a	90.6c
Non-inoculated	0a	0a	0a	0a	0a	0a	0a
Bravo Ultrex	0a	0.3a	0a	87.5cd	68.8b	8.1a	9.4a
Ridomil Gold SL	0.3a	0a	0a	68.8bc	78.8bc	11.3a	2.5a
Revus	1.6a	0.3a	0a	75bcd	82.8bc	10.3a	NA
Zonix	12.8a	22.2b	1.6a	84.4cd	100c	13.8a	68.8bc
EF400 low	39.1b	20.0ab	1.3a	85.9cd	98.4c	17.5a	71.9bc
EF400 high	44.4b	24.4b	2.2ab	92.2d	100c	15.9a	59.4b
Phostrol	50.9b	24.4b	3.8abc	58.4b	90.6c	4.7a	78.1bc
Mycostat low	100c	51.6c	26.6abcde	93.8d	98.4c	14.4a	NA
Champ high	95.3c	75.0d	10.3abcd	90.6d	98.4c	12.2a	87.5bc
Baking Soda	89.1c	84.4de	19.1abcde	93.8d	98.4c	13.1a	NA
Champ low	92.2c	84.4de	8.8abcd	87.5cd	100c	11.6a	81.3bc
Regalia	98.4c	87.5de	23.4bcde	90.6d	96.9c	11.6a	NA
Serenade Soil	100c	92.9de	26.6de	93.8d	96.9c	11.3a	NA
Oxidate	100c	93.8de	10.0abcd	84.4cd	100c	11.6a	NA
Mycostat high	100c	95.3de	33.4e	89.1cd	98.4c	13.4a	NA

[v] The designation “high” or “low” refers to either the higher or lower rate of each fungicide, as specified in Table 1.

[w] Fungicides were applied to detached tomato leaves and allowed to dry before inoculation with *P. infestans*.

[x] Fully expanded detached tomato leaves were inoculated with *P. infestans* two days before applying each treatment.

[y] Three to four week old potted tomato plants were sprayed with fungicides and allowed to dry before inoculation with *P. infestans*. Only a subset of fungicides was evaluated for the potted plant experiment; those not tested are represented by NA.

[z] Numbers are mean disease ratings 7 days after inoculation and means for each lineage within each experiment followed by the same letters are statistically similar (Tukey test, P=0.05)

Fig. 1. Efficacy of fungicides applied (A) preventatively, or (B) two days after inoculation with the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* on detached tomato leaves. Late blight disease ratings were taken 7 days after inoculation. Letters indicate significant differences between the three clonal lineages for each treatment (Tukey test, P=0.05). The designation "high" or "low" refers to either the higher or lower rate of each fungicide, as specified in Table 1.

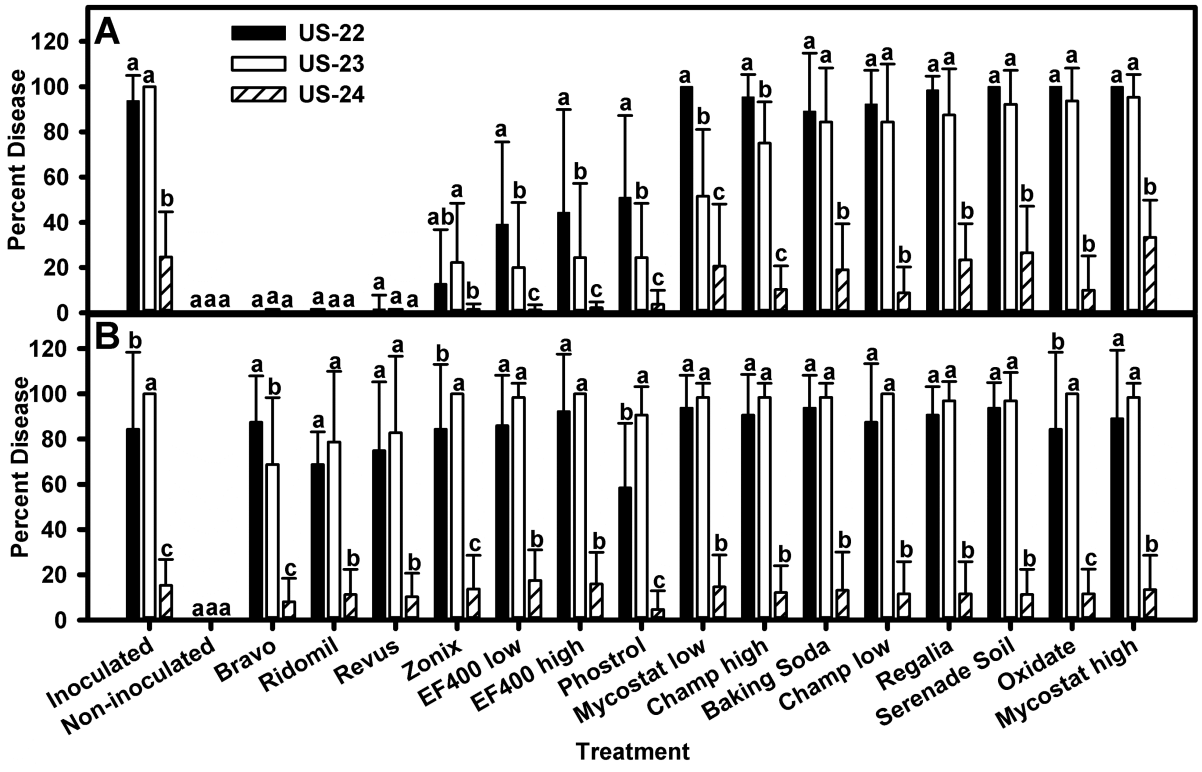
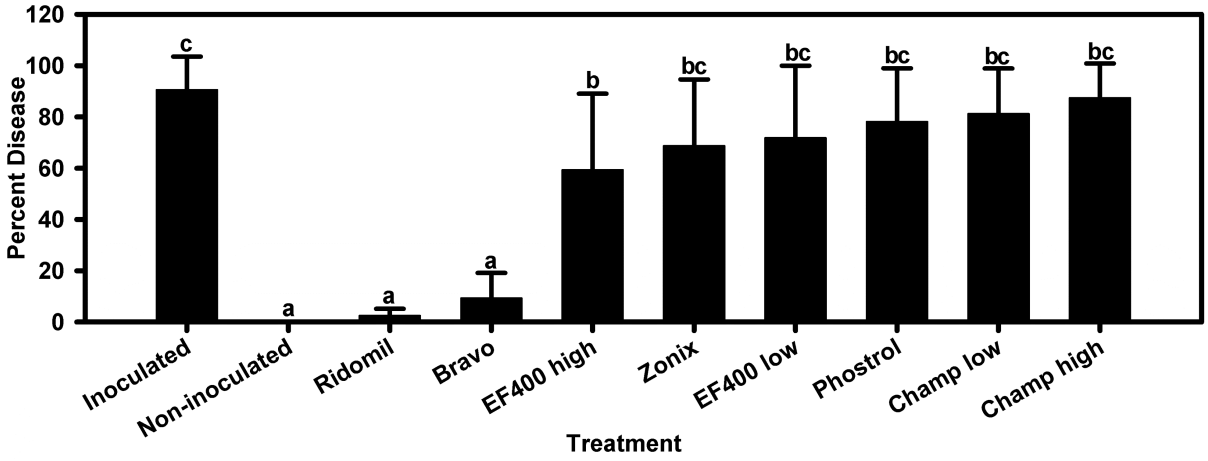


Fig. 2. Efficacy of fungicides applied before inoculation with the US-23 clonal lineage of *P. infestans* on potted tomato plants. Disease ratings were taken at 7 days after inoculation. Bars with the same letters are not significantly different (Tukey test, P=0.05)



CHAPTER 7:

Investigating the effects of temperature and sporulation on late blight epidemiology caused by the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* using the LATEBLIGHT simulation model

Abstract

Epidemics of late blight, caused by *Phytophthora infestans* (Mont.) deBary have been studied by plant pathologists and regarded with great concern by potato and tomato growers since the disease was discovered as the cause of the Irish potato famine of the 1840s. We experimentally determined sporulation rates on potato and tomato and the effect of temperature on lesion growth rate on tomato for three recently identified clonal lineages of *P. infestans,* US-22, US-23, and US-24. Results were input to the LATEBLIGHT simulation model using Wisconsin environmental conditions from four growing seasons (2009 to 2012) to investigate the effect of these lineages on late blight epidemiology. The US-22 and US-23 lineages had higher lesion growth rates on tomato than the US-24 lineage, resulting in larger epidemics in all years of weather data tested, particularly in 2011. Sporulation rates for all lineages were higher on potato than tomato, causing potato epidemics to progress faster than tomato epidemics. However, the US-23 lineage had higher sporulation rates on both tomato and potato than the other lineages and than the default pathogen parameters included with the model. Additionally, US-23 always caused large epidemics when the pathogen parameters were investigated singly or collectively. Sporangial size of the US-23 lineage was significantly smaller than that of the US-22 and US-24 lineage, which may result in more efficient release of sporangia from the tomato or potato canopy and enhance long-distance spread of this lineage. Our results suggest that US-23 may be the lineage with the greatest fitness and the most likely lineage to persist in the *P. infestans* population.

Introduction

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) deBary, is one of the most devastating diseases of potato and tomato production worldwide, costing an estimated $6.7 billion to potato production (34). In U.S. potato production, it is estimated that $77.1 million per year is spent on late blight control, yet losses are still estimated at $287.8 million annually (33).

Genetic change of this pathogen in northern North America has been relatively limited, due mainly to the lack of widespread sexual recombination (15, 21, 29, 37, 48). As such, the concept of clonal lineages, or asexual descendants of a single genotype, has been useful in studying the population (19). Isolates within a clonal lineage generally have identical genotypic characteristics such as restriction fragment length polymorphism fingerprints at the RG57 locus (27) and allozyme genotypes (28), and share similar phenotypic characteristics such as sensitivity to the systemic fungicide mefenoxam and mating type (14, 31, 42, 48). These and a few other genotypic characteristics are typically promptly determined for each new lineage discovered (22-24, 39) but other epidemiologically important phenotypic characteristics such as sporangial production, lesion growth rate, and temperature preference are less routinely explored. Sporangial production and lesion growth rate by *P. infestans* has been explored for several lineages including US-1 (41, 43, 44), US-6 (41), US-7 (41, 43, 44), US-8 (41, 43, 44), US-11 (43) and multiple isolates not assigned to a US-# lineage (4, 36, 41, 43, 50). The effect of temperature on various life stages of the pathogen has been less widely studied. In 1934, Crosier (12) investigated the influence of temperature on lesion growth and sporulation but it is likely that he used isolates from a single clonal lineage, US-1, which was widespread and believed to be the only lineage present at the time. More recently, Mizubuti and Fry (44) explored the effect of temperature on incubation period, lesion area and sporangial production on the US-1, US-7

and US-8 clonal lineages. However, the US-1 clonal lineage of *P. infestans* has not been found in the U.S. in recent years and the US-7 and US-8 lineages have declined to be nearly absent (13, 35, 48). Instead, the US-22, US-23, and US-24 lineages have become prominent in the U.S. since 2009 (13, 48).

The concept of fitness includes, foremost, the ability of an organism to survive and reproduce (1). For plant pathogens, including *P. infestans*, many biological parameters, including, but not limited to, the ability of a particular isolate or clonal lineage to produce more sporangia (13, 44), survive at greater temperature ranges (12, 44), infect a broader range of hosts (25, 30), or have higher rates of direct or indirect germination (13), can all increase the probability that a particular isolate or clonal lineage will be able to successfully survive and reproduce. Because plant epidemics are highly dependent on environmental conditions (1), these conditions must be considered when judging the degree of fitness of an isolate or clonal lineage.

Simulation modeling can be used to analyze the effects of changes in pathogen fitness and the effects of fungicides on the overall progression of late blight epidemics (9, 16, 38). One of the first models, called LATEBLIGHT, was developed in 1981 by Bruhn and Fry (9) to study the effect of rate reducing resistance combined with different fungicide schedules in potato to determine the combination that would optimize disease management. This model was further refined by Doster et al. (16) by adding a parameter for changes in leaf area index and refining some of the existing parameters. The model was then used to analyze combinations of fungicides and plant resistance that would minimize selection of isolates of *P. infestans* insensitive to the fungicide active ingredient metalaxyl (16). In both studies, the model was compared to actual late blight epidemics in New York for validation. More recently, the

LATEBLIGHT model was again revisited and further refined by Andrade-Piedra et al. (4). In this study, the effect of temperature on latent period was determined and added to the model, and the equations for the effect of temperature on lesion growth rate and sporulation rate were re-determined using lineages more recently emerged than the US-1 lineage on which that the equations had previously been based (4). Additionally, the revised model was validated using actual field epidemics in the Peruvian Andes (5). Since then, it has been validated against epidemics in Ecuador, Israel, Mexico, Nicaragua, and the U.S. and has been found to accurately predict field epidemics across this broad range of environmental conditions (3, 8).

In this study, we measured the effect of sporulation rate on potato and tomato, and lesion growth rate at different temperatures on the epidemiology of three recently identified clonal lineages of *P. infestans,* US-22, US-23, and US-24. To further explore other epidemiologically important parameters, we also determined the average sporangial size of each lineage. To quantify the effect of these pathogen parameters on late blight epidemiology, we employed the LATEBLIGHT simulation model (4) and modified parameters for each lineage based on our experimental results. These data will offer insight into the biology and epidemiology of a changing *P. infestans* population and lead to enhanced management of late blight.

Materials and Methods

P. infestans *isolates*. All isolates of *P. infestans* were collected from Wisconsin potato and tomato fields or home gardens during 2009, 2010, and 2011. Five isolates each of US-22, US-23, and US-24 were chosen as representatives from a larger collection (48). The fifteen selected isolates were collected from different years and locations and from multiple host and

plant parts (Table 1). Isolates are maintained in hemp seed long-term storage vials (40) in the Vegetable Pathology pathogen collection of Dr. Amanda Gevens at the University of Wisconsin-Madison in the Plant Pathology Department. Selected isolates were transferred minimally in culture and all lesion growth rate experiments were performed within 18 months of collection of the original isolate to reduce the probability of losing the original virulence. Most of the sporangia quantification experiments were performed at a later date, and to ensure virulence, each of these older isolates was passed through potato and tomato leaf tissue twice before inoculating the potato and tomato leaves from which sporangia were quantified.

Temperature effect on lesion growth rate. Inoculum was prepared from cultures actively growing on rye A medium (10), which due to differences in growth rates of different isolates, were between 2 and 4 weeks old. Plates were flooded with sterile distilled water, brushed gently with a sterile glass rod to dislodge sporangia, and the resulting suspension placed in sterile plastic 50-ml centrifuge tubes. Suspensions were quantified using a hemacytometer and adjusted to concentrations of 50,000 sporangia/ml using sterile distilled water.

Six tomato leaflets (cv. Brandywine Red, Reimer Seed, Saint Leonard, MD) were cut from 4- to 6-week-old tomato plants grown in a growth chamber at 28°C with a 14-hour photoperiod and immediately distributed, two leaflets per plate, among three petri plates containing a thin layer of water agar. For each isolate, five of the leaflets were inoculated with one 10-μl droplet of the inoculum suspension placed on the midvein of the leaf. The sixth leaflet was similarly inoculated, but with a 10-μl droplet of sterile distilled water as a negative control. Six sets of three petri dishes were prepared for each isolate and one set each was incubated at 12, 16, 20, and 24°C. The remaining two sets of petri dishes were incubated at

20°C for two days, after which they were moved to 8 and 28°C. Preliminary experiments showed that very little infection occurred at 8 and 28°C. Therefore, two sets of petri dishes were incubated at 20°C to allow infection to occur so that pathogen growth could be measured at the temperature extremes. Separate identical incubators (Percival Scientific Inc., Perry IA) without lighting were used for each temperature setting. At 0, 5, 7, 9, 12, and 14 days after inoculation (dai), lesion length, defined as the farthest extent of either mycelial/sporangial growth and/or necrotic/chlorotic leaf tissue along the midvein, was measured using a ruler. The experiment was performed twice, each initiated on different days, for each isolate.

Temperature effect on colony growth on rye A medium. Two 8-mm-diameter plugs of mycelia/sporangia from an actively growing 2- to 3-week old *P. infestans* culture were placed in the center of two separate petri dishes containing 17 ml of rye A media (10). For each isolate, eight separate sets of two plates each were prepared and placed in plastic petri dish sleeves secured with a twist tie before placing in dark incubators at 4, 8, 12, 16, 18, 20, 24, and 28°C. All temperature experiments were performed in identical incubators (Percival Scientific Inc., Perry IA) except for the 4°C temperature, for which a refrigerator equipped with a Ranco electronic temperature control (Medford, MA) was used. Colony diameter was measured at 3, 5, 7, 10, 12, and 14 dai using a ruler. The experiment was performed twice, each initiated on different days, for each isolate.

Sporangia quantification. For the few isolates that were quantified within 18 months of original isolation, inoculum was prepared from rye A medium as outlined for the lesion growth rate assay. Isolates that were not quantified within 18 months of collection were passed through both tomato and potato leaf disks twice, in parallel. The inoculum suspension was prepared

directly from the second set of tomato and potato leaf tissue by agitating sporulating portions of inoculated leaves in 50-ml plastic centrifuge tubes containing sterile distilled water. The leaf portions were removed and the resulting suspension was adjusted to a concentration of 50,000 sporangia/ml with sterile distilled water.

Tomato plants (cv. Brandywine Red, Reimer Seed, Saint Leonard, MD) were grown in a 28°C growth chamber with a 14-hour photoperiod. Potato plants (cv. Katahdin) were grown from cuttings in a greenhouse with a daytime temperature of 18 to 21°C and a nighttime temperature of 21 to 24°C in an 18-hour photoperiod. Fully expanded leaflets were detached from the upper half of 4-to 6-week-old tomato and potato plants and 15-mm-diameter leaf disks cut from along the midvein of each leaf using a cork borer. Leaf disks, nine each of tomato and potato, were placed in separate 9-cm-diameter petri dishes directly on a thin layer of water agar and eight of the leaf disks for each species were inoculated with a 10-μl droplet of the inoculum suspension prepared as outlined above. The ninth leaf disk was similarly inoculated, but with a 10-μl droplet of sterile distilled water as a negative control. Plates were placed in a single layer in clear plastic boxes with non-sealing lids in a 20°C incubator with a 12-hour photoperiod. After 9 days, four sporulating leaf disks were removed from each plate and placed in separate 25-ml plastic centrifuge tubes containing 2 ml of sterile distilled water and 1 drop (12 to 18μl) of Tween 20 (Agdia Inc. Elkhart, IN). The Tween 20 was necessary to break the surface tension of water around plant trichomes and achieve accurate and comparable results between hosts and pathogen isolates (49). Each suspension was agitated with a vortex and quantified within 1 hour using a hemacytometer. The experiment was performed twice for each isolate and the data were analyzed using an analysis of variance (ANOVA).

Measurement of sporangial sizes. Sporangia were harvested from 2- to 4-week old cultures using the method described for inoculum preparation. Drops of the suspension were placed on a microscope slide under a cover slip. Photographs were taken of sporangia at 200x magnification using a Zeiss Axio Scope.A1 microscope (Göttingen, Germany) equipped with a Zeiss AxioCam MRc camera and the AxioVision Release 4.8 software included with the camera. The measurement tool included with the camera software was used to determine length and width of 50 sporangia for each isolate. As a summary statistic, the two dimensional area of each spore was calculated, assuming each spore was an ellipse, using the standard equation for the area of an ellipse: Area = π*A*B, where A is half of the major axis and B is half of the minor axis. Results were analyzed using a 1-way ANOVA.

Estimating relative and maximum lesion growth rate. Linear models were used to fit lesion growth as a function of time for the experiments conducted at different temperatures on tomato leaflets. Slopes were fit for each isolate and experimental replicate and the slopes of the linear models were used to represent the lesion growth rates. The relative lesion growth rates (rLGR) were then calculated for each clonal lineage by dividing the lesion growth rates (slopes) by the maximum calculated lesion growth rate. The relative lesion growth rate estimates were then plotted versus temperature and linear (polynomial) regression was used to model relative lesion growth as a function of temperature for each clonal lineage. All analyses were conducted in R version 2.9.2 (46). The polynomials generated for each lineage were used in the model for rLGR when the temperature was greater than 0°C but less than 28°C. For temperatures over 28°C, an equation for a line of constant slope between the y-value of the curve at x=28 and 32.9

was used. The value of 32.94 was the maximum growth temperature value provided in the model.

The maximum lesion growth rate (LGR) of a specific combination isolate and host was calculated using data from the lesion growth rate temperature experiment at 16°C. For each isolate, the lesion radius at 9 dai (in meters) was divided by the difference between the infection period, defined as the first appearance of necrotic spots, and 9 days to obtain a value for the lesion growth rate in meters per day (4). According to the rLGR curves calculated above, the temperature of 16°C was not optimal for growth for all lineages. To obtain an estimate of the LGR at the temperature where optimal growth occurs, the polynomial fit for the rLGR was solved at temperature=16°C and the mean of the growth rate for each lineage at 16°C divided by the result.

Estimating sporulation rate. To prepare the sporangial production data for the LATEBLIGHT simulation model, the counts from each leaf disk were averaged to obtain the number of sporangia produced per leaf disk and sporangia per square meter of leaf area was calculated using the equation: concentration of sporangia in suspension/area of the leaf disk. An average value for each lineage was calculated from the five isolates of each lineage tested. A latent period of more than 2 days has been observed at 20°C for isolates of the US-22, US-23, and US-24 lineages (unpublished data), so the latent period was estimated at a conservative value of 2.5 days. The average sporulation values for each lineage were divided by 6.5 (9 days total minus a latent period of 2.5) to obtain a value for sporangia production per square meter per day. The parameter SR (sporulation rate) is the maximum sporulation rate for a particular clonal lineage/host interaction. The sporulation experiment was performed at 20°C, which, according

to the relative sporulation rate curve in the provided in the LATEBLIGHT model (4), is greater than the optimal temperature. To obtain the maximum sporulation rate (SR), the equation was solved for temperature=20°C and the mean sporulation rate per day for each clonal lineage and host divided by the result.

Weather data for the LATEBLIGHT model. Integrated surface weather data for the 2009 to 2012 growing seasons were downloaded from the National Oceanic Atmospheric Association (NOAA) integrated surface database of hourly weather data (<ftp://ftp.ncdc.noaa.gov/pub/data/noaa/isd-lite/>). Due to its close proximity to the potato growing region of Wisconsin, data from the Stevens Point, Wisconsin weather station, USAF-WBAN Station ID:726426-04895 were selected to be input to the LATEBLIGHT model. These weather data include hourly observations of temperature, precipitation, and dew point temperature. The downloaded weather data were arranged as needed for input into the LATEBLIGHT program and, when observations were missing from the data file, linear interpolation was used to provide an estimate for the missing data. Relative humidity value was calculated from the observed dewpoint temperature and temperature as follows: Relative Humidity = 100 * exp((17.625*DP)/(243.04+DP)) / exp((17.625*T)/(243.04+T))), where DP is the dewpoint temperature, and T is the temperature.

LATEBLIGHT simulation. The LATEBLIGHT simulation model was downloaded from the CIP website, <https://research.cip.cgiar.org/confluence/display/GILBWEB/Download+LATEBLIGHT+Simulator> and set up and run using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina) according to the instructions provided. For each year, the weather data were input and the

EmergDate parameter in the file "Estimating initial inoculum" was set to reflect the date of 50% emergence of mid-planted potatoes for the Stevens Point, Wisconsin area for each year (Table 2). The threshold for relative humidity was set to 85% according to the instructions for the simulation, because the weather data was collected from outside of the plant canopy. Initial inoculum was estimated following the instructions provided in the "Estimating initial inoculum" file. Commercial fields are typically scouted every week, so the parameter t0Date, or the last day no lesions were seen was set to 7 days before the parameter t1Date, which is the first day lesions were detected (Table 2). An exception was in 2009 were the t0 date was set earlier due to the known likelihood that late blight was introduced on tomato transplants much earlier than the date the disease was actually recognized (47). The cultivar used was set to a susceptible long-season cultivar and the fungicide program was set as "no fungicides."

For each year of weather data (2009 to 2012), the epidemic was run with the default pathogen parameters provided in the downloaded version, where SR was 292,000,000 and LGR was 0.00410, and the equations and domains for relative lesion growth rate (rLGR) set as in Andrade-Piedra et al. (4). In subsequent simulations, the SR and/or LGR and rLGR were changed to reflect the new sporulation and growth rates obtained for each clonal lineage in the experiments outlined above. For example, the SR parameter and the lesion growth rate parameters (LGR and rLGR) for US-22 on tomato were first changed separately to examine the effect of changing either the SR or LGR on epidemic progression. The SR and LGR parameters were then changed in combination to further examine the effect of changing both sporulation and lesion growth on epidemic progression. Since lesion growth rate parameters were not measured on potato, only the effect of SR was examined for the clonal lineage/potato combination. All

other parameters in the model were set to the default values. Epidemic simulations were run and disease severity data output and concatenated in R version 2.9.2 (46), which was used to calculate the summary statistics of AUDPC, time to 50% severity, time to 100% severity, and rate of epidemic progression.

Results

Growth rate assays. There were numerically different results between the growth experiments on medium and the experiments on tomato leaves. Most notably, growth on medium declined to nearly zero at 28°C, particularly for the US-23 and US-24 clonal lineages (Fig. 1). In contrast, lesions on tomato leaves grew quickly at 28°C after the 20°C establishment period of two days, particularly for the US-22 and US-23 clonal lineages, with growth only slightly less than that at the optimum temperature (Fig 1). There were also numerical differences between experiments on media and experiments on tomato leaves in the optimal growth temperature of each lineage. Based on the growth curves, the optimal temperatures on media were 19.5, 19.9 and 19.9°C for the US-22, US-23, and US-24 lineages, respectively. On tomato leaves, the optimal temperatures for lesion expansion were 15.8, 21.5, and 16.2°C for the US-22, US-23, and US-24 lineages, respectively.

Sporangia quantification. There was no significant difference between the two repetitions of the experiment (P=0.466) so they were pooled before a two-way ANOVA was performed. One isolate each from the US-22 and US-24 clonal lineages failed to infect or sporulate on either potato or tomato leaves so the analysis was performed on the remaining four isolates for each of these lineages. For all of the lineages, there were always significantly fewer

sporangia produced per square meter per day on tomato than on potato, but the greatest difference between the two hosts occurred when inoculated with the US-24 clonal lineage of *P. infestans* (Fig. 2). On tomato, the US-23 lineage produced significantly more sporangia than the US-22 ($P=0.001$) and US-24 lineages ($P<0.001$) (Fig. 2). On potato, sporulation by the US-23 lineage was also high, but not significantly different from that by the US-24 lineage on potato ($P=0.455$) (Fig. 2).

Sporangial size. Considering the two dimensional area of each sporangium, sporangia from isolates of the US-23 clonal lineage were significantly smaller than sporangia from the US-22 and US-24 clonal lineages ($P<0.001$ in both cases) (Fig. 3). Sporangia of the US-23 clonal lineage had a mean area of 357.8 μm^2 (sd=115.4) while those of the US-22 and US-24 lineages had areas of 416.1 μm^2 (sd=122.4) and 484.6 μm^2 (sd=128.4), respectively (Fig. 3).

LATEBLIGHT simulation modeling. The pathogen parameters of LGR and SR (Table 3) and equations for rLGR (Table 4) were modified for input into the LATEBLIGHT model. In Wisconsin, the 2009 growing season was characterized by temperatures mostly below average and moderate rainfall (Fig. 4). The 2010 growing season had average temperatures but abnormally high rainfall amounts, particularly during mid- to late summer (Fig. 4). The 2011 growing season was characterized by slightly above average temperatures and moderate rainfall (Fig. 4). The 2012 growing season had many abnormally high temperatures, particularly in the beginning of the season, and very little rainfall, particularly in the middle of the growing season (Fig. 4). These differences in weather parameters were reflected in the number of hours each year that the relative humidity was above 85%, an important consideration for *P. infestans* growth and referred to as HumidHrs in the LATEBLIGHT simulation model (Fig. 4). The year

2010 accumulated the most HumidHrs, followed closely by 2011 and then 2009 (Fig. 4). The year 2012 accumulated many fewer HumidHrs than the other years (Fig. 4).

When the default pathogen parameters, which are based mostly on the EC-1 clonal lineage (4) were used, AUDPC values were greatest in 2010 and least in 2011 (Table 5, Fig. 5). These values are dependent on environmental conditions, but also on the date used for initial inoculation, which is calculated by the model from the actual date of late blight appearance (Table 2). Epidemics with the default parameters increased to greater than 50% severity in all years analyzed and reached 100% severity in all years except 2011 (Table 5, Fig. 5). The rates of epidemic progression were highest in 2010, followed by 2009, then 2012, and then 2011 (Table 5).

For the US-22 clonal lineage, changing SR to that for tomato decreased the AUDPC value compared to the default epidemic, but adjusting SR to that for potato always increased the AUDCP (Table 6, Fig. 5); the greatest decrease and increase, respectively, was in the 2011 season. Changing the lesion growth rate parameters (rLGR and LGR) to reflect that of the US-22 lineage increased the AUDPC in every year except in 2010, where it had essentially no effect on AUDPC (Table 5). Changing the lesion growth rate parameters had a greater effect on increasing the AUDPC than the effect of SR of potato. When this parameter was changed in 2011 for the US-22 lineage, this was the only epidemic simulation that achieved 100% severity (Table 5, Fig. 5). When both SR and the lesion growth rate parameters were changed for the US-22 lineage, AUDPC was lowered in 2010, even though this combination increased the AUDPC in every other year, even by as much as 110% in 2011 (Table 6). This increase in AUDPC

because of lesion growth rate parameters greater than the default parameters appeared to negate the effect of an SR lower than the default in 2011.

For the US-23 clonal lineage, all AUDPC values were the lowest in 2011 and the highest in 2010 (Table 5, Fig. 5). Changing the SR parameter to that for either tomato or potato always increased the AUDPC for epidemics in every year, but the greatest effect was seen in 2011. Changing SR for potato increased AUDPC more than for tomato (Table 5). Changing the lesion growth rate parameters (rLGR and LGR) for the US-23 lineage also increased AUDPC above the default value, and these parameters had a greater effect on AUDPC than either of the sporulation parameters (Table 6). Combining the lesion growth rate parameters with SR had the greatest effect of all the changes; in 2011 where the greatest increase of each parameter was seen, the AUDPC value increased to 188% of the default (Table 6).

For the US-24 clonal lineage, changing each parameter to be specific for this lineage resulted in lower AUDPC values than the default, except when SR was changed to that for potato (Table 5). In 2011, where the greatest effect was seen, using the SR for US-24 on potato increased the AUDPC to 135% of the default (Table 6). The 2011 season also had the greatest effect on lowering AUDPC values for each of the other pathogen parameters and combinations. In every season, the lesion growth rate parameters (rLGR and LGR) had a greater effect on lowering the AUDPC values than SR for tomato (Table 5). When both SR and the lesion growth rate parameters were combined, epidemics progressed very little, with AUDPC values as low as 90 in 2011 (Table 5). In 2011 and 2012, the epidemic never reached 50% severity when these two parameters were combined for the US-24 lineage (Table 5).

While the AUDPC partly reflects the length of the epidemic, the rate of progression for each epidemic is independent of epidemic length. When SR was changed to that for tomato, the US-22 and US-24 lineages both saw decreases in the rate of epidemic progression in every year, with the greatest decrease in 2010 and the least in 2009 (Table 6). When SR for the US-23 lineage on tomato was changed, increases in the rate were seen in every year, with the greatest increase in 2011 and the least in 2012 (Table 5). Changing the SR parameter to that for potato also resulted in increases in the rate of epidemic progression over the default in every year for each clonal lineage, with the greatest increase in 2011 and the least in 2012 (Table 5).

When the lesion growth rate parameters (LGR and rLGR) were changed, the US-22 lineage had a large increase in the rate of epidemic progression over the default parameters (143%) even though there was no change in 2010 and 2012, and a decrease in 2009 (Table 6, Fig. 5). The US-23 lineage also had a large increase in the rate of the simulated epidemic in 2011 compared to the default when the lesion growth rate parameters were changed, but there was no affect on the epidemic rate in 2009 and only slight increases in the other years. When these parameters were changed for the US-24 lineage, the rate always decreased, with the biggest effect seen in 2010 and the smallest in 2009 (Table 6).

When the lesion growth rate parameters were combined with changes in SR, this resulted in a decrease in the rate of epidemic progress for the US-22 lineage in all years except 2011 where there was an increase of 119% over the default parameters (Table 6). For the US-23 lineage, a large increase in the rate of epidemic progression was similarly seen in 2011, but unlike the rates for the US-22 lineage, there were increases in all years, except in 2009 where there was no effect (Table 5, Fig. 5). For the US-24 lineage, changing both parameters caused

decreases the rate relative to the default in every year, with the biggest decrease in 2010 and the smallest in 2009 (Table 6).

Discussion

Changes in the biology of plant pathogens can have specific impacts on epidemic progression (1). Some of the most obvious of these may be insensitivity to fungicides (11, 14, 32) or changes in the race structure (18, 45), as these often have an immediate and sometimes marked impact on the efficacy of currently common management strategies. Subtle changes in sporangial size or production or optimal temperatures for lesion expansion may be harder to quantify in a field setting, but simulation modeling can provide insight into the effect of these changes in the pathogen population. In the current study, we have shown that differences in sporulation rate and lesion growth rate between the US-22, US-23, and US-24 clonal lineages of *P. infestans* affect the progression of late blight epidemics simulated using the LATEBLIGHT model, but this is also dependent on the length of the epidemic and differing environmental conditions.

Scale is an important consideration of the LATEBLIGHT simulation model. The model simulates each late blight epidemic on one square meter of a potato field. In many cases, fields may be inoculated with a sporangial shower, with sporangia deposited evenly over an entire large field. In such an instance, there may well be one to a few sporangia for each square meter of the field, and the entire field will reach 100% severity at the same time that any given square meter reaches 100% severity. In this case, the simulation results from one square meter should give an accurate approximation of a large-scale field epidemic. However, fields are often

diligently sprayed with fungicides, thereby limiting the effects of a spore shower, but plants directly around implements such as irrigation heads may be missed, resulting in a single disease focus in a field. In these cases, only one or a few square meter around the inoculation point would be likely to reach 100% severity as simulated, but the entire field would take much longer to reach 100% severity as sporangia gradually spread from the initial focus. Therefore, the scale and method of initial inoculation is important to consider when predicting the reliability of the simulation results in predicting field epidemics.

The two metrics of AUDPC and rate of epidemic progression were useful for analyzing different aspects of the results of each simulated epidemic. The AUDPC values were highly influenced by epidemic length and not as much by environmental conditions; HumidHrs, one measure of a conducive environment, were lowest in 2012, yet the default epidemic that had the lowest AUDPC values was for 2011. Considering all the lineages, changing fitness parameters had the most effect in 2011, where the epidemic length was the shortest. Certain parameters for each lineage always caused a decrease in the AUDPC value relative to the default parameters and this amount of decrease was always magnified in 2011. Similarly, increases in AUDPC values relative to the default were increased the most in 2011. This suggests that isolates of *P. infestans* that have increased fitness may have the greatest advantage in locations with shorter growing seasons rather than in regions with endemic populations of *P. infestans* that would see epidemics initiated earlier and lasting longer. Similarly, isolates with decreased fitness would be more quickly outcompeted in locations that typically have shorter late blight epidemics.

Unlike AUDPC, the rate of epidemic progression was not affected by the epidemic length. Instead, the rate reflects the interaction between the pathogen and the environmental

conditions. The effect of temperature on the rate of epidemic progression for each clonal lineage was most evident when the lesion growth rate parameters, which depend on temperature, were input in the model. The values input for LGR for the US-22 and US-23 lineages were much larger than those values for the default and these large values likely contributed to the increases seen. However, the effect of temperature was also included in the lesion growth rate parameter changes by the equations determined for rLGR. The US-22 lineage in particular demonstrated growth near the maximum at 28°C, which is higher than that for the default rLGR curve value (4). In the simulated epidemics in 2009, where temperatures were generally cool, the US-22 lineage had a rate of epidemic progression lower than the default epidemic when the lesion growth rate parameters were changed. However, in 2011, when temperatures were higher, the US-22 epidemic increased at a rate higher than the default, suggesting that this lineage may have a fitness advantage in warmer environmental conditions. The epidemic caused by the US-23 lineage did not proceed at a rate higher than the default in 2009, suggesting that this lineage also does not have a fitness advantage at lower temperatures. That an increase in epidemic progression was seen by these lineages at higher temperatures but not low temperatures suggests that these lineages may predominate in warmer climactic regions or regions that may be becoming warmer due to global climate change (2).

For all of the parameters tested, considering the metrics of both AUDPC and the rate of epidemic progression, the US-23 clonal lineage consistently had larger and faster simulated epidemics regardless of epidemic length or environmental conditions than either the US-22 or US-24 lineages or the epidemic simulated by the default parameters. Additionally, of the lineages tested, US-23 had the highest sporulation rates on both potato and tomato. US-22

readily infected both hosts, but sprorulation by this lineage was overall less. The US-24 lineage, while having a high sporulation rate on potato, had the lowest rate of the lineages tested on tomato. The large and fast simulated epidemics produced by US-23 on both potato and tomato suggest that this lineage is the most fit and is the most likely to predominate the pathogen population. Indeed, this has been observed in Wisconsin and across the U.S. in the past several years. In Wisconsin, the US-23 lineage first appeared in a single location in 2009 (48). Over the following three years, the proportion of late blight samples collected that yielded the US-23 lineage steadily increased and the prevalence of the US-22 and US-24 lineages decreased until by 2012 US-23 was the only lineage collected (48). Similarly, across the U.S., US-22 was the predominant lineage reported in 2009, but by 2012 almost all of the reports of late blight were caused by US-23 (6). That US-23 is simulated to do well in short or long seasons and over a range of environmental conditions and on both tomato and potato suggests that this lineage may likely persist in the U.S. pathogen population for many years.

The differences among the three clonal lineages may affect management of late blight at the field level. US-24 sporulates poorly on tomato, and when this is combined with an attenuated lesion growth rate, simulated epidemics progressed little. This would suggest that if US-24 was the only lineage in a region, tomato growers could concentrate their management efforts on other issues that may have more effect on the crop than the US-24 lineage. Conversely, if the US-23 lineage was in the region, late blight control measures would be warranted, as this lineage sporulates and grows well on tomato. Additionally, tomato or potato crops that are already infected may benefit from different management practices depending on the lineage present. From the simulation results, US-23 causes an explosive epidemic, while that

caused by US-24 is much less and slower, suggesting that US-23 might be best controlled by crop destruction, but the US-24 lineage might be kept from spreading through the diligent use of fungicides.

Actual late blight epidemics may progress faster than those simulated in this work due to the effects of irrigation. The simulation modeling was performed for four years of weather data from a NOAA weather station that was not located in the potato or tomato canopy and did not include the effects of irrigation. There is a provision in the model to lower the relative humidity threshold from 90% to 85% if the environmental data are collected from above the plant canopy. This change was made, but irrigation is relied upon heavily in the light sandy soils of central Wisconsin, likely producing far more hours of favorable relative humidity than can be accounted for simply by changing the threshold to 85%. Therefore, the potential for large epidemics, particularly by the US-23 clonal lineage, may be even higher than estimated in the simulations.

The higher variation observed by the US-24 clonal lineage in the tomato leaf temperature experiment might be partly explained by a lack of virulence on tomato. The low growth curve partly reflects a low infection rate. Many of the inoculated leaflets never showed any sign or symptom of disease. In other cases, lesions formed, but remained small and never progressed to cover the whole leaflet. The US-24 lineage has never been isolated from tomato in Wisconsin (48) and has only seldom been isolated from tomato elsewhere (13, 35). Other studies have also suggested that US-24 is a poor tomato pathogen (13, 35). Under ideal laboratory conditions, lesions and sporulation were produced by US-24 on tomato, but in a field situation the probability that sporangia of US-24 will successfully infect tomato leaves may be even lower than demonstrated, resulting in epidemics with lower disease severity values than simulated.

The optimal temperatures for growth varied between lineages and between the tomato leaf tissue and media experiments. On medium, the optimal temperatures for growth were very similar for each lineage, at 19.5 for US-22 and 19.9 for US-23 and US-24. On tomato leaves, there was more variation among the lineages, with US-22 and US-24 showing optimums below that seen on media (15.8 and 16.2°C, respectively), and US-23 above the optimum seen on media, at 21.5°C. However, for the US-22 lineage, nearly optimal growth was also observed at 28°C on tomato leaves, and for both the US-22 and US-23 lineages, growth was relatively high at 28°C on tomato leaves, but very low on media. In several secondary research documents, including a *Phytophthora* key, the maximum temperature for growth is reported at 26°C (17, 20), which is inconsistent with our data from experiments on media and completely unsupported by our data from leaf tissue. This contrast underscores, first, the importance of performing experiments on living plant tissue rather than artificial media, and, secondly, the importance of continuing to monitor new pathogen populations for changes in their basic biology.

The dip in the growth curve for the US-22 lineage at 24°C on tomato leaves may indicate a low rate of direct germination of sporangia for this lineage. Indirect germination via zoospores occurs at lower temperatures (17), but direct germination was shown to be more common at temperatures near 24°C for these lineages (13). Little or no infection of leaves at 28°C was observed in preliminary experiments, so to collect data at this temperature, the leaves were incubated at 20°C for 2 days before moving to 28°C. This was not done for the 24°C treatments, so the dip in the growth curve may simply reflect a low rate of indirect spore germination. Interestingly, this was not observed for the US-23 or US-24 lineages. This suggests that the US-23 and US-24 lineages have a greater direct germination efficiency than the US-22 lineage.

The effect of sporangial size on epidemic progression is not currently incorporated into the LATEBLIGHT simulation model. There are significant differences in average sporangial size between the US-22, US-23, and US-24 lineages. Smaller spores may be lifted from the leaf surface and escape the plant canopy more easily than large spores, and may have further aerobiological implications (7). This would give the US-23 lineage a selective advantage over the other two lineages, particularly over the US-24 lineage, which had the largest sporangia. Smaller sporangia may be carried farther on air currents and thereby infect fields at a greater distance than clonal lineages that have large sporangia. Anecdotal evidence from the 2010 and 2012 growing seasons in Wisconsin suggest that the US-23 lineage may travel farther than the US-24 lineage. In 2010, isolates of the US-24 lineage of *P. infestans* were collected from eight fields in Wisconsin, but all of these fields were within a 2-county area in the center of the state (48). In contrast, in 2012, isolates of the US-23 clonal lineage were collected from many fields over 10 different Wisconsin counties (48). The possibility of more than one inoculation source in 2012 cannot be eliminated, but the anecdotal evidence suggests that the US-23 clonal lineage may have a higher potential for long-distance travel than the US-22 and US-24 clonal lineages, and this may be due to smaller sporangia. Further experimental field data would be needed to confirm this, but if confirmed, the parameter of sporangial size should be considered in future revisions of the LATEBLIGHT simulation model.

The results of the simulated epidemics suggest that grower management practices in the state are effectively limiting disease. The cumulative HumidHrs in 2011 were just slightly below those in 2010, the year that had the maximum number, but AUDPC values were the lowest in 2011 of any of the years tested and the first report of late blight was as late as 25 August. This

late epidemic start in spite of a high accumulation of HumidHrs suggests that grower applications of fungicides to protect the crop were highly effective because they were able to delay an epidemic until 25 August.

The simulation model was originally designed for use on potato but was extended to the tomato crop in this study. Several assumptions were made, including that temperature has the same effect on sporulation on tomato as on potato, but we also experimentally determined some key parameters, including rLGR, to make the model applicable to tomato. While US-1 was historically the only lineage in the U.S and this was not highly virulent on tomato (26, 29, 42), many lineages that have been identified in the past 25 years, including those in the present study, that are highly virulent on both potato and tomato (29). Late blight is a community disease, easily spread from field to field; therefore, virulence on tomato should not be ignored when considering the overall epidemiology of the late blight pathogen. Additionally, a state like Wisconsin has many hectares of potatoes but also significant tomato production, so consideration of tomato in overall late blight epidemics is crucial to understanding the epidemiology. Field studies would validate the application of the LATEBLIGHT model to tomato production, but this model in its current form can still contribute to our understanding of tomato and potato late blight epidemiology.

This study has explored the effects of the fitness parameters of sporulation and the effect of temperature on lesion growth of three recently emerged clonal lineages of *P. infestans* on the epidemiology of late blight through simulation modeling. The results of the simulations suggest that the US-23 clonal lineage may be more fit than either the US-22 or US-24 lineage as the higher sporulation rates on both potato and tomato and relatively high lesion growth rate resulted

in the largest and fastest simulated epidemics. These results will further an understanding of a changing *P. infestans* pathogen population and will lead to enhanced management of late blight on both potato and tomato.

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Table 1. Characteristics of the *Phytophthora infestans* isolates used in this study. Five isolates from each lineage representing a range of years, locations, and original hosts and plant parts were chosen from a larger collection from Wisconsin (48).

Clonal lineage	Isolate#	Year	Host	Plant part	County	Mating type	Mefenoxam sensitivity
US-22	Pi7-09a	2009	Tomato	Fruit	Dane	A2	Sensitive
	Pi14-09a	2009	Potato	Tuber	Langlade	A2	Sensitive
	Pi15-10a	2010	Potato	Leaf	Marquette	A2	Sensitive
	Pi23-10a	2010	Tomato	Leaf	Vernon	A2	Sensitive
	Pi29-10a	2010	Tomato	Leaf	Manitowoc	A2	Sensitive
US-23	Pi1-09a	2009	Potato	Leaf	Vernon	A1	Sensitive
	Pi16-10a	2010	Tomato	Fruit	Waukesha	A1	Intermediate
	Pi30-10a	2010	Tomato	Fruit	Ozaukee	A1	Intermediate
	Pi35.2-11a	2011	Tomato	Leaf	Waukesha	A1	Intermediate
	Pi36.2-11a	2011	Potato	Leaf	Adams	A1	Intermediate
US-24	Pi21-10a	2010	Potato	Leaf	Waushara	A1	Intermediate
	Pi27-10a	2010	Potato	Leaf	Portage	A1	Intermediate
	Pi34-10a	2010	Potato	Tuber	Portage	A1	Intermediate
	Pi38.1-11a	2011	Potato	Leaf	Adams	A1	Intermediate
	Pi40.1-11a	2011	Potato	Tuber	Waushara	A1	Intermediate

Table 2. Parameters input for the LATEBLIGHT simulations for each epidemic year.

Year	Emergence date[a]	First late blight[b]	InocDate[c]	InMicCol[d]
2009	20 May	29 July	10 July	4
2010	18 May	14 July	9 July	5
2011	20 May	25 August	20 August	5
2012	10 May	31 July	26 July	5

[a] Dates for 50% emergence of mid-planted potatoes for the Stevens Point, Wisconsin area.

[b] The date that late blight was first reported in or near the central sands area of Wisconsin each year based on samples submitted to our lab by growers or crop consultants.

[c] Inoculation Date, as defined and estimated by the program "Estimating initial inoculum" included with the LATEBLIGHT simulation.

[d] Initial Microcolonies, as defined and estimated by the program "Estimating initial inoculum" included with the LATEBLIGHT simulation.

Table 3. Experimental results obtained for the values of lesion growth rate (LGR, meters per day) and sporulation rate (SR, sporangia per square meter per day) used in the LATEBLIGHT model.

Parameter	Clonal lineage	Host	Mean[a]	Adjusted mean[a]
SR	US-22	Tomato	122,220,315	240,547,134
		Potato	162,577,058	319,975,001
	US-23	Tomato	185,008,679	364,123,653
		Potato	224,404,824	441,660,924
	US-24	Tomato	87,482,318	172,177,767
		Potato	202,383,837	398,320,459
LGR ($\times 10^{-3}$)	US-22	Tomato	4.80	6.33
	US-23	Tomato	4.65	5.86
	US-24	Tomato	2.52	4.01

[a] The LGR experiment was performed on detached leaflets in moist chamber petri plates at 16°C in darkness and the SR experiment was performed on leaf disks in moist chamber petri plates at 20°C with a 12-hour photoperiod.

[b] LGR represents a maximum lesion growth rate for a particular lineage-host interaction, so the means obtained at 16°C were adjusted to maximums by solving the equation for rLGR at x=16°C and dividing the mean by the result. SR similarly is a maximum value, so the equation for rSR provided in the model (4) was solved for 20°C and the mean divided by the result to obtain the adjusted mean used in the simulation modeling.

Table 4. Equations used for modifying the relative lesion growth rate (rLGR) parameter in the LATEBLIGHT model (4)

Clonal lineage	Modified rLGR equations	Range	R^2
US-22			
	$-2.9581056 + 0.6044356 \times T - 0.0318121 \times T^2 + 0.0005345 \times T^3$	0°C≤T≥28°C	0.431
	$-0.156599 \times T + 5.138916$	28°C<T>32.94°C	…
US-23			
	$-0.8358 + 0.1604 \times T - 0.003604 \times T^2 - 0.000003845 \times T^3$	0°C≤T≥28°C	0.669
	$-0.150903 \times T + 4.970744$	28°C<T>32.94°C	…
US-24			
	$-1.9610821 + 0.3890977 \times T - 0.0183672 \times T^2 + 0.0002602 \times T^3$	0°C≤T≥28°C	0.265
	$0.0572259 \times T + 1.88502$	28°C<T>32.94°C	…

Table 5. Results of the LATEBLIGHT simulations for each clonal lineage and each year of weather data from Stevens Point, Wisconsin.

Year	Parameter[a]	US-22				US-23				US-24			
		AUDPC[b]	50%[c]	100%[d]	Rate[e]	AUDPC	50%	100%	Rate	AUDPC	50%	100%	Rate
2009													
	Default	5039	21	26	3.85	5039	21	26	3.85	5039	21	26	3.85
	SR Potato	5092	21	25	4.00	5272	19	23	4.35	5214	19	24	4.17
	SR Tomato	4923	22	27	3.70	5163	20	24	4.17	4675	24	33	3.03
	LGR	5297	20	27	3.70	5495	19	26	3.85	3892	28	39	2.56
	SR+LGR	5172	23	29	3.45	5631	18	24	4.17	3254	34	45	2.22
2010													
	Default	5796	19	25	4.00	5796	19	25	4.00	5796	19	25	4.00
	SR Potato	5850	18	24	4.17	6004	17	21	4.76	5958	17	22	4.55
	SR Tomato	5639	20	32	3.13	5915	18	23	4.35	4720	22	…	1.46
	LGR	5797	19	25	4.00	6108	17	21	4.76	2973	36	…	1.26
	SR+LGR	5646	20	31	3.23	6200	16	20	5.00	1968	48	…	1.16
2011													
	Default	1068	22	…	2.33	1068	22	…	2.33	1068	22	…	2.33
	SR Potato	1166	21	…	2.44	1561	16	…	2.95	1427	16	…	2.79
	SR Tomato	872	25	…	2.08	1314	17	…	2.62	584	32	…	1.63

	LGR	1592	16	30	3.33	1871	16	23	4.35	257	…	…	1.48
	SR+LGR	1171	21	…	2.77	2004	15	19	5.26	90	…	…	0.56
2012													
	Default	3335	28	36	2.78	3335	28	36	2.78	3335	28	36	2.78
	SR Potato	3405	27	35	2.86	3660	24	33	3.03	3576	25	34	2.94
	SR Tomato	3140	29	44	2.27	3507	26	34	2.94	2558	32	…	1.61
	LGR	3580	24	36	2.78	3936	20	35	2.86	1603	35	…	1.82
	SR+LGR	3412	27	38	2.63	4144	18	33	3.03	655	…	…	0.85

[a] The default parameters were the pathogen parameters that were included with the LATEBLIGHT model. For ease of comparison, the default values for each year are repeated with data for each clonal lineage. SR is the maximum sporulation rate by each lineage. This was experimentally determined for tomato and potato and the parameters analyzed separately in the model. The LGR parameter includes the maximum lesion growth rate of a particular isolate (LGR) on tomato and potato, and cubic polynomial equations representing the effect of temperature on lesion growth (rLGR). The SR+LGR parameter change includes the effect of SR on tomato and the lesion growth rate parameters (LGR+rLGR) for each lineage.

[b] Area under the disease progress curve.

[c] Time from the initiation of the epidemic to 50% severity.

[d] Time from the initiation of the epidemic to 100% severity.

[e] The rate of epidemic progression. Calculated by determining the slope of a line between the first onset of disease and the maximum value.

Table 6. The percent change observed in AUDPC values between the default pathogen parameters and those used for each year and clonal lineage combination.

		AUDPC % of default			Rate % of default		
Year	**Treatment[a]**	**US-22**	**US-23**	**US-24**	**US-22**	**US-23**	**US-24**
2009							
	SR Potato	101%	105%	103%	104%	113%	108%
	SR Tomato	98%	102%	93%	96%	108%	79%
	LGR	105%	109%	77%	96%	100%	67%
	SR+LGR	103%	112%	65%	90%	108%	58%
2010							
	SR Potato	101%	104%	103%	104%	119%	114%
	SR Tomato	97%	102%	81%	78%	109%	37%
	LGR	100%	105%	51%	100%	119%	31%
	SR+LGR	97%	107%	34%	81%	125%	29%
2011							
	SR Potato	109%	146%	134%	105%	127%	120%
	SR Tomato	82%	123%	55%	89%	113%	70%
	LGR	149%	175%	24%	143%	187%	64%
	SR+LGR	110%	188%	8%	119%	226%	24%
2012							
	SR Potato	102%	110%	107%	103%	109%	106%
	SR Tomato	94%	105%	77%	82%	106%	58%

LGR	107%	118%	48%	100%	103%	66%
SR+LGR	102%	124%	20%	95%	109%	31%

[a] SR is the sporulation rate, and this was tested separately for potato and tomato. LGR incudes the effect of temperature on lesion growth rate for each lineage on tomato leaf tissue. SR+LGR is both of the factors combined in the LATEBLIGHT simulation model.

Figure 1. Growth curves for the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* at different temperatures. The top panels illustrate growth on rye A media based on colony diameter data collected at 4, 8, 12, 16, 18, 20, 24, and 28°C. The bottom panels show growth on tomato leaves based on lesion length data collected at 8, 12, 16, 20, 24, and 28°C. Each curve is an average of five isolates from each clonal lineage.

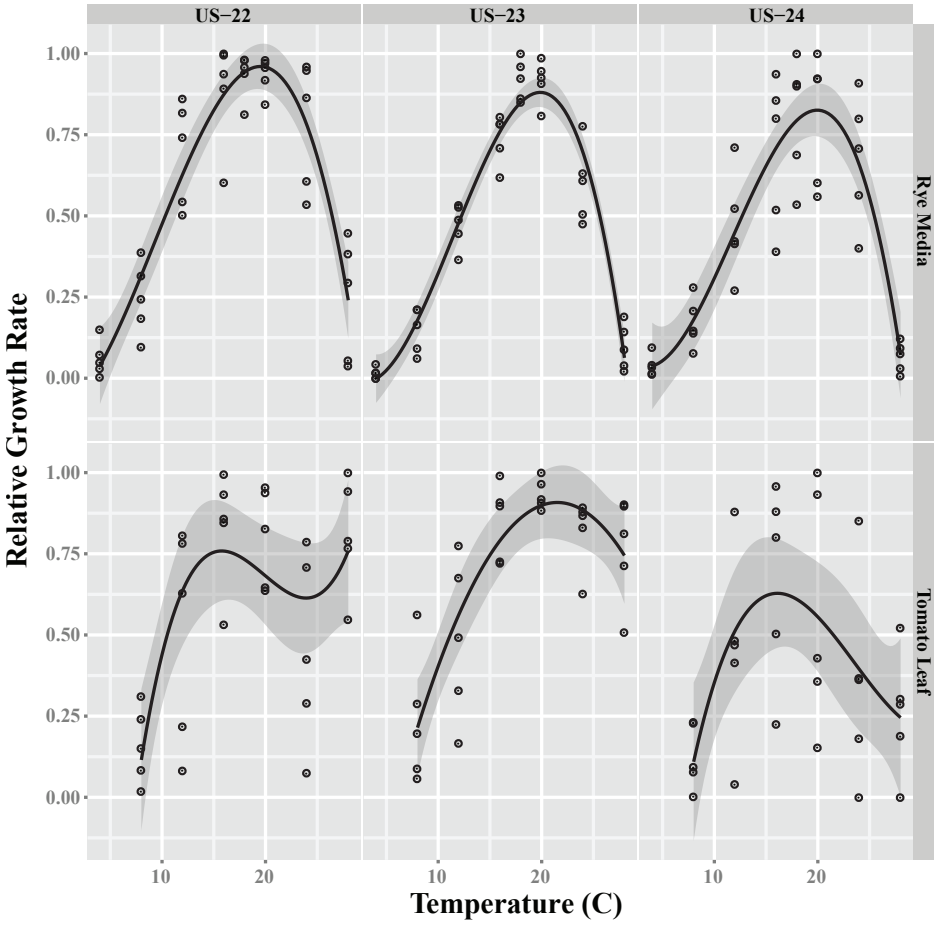


Fig. 2. Sporulation rate of the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* on tomato and potato at 20°C. Bars represent the mean of four to five isolates of each lineage and error bars are standard deviations. Each lineage had a statistically higher sporulation rate on potato than on tomato. Different letters over the bars indicate significant differences between the clonal lineages for each host (lowercase for tomato and uppercase for potato). Tukey tests (P=0.05) were used for all statistical analyses.

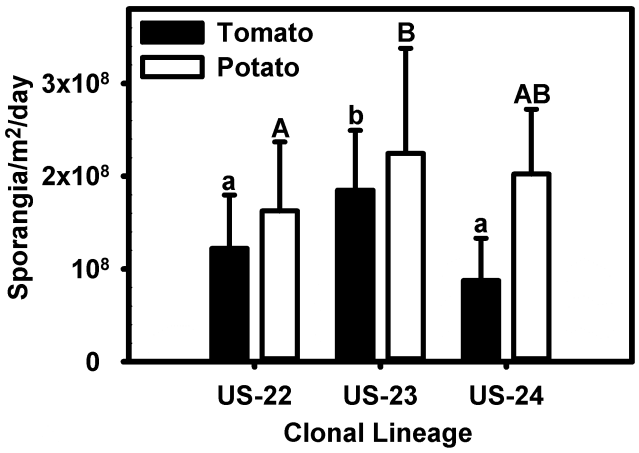


Fig. 3. Sporangial sizes for the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans*. Each bar represents the mean of five isolates from each clonal lineage and the error bars are standard deviations. Different letters over the bars indicate significant differences (Tukey test, P=0.05).

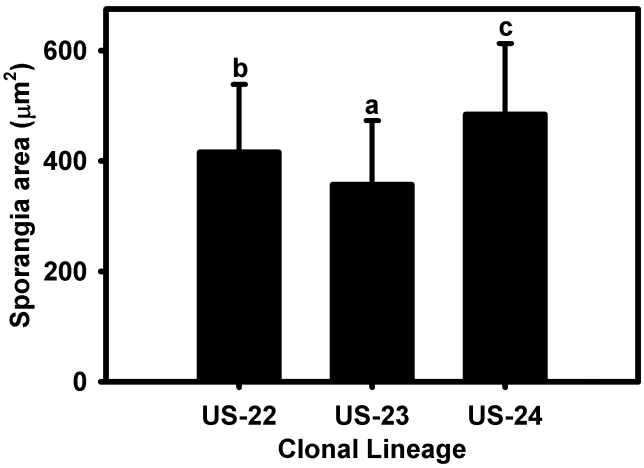


Figure 4. Average daily temperatures (A), cumulative rainfall (B), and cumulative hours when relative humidity was above 85% (C) at Stevens Point, Wisconsin for each of the 2009 through 2012 growing seasons. The bold line in (A) represents a 30-year average. The vertical lines in each plot signify the inoculation date used in the LATEBLIGHT model each year.

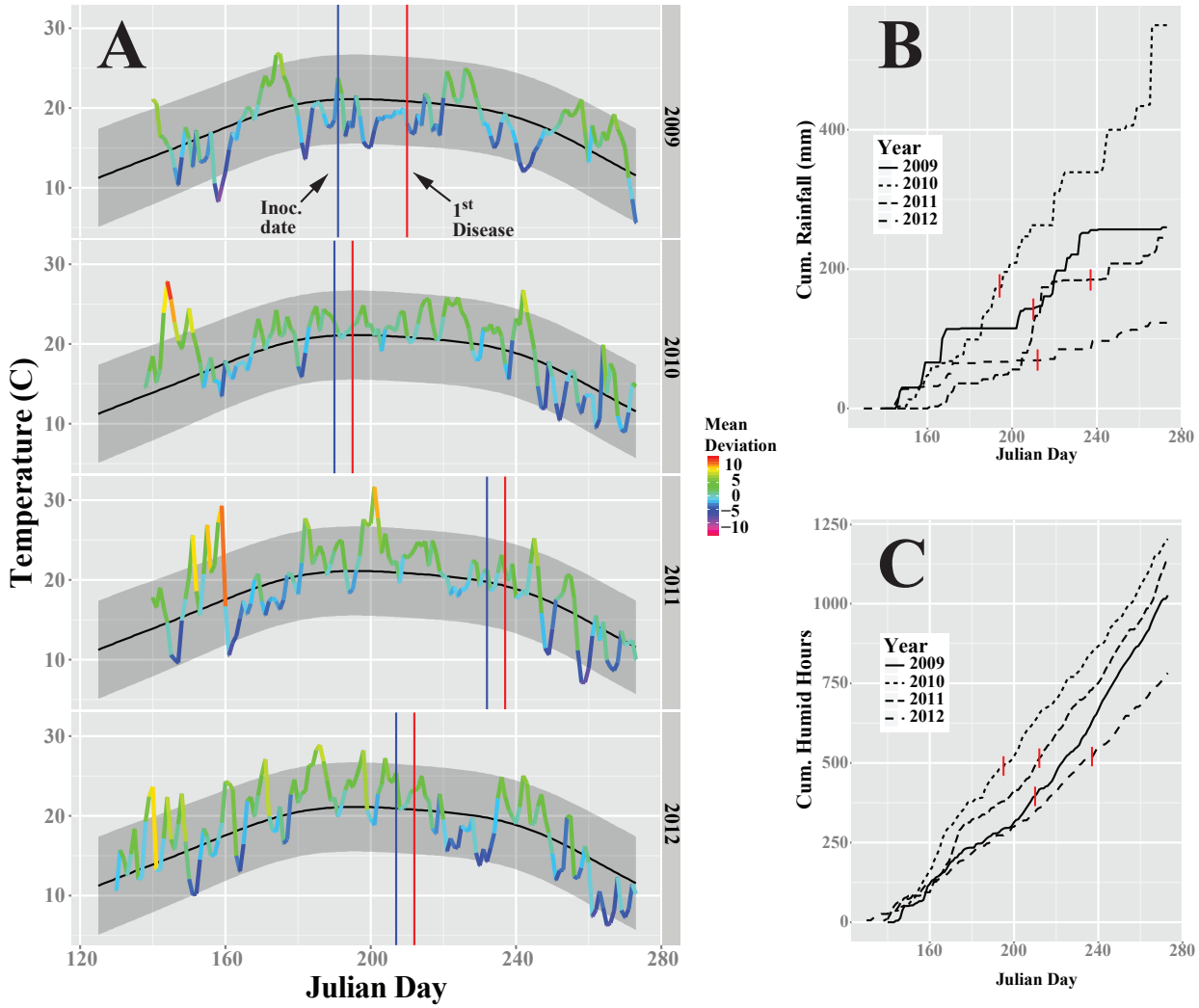
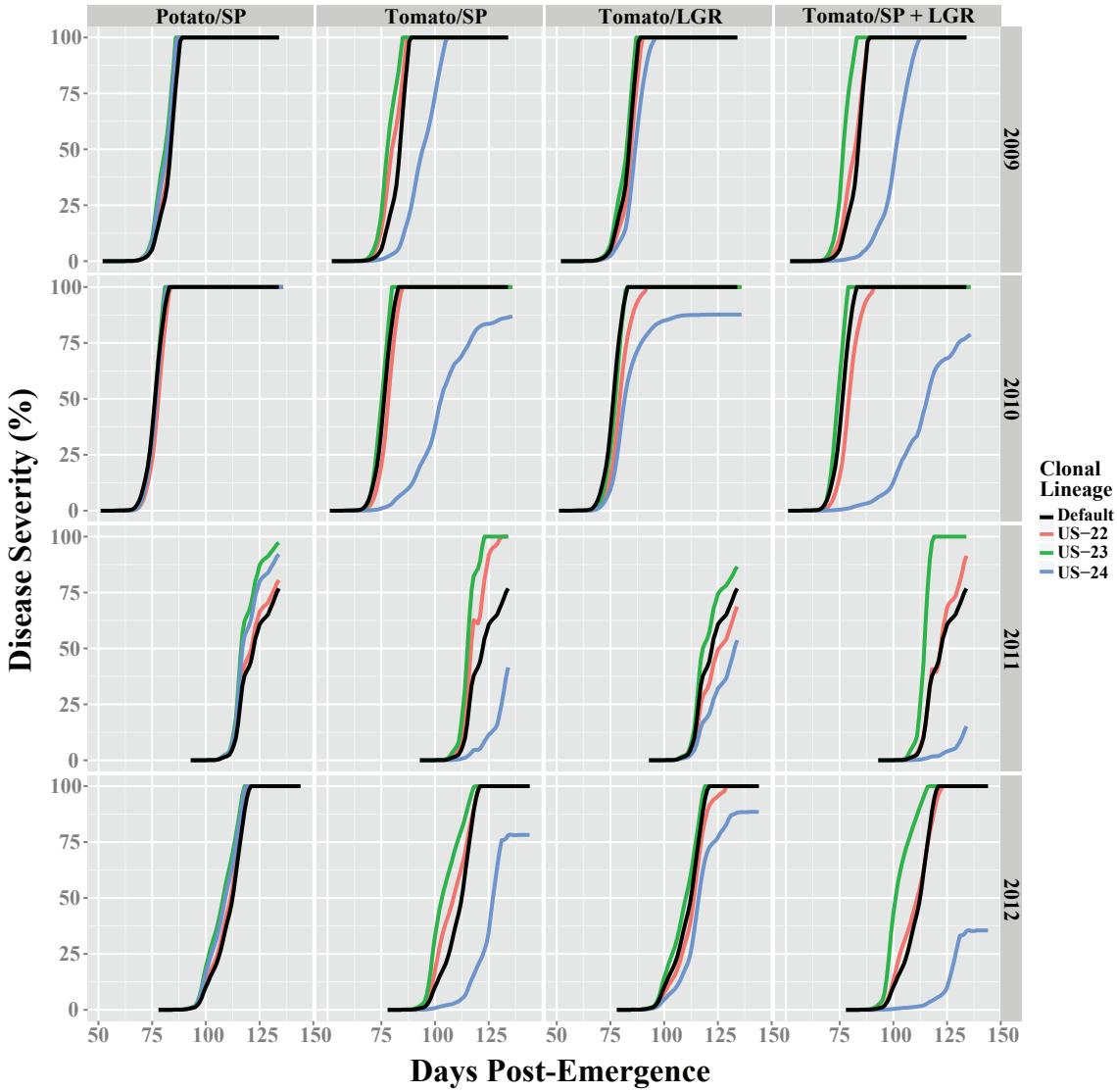


Figure 5. Results of late blight epidemic simulation using the LATEBLIGHT model (Andrade-Piedra). The parameters of sporulation rate (SR) on potato and tomato and relative lesion growth rate (rLGR) on tomato were changed first separately and then together for the US-22, US-23, and US-24 clonal lineages of *Phytophthora infestans* for each of four years of weather data from Stevens Point, Wisconsin. Different line colors indicate the different simulated epidemics produced by each lineage for each combination of modified parameters.



CHAPTER 8:

Synthesis

Phytophthora infestans causes late blight of potato and tomato, a disease that has been estimated to cost U.S. potato growers $287.8 million annually. We collected isolates of *P. infestans* from Wisconsin from 2009 to 2012 and determined distribution of clonal lineages and mating types and sensitivity to the systemic fungicide mefenoxam. We also sought to evaluate the current utility of an analysis of the *Glucose-6-phosphate isomerase* (*Gpi*) allozyme locus for predicting mefenoxam sensitivity with the aim of delivering timely information to growers. Overall, 143 isolates were collected from 52 locations in 20 Wisconsin counties from 2009 to 2012. Three clonal lineages, US-22, US-23, and US-24, were identified and were novel to Wisconsin and the U.S. US-22 is of the A2 mating type and sensitive to mefenoxam, with *Gpi* 100/122. US-23 and US-24 are of the A1 mating type and primarily intermediately sensitive to mefenoxam, with *Gpi* 100/100 and 100/100/111, respectively. Because of this close correlation and the unique *Gpi* patterns for each lineage present, we were able to predict mefenoxam sensitivity directly from samples using the allozyme assay and quickly deliver management information to growers. Both mating types were present in Wisconsin in 2009 and 2010 but were spatially separated and no evidence of sexual recombination or soil persistence was detected. The presence of new clonal lineages of *P. infestans* in Wisconsin indicates a need for continued close monitoring of late blight to facilitate generation of timely information for enhanced short-term and long-term late blight management.

While potato and tomato are the most commonly infected solanaceous hosts for *P. infestans*, new lineages may have a broader or different host range. We determined the host range of US-22, US-23, and US-24 on cultivated solanaceous plants and solanaceous weeds common to the upper midwestern production region. None of the clonal lineages produced late

blight symptoms or pathogen signs on foliage of selected cultivars of eggplant, pepper, tomatillo, or ground cherry. Symptoms and signs were evident on the potato and tomato cultivars tested, though with US-24, infection on tomato was limited. None of the lineages sporulated on the common weed black nightshade, but sporulation and disease was observed with all lineages on bittersweet nightshade. Hairy nightshade hosted abundant pathogen signs and symptoms, and sporangial production was not significantly different than that on tomato for each of the three lineages, indicating the potential for this weed to produce inoculum and contribute to late blight epidemics. Interestingly, black nightshade had the highest incidence of sporulation on berries, but the lowest on leaves, suggesting the importance of testing multiple plant organs when determining susceptibility of a species. Our results update knowledge of the host range of an ever-changing *P. infestans* population and will help to inform management strategies for enhanced efficacy of late blight control.

Asexual survival of *P. infestans*, is generally limited under freezing conditions due to the inability of this phase of the pathogen to survive in the absence of living plant tissues. Potato tubers insulated in cull piles can harbor the asexual stages of this pathogen overwinter, but overwintering in tomato is thought to be negligible and has been little researched. Because late blight infected tomatoes can be buried in a production field and harbor wet infested seeds, we tested survival on wet seeds for up to 112 days at temperatures of 18, 4, 0, -5, and -3°C using isolates of three recently identified clonal lineages of *P. infestans,* US-22, US-23, and US-24. To facilitate the exploration of four applied examples, we compared the data to recent winter soil temperatures in four USDA hardiness zones in the upper Midwestern United States. All lineages survived for 112 days at 18 and 4°C and for 84 days at 0°C. The US-23 lineage showed the

greatest cold tolerance, surviving for 21 days at -3°C and 7 days at -5°C on wet tomato seeds. Comparing the survival data from each lineage to 5-cm winter soil temperatures suggests that overwintering of each of the three lineages at each of the four example locations may be possible, but is more likely in the Zones 5a and 5b locations than in Zones 4a and 4b locations. Together, these data suggest that *P. infestans* has the potential to overwinter asexually in tomato production systems in northern regions. Seedlings arising from buried infected fruit should be monitored and managed to prevent the initiation of tomato and potato late blight epidemics.

Application of fungicides throughout the growing season is a primary means of tomato and potato late blight control in many regions, but cultivar resistance, primarily through *Ph* resistance genes from *Solanum pimpinellifolium*, can provide a cost-effective and environmentally friendly foundation to an overall disease management program. Due to highly adaptable pathogen populations, cultivar resistance against late blight is often short-lived. We evaluated 11 tomato cultivars against three clonal lineages (US-22, US-23 and US-24) of *P. infestans* novel to the U.S. to determine the efficacy of currently deployed *Ph* genes in hybrid cultivars and the validity of claims of late blight resistance in heirloom cultivars. The US-24 clonal lineage showed a low level of virulence on all the cultivars tested, but US-22 and US-23 were highly virulent on tomato. 'Plum Regal' (*Ph-3*) and 'Legend' (*Ph-2*) demonstrated some resistance against US-23 but both were susceptible to US-22. Interestingly, when the *Ph-2* and *Ph-3* resistance genes are combined in 'Mountain Magic', resistance was effective against all three lineages, including US-22, which may be explained by the different genetic backgrounds in the cultivars, or a possible residual effect of broken resistance genes. Surprisingly, three heirloom cultivars, 'Wapsipinicon Peach', 'Matt's Wild Cherry', and 'Pruden's Purple' showed

resistance to all three clonal lineages, suggesting broad-spectrum resistance unlike that conferred by the currently deployed *Ph* genes. Wapsipinicon Peach may have *S. pimpinellifolium* in its background and Matt's Wild Cherry may have been subjected to selection pressure in Mexico, but the resistance in Pruden's Purple is currently without explanation. The heirloom cultivars may be useful for future tomato late blight breeding efforts. All of the cultivars investigated in this work are currently commercially available to growers and home gardeners to limit losses to tomato late blight and reduce reliance on fungicides. Additionally, resistant cultivars limit the production of inoculum, reducing overall late blight risk in tomato and potato.

Repeated preventative application of fungicides is the primary means of control of late blight on susceptible tomato and potato crops. For organic production, fungicide choices are limited, and little efficacy data are available from which to base control recommendations. Twelve fungicides, including organic and conventional standards, were evaluated for control of three recently identified *P. infestans* clonal lineages, US-22, US-23, and US-24, using a detached tomato leaf assay. A subset of the most effective fungicides was also tested against US-23 on potted tomato plants under laboratory conditions. Fungicide applications made 2 days after inoculation failed to control late blight on detached leaves. Compared to US-22 and US-23, US-24 demonstrated a low level of virulence on tomato leaves, and all fungicides applied preventatively effectively limited disease. The biopesticides EF400 and Zonix, and Phostrol applied prior to inoculation with US-22 or US-23 resulted in significantly less disease than the copper hydroxide treatments. All conventional fungicide treatments demonstrated a high degree of efficacy when applied two days before inoculation. Similar trends with all fungicides were seen with control of US-23 in potted tomato plant assays. These results indicate that currently

available conventional and organic fungicides can effectively control late blight caused by new clonal lineages of *P. infestans*.

Epidemics of late blight have been studied by plant pathologists and regarded with great concern by potato and tomato growers since the disease was discovered as the cause of the Irish potato famine of the 1840s. We experimentally determined sporulation rates on potato and tomato and the effect of temperature on lesion growth rate on tomato for three recently identified clonal lineages of *P. infestans,* US-22, US-23, and US-24. Results were input to the LATEBLIGHT simulation model using Wisconsin environmental conditions from four growing seasons (2009 to 2012) to investigate the effect of these lineages on late blight epidemiology. The US-22 and US-23 lineages had higher lesion growth rates on tomato than the US-24 lineage, resulting in larger epidemics in all years of weather data tested, particularly in 2011. Sporulation rates for all lineages were higher on potato than tomato, causing potato epidemics to progress faster than tomato epidemics. However, the US-23 lineage had higher sporulation rates on both tomato and potato than the other lineages and than the default pathogen parameters included with the model. Additionally, US-23 always caused large epidemics when the pathogen parameters were investigated singly or collectively. Sporangial size of the US-23 lineage was significantly smaller than that of the US-22 and US-24 lineage, which may result in more efficient release of sporangia from the tomato or potato canopy and enhance long-distance spread of this lineage. Our results suggest that US-23 may be the lineage with the greatest fitness and the most likely lineage to persist in the *P. infestans* population.

Broadly, the results of this dissertation will contribute to an increased understanding of *P. infestans*. Generalizations regarding the biology, epidemiology, and management of are often

made about *P. infestans* from research performed on older lineages, even though genetic diversity among clonal lineages is acknowledged. This work has explored several key aspects of the biology and epidemiology of recently identified clonal lineages of *P. infestans*, and this will offer new insight into the organism as a whole. Additionally, it is the intention of this author that the discoveries of this work will help the tomato and potato growers of Wisconsin and across the U.S. to better manage late blight and reduce losses to this potentially devastating disease.